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ASPECTS OF ELECTRON TRANSFER IN XANTHINE OXIDASE

Rice University

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ASPECTS OF ELECTRON TRANSFER
IN XANTHINE OXIDASE

by

Arturo Guillermo Porras

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ABSTRACT

The characteristics of the oxidation reaction and of the thermodynamics of electron transfer in xanthine oxidase were analyzed by stopped flow spectrophotometry and by room temperature potentiometric titrations.

Product formation during the oxidation of xanthine oxidase was examined directly by using cytochrome c peroxidase as a trapping agent for hydrogen peroxide and the reduction of cytochrome c as a measure of superoxide formation. When fully reduced enzyme is mixed with high concentrations of oxygen, 2 molecules of $\text{H}_2\text{O}_2$/flavin are produced rapidly, while 1 molecule of $\text{O}_2$/flavin is produced rapidly and another much more slowly. Time courses for superoxide formation and those for the absorbance changes due to enzyme oxidation were fitted successfully to the mechanism proposed earlier (Olson, J. S., Ballou, D. P., Palmer, G., and Massey, V., (1974), J. Biol. Chem. 249, 4363-4382). In this scheme, each oxidative step is initiated by the very rapid and reversible formation of an oxygen-FAD$\text{H}_2$ complex (the apparent $K_D = 2.2 \times 10^{-4}$ M at 20 °C, pH 8.3). In the cases of 6- and 4-electron-reduced enzyme, 2 electrons are transferred rapidly ($k_e = 60 \text{ s}^{-1}$) to generate hydrogen peroxide and partially reduced enzyme. In the case of the 2-electron-reduced enzyme, only 1 electron is transferred rapidly and superoxide is produced. The remaining electron remains in the iron-sulfur centers and is removed slowly by a second order process ($k_s = 1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$).

When the pH is decreased from 9.9 to 6.2, both the apparent $K_D$ for oxygen binding and the rapid rate of electron transfer are decreased about 20 fold. This is suggestive of uncompetitive inhibition and implies that
proton binding to the enzyme-flavin active site affects primarily the rate of electron transfer, not the formation of the initial oxygen complex.

A series of potentiometric titrations of xanthine oxidase was carried out at room temperature in the pH range 6.2-9.9. Reduction of the two Fe/S centers was monitored by CD, and that of the FAD and Mo center by EPR. The Fe/S centers behave as centers having a protonable group whose $pK_a$ changes with reduction state ($E = -344$ mV, $pK_o = 6.4$, $pK_r = 8.1$ for Fe/S I; and $E = -249$ mV, $pK_o = 6.4$, $pK_r = 8.0$ for Fe/S II). The flavin and the two types of molybdenum centers show varying behavior but in all cases electron addition is accompanied by protonation. The sequence for FAD is: reduction, protonation, reduction, protonation with $E_1 = -398$ mV, $E_2 = -240$ mV, $pK_1 = 9.5$, $pK_2 = 7.4$. For "rapid" molybdenum the sequence is: protonation, reduction, protonation, reduction with $E_1 = -369$ mV, $E_2 = -301$ mV, $pK_1 = 7.9$, $pK_2 = 8.4$; and for "slow" molybdenum: protonation, reduction, reduction, protonation with $E_1 = -320$ mV, $E_2 = -477$ mV, $pK_1 = 7.5$, $pK_2 = 9.5$. Comparison to data obtained previously at cryogenic temperatures (Cannack, R., Barber, M. J., and Bray, R. C., (1976), Biochem. J. 157, 469-475 and Barber, M. J., and Siegel, L. M., (1981) in "Flavins and Flavoproteins", (Kassey, V., and Williams, C. H., eds.) in press) showed the centers to have significant temperature dependence. This finding calls for reevaluation of conclusions reached using cryogenic techniques (eg. rapid freeze). The optical absorbance characteristics of the enzyme were also investigated and a possible absorbance spectrum for the molybdenum suggested.
I would like to thank my advisor, Dr. Granam Palmer, for his support and sound scientific advice during my tenure as a graduate student. I also wish to thank Dr. John S. Gilson for providing the facilities and technical and theoretical expertise necessary to perform the kinetic experiments described in this work, as well as for innumerable enjoyable discussions on topics taken from the many fields of human endeavor.

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CHAPTER I
General Introduction

Xanthine oxidase can be said to be one of the most studied of all enzymes, and it was among the first to be investigated in any detail (1, 2). In 1902, Schardinger observed that a substance in fresh milk catalyzed the reduction of methylene blue by formaldehyde (3). This substance became known as "Schardinger's enzyme". A few years later the same activity was detected in other biological sources, and hypoxanthine was shown to be a substrate for the enzyme (4). Milk xanthine oxidase was first purified in 1939 (5, 6), and though it was then recognized to contain flavin (6), it was not until 1953 that any other prosthetic groups were identified. In that year the enzyme was reported to contain molybdenum (7). The presence of iron in the system was recognized in 1954 (8). The following year the enzyme was first crystallized, but unfortunately, the crystals which have been obtained so far are unsuitable for crystallographic work (9).

Purification methods for milk xanthine oxidase are numerous (9, 10, 11, 12, 13, 14, 15, 16), but they are all variations of the method of Avis et al. (9). All use either cream of buttermilk as a starting material. The most widely used is the one developed by Massey and coworkers (11). This procedure involves a proteolytic step with pancreatin, which aids the release of the enzyme from the fat globule to which it is normally attached and increases the yield tremendously. The enzyme isolated by this method has been shown not to differ from that isolated without proteolysis in its spectral, electrophoretic and catalytic characteristics (11). In all the preparations, some of the enzyme obtained is inca-
pable of catalyzing the hydroxylation of purines and is known as inactive. The capable enzyme is, of course, known as active. The exact proportions of these forms obtained being subject to variation from preparation to preparation (23).

Molecular Properties:

The molecular weight of xanthine oxidase has been investigated in some detail by a number of workers. Avis et al. (17) have calculated a molecular weight of 290,000 daltons from sedimentation and diffusion studies, reporting two monomers, each with an individual weight of 145,000 daltons. Nelson and Handler (10) have also reported two identical subunits which they calculate to weigh 150,000 daltons each. Massey et al. (11), in turn, have reported a weight of 180,000 daltons per monomer. More recently, enzyme isolated without the use of proteases has been reported to have a molecular weight of 303,000 daltons with two subunits of 150,000 daltons (14).

The work of Massey and coworkers has provided detailed analytical data about the composition of xanthine oxidase (11). This enzyme contains FAD, molybdenum, iron and acid labile sulfur in the ratios 1:1:4:4. In addition, a number of groups in the enzyme have been shown to be related to its activity: An amino group with a $pK_a$ of 10.6 located near the molybdenum site (18), a sulfur atom at the molybdenum site (19), and a thiol group near the flavin (20).

As noted above, the enzyme has been reported by several groups to be composed of two subunits with very similar molecular weights. Inhibitor studies with alloxanthine show that active xanthine oxidase binds
two moles of inhibitor per 360,000 grams of enzyme, that is one inhibitor molecule per molybdenum atom (13, 21). This data suggest that the system is composed of two identical, independently active subunits, each containing one molybdenum atom, one FAD and four iron atoms, as well as four acid labile sulfurs. The structures of the Fe/S and Mo centers are not known with certitude, however preliminary evidence has led to some speculation in this regard. The proposed structures and the known redox states of the different centers are illustrated in Fig. 1.1.

The iron and acid labile sulfur in xanthine oxidase, appear to be organized in two ferredoxin-type 2 Fe-2 S clusters referred to as Fe/S I and Fe/S II respectively. This view is supported first, by the spectral similarity of flavin-free xanthine oxidase to spinach ferredoxin. Furthermore, the extinction coefficient of flavin-free oxidase at the wavelength of maximum absorption has roughly twice the value of the corresponding coefficient in spinach ferredoxin (22). Second, the EPR of the reduced Fe/S I cluster closely resembles those typical of ferredoxin type centers (23). Third, core extrusion experiments have also shown the Fe/S centers in xanthine oxidase to be very similar to that of spinach ferredoxin (24, 25). In contrast, the EPR spectrum of the Fe/S II center is very different from that of any other iron-sulfur center known. This is cited as an argument against the proposed structure (23).

Unlike the structure of the iron-sulfur centers in xanthine oxidase, that of the molybdenum remains largely unknown. Efforts to elucidate this structure have been hampered by the resistance to isolation of the molybdenum center and by the scarcity in nature of simple molybdenoproteins which would allow the study of this type of center without interference from other prosthetic groups (26). In spite of these difficul-
FIGURE 1.1

Structure and Oxidation States of the Fe/S centers of Xanthine Oxidase
Structure and Oxidation States of Flavin Adenine Dinucleotide (FAD)

\[ R = \text{CH}_2 \text{CH} = \text{CH}_2 \text{OH} \]

\[ R = \text{CH} = \text{CH}_2 \text{OH} \]
Oxidation States and Proposed Structures of the Molybdenum Centers of Xanthine Oxidase

\[ O = Mo^{\text{VIII}} - S \rightarrow O = Mo^{\text{V}} - S \rightarrow O = Mo^{\text{IV}} - S \]

\( X = S, \) Active Molybdenum

\( O, \) Inactive Molybdenum
ties, some information regarding the structure of the molybdenum center does exist. Massey and Edmonson have found that upon addition of cyanide, sulfur is liberated in the form of thiocyanate, while the enzyme is inactivated. They also found that this inactivation could be reversed by incubation with sodium sulfide, with sulfur being incorporated into the enzyme. This led them to suggest that the enzyme contains a persulfide group at the molybdenum center which is essential for activity (19).

This hypothesis, however, has been criticized on grounds of the instability of persulfides and the fact that the group in question does not show other reactions characteristic of persulfides (23). An alternative explanation has been suggested by Coughlan who proposed that the cyano-lyzable sulfur is actually a thiol group which is bound to the molybdenum atom (27). More recent EPR work has supported the suggestion of a sulfur atom directly bound to the molybdenum (28). The presence of an oxygen atom directly bound to molybdenum has also been suggested by EPR work (29). Preliminary work by Johnson et al. (30) indicates that there is a pteridine present as a structural component of the molybdenum center. The nature of this pteridine, however, has not been determined.

The most promising method for determining the structure of the molybdenum center of xanthine oxidase is the technique of Extended X Ray Absorbance Fine Structure (EXAFS). This technique is sensitive to the chemical nature of the substituents around the atom being investigated, and in conjunction with the use of model systems can be used to study the structure around a site. Preliminary evidence from two laboratories using this technique (31, 32, 33) indicates that the molybdenum center of resting enzyme has five to six ligands arranged in an octahedral fashion around the molybdenum, with two oxo groups, which are cis to each
other, and trans to two thiol groups. At the fifth and sixth possitions, respectively, there appear to be a third sulfur group and an undetermined sixth ligand, perhaps a nitrogen. The substituent on the fifth position, could well be the cyanolizable sulfur, while that on the sixth position could arise from the pteridine reported by Johnson et al. (30). It is clear, however, that in spite of this information much work remains to be done before the structure of the center is known with certitude.

The arrangement of the various redox centers of xanthine oxidase within the enzyme is not presently known. There is a modicum of information available to this respect, but it all pertains to minimum distances between the individual prosthetic groups. Komai et al. (34) and Edmonson et al. (35) have prepared flavin free enzyme and after investigation of its spectral properties concluded that the flavin in milk xanthine oxidase is not directly bound to the molybdenum or to either of the iron sulfur centers. Other workers (36) have calculated from the maximum observable anisotropy of the molybdenum EPR signal, that the molybdenum and the iron sulfur centers are probably at least 25 Å apart. Distances of this magnitude pose a problem with respect to the elucidation of a mechanism of electron transfer between the groups, since all the known mechanisms for electron transfer either do not operate or operate too slowly to explain the kinetics of the enzyme when distances this large are involved (37).

Modified Enzyme Forms:

Besides the more important oxidase form of the enzyme, there are other forms which deserve attention. Most important of these for inves-
tigative purposes has been deflavo xanthine oxidase, that is enzyme from
which the flavin has been removed by chemical treatment. It was first
prepared by Komai et al. (34) by a method involving treatment with 2 M
calcium chloride at pH 8.0 and 20°C for 90 minutes. The product con-
tained no detectable FAD but had undiminished amounts of Mo, Fe and a-
cid labile sulfur. The spectrum of this enzyme is markedly different
from that of the native form and is strikingly similar to that of spi-
nach ferredoxin (23). The deflavo form of the enzyme has no reactivity
toward oxygen, but retains full hydroxylase activity. The removal of
the flavin is reversible and one can recover 80% of the oxidase activ-
ity on reconstitution (34).

The hydroxylase activity of xanthine oxidase is often found in the
form of xanthine dehydrogenase (23, 37). Della Corte and Stirpe (38, 39)
have shown that many xanthine oxidizing enzyme exists in vivo as Na+/ 
dependent dehydrogenases, and during isolation and storage convert to
the oxidase form. This conversion can be brought about by proteolysis,
heating, storage at temperatures as low as -20°C for an extended period
of time, oxygen, organic solvents, certain subcellular fragments and
sulfhydryl modifying reagents (37, 38, 39). Batelli and coworkers (40)
have reported that incubation of the oxidase form of the enzyme, ob-
tained by a procedure involving no proteolytic degradation, with 10 mM
dithiothreitol for 20 minutes at 37°C converts the protein to the dehy-
drogenase form. These findings have led to the suggestion that a speci-
fic thiol group near the FAD is responsible for the oxidase-dehydrogen-
ase interconversion (40, 41). Modification or elimination of the thiol
group causes a conversion to the oxidase form (23).
Nonfunctional Forms:

In 1956 Avis and coworkers (42) showed that two kinds of nonfunctional enzyme occur naturally in some preparations of xanthine oxidase. Of these nonfunctional forms, one lacks molybdenum and is known as "demolybdo", and the other one, known as "desulfo", is thought to differ from the active form by the lack of the cyanolyzable sulfur in the active site. Bray (23) has proposed that most preparations of xanthine oxidase are contaminated with the demolybdo form and that the proportion of this form present in a preparation is a factor of the nutritional status of the organism. Hart et al. (43) have been able to eliminate demolybdo enzyme from their preparations by selective denaturation with salicylate. This form is also removable by affinity chromatography (37). Demolybdo enzyme, however, is not present in all preparations (11, 37) so that it is not always a problem.

The inactivation of xanthine oxidase by cyanide was first reported by Dixon and Keilin (44). Massey and Edmonson (19) later determined that the effect of the cyanide was to eliminate a sulfur atom from the active site of the enzyme. More work by the same group (13) has shown that the nonfunctional enzyme which is isolated by affinity chromatography has no cyanolyzable sulfur. The absence of this sulfur atom affects the EPR spectra arising from the molybdenum center, and the ability of the latter to react with purine substrates, while those characteristics of the enzyme which are chiefly associated with the flavin and Fe/S centers remain unaffected (37).
Spectral Properties:

The structure and function of xanthine oxidase can be investigated by a number of spectral methods, among which are optical absorption, EPR and circular dichroism. All of the various redox centers can be observed by one or more of these techniques in at least one of their oxidation states.

Optical Spectra: The optical spectra of native oxidized and reduced, as well as deflavo xanthine oxidase are shown in Fig. 1.2. The difference spectrum of native minus deflavo enzyme, which is also shown in the same figure, was first determined by Komai et al. (34). It has marked similarity to that of other flavoproteins. Furthermore, the semiquinone form of the flavin produced during partial reduction of the enzyme has an absorbance centered around 610 nm (19), which is similar to other flavin systems. Since the molybdenum center is believed to show little or no visible absorbance, the spectrum of deflavo enzyme in the region 350-750 nm must therefore arise almost totally from the Fe/S centers. This latter spectrum is similar to that of other ferredoxin like Fe/S proteins (23). The individual contributions of the two Fe/S centers have not yet been unambiguously determined, but Olson et al. (45) have concluded from analysis of stoichiometric titrations that the ratio of the Fe/S I and Fe/S II contributions to the absorbance changes seen on complete reduction at 450 and 550 nm are approximately 4:1. Finally, even though no absorption spectrum has been identified for the molybdenum center, a charge transfer complex of xanthine oxidase with violapterin has been recently reported (47). This complex shows a broad absorption band centered about 650 nm and it is suggested to arise from complexa-
FIGURE 1.2

Absorption Spectra for Oxidized and Reduced Native Xanthine Oxidase and of Oxidized Deflavo Enzyme at pH 8.3. The lower dashed line represents the difference spectrum calculated between oxidized native and deflavo enzymes.
tion of the molybdenum center by the pteridine. The use of the complex
should be of help in the attempts to elucidate the structure and func-
tion of the molybdenum site.

Circular Dichroism: The circular dichroism spectra of both oxidized
and reduced xanthine oxidase have been recorded by Palmer and Massey
(48). Both spectra are due to the Fe/S centers and show great similarity
to those of typical iron-sulfur proteins like the ferredoxins and adre-
nodoxin. The enzyme shows exceedingly weak CD spectra.

EPR: All the redox centers in xanthine oxidase exhibit EPR spectra
in at least one of their reduction states. FAD is capable of exhibiting
3 reduction states both in its enzyme bound and free states (49). Of
these states, only the semiquinone exhibits an EPR spectrum. This signal
exhibits a $g$ value of 2.004 (50) and a linewidth of 19 gauss (48). The
last value is characteristic of the neutral or blue semiquinone form
(48, 51).

The two different 2 Fe-2 S centers in the enzyme give rise to two
widely different EPR signals. Neither exhibits a signal when oxidized,
but both exhibit intense resonances when reduced (23). One of the Fe/S
centers exhibits $g_{av}$=2.01 and is referred to as Fe/S II system. The o-
ther one of the Fe/S centers exhibits $g_{av}$=1.95 and is referred to as
Fe/S I system (23, 35). These EPR signals are only detectable at very
low temperatures. The Fe/S I signal can be observed at liquid nitrogen
temperatures (52), whereas that of the Fe/S II system can only be seen
at liquid helium temperatures (48).

The $g$ values of the signal arising from the Fe/S I system are 2.022,
1.935, and 1.899 (52). These values are typical of Fe/S proteins and
stand in agreement with the presumption stated earlier that the struc-
ture of the centers is similar to that of the center of spinach ferredoxin (23). The signal from Fe/S II, on the other hand, has g values of 2.12, 2.007 and 1.91 (54). These signal is unique among iron-sulfur proteins in having only one g value smaller than the free electron value. While it is not clear what structural information can be derived from this peculiarity, this is often cited as an argument against xanthine oxidase having two ferredoxin type Fe/S centers (23).

Molybdenum: Unlike the flavin and iron-sulfur centers, the molybdenum moiety in xanthine oxidase has proven inaccessible to most common forms of spectroscopy other than EPR. As a result this technique has provided most of the information available on the behavior and structure of this center. Fortunately, there are several different molybdenum EPR spectra which arise from xanthine oxidase. These spectra are each associated with a distinct chemical species, and are all believed to arise from Mo(V) (23).

The molybdenum EPR signals from xanthine oxidase have been classified by Bray and Vangard (50) into Very Rapid, Rapid, Slow and Inhibited. The first three according to their order of appearance during kinetic studies, and the last one because it is observed after the enzyme is reacted with some inhibitors like methanol. All these signals except the Rapid correspond to just one chemical species each, while the Rapid signal is associated with a set of closely related species (55). The chief technique used for the study of these signals has been rapid freeze-EPR (56). It has been specially useful for the study of transient phenomena like the appearance of catalytic intermediates during turnover (45, 46, 57, 58), and proton transfer and exchange (59).

The spectra obtained during EPR studies of the molybdenum center
are a combination of the individual signals, since usually more than one chemical species is present in solution (56). To alleviate this problem Bray et al. (60) have used numerical manipulation of standard spectra to resolve the contributions of the various chemical species to the observed spectra.

The Very Rapid signal is the first one to appear during kinetic experiments on reduction of the enzyme with xanthine. It is always generated as a transient signal (56) and Olson et al. (45) assigned it to a substrate-molybdenum complex intermediate. This assignment has been substantiated by Tanner et al. (61) in experiments using $^{14}$C labeled xanthine to produce a hyperfine splitting of the signal. Bsson et al. (35) had suggested that the species giving rise to this signal was in protonic equilibrium with that which originates the Rapid signal. Further experiments by other workers, however, have shown this not to be the case (56, 59).

Unlike the others, the Rapid is actually a family of very similar signals which arise from a group of closely related chemical species (56). This set of signals has been further subdivided into 3 groups: aquo, type 1 and type 2. These groups arise from interactions of the molybdenum with ligands and with two proximal protons which account for much of the fine structure observed (58, 60). The aquo signal has been suggested by Gutteridge et al. (58) to be produced by ligand-free enzyme, the "aquo" name arising from the presumption that in the absence of a ligand, water is likely bound at the active site. Gutteridge and Bray (53, 58) have proposed that Rapid type 1 and 2 signals are due to substrate, product and/or anion molecules bound at the active site. They report that the precise structure of the signal reflects the na-
ture of the ligand present. According to these authors, type 1 is always seen and can be obtained spectrally pure by reducing the enzyme with 1-methylxanthine or nitrate. Type 2 signal is always seen together with type 1 and can be induced by xanthine and by certain anions. While the significance of these two types of signals and their interrelationship is not clear, Gutteridge and Bray (53) suggest that they reflect possible allowed orientations for the bound ligands and degrees of proximity to the molybdenum.

The name of the Slow signal arises from the fact that it is the last signal to appear during the reduction of xanthine oxidase with xanthine. Its time scale of appearance is much larger than that of the Very Rapid and Rapid signals and it has been proposed by Olson et al. (45) to have no catalytic significance. Tanner and coworkers (61) have reported that the species which gives rise to this signal shows a rate of proton exchange 200 times smaller than that of the species producing the Rapid signal. From the exchange rates they measure (65/sec. and 0.4/sec. respectively), they calculate $pK_a$ values of 7.8 and 10.1 for the corresponding species. They propose (in analogy to the relationship of the $pK_a$'s of thioacids and oxoacids) that the Rapid signal arises from an active site having the group Mo=S, while the Slow signal arises from the group Mo=O being present in the inactive site. This is in accordance with their observation that the slow signal is the only one produced on reduction of desulfo enzyme.

The Inhibited signal is produced by reacting the enzyme with either methanol or formaldehyde (62). These reagents render the enzyme catalytically inactive and Fick et al. (62) have suggested that there is a formyl group bound to the active center. This signal, unlike the others
is stable to air, presumably because of having a much higher redox potential than the rest of the enzyme. It is of no catalytic significance.

Catalytic Properties:

Xanthine oxidase is an enzyme of very low specificity toward both oxidative and reductive substrates. It can catalyze the hydroxylation of a wide variety of compounds, among which are a large number of purines, including hydroxylated, amino, methyl, mercapto, halogenated and N-oxide derivatives. The enzyme will also react readily with 2- and 8-azapurines. The imidazole ring of purines can also be replaced by a pyrazolo ring without significant loss of reactivity. A number of compounds with other heterocyclic rings as well as aldehydes have also been shown to be reactive (22, 23, 63, 64). There is however a large variation in the specific activity of the enzyme for these compounds (23). For example, methylation of the N\textsuperscript{3} position of purines reduces the reactivity of the substrate toward hydroxylation by several orders of magnitude (65). Furthermore, in the case of substrates which can be hydroxylated more than once, most notably purines and pteridines, hydroxylation of the different positions takes place in a specific sequence. The order of this sequence is dependent on the substrate and on the source of the enzyme. For example, purine is hydroxylated by bovine milk xanthine oxidase in the sequence O\textsuperscript{6}, O\textsuperscript{2}, O\textsuperscript{8} (66), while enzyme obtained from C. Cylindrosporum follows the sequence O\textsuperscript{8}, O\textsuperscript{6}, O\textsuperscript{2} with the same substrate (67). Since there is an accumulation of substrate intermediates (23, 37, 68, 69, 70), it appears likely that the product of each hydroxylation step is released after the reaction and must compete
with other substrates for being rebound, presumably in a different configuration, before being oxidized further.

Besides oxidative hydroxylation reactions, xanthine oxidase is also capable of catalyzing the reduction of a wide variety of substrates. Among these are molecular oxygen, NAD, ferredoxin, iodine, dichlorophenol indophenol, an assortment of quinones, ferricyanide, cytochrome g, alloxan, phenazine methosulfate, trinitrobenzene sulfonate, methylene blue, furacin, tetrazolium salts and a number of aromatic nitro compounds (22, 23, 37, 71). Again the ability of the enzyme to transfer electrons varies with the substrate and the source of the enzyme (37). The physiological electron acceptors are oxygen for the oxidase form of the enzyme and NAD for the dehydrogenase form (23, 37, 72). Recently, evidence has been presented that both the oxidase and dehydrogenase forms coexist in the same organism (73).

**Mechanism of Action:**

The mechanism of action of xanthine oxidase has been the subject of intense investigation over a long period of time. This enzyme has often been considered to be the prototype for multicomponent redox enzymes and many of the concepts which have been developed in the study of its actions are currently being applied in attempts to understand the workings of other similar complex redox systems, most notably nitrogenase and the components of the mitochondrial electron transport chain.

Xanthine oxidase can catalyze both the oxidation and reduction of substrates. These activities are found largely associated with the molybdenum and flavin sites respectively. Thus while a first look
at the steady state kinetics would seem to indicate a classical ping-pong mechanism (11, 74). Closer examination shows that oxidative substrates do not compete with reducing substrates and vice-versa (23, 75). Moreover, removal of FAD destroys the enzyme's ability to reduce oxygen and NAD without affecting its ability to oxidize xanthine (34). Inhibition (21) or modification (19) of the molybdenum center prevents reduction of the enzyme without modifying its behavior toward oxidizing substrates. Thus the enzyme has two independently acting sites and presents a "hybrid ping-pong rapid equilibrium random mechanism" (37). That is the enzyme accepts electrons from one substrate and transfers them to another in the manner of a classical ping-pong mechanism; however, there is no requirement for a strict reaction sequence.

During catalysis one molecule of xanthine oxidase can receive and store as many as six electrons which are accepted two each in the flavin and molybdenum centers and one in each of the iron-sulfur centers. The rate of equilibration between these centers appears to be very rapid \((k > 100 \, \text{s}^{-1})\) (22, 35) and therefore a partially reduced enzyme mole- cule can exist in any one of the series of microstates shown in Fig. 1.3 (46). The distribution of molecules among these microstates is defined by six redox potentials, corresponding to each of the six redox pairs in the enzyme. The relative values of these constants were first obtained from stoichiometric titrations with dithionite by Olson et al. (46) and are presented in Table 1.1. In these experiments the formation of Mo(V), FADH', and the reduced iron-sulfur centers was monitored by low temperature EPR, that of FADH₂ by optical spectroscopy and that of Mo(IV) by quantitation of the reductant added. The values of the electron affini- nities were then established from the titration curves by determining the
FIGURE 1.3

Possible Reduction States of the Prosthetic Groups of Individual Molecules of Xanthine Oxidase at Various Overall Reduction States. The various intermediates represent the electron distributions possible for the overall reduction state attributed to that column. With the exception of fully oxidized enzyme, only the reduced forms of the prosthetic groups are shown. Taken from Olson et al. (46).
### TABLE 1.1

Midpoint Oxidation-Reduction Potentials for Milk Xanthine Oxidase.

Values for the midpoint potentials are given in millivolts. The values in parenthesis represent the values calculated from the relative potentials of Olson et al. (46) by assuming a value of -303 mV for the midpoint potential of Fe/S II. A, Xanthine oxidase in 0.1 M pyrophosphate pH 8.5, 21 °C. From Olson et al. (46). B, in 0.055 M pyrophosphate pH 8.2. From Cammack et al. (76). C, in 0.1 M Tris pH 8.2. From Cammack et al. (76).

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe/S I</td>
<td>-24</td>
<td>-343</td>
<td>-330</td>
</tr>
<tr>
<td></td>
<td>(-327)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe/S II</td>
<td>0</td>
<td>-303</td>
<td>-255</td>
</tr>
<tr>
<td></td>
<td>(-303)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAD/FADH</td>
<td>-60</td>
<td>-351</td>
<td>-378</td>
</tr>
<tr>
<td></td>
<td>(-363)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FADH/FADH₂</td>
<td>+60</td>
<td>-236</td>
<td>-223</td>
</tr>
<tr>
<td></td>
<td>(-343)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mo(VI)/Mo(V)</td>
<td>-60</td>
<td>-355</td>
<td>-397</td>
</tr>
<tr>
<td></td>
<td>(-363)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mo(V)/Mo(IV)</td>
<td>-31</td>
<td>-355</td>
<td>-405</td>
</tr>
<tr>
<td></td>
<td>(-334)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
relative potentials necessary to achieve the observed electron distributions. An arbitrary value of 1.0 was assigned to the electron affinity associated with the Fe/S II redox couple.

The values of the redox potentials relative to the Standard Hydrogen Electrode associated with the different centers have since been established by potentiometric titrations using low temperature EPR spectroscopy (76, 77). These results, shown in Table 1.1 are in substantial agreement with the relative potentials calculated by Olson et al. (46), Barber and Siegel (77), in a study carried out simultaneously with the work presented in this thesis, have gone further than this and have carried out a thorough survey of the values of the midpoint potentials as a function of pH. They found a strong influence of the pH on the value of all the midpoint potentials associated with the centers in xanthine oxidase (See Table 1.2). To explain this behavior they proposed a set of protonation schemes associated with all the centers (Fig. 1.4). By fitting the equations derived from these schemes, they obtained values for the pH independent potentials and for the ionization constants of the groups which become protonated. The values obtained are shown in Table 1.3. While the variations of the midpoint potentials for the different groups are complicated, some patterns can be readily discerned. Throughout the pH range the midpoint potentials for Mo remain the most negative, while for most of the range the Fe/S centers occupy an intermediate position and the flavin has widely separate potentials. Even though this generalization breaks down somewhat at the highest pH values, it is a good approximation for the pH range as a whole. Since most mechanistic work with xanthine oxidase, in particular the work of Olson et al. (45, 46), has been carried out at pH 8.2, and this pH is not too
### TABLE 1.2

Midpoint Potentials of the Prosthetic Groups of Xanthine Oxidase as a Function of pH. The values of the potentials are given in millivolts. The data were obtained by a combination of room-temperature potentiometric titrations with low-temperature EPR. Taken from Barber and Siegel (77).

<table>
<thead>
<tr>
<th>pH</th>
<th>Fe/S I (mV)</th>
<th>Fe/S II (mV)</th>
<th>FAD/FADH</th>
<th>FADH/FADH&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Mo(VI)/Mo(V) (mV)</th>
<th>Mo(V)/Mo(IV) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.7</td>
<td>-260</td>
<td>-250</td>
<td>-214</td>
<td>-170</td>
<td>-300</td>
<td>-276</td>
</tr>
<tr>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-283</td>
<td>-197</td>
</tr>
<tr>
<td>6.5</td>
<td>-290</td>
<td>-245</td>
<td>-235</td>
<td>-229</td>
<td>-327</td>
<td>-321</td>
</tr>
<tr>
<td>6.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-318</td>
<td>-282</td>
</tr>
<tr>
<td>7.1</td>
<td>-300</td>
<td>-255</td>
<td>-280</td>
<td>-244</td>
<td>-355</td>
<td>-356</td>
</tr>
<tr>
<td>7.7</td>
<td>-310</td>
<td>-255</td>
<td>-332</td>
<td>-234</td>
<td>-373</td>
<td>-392</td>
</tr>
<tr>
<td>7.7*</td>
<td>-320</td>
<td>-270</td>
<td>-343</td>
<td>-223</td>
<td>-369</td>
<td>-271</td>
</tr>
<tr>
<td>8.2</td>
<td>-330</td>
<td>-255</td>
<td>-378</td>
<td>-223</td>
<td>-397</td>
<td>-405</td>
</tr>
<tr>
<td>8.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-432</td>
<td>-468</td>
</tr>
<tr>
<td>9.3</td>
<td>-360</td>
<td>-290</td>
<td>-410</td>
<td>-290</td>
<td>-472</td>
<td>-428</td>
</tr>
<tr>
<td>9.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-490</td>
<td>-506</td>
</tr>
<tr>
<td>9.8</td>
<td>-365</td>
<td>-282</td>
<td>-402</td>
<td>-296</td>
<td>-505</td>
<td>-439</td>
</tr>
<tr>
<td>10.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-595</td>
<td>-585</td>
</tr>
<tr>
<td>10.9</td>
<td>-370</td>
<td>-320</td>
<td>-407</td>
<td>-293</td>
<td>-605</td>
<td>-405</td>
</tr>
</tbody>
</table>

* With 2 mM uric acid added.
TABLE 1.3

<table>
<thead>
<tr>
<th></th>
<th>$pK_o$</th>
<th>$pK_r$</th>
<th>$E_0$</th>
<th>$E_1$</th>
<th>$E_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe/S I</td>
<td>7.0</td>
<td>8.5</td>
<td>-363 mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe/S II</td>
<td>8.2</td>
<td>9.0</td>
<td>-297 mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td>8.8</td>
<td>6.7</td>
<td>-410 mV</td>
<td>290 mV</td>
<td>235 mV</td>
</tr>
<tr>
<td>Mo(Rapid)</td>
<td>6.2</td>
<td>8.5</td>
<td></td>
<td>290 mV</td>
<td>-440 mV</td>
</tr>
<tr>
<td>Mo(Slow)</td>
<td></td>
<td>7.6</td>
<td>64 mV</td>
<td></td>
<td>493 mV</td>
</tr>
</tbody>
</table>

$pH$ Independent Midpoint Potentials and $pK_a$ Values for the Dissociable Protons Associated with Reduction of the Prosthetic Groups of Xanthine Oxidase. The values correspond to the midpoint potential and $pK_a$ of the centers according to the schemes presented in Fig. 1.4. Taken from Barber and Siegel (77).
FIGURE 1.4
Protonation Schemes Associated with the Reduction of the Different Prosthetic Groups of Xanthine Oxidase Proposed by Barber and Siegel (77).
far away from physiological pH we will restrict ourselves in this dis-
ッション to that value.

As seen in Table 1.1, the midpoint potentials associated with the
redox centers of xanthine oxidase at pH 8.2 are all contained within a
range of 120 mV and are centered around the values of -303 mV. As out-
lined above, both potentials for the Mo center are at the lowest end of
the range with the Fe/S centers being close to the average potential.
The potentials for FAH/FADH and FADH/FADH₂ lie at the extremes of the
range, with the latter being considerably more positive than that of any
other group in the enzyme. This arrangement of the relative values of
the potentials has the following consequences:

Because of the closeness of the potentials, all of the possible
microstates shown in Fig. 1.3 for a given reduction state are populated.
Since the electron distribution among the enzyme molecules obeys statis-
tical constraints, a partially reduced sample of the enzyme contains a
distribution of molecules in the different possible reduction states
(0, 1, 2, ..., 6 electrons). The shape of this distribution is deter-
mined by the overall state of reduction of the sample and the individ-
ual electron affinities of the different reduction states.

Since both the molybdenum potentials are very low, this center re-
mains substantially oxidized in most intermediate reduction states. As
calculated by Olson et al. (46) four-electron reduced enzyme contains
as much as 70% Mo(VI) with almost no Mo(IV) being present (46, 78).
This situation suggests that the potentials of this center have been
adjusted to provide the most efficient oxidative activity with respect
to purine substrates.

Since the potential of FAH/FADH₂ is considerably more positive
than that of any other redox couple in xanthine oxidase including that of the FAD/FADH couple, little flavin semiquinone is formed (46, 78). In reduction states higher than 1-electron reduced enzyme, most of the flavin is present as FADH₂ (as much as 95% for 4-electron reduced enzyme) (78). This again, suggests adjustment of the redox potentials of the enzyme to provide optimum catalytic activity. Thus the enzyme is capable of providing maximum activity over a wide range of oxidative and reductive substrate concentrations by maintaining its Mₐ site oxidized and its flavin site reduced with the Fe/S centers functioning as buffering electron storage sites.

Although the findings delineated above lead to an attractive picture of how the enzyme works, there are some problems associated with the manner in which some of the data have been collected. As shown in Table 1.1 the use of different buffers leads to different values for the midpoint potentials (76). This phenomenon has been attributed to the pH shift experienced by the buffers on freezing (79). Another possible source of error is the temperature dependence of midpoint potentials if electrons are capable of reequilibration in the frozen enzyme solutions. This problem will be addressed more fully later in this work.

Mechanism of Reduction:

The reduction of xanthine oxidase by xanthine is a deceptively simple process when monitored by visible spectrophotometry. When the time course of the reaction is monitored at 450 nm, the reaction exhibits roughly exponential behavior. In contrast, the sequence of appearance of EPR signals during reduction led workers initially to propose a
complex linear pathway for electron flow (57):

\[
Xanthine \rightarrow \text{Mo} \rightarrow \text{FAD} \rightarrow \text{Fe/S}
\]

The work of Olson et al. (46) led to a much different picture, and to the proposal that the peculiarities of the kinetics of the reaction of the enzyme with xanthine are largely dependent on the fact that 3 molecules of substrate are necessary to achieve complete reduction of the enzyme. Edmondson et al. (35) first proposed that the rate limiting step during the reaction of one molecule of xanthine with one enzyme molecule is the dissociation of the product, and that the rate of this step is the same for all 3 equivalents of xanthine. The observation that the Rapid molybdenum EPR signal appears and disappears during reaction with one mole of xanthine, together with the accelerating behavior of the reaction, which is characteristic of certain two step mechanisms, led Olson et al. (46) to refine the ideas of Edmondson et al. and propose that each of the reactions is composed of the following steps:

\[
E(i)+X \xrightleftharpoons[k_D]{K_D} EX(i) \xrightarrow[k_2(1)]{} E'U(i+2) \xrightarrow[k_3]{k_3} E(i+2)+U
\]

where \(E(i)\) represents enzyme containing \(i\) electrons, \(X\) represents xanthine and \(U\) represents uric acid. They interpreted the first intermediate to be a species in which the substrate is bound to the active site but does not interact directly with the molybdenum. In the second step, the substrate transfers two electrons to the molybdenum center generating the Very Rapid molybdenum signal. The presence of product in the binding site stabilizes the reduced form of the molybdenum, inhibiting the transfer of electrons to the flavin and the iron-sulfur centers. The proposed irreversibility of this step is justified by the large discrepancy between the redox potential of the xanthine/urate couple and those
of the active centers of the enzyme. The final step is the release of product from the active site. This release frees the electrons in the No for redistribution throughout the rest of the enzyme molecule, and is accompanied by the disappearance of the Very Rapid molybdenum signal. Since the rate limiting step of the reaction is the release of product, it is readily understood why there is no observable isotope effect on the V_max of the reaction when deuterated substrate is used. Simonson et al. (35) have proposed that the last step involves the hydroxylation of the substrate by nucleophilic attack of hydroxyde upon the enzyme-substrate complex. The fact that this step shows no pH dependence, however, makes this an unlikely proposition.

The success of this scheme in explaining the kinetic behavior of the enzyme is dependent on the assumption that the bound product is able to stabilize the reduced molybdenum and trap the electrons in it. In support of this proposal, Barber and coworkers (62) have reported that addition of uric acid to a potentiometric titration increases the midpoint potentials associated with the molybdenum center. Although the increase they found is not as big as required by the proposal of Olson et al. (46), this can be explained by the fact that Barber et al. (62) were unable to use saturating concentrations of uric acid.

The same scheme can also be used to explain the discrepancy observed between the kinetic parameters for the individual steps obtained from steady state measurement and the overall apparent rate of decay of the fully reduced enzyme in stopped flow experiments (45, 46). The rapid kinetic measurement yield smaller kinetic constants when analyzed as exponential decays. This occurs because each of the reduction steps in xanthine oxidase is irreversible, and when reduction of the enzyme
is carried out by 2 or 3 xanthine molecules, the various states of the enzyme being simultaneously formed \( E(2), E(4), E(6) \), yield erroneous values for the kinetic parameters \( (46) \).

The final item with regard to the reduction of the enzyme is the sequence of appearance of the EPR signals associated with the several redox centers. As explained above, the first signal to appear is the Very Rapid molybdenum which is formed immediately upon reaction and remains until the electrons are allowed to equilibrate with the rest of the enzyme. Since the rate of this equilibration is very large \( (k > 100 \text{ s}^{-1}) \) \( (46) \), the appearance of the other signals is determined by the relative redox potentials of the prosthetic groups, so that instead of reflecting the existence of a well defined electronic pathway, the order of appearance of the signals reflects the individual electron affinities of the different groups within the enzyme.

In evaluating the overall effectiveness of the model it must be said that although it is not perfect and discrepancies can still be found between the predictions and experimental data, Olson and coworkers enjoyed remarkable success in explaining hitherto mysterious phenomena and in predicting the kinetic behavior of the enzyme in a quantitative fashion. Further work on this subject has succeeded in refining this mechanisms but the basic features remain unchanged \( (47) \).

**Mechanism of Oxidation:**

The reaction of oxygen with reduced xanthine oxidase exhibits a markedly biphasic time course with the rate of the fast phase being approximately ten times that of the slow phase \( (45, 46) \). The relative am-
Plitudes of these two phases are dependent on the wavelength of observation. At 450 nm the fast phase comprises about 85% of the total absorbance change, while at 550 nm each phase comprises some 50% of the extinction change. As in the case of reduction the overall oxidation presents a stoichiometry of three substrate molecules per active site. Olson et al. (46) suggested that the fast phase accounts for the removal of 5 electrons from the enzyme, with the remaining electron being removed in the slow phase. The absorbance changes associated with the slow phase are very similar to the difference spectrum obtained from the oxidation of the iron sulfur centers, whereas the changes associated with the fast phase resemble a combination of flavin and iron-sulfur centers difference spectra.

Since these phenomena are not readily explained by any simple scheme, Olson et al. (46) proposed a scheme in which the reoxidation occurs by way of a combination of one and two electron steps to give both peroxide and superoxide as products:

\[
\begin{align*}
XO(5) & \xrightarrow{K_1} XO(3) \\
XO(6) & \xrightarrow{K_1} XO(4) \\
XO(4) & \xrightarrow{K_1} XO(2) \\
XO(2) & \xrightarrow{K_1} XO(1) \\
XO(1) & \xrightarrow{K_2} XO(0) \\
H_2O_2 & \xrightarrow{K_1} XO(2) \\
H_2O_2 & \xrightarrow{K_1} XO(4) \\
H_2O_2 & \xrightarrow{K_1} XO(0)
\end{align*}
\]

Scheme 1

Again E(i) represents a reduced enzyme species containing i electrons. In each of these steps, except the last, oxygen binds rapidly to fully reduced flavin and then an electron is transferred to generate a flavin
semiquinone-superoxide complex:

\[ E(1) - \text{FADH}_2 \rightleftharpoons E(1) - \text{FADH} \cdots \cdot O_2^- \]

When the enzyme still contains 3 or more electrons, \( \text{FADH}_2 \) is regenerated very rapidly by intramolecular transfer from the reduced iron-sulfur centers and molybdenum. The rate of this latter process is postulated to be greater than the rate of superoxide diffusion out of the active site. A second electron is then transferred to superoxide to produce hydrogen peroxide. Because of its high midpoint potential for complete reduction the flavin will remain reduced as long as electrons are available for replenishing \( \text{FADH}_2 \). This two-electron oxidation process occurs until \( E(2) \) is produced. At this stage oxygen again binds rapidly and accepts an electron but, in the resulting complex, fully reduced flavin cannot be regenerated at this step. In addition the remaining electron does not stay in the flavin because of the greater electron affinity of the iron-sulfur centers with respect to oxidized flavin. This in one-electron reduced enzyme almost all of the reducing equivalents are present in the iron atoms and superoxide is released.

The removal of the last electron from xanthine oxidase also produces superoxide. The rate of this last step is considerably slower than that of the previous reactions since fully reduced flavin cannot be formed. The amount of semiquinone present is also quite small \((46)\) and oxygen reacts extremely slowly, if at all, with the iron-sulfur centers \((34)\).

As with the model for the reductive reaction, Olson et al. \((46)\) enjoyed great success in explaining and predicting the various kinetic features of the oxidative half reaction. Some problems, however, remain unresolved. Scheme 1 predicts that complete oxidation of 6-electron reduced enzyme should produce two molecules of hydrogen peroxide and one
of superoxide during the fast phase and one molecule of superoxide during the slow phase. Attempts to quantitate the production of superoxide by rapid freezing techniques yielded values no greater than 0.1 O$_2^-$/FAD. In addition to being inconsistent with the proposal of Olson et al. (46) this result contradicts other work which has suggested that O$_2^-$ is the initial product of the oxidative steps and that peroxide production takes place only by dismutation of O$_2^-$(79). In addition, the rate of hydrogen peroxide production during the oxidation has never been measured. Investigation of these phenomena should provide a good test of the relevance of the mechanism just described.

In order to improve our understanding of the action of xanthine oxidase I have carried out an investigation of the kinetics of reoxidation of the enzyme at a wide variety of conditions. In particular, I monitored the production of H$_2$O$_2$ and O$_2^-$ at different pH values and oxygen concentrations. I then fit the data obtained to a proposed mechanistic scheme. The results, given in Chapter 3, have been published elsewhere (Porras, A. G., Olson, J. S., and Palmer, G., (1981), J. Biol. Chem. 256, 9096-9103). I have also carried out an investigation of the potentiometric behavior of the same protein at a variety of pH values and have fit the behavior of the potentials of the various redox centers with changing pH to appropriate reduction-protonation schemes. These results are given in Chapter 4.
CHAPTER II
Materials and Methods

Enzyme Preparation:

Xanthine oxidase was prepared by a modified version of the method of Massey et al. (11). Six gallons of fresh raw cream (Carnation Corporation, Houston, Texas) were churned at 16 °C, in two batches of 3 gallons each. The buttermilk obtained was separated from the butter by filtration through cheesecloth. The following substances were then added to the buttermilk: Salicylic acid (2.08 g/l), Cysteine-HCl (0.312 g/l), EDTA (0.115 g/l) and K₂HPO₄ (20.1 g/l). The solution was titrated to pH 7.5 and warmed up to 40 °C in a shaker-water bath. Pancreatin (1.64 g/l of buttermilk) (BDH Biochemicals), dissolved in a minimal volume of distilled water, was then added to the solution which was then incubated for 3 1/2 hours at 40 °C. The buttermilk was then placed at 4 °C and allowed to stand overnight.

The next morning the following were added to the treated buttermilk: Butanol (170 ml/l of buttermilk) and ammonium sulfate (149 g/l of buttermilk). The mixture was then allowed to stand for 30 min. at 4 °C and subsequently centrifuged for 30 min. at 4000 g. This procedure separated the mixture into a brown liquid layer sandwiched between two solid layers. The liquid layer was collected by suction. Ammonium sulfate (126 g/l) was added to the solution and the liquid was allowed to stand for 1 hour and then centrifuged under the same conditions as before. The resultant green supernatant was removed by suction and discarded, and the brown pellet was resuspended in 0.1 M pyrophosphate +
0.3 mM EDTA + 1 mM salicylate, pH 8.3. Homogenization was used to achieve full resuspension in as small a volume as feasible. The resuspended xanthine oxidase was then ultracentrifuged at 75,000 g for 1 hour. This procedure separated the suspension into four layers: the top one, a yellow liquid which consisted mainly of butanol, was removed by suction and discarded. Underneath this layer there were two solid layers separated by a brown liquid layer containing the enzyme. This liquid was decanted and filtered through several layers of cheesecloth and was then thoroughly dialyzed against 5 mM KPi + 0.3 mM EDTA + 1 mM salicylate, pH 6.3. After dialysis the enzyme was centrifuged to remove the precipitate formed during the preceding step and was then loaded on an 8 x 25 cm hydroxyapatite/cellulose column (100 g of hydroxyapatite + 100 g of cellulose) which had been equilibrated with the same buffer used to dialyze the enzyme solution. After the enzyme solution was loaded, the column was washed with at least 5-6 volumes of 0.1 M KPi + 0.3 mM EDTA + 1 mM salicylate, pH 6.2. The enzyme was then eluted with the same buffer which had been made 5% in ammonium sulfate, and 15 ml fractions were collected. The fractions comprising the central 2/3 of the peak were then pooled. A small sample of the enzyme was desalted in a Biogel P-6 column (Bio-Rad) to remove the salicylate, and the ratio of the absorbances at 280 and 450 nm was determined. If this ratio was larger than 5.5, all the enzyme fractions were pooled together and the hydroxyapatite step repeated until the desired absorbance ratio was obtained. The purified enzyme was concentrated in an Amicon ultrafiltration apparatus and stored under liquid nitrogen until used.
Xanthine Oxidase Characterization:

The enzyme prepared by this method contained Mo, Fe and acid labile sulfur in the ratios 1.07 : 4.0 : 4.4 per FAD (cf. 47), which are very close to the accepted 1 : 4 : 4 for the native enzyme (23).

The activity of xanthine oxidase was determined by the method of Massey et al. (11): a known amount of enzyme was added to 3 ml of 0.1 M sodium pyrophosphate buffer, pH 8.3, containing 0.3 mM EDTA and 0.1 mM xanthine, which had been air equilibrated at 25 °C. The conversion of the xanthine to uric acid was followed at 295 nm. The percentage of active enzyme was determined from the activity to flavin ratio (AFR). AFR values were calculated by dividing the absorbance change per minute at 295 nm by the absorbance at 450 nm of the enzyme used in the assay. My enzyme preparations showed AFR values of 90-130. AFR values of 120 have been obtained by extrapolation for fully active enzyme preparations under the same conditions (13). Enzyme concentrations were measured spectrophotometrically at 450 nm using the value of 37,800 M⁻¹ cm⁻¹ for the molar extinction coefficient at this wavelength.

Purification of Cytochrome c Peroxidase:

Cytochrome c peroxidase was prepared by a modified version of the method of Nelson et al. (80). Eleven pounds of fresh commercial brewers yeast (Red Star, Universal Foods Corp., Houston, Texas) were crumbled and spread on aluminum foil over a large surface. The yeast was allowed to dry for 12 hours at room temperature, and it was turned over and mixed 2 to 3 times per hour. The dried yeast was then kneaded with
900 ml of ethyl acetate until an even and smooth consistency was attained. The mixture was stored overnight (10-12 hours) in a closed container at 4 °C. The next morning the autolyzed yeast was suspended in 5 liters of cold 500 mM sodium acetate + 1.25 mM EDTA + 1.25 mM sodium metabisulfite, pH 5.0 and stirred for 4 hours. This suspension was centrifuged for 30 min. at 4000 g and the precipitate discarded. The supernatant (about 4 l) was filtered through Miracloth and dialyzed twice against a tenfold volume of distilled water. The precipitate formed during this step was removed by centrifugation at 4000 g for 30 min. The enzyme was then loaded on a 5 x 15 cm DEAE cellulose (DE-52) column which had been equilibrated with 5 mM potassium acetate, pH 5.0. The cytochrome c peroxidase collected at the top of the column as a dark brown band and the eluant, containing cytochrome c was collected for further purification of this protein. The column was washed with 100 mM potassium acetate, pH 5.0 while the brown band at the top slowly broadened until it reached the bottom of the column. The enzyme was eluted with 500 mM potassium acetate, pH 5.0 and concentrated by ultrafiltration. The concentrated solution was applied to a 5 x 100 cm column of Sephadex G-75 (Sigma Chemical Co.) which had been equilibrated with 100 mM potassium acetate, pH 5.0 and eluted with the same buffer with a hydrostatic head of about 120 cm. Fractions of 5 ml were collected and those showing a 408/280 nm absorbance ratio greater than 1.6 were pooled and concentrated by ultrafiltration.

Experimental Procedures:

Before use, the proteins were transferred to one of the following
buffers by desalting in a Bio-Gel P-6 column, and were then diluted to the appropriate concentrations: 0.1 M potassium 2-[N-morpholino] ethane sulfonate (MES), pH 6.15; 0.1 M potassium morpholino propane sulfonate (MOPS), pH 7.2; 0.1 M potassium N, N-bis[2-hydroxyethyl] glycinate (Bicine), pHs 7.7 and 8.3; 0.1 M 2-[N-cyclohexyl amino] ethane sulfonate (CHES), pH 9.2 and 0.1 M potassium glycinate, pH 9.9.

Stopped Flow Experiments:

Xanthine oxidase and cytochrome c peroxidase were prepared as described above. Sodium dithionite was obtained from Virginia Snellting Co., and the buffer salts and cytochrome c (type VI) were all purchased from Sigma Chemical Co.

Rapid mixing experiments were performed using a Gibson-Durrum stopped-flow spectrometer interfaced to a Nova computer by means of a high-speed 12-bit A/D converter. The data were collected as voltage readings from the photomultiplier tube and converted to absorbance changes by means of software. In order to increase the signal-to-noise ratio, each experimental record consisted of the average of 4-10 consecutive measurements.

Standard oxygen solutions were prepared by bubbling syringes containing the appropriate buffer with either air or pure O$_2$ in an open vessel at room temperature for about 30 minutes. Anaerobic buffer was prepared in the same way using 99.99% nitrogen. Solutions at other O$_2$ concentrations were prepared by diluting the standard oxygen solutions with the anaerobic buffer in the gas tight syringes.

Reduced xanthine oxidase was prepared in a glass tonometer equipped
with a standard taper joint to accept a syringe containing the reductant and a 10 mm optical cuvette for direct spectrophotometry. The enzyme solution was made anaerobic by equilibration with an argon atmosphere containing less than 0.2 ppm oxygen (81). Reduction was carried out by titrating to the desired level with a 20 mM solution of sodium dithionite (in 0.1 M pyrophosphate, pH 8.3), while monitoring the absorbance change at 450 nm (Δε = 26,600 M⁻¹ cm⁻¹).

**Potentiometric Titrations:**

To ensure rapid equilibration between the system and the electrode used to measure the potentials the following mediator dyes were used: Indigo tetrasulfonic acid (-46 mV, Pfalz & Bauer), anthraquinone-1,5-disulfonic acid (-170 mV, ICN Pharmaceuticals), 2-hydroxy-1,4-naphthoquinone (-145 mV, ICN Pharmaceuticals), phenosafranine (-255 mV, ICN Pharmaceuticals), safranine T (-289 mV, Pfalz & Bauer), benzyl viologen (-311 mV, Mann Research Laboratories), methyl viologen (-440 mV, Mann Research Laboratories), cresol red (-405 mV Allied Chemical Corp.), bromocresol purple (-410 mV, Kodak).

The potentials were measured with a Metrohm EA224 combination platinum electrode with Ag/AgCl reference system. This electrode has a ground joint so that it can be conveniently installed in an all-glass titration vessel.

**CD Titrations:**

The changes in optical activity during the potentiometric-CD ti-
trations were monitored using a Jasco-J500 circular dichroism spectrometer. The titrations were carried out using a Metrohm EA880-V titration vessel which had been fitted with a 1 cm pathlength spectrophotometric cell attached by means of a glass sidearm. The oxidation-reduction titrations were carried out with 5 ml of approximately 20 mM xanthine oxidase in the appropriate buffer containing the mediator dyes (25 μM each). Initially, the sample was equilibrated with an argon atmosphere containing less than 0.2 ppm oxygen (81). The redox potential of the reaction mixture was then adjusted by additions of 0.1 N sodium dithionite (in 0.1 N pyrophosphate, pH 8.3) and the solution was stirred until the potential reading stabilized (1-2 minutes). Addition of the amounts of dithionite needed to complete a titration had no detectable effect on the pH of the sample under study, and its effects on the system's potential and spectroscopic changes were readily reversed by addition of oxygen to the solution. The sample was then transferred to the spectrophotometric cell and the CD spectrum recorded. The titrations were performed in the reductive and oxidative directions and identical results were obtained in both cases.

EPR Titrations:

In order to carry out room temperature potentiometric titrations of the molybdenum and flavin centers in xanthine oxidase, an appropriate titrator was built as follows (Fig. 2.1): A standard potentiometric vessel (Metrohm EA880-V) was modified by attaching a 7/25 female joint, close to the base. The titrator was then connected to one end of a length of standard flexible quartz GLC capillary tubing provided with
FIGURE 2.1
Schematic Diagram of the EPR Potentiometric Assembly. The individual parts are labeled as follows: (A), Potentiometric vessel; (B), Enzyme solution; (C), Magnetic stirrer; (D), Measuring electrode; (E), Titration cap; (F), Syringe containing reductant (sodium dithionite); (G), Capillary connector; (H), EPR flat cell; (I), Airtight syringe for driving the enzyme solution in and out of the flat cell; (J), Three-way stopcock leading from the titration setup to either the atmosphere or a nitrogen cylinder under pressure; (K), EPR cavity. The setup is operated as described in the text.
a 7/25 male joint. The other end of the capillary tube was provided with a 4/20 male joint which was connected to the bottom end of a Varian 4548 EPR flat cell mounted in a Varian E231 rectangular EPR cavity. The top of the flat cell was connected to an airtight syringe and to a 3-way stopcock leading to a Nitrogen cylinder and to the atmosphere. The titrator was fitted with the electrode described previously for measuring the system potential, and a Hamilton gas-tight syringe provided for adding the reductant.

A potentiometric titration is performed as follows: Before the beginning of the experiment, the titration ensemble is assembled in place and thoroughly flushed with nitrogen gas. The enzyme solution (B) is then added to the body of the titrator (A). The solution in the vessel is stirred throughout the experiment by a magnetic stirrer (C). To record a measurement, enough titrant is added from the syringe containing the reductant (F) to poise the system potential at or near a desired value which is measured by the attached electrode (D). The enzyme solution is then drawn through the capillary tubing (G) into the flat cell (H) located in the EPR cavity (K) by applying a vacuum with an airtight syringe (I) until the body of the cell is completely filled with liquid. During this manipulation the stopcock (J) attached to the flat cell remains closed to both the atmosphere and the source of nitrogen gas.

When this operation is complete the syringe (I) is filled with nitrogen by opening the attached stopcock (J) to the line leading to the gas cylinder. The stopcock is again closed and by means of the syringe (I), a positive pressure is applied to the top of the solution in the flat cell and the enzyme is driven back into the body of the titrator. The syringe attached to the flat cell is then emptied of gas by opening the stopcock
(J) to the atmosphere. After flushing in this manner, the enzyme solution is again drawn into the flat cell and is allowed to fill the upper tube of the flat cell to about 1/3 of its height. The EPR spectrum of the sample is then recorded and the sample subsequently returned to the titrator vessel by applying pressure through the syringe (I) as described before. This cycle is repeated for every potential at which a reading is made. The changes observed during each step of the titration are readily reversed by addition of oxygen to the reaction mixture. If the system's overall potential is not changed between two successive EPR measurements even when the sample is cycled as described above, the spectra obtained are essentially identical. Mo(V) and FADH EPR spectra were recorded simultaneously using 5 gauss field modulation, and 5 mW microwave power. The gain for each run was adjusted to obtain the largest possible signal amplitude. The spectra were recorded using a Varian E6 EPR spectrometer interfaced to an Interdata 7-16 computer and stored on disc. Reductive and oxidative titrations were performed with identical results.

The EPR spectra collected in this manner present one set of complications. First, some of the potentiometric mediator dyes used in the experiment produce detectable free radical intermediates. Fortunately, in the range of potentials being investigated these signals are not unmanageably large, and the apparent linewidth of the dye signal (5 gauss) is substantially different from that of the flavin radical signal, which ranges from 19 gauss at low pH to 15 gauss at the highest pH value studied. By taking advantage of this difference, the contribution of the dyes can be subtracted and this problem minimized. Second, the presence of active and inactive molybdenum simultaneously, gives rise
to a spectrum which is a mixture of the two signals (35). These contributions to the spectrum can be separated by computer subtraction of independently obtained difference spectra. However, because the EPR linewidth of the species is somewhat broader at room temperature, this separation is not as clean as with data obtained at liquid nitrogen temperatures. Thus, the signals of interest were isolated by subtraction of standard spectra of appropriate size of all the other contributing signals and the resulting spectra were then quantitated by integration, using nitrosyl disulfonate as standard (83).

Both the FAD and the molybdenum center in xanthine oxidase are capable of undergoing one-electron reduction to the semiquinone forms. The complete reduction of each of these systems can therefore be regarded as a process with two one-electron reduction steps:

\[
\begin{align*}
\text{FAD} & \xrightarrow{e^-} \text{FADH} \xrightarrow{e^-} \text{FADH}_2 \\
\text{Mo(VI)} & \xrightarrow{e^-} \text{Mo(V)} \xrightarrow{e^-} \text{Mo(IV)}
\end{align*}
\]

the equation describing the behavior of this type of system was fitted to the appearance and disappearance of the flavin semiquinone and of the Mo(V) to obtain the corresponding midpoint potentials at the different pH values.

Optical Stoichiometric Titrations:

Optical stoichiometric titrations were carried out in an all glass anaerobic vessel provided with an optical cuvette with a 1 cm path-length for absorbance measurements, and a sidearm to which a Hamilton syringe containing a standard dithionite solution could be attached.
After each addition of reductant, the absorbance change was monitored until no further changes took place. The optical spectrum was then recorded using a Cary 17 spectrophotometer.
CHAPTER III

The Reaction of Reduced Xanthine Oxidase with Oxygen:
Kinetics of Peroxide and Superoxide Formation.

The reaction of reduced xanthine oxidase with oxygen exhibits a markedly biphasic time course, with the rate of the fast phase being approximately 10 times that of the slow phase \( (45, 46) \). The relative amplitudes of these two phases are dependent on the wavelength of observation. The absorbance changes observed in the slow phase are very similar to the difference spectrum associated with the oxidation of the iron-sulfur centers, whereas the changes associated with the fast phase resemble a combination of flavin and iron-sulfur centers difference spectra.

To explain these observations, Olson et al. \( (46) \) proposed a mechanism in which the reoxidation occurs by way of a combination of 1- and 2-electron transfer steps to give both hydrogen peroxide and superoxide as products:

\[
\begin{align*}
XO(6) & \xrightarrow[k_1]{} XO(4) & \xrightarrow[k_1]{} XO(2) & \xrightarrow[k_1]{} XO(1) & \xrightarrow[k_2]{} XO(0) \\
XO(5) & \xrightarrow[k_1]{} XO(3) & \xrightarrow[k_1]{} & & \\
H_2O_2 & & & & \text{H}_2O_2
\end{align*}
\]

Scheme 1

This mechanism predicts that complete oxidation of 6-electron reduced enzyme should produce 2 equivalents of hydrogen peroxide and 1 of
superoxide during the fast phase and 1 equivalent of superoxide in the slow phase. Olson et al. (45, 46) proposed this sequence and stoichiometry to account for the 5:1 ratio of absorbances at 450 nm of the fast and slow phases. However, attempts to quantitate the production of superoxide by rapid freezing techniques yielded values no greater than 0.1 eq./FAD for the complete oxidation of fully reduced enzyme. In addition to being inconsistent with the proposed mechanism, this result also contradicts other work which has suggested that $O_2^-$ is the initial product of the oxidative step and that $H_2O_2$ is only produced by dismutation (79). In order to resolve this problem, I have measured directly the production of $H_2O_2$ and $O_2^-$ under a wide variety of conditions. Cytochrome c peroxidase was used as a trapping agent for $H_2O_2$ and the reduction of cytochrome c was used to monitor the formation of superoxide.

Peroxide Formation:

Yonetani (84) and Yonetani and Ray (85) have reported that cytochrome c peroxidase reacts quantitatively and rapidly with hydrogen peroxide, producing large absorbance changes in the Soret region. The difference spectrum between the peroxidase and its hydrogen peroxide adduct has a maximum at 424 nm and an isosbestic point at 454 nm. This latter wavelength coincides closely with that of the maximum absorbance of xanthine oxidase. Thus, it appeared that binding to cytochrome c peroxidase could be used to monitor the formation of $H_2O_2$ during the oxidation of reduced xanthine oxidase.

Loo and Erman (86) have measured the binding of hydrogen peroxide to cytochrome c peroxidase and found a bimolecular constant for this
process of $4.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. They assumed the binding to be irreversible and reported that the enzyme was unstable above pH 8. I have repeated their work and have obtained results which are quite similar. Data were collected at 424 nm under second order conditions and subsequently fit to a reversible second order reaction (see Fig. 3.1). The association rate was independent of pH and equal to $1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. This value is somewhat smaller than that reported by Loo and Erman (86) but still sufficiently fast for the reaction to serve as a probe for the production of hydrogen peroxide by xanthine oxidase.

No reaction could be detected when cytochrome $c$ peroxidase was mixed with reduced xanthine oxidase anaerobically. More importantly, the kinetics of reoxidation of 2.5 μM reduced xanthine oxidase observed at 454 nm were unaffected by addition of 8.5 μM cytochrome $c$ peroxidase to the oxygen solution (Fig. 3.2). Thus, the peroxidase in the Fe(III) oxidation state does not accept electrons directly from xanthine oxidase nor does it perturb the normal reoxidation reaction.

The production of $H_2O_2$ during the reaction of xanthine oxidase with $O_2$ was followed as the difference in the absorbance changes at 424 nm between reaction mixtures with and without added peroxidase. This difference in the absorbance changes at 424 nm was calculated from the results of two successive experiments, the kinetic traces from each having been manipulated and preserved by means of the on-line data system. As shown in Fig. 3.3a, the time course for the appearance of the $H_2O_2$-peroxidase complex at pH 8.3 exhibits a single phase which roughly parallels the fast phase observed for the reoxidation of xanthine oxidase monitored at 454 nm (see Fig. 3.5). Addition of superoxide dismutase had no effect on the data (see Fig. 3.3b).
FIGURE 3.1

Time Course for the Reaction of Cytochrome c Peroxidase with Hydrogen Peroxide. The experiment was performed in 0.1 M Bicine, pH 8.3 at 25 °C. The concentrations after mixing were 0.5 μM cytochrome c peroxidase and 2.5 μM hydrogen peroxide. The absorbance change was monitored at 424 nm. The symbols represent the experimental data and the line represents a second order simulation of the binding reaction performed as described in the text.
FIGURE 3.2

Time Course of Xanthine Oxidase Reoxidation in the Presence and Absence of Cytochrome c Peroxidase. The experiments were performed in 0.1 M Bicine, pH 8.3, at 25 °C. The concentrations after mixing were 2.5 μM xanthine oxidase, 8.5 μM cytochrome c peroxidase (CCP), 125 μM oxygen. The absorbance changes were monitored at 454 nm where cytochrome c peroxidase has an isosbestic point.
Scheme 1 predicts the formation of 2 molecules of $\text{H}_2\text{O}_2$/flavin in the fast phase and 1 molecule of $\text{O}_2$/flavin in both the fast and slow phases of reoxidation. Since each superoxide is expected to dismute rapidly to produce hydrogen peroxide and oxygen, the absorbance change expected in this experiment should be consistent with the formation of 2.5 equivalents of the peroxidase-$\text{H}_2\text{O}_2$ complex during the fast phase and an additional 0.5 equivalents during the slow phase. However, only a single fast phase is observed experimentally. This discrepancy appears to be due to a reaction of the heme-$\text{H}_2\text{O}_2$ compound with the reduced xanthine oxidase.

In contrast to the experiment with untreated cytochrome c peroxidase, a slow ($t_\frac{1}{2} \approx 10 \text{ s}^{-1}$) decrease in absorbance at 424 nm is observed when 30 μM peroxidase-$\text{H}_2\text{O}_2$ complex is mixed anaerobically with 5 μM reduced xanthine oxidase (Fig. 3.3c). I interpret this absorbance change to represent direct electron transfer from the flavoprotein to the hemoprotein, since a decrease in absorbance accompanies the reduction of the cytochrome c peroxidase-$\text{H}_2\text{O}_2$ complex with dithionite. In addition, an absorbance increase at 454 nm occurs, which indicates that anaerobic reoxidation of xanthine oxidase is taking place. Because this competing reaction is slow, it only affects the peroxidase absorbance changes toward the end of the reaction of $\text{O}_2$ with xanthine oxidase, when the concentration of the heme-peroxide complex is relatively high. Nevertheless, it does complicate the interpretation of the results by eliminating the slow phase of $\text{H}_2\text{O}_2$ production and by reducing the amount of $\text{H}_2\text{O}_2$ detected during the fast phase. The amount of $\text{H}_2\text{O}_2$ observed was calculated from the total absorbance change at 424 nm, using a value of 48,000 M$^{-1}$cm$^{-1}$ for the extinction coefficient change associated with
FIGURE 3.3

Time Courses for the Interactions of Cytochrome c Peroxidase with Xanthine Oxidase. A, time course for hydrogen peroxide production during the reoxidation of xanthine oxidase. The reaction was carried out in 0.1 M Bicine, pH 8.3, at 25 °C. The following concentrations after mixing were used: 5 μM xanthine oxidase, 37.5 μM cytochrome c peroxidase, 125 μM oxygen. The squares represent the difference between traces taken at 424 nm in the presence and absence of peroxidase. This difference represents the absorbance change due to the formation of the peroxidase-H₂O₂ complex. The continuous line is the expected production of H₂O₂ as predicted by Scheme 1. The discrepancy between the two traces is explained in the text. Quantitation of the absorbance change of cytochrome c peroxidase indicated that 2.3 molecules of H₂O₂ were detected during the oxidation of fully reduced xanthine oxidase. B, Observed absorbance change for the production of hydrogen peroxide in the presence (solid line) and absence of superoxide dismutase. C, Time course for the interaction of reduced xanthine oxidase with cytochrome c peroxidase-H₂O₂ complex. The reaction was performed in the absence of oxygen in 0.1 M Bicine, pH 8.3, at 25 °C. Concentrations after mixing were 10 μM xanthine oxidase, 15 μM cytochrome c peroxidase. The peroxidase-H₂O₂ complex was prepared by titrating anaerobic cytochrome c peroxidase with a stock solution containing approximately 5 nM hydrogen peroxide. The time course was followed at 424 nm. The small and rapid increase in absorbance observed initially is due to contamination of the stock peroxide solution with oxygen, which oxidizes a small fraction of the xanthine oxidase. The large decrease in absorbance is due to a breakdown of the cytochrome c peroxidase-H₂O₂ complex which occurs much more slowly.
the CCP-H₂O₂ reaction. This quantitation yielded values of 2.3 mol of 
H₂O₂/flavin at pH 8.3 and 2.0 mol of H₂O₂/flavin at pH 6.15. These num-
bbers agree reasonably well with the values of 2.5 mol predicted for the 
fast phase of reoxidation of totally reduced xanthine oxidase (Scheme 1).

Superoxide Formation:

The reduction of cytochrome c by O₂⁻ has often been used as a method of detection of superoxide in solution (87, 88). This reaction proceeds quite rapidly with a bimolecular rate constant of 10⁶ M⁻¹ s⁻¹ at pH 7.2 (88). At this pH, the cytochrome c reaction competes favorably with superoxide dismutation. A value of 2.1 x 10⁵ M⁻¹ s⁻¹ for the dismutation reaction at pH 7.2, has been measured by Rabani and Nielsen using flash photolysis (89).

Cytochrome c reduction was followed at 600 nm to optimize the sig-
nal-to-noise ratio, since high concentrations of protein were required (15 μM xanthine oxidase, 0.5 mM cytochrome c). This wavelength afforded the largest extinction change for cytochrome c reduction while still maintaining a total absorbance of less than 2.0. I routinely employed 0.5 mM cytochrome c as the superoxide scavenging system at all the pH values studied. In each case, controls using 0.125 and 0.25 mM cyto-
chrome c were performed, and the experimental traces were always found to be independent of cytochrome c concentration.

The time courses observed for the production of reduced cytochrome 
c during the reaction of xanthine oxidase with O₂ are complex (Fig. 3.4a) 
There are several reasons for this situation. First, the oxidation of 
xanthine oxidase and the reduction of cytochrome c proceed with differ-
FIGURE 3.4

Time Courses for the Reaction of Reduced Xanthine Oxidase with Oxygen
Measured in the Presence of Cytochrome c$^{3+}$ at 600 nm. The reactions
were carried out in 0.1 M Bicine, pH 8.3, at 25°C. The following con-
centrations after mixing were used: 15 μM xanthine oxidase, 10 μM super-
oxide dismutase, 125 μM oxygen, 0.5 mM cytochrome c$^{3+}$. The first 100
points were collected from 0.2-2.2 s. A, observed absorbance changes in
the absence (top) and presence (bottom) of superoxide dismutase. B, dif-
fERENCE between the traces taken in the presence and absence of super-
oxide dismutase which represents cytochrome c reduction by superoxide
only. C, time course for the direct electron transfer from reduced
xanthine oxidase to cytochrome c$^{3+}$. The reaction was carried out ana-
erobically in 0.1 M Bicine, pH 8.3, at 25°C. The concentrations after
mixing were 10 μM xanthine oxidase, 0.5 mM cytochrome c. The reaction
was followed at 600 nm.
ent time dependencies and yield absorbance changes of opposite signs at 600 nm. Second, there is a direct electron transfer from reduced xanthine oxidase to cytochrome $c$. As shown in Fig. 3.4c, an absorbance decrease occurs at 600 nm when cytochrome $c$ is mixed with reduced xanthine oxidase anaerobically. Fortunately this reaction is quite slow compared to the reoxidation of the enzyme (see Figs. 3.4c and 3.5). Third, hydrogen peroxide appears to destroy cytochrome $c$. Incubation of ferricytochrome $c$ with levels of $H_2O_2$ comparable to those produced in the overall reoxidation of xanthine oxidase causes bleaching of the heme $c$ absorbance at a rate of $10^3 \text{ s}^{-1}$. Addition of catalase to the reaction mixture eliminates some of the absorbance decrease at 600 nm. (see Fig. 3.6). Since oxidation of $c^{+2}$ leads to an absorbance increase at this wavelength, the hydrogen peroxide reaction does not represent simple reoxidation of newly formed reduced cytochrome. Even though the addition of catalase modifies slightly the time courses obtained in the presence and absence of superoxide dismutase, it has little effect on the difference between these traces. This indicates that the reaction of $H_2O_2$ with cytochrome $c$ is essentially the same in both cases.

These complications can be overcome by performing the reaction both in the presence and in the absence of superoxide dismutase. Since all the interfering processes should remain unaffected, the differences in the kinetic data between the two experiments yield absorbance changes which are due exclusively to the interaction of cytochrome $c^{+3}$ with superoxide (Fig. 3.4b). These data are independent of cytochrome $c$ concentration in the range 0.125-0.5 mM and of superoxide dismutase concentration in the range 1-10 uM. As shown in Fig. 3.4b, the time course for superoxide formation at pH 8.3 consists of two distinct phases of
equal amplitude. This result agrees with the idea that 1 molecule of $O_2^-$ is produced in the rapid phase of reoxidation and one is produced in the slow phase.

Effects of the Degree of Reduction of Xanthine Oxidase on $O_2^-$ Production:

Scheme 1 predicts that superoxide is generated only when the last 2 electrons are removed from xanthine oxidase. Thus, the amount of $O_2^-$ produced during reoxidation should be independent of the level of reduction of the enzyme once 2 electrons have been added. This idea was tested directly by mixing partially reduced xanthine oxidase with oxygen solutions containing cytochrome c. As shown in Fig. 3.5, the amount of superoxide generated increases by only 20% in going from 2- to 6-electron reduced enzyme. In agreement with Scheme 1, there is also a marked lag in the time courses of cytochrome c reduction when oxygen is reacted with enzyme containing 4.5 and 6 electrons/flavin (Fig. 3.5b, upper curves).

The small decrease in superoxide production which is observed for 2-electron-reduced xanthine oxidase (Fig. 3.5b, lower curve) is a result of the distribution of enzyme species which are present in this partially reduced sample. Olson et al. (46) have shown that small but significant amounts of XO(3) and XO(1) will be present at equilibrium when 2 electrons/flavin are added to xanthine oxidase. As shown in Scheme 1, oxidation of XO(3) and XO(1) produces only 1 molecule of $O_2^-$ whereas the oxidation of XO(2) produces 2. Thus, enzyme which is reacted with only 1 equivalent of dithionite is both expected and observed to exhibit less $O_2^-$ production than when it is fully reduced.
FIGURE 3.5

Oxidation of Partially Reduced Xanthine Oxidase. All reactions were carried out in 0.1 M Bicine, pH 8.3, at 25 °C. The concentrations after mixing were: 15 μM xanthine oxidase, 0.5 mM cytochrome $c^+\text{3}$, 125 μM oxygen. The levels of reduction of the xanthine oxidase solutions before mixing were 2 electrons/flavin (♦), 4.5 electrons/flavin (▲), and 6 electrons/flavin (■). A, absorbance change for xanthine oxidase oxidation measured at 454 nm. B, absorbance change for cytochrome $c^+\text{3}$ reduction measured at 600 nm. The data in B were obtained as described in Fig. 3.4b and represent the difference between absorbance changes measured in the absence and in the presence of superoxide dismutase (5 μM after mixing). The data were collected at two time scales, 0-0.2 and 0.2-2.2 s. The symbols represent experimental data which are expressed as a percentage of the total absorbance change observed with fully reduced xanthine oxidase. The lines are the time courses predicted by Scheme 1 and Equation 3.2.
FIGURE 3.6

Time Course of Cytochrome c Reduction in the Presence and Absence of Catalase. The experiments were performed in 0.1 M Bicine, pH 8.3, at 25 °C. The concentrations after mixing were 10 μM xanthine oxidase, 0.5 mM cytochrome c, 5 μM catalase, and 125 μM oxygen. The absorbance change was monitored at 600 nm.
Dependence of Superoxide Production on Oxygen Concentration:

A second test was designed to verify the reaction mechanism shown in Scheme 1. Xanthine oxidase was reacted with varying amounts of oxygen. When fully reduced enzyme is mixed with only 1 equivalent of $O_2$ (Fig. 3.7), little or no superoxide is produced as evidenced by the lack of absorbance change at 600 nm. This observation is consistent with Scheme 1 since oxidation of XO(6) to XO(4) is postulated to yield $H_2O_2$ exclusively. As a control, the enzyme solution was reacted with a 10-fold excess of oxygen and the typical result was obtained. In contrast, when the enzyme sample was reacted with 2 equivalents of $O_2$, the absorbance stayed essentially constant for 100 ms and then increased slowly (Fig. 3.7, lower curve).

Although unexpected, this result can also be explained by Scheme 1. The reaction of 6-electron-reduced xanthine oxidase with 2 equivalents of $O_2$ is not synchronous for all molecules. Small fractions of enzyme are oxidized to the level of XO(1) and XO(0) leading to the formation of small amounts of $O_2^-$. This superoxide is scavenged by the cytochrome $c^3$ regenerating $O_2$. As a result, the amount of oxidizing equivalents available under these conditions exceeds the original oxygen concentration. In the presence of excess superoxide dismutase, this catalytic reduction of cytochrome $c$ by $O_2^-$ is eliminated and the total oxidizing capacity is fixed by the amount of oxygen present. Thus, in the absence of dismutase, xanthine oxidase is oxidized to a greater extent than in its presence. During oxidation, the absorbance of xanthine oxidase at 600 nm increases, while reduction of cytochrome $c$ is accompanied by a decrease in absorbance. Since the absorbance change of xanthine oxidase at 600 nm
FIGURE 3.7

Oxidation of Fully Reduced Xanthine Oxidase by Various Levels of O₂.
The data at 454 and 600 nm were obtained as described in Figs. 3.3b and 3.5. The experiments were carried out in 0.1 M Bicine, pH 8.3, at 25 °C. The protein concentrations after mixing were 15 μM xanthine oxidase, 0.5 mM cytochrome c₃. The top line represents the trace taken with a 9-fold excess of oxygen (125 μM after mixing). The intermediate trace corresponds to the absorbance change when roughly 1 eq. of oxygen/flavin was used (12.5 μM after mixing). The bottom line represents the trace obtained when approximately 2 eq. of oxygen/flavin were used (25 μM after mixing).
per electron is 50% larger than that of cytochrome c a net increase in absorbance is observed when the time courses in the presence and absence of superoxide dismutase are subtracted (Fig. 3.7, lower curve).

Effect of pH on Superoxide Production:

Samples of fully reduced xanthine oxidase at pH values ranging from 6.15-9.9 were reacted with various concentrations of oxygen (63, 125, 312, 375, and 625 μM). Typical kinetic traces for reoxidation of the enzyme and for superoxide production are shown in Fig. 3.8. The cytochrome c absorbance changes associated with the $O_2$ formation were quantitated, and the results are presented in Table 3.1.

DISCUSSION

Quantitative Analysis:

Scheme 1 has been investigated numerically on the simplifying assumption that the rate constants for all the reoxidation steps except the last one are identical. Each oxidative stage is assumed to be a two step process composed of a rapid binding step followed by electron transfer:

$$XO(n) + O_2 \xleftrightarrow{k_f} XO(n) \cdot O_2 \xrightarrow{k_e} XO(n-2) + O_2$$

Scheme 2
FIGURE 3.8

pH Dependence of Enzyme Oxidation and Superoxide Production. The reactions were carried out at 25 °C. Using the following concentrations after mixing: 15 µM xanthine oxidase, 0.5 mM cytochrome c, 125 µM oxygen. The symbols represent the absorbance changes of xanthine oxidase at 454 nm (■) and those of cytochrome c at 600 nm (▲) obtained as described in Fig. 3.3. The data are expressed as a percentage of the total absorbance change observed for each trace. The line represents theoretical curves computed using Equation 3.2. The buffer systems used are given in Chapter 2.
pH 6.15

% A bsorbance

TIME, msec.

0 20 40 60 80 100

0 500 1500 2000 2500
The concentration of the oxygenated intermediate is assumed to be small and to remain relatively constant throughout the reaction. Under these conditions, the observed rate is given by

\[
k_1 = \frac{k_e [O_2]}{[O_2]^+ \frac{k_f}{k_r}}
\]  

(3.1)

Since the data to be fitted were obtained under pseudo-first order conditions, the oxygen concentration remains essentially constant throughout the reaction and the observed rate \( k_1 \), for the oxidative step is given by Equation 3.1. The last step, \( X_0(1) \rightarrow X_0(0) \), is defined as a simple second order process with the pseudo-first order rate, \( k_2 \), equal to \( k_e [O_2] \) (45). The time courses for the disappearance of the reacting species, using all the assumptions above, are given by

\[
X_0(6) = X_0(6) I e^{-k_1 t} \quad X_0(5) = X_0(5) I e^{-k_1 t}
\]

\[
X_0(4) = X_0(6) I k_1 t e^{-k_1 t} \quad X_0(3) = X_0(5) I k_1 t e^{-k_1 t}
\]

\[
X_0(2) = X_0(6) I \frac{k_2^2 t^2}{2} e^{-k_1 t}
\]

\[
X_0(1) = X_0(6) I k_1^2 \left( \frac{t^2}{2(k_2 - k_1)} - \frac{t}{(k_2 - k_1)^2} + \frac{1}{(k_2 - k_1)^3} \right) e^{-k_1 t} - \frac{e^{-k_2 t}}{(k_2 - k_1)^3} + X_0(5) I k_1^2 \left( \frac{t}{(k_2 - k_1)} - \frac{1}{(k_2 - k_1)^2} \right) e^{-k_1 t} - \frac{e^{-k_2 t}}{(k_2 - k_1)^3}
\]  

(3.2)

where \( X_0(n) \) represents the concentration of \( n \)-electron-reduced enzyme.

It should be noted that when fully reduced enzyme is used, \( X_0(5) \) = 0, no \( X_0(3) \) is produced, and the expression for \( X_0(1) \) reduces to the first group of terms. More complicated expressions are required when partially reduced enzyme is reacted with oxygen since in that case the initial concentrations of all the reduction states must be considered. However,
the form of these equations is similar to those of the equations given above. Absorbance changes at 454 nm were computed by combining the time courses of the various intermediates using the spectral weights reported by Olson et al. (46). The production of $H_2O_2$ is associated with the disappearance of XO(6), XO(5), XO(4), and XO(3), and the production of $O_2^-$ is associated with the disappearance of XO(2) and XO(1).

Equation 3.2 was fitted to the observed time courses for the absorbance changes at 454 nm using standard nonlinear least squares methods; only $k_1$ and $k_2$ were allowed to vary. The fitted values of $k_1$ and $k_2$ were then used to predict the time courses for formation of $H_2O_2$ and $O_2^-$. Typical results of this analysis are shown in Figs. 3.5 and 3.6. It should be noted that, at each pH, 5 different oxygen concentrations were examined (63, 125, 312, 375, and 625 μM) and Equation 3.2 was fitted to the resulting traces. For the most part, the observed and calculated curves are in very close agreement. The poorest fits were obtained for the time courses of cytochrome c reduction at pH 6.15 and 7.2. This is primarily a result of the small absorbance changes observed at these pH values and of the close similarity between the fast and slow rates.

The dependence of the pseudo-first order rates, $k_1$ and $k_2$, on oxygen concentration and pH is shown in Fig. 3.9. In agreement with earlier work (45), both rates vary with pH. The rate of the fast phase, $k_1$, exhibits a hyperbolic dependence on oxygen concentration which is consistent with Scheme 2. The intrinsic electron transfer rate $k_c$ was obtained from the y-intercept of the double reciprocal plots shown in Fig. 3.9, while the apparent $K_D$ for the enzyme-$O_2$ binary complex, $(k_c + k_e)/k_e$ was obtained from the slope. As shown in Table 3.1, the apparent affinity of reduced flavin for $O_2$ increases 20-fold as the pH
FIGURE 3.9

Dependence of the Apparent Rates of Oxidation on C₂ Concentration and pH.

The reactions were carried out as described in Fig. 3.8. The rates were obtained by fitting the absorbance changes at 454 nm to Equation 3.2.

The pH values used were 6.15 (■), 7.2 (▲), 8.3 (◇), 9.2 (●), and 9.9 (○). A, double reciprocal plot of the rate constants for the fast phase (k₁ in Scheme 1) versus oxygen concentration. The lines represent least squares fits to the data. B, rate constants for the slow phase (k₂ in Scheme 1) versus oxygen concentration.
TABLE 3.1

Rate Constants for the Reoxidation of Xanthine Oxidase. Rate constants for the fast steps of oxidation (Equation 3.1) and the second order rate constants for the slow phase were obtained from an analysis of the data in Fig. 3.9 as described in the text. The quantity of superoxide produced was obtained from the magnitude of the absorbance change at 600 nm due to $O_2^-$ reduction of cytochrome c ($\Delta$e for cytochrome c reduction at 600 nm = $-1000 \text{ mol}^{-1} \text{ cm}^{-1}$).

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_w$ (mM)</th>
<th>$k_c$ ($s^{-1}$)</th>
<th>$O_2^-$/flavin</th>
<th>Slow Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$k_e$ ($s^{-1}$)</td>
</tr>
<tr>
<td>6.15</td>
<td>$7.7 \times 10^{-5}$</td>
<td>13</td>
<td>0.4</td>
<td>5 $\times 10^{-3}$</td>
</tr>
<tr>
<td>7.2</td>
<td>$1.1 \times 10^{-4}$</td>
<td>25</td>
<td>0.3</td>
<td>13 $\times 10^{-3}$</td>
</tr>
<tr>
<td>8.3</td>
<td>$2.2 \times 10^{-4}$</td>
<td>60</td>
<td>0.7</td>
<td>10 $\times 10^{-3}$</td>
</tr>
<tr>
<td>9.2</td>
<td>$1.4 \times 10^{-3}$</td>
<td>350</td>
<td>0.9</td>
<td>6.3 $\times 10^{-3}$</td>
</tr>
<tr>
<td>9.9</td>
<td>$1.4 \times 10^{-3}$</td>
<td>350</td>
<td>0.3</td>
<td>6 $\times 10^{-3}$</td>
</tr>
</tbody>
</table>
is lowered from 9.9 to 6.15, whereas $k_e$ decreases by 25-fold over the same range. Since $pH$ affects both the electron transfer rate and the apparent $k_d$ to about the same extent, the double reciprocal plots are parallel and therefore suggestive of uncompetitive inhibition (i.e., proton binding to the enzyme-$O_2$ complex inhibits the electron transfer and release of $H_2O_2$ step).

The rate of the slow phase, $k_2$, is plotted with respect to oxygen concentrations (in Fig. 3.9b). A large amount of scatter is observed at pH 6.15 and 7.2. This is due to the difficulty of determining the rate with precision since the slow phase at these pH values is only about 5% of the total absorption change (cf. Table 3.1). In general, however, the rate of the slow phase appears to be directly proportional to the $O_2$ concentration, and, with the possible exception of the lowest pH, there is no evidence for rate saturation at the highest oxygen concentrations. This agrees with the earlier results of Olson et al. (45). Clearly, the complex between $O_2$ and 1-electron-reduced enzyme must be rather weak, if it exists at all.

Hydrogen Peroxide and Superoxide Production:

The release of $H_2O_2$ is confined to the fastest phase of reoxidation (Fig. 3.3) while superoxide production (Figs. 3.5, 3.7 and 3.9) occurs only toward the end of the fastest phase and during the subsequent slow phase. This behavior is precisely that postulated by Olson et al. (46) as outlined in Scheme 1. The production of $H_2O_2$ occurs exclusively during the steps $X(6) \rightarrow X(4)$ and $X(4) \rightarrow X(2)$; superoxide production is confined to the steps $X(2) \rightarrow X(1)$ and $X(1) \rightarrow X(0)$. Thus 2 equivalents
of peroxide and 2 equivalents of superoxide are the anticipated yield from the reoxidation of fully reduced enzyme. The superoxide should subsequently dismute to yield 1 equivalent of $\text{H}_2\text{O}_2$, half of which should be formed rapidly, with the remainder formed more slowly.

My data are not completely consistent with this expectation. Typically, I find 2.0-2.3 mol of $\text{H}_2\text{O}_2$ formed rapidly at the different pH values examined with a time course essentially identical with that for the fast phase of reoxidation of the enzyme (Fig. 3.3). No production of peroxide was observed during the slow phase of reoxidation. This discrepancy is attributed to an unanticipated reaction of the cytochrome $\epsilon$ peroxidase-$\text{H}_2\text{O}_2$ adduct with reduced xanthine oxidase. Fortunately, this process is only significant during the closing stages of reoxidation when substantial quantities of the peroxidase-$\text{H}_2\text{O}_2$ complex have accumulated. In view of this complication, the close to theoretical yields of $\text{H}_2\text{O}_2$ observed during the fast phase are rather satisfactory.

Quantitation of superoxide release is also complicated by the direct reaction of cytochrome $\epsilon^{+3}$ with reduced xanthine oxidase. The use of superoxide dismutase to resolve the direct and the superoxide-elicted reduction processes revealed that $\text{O}_2^-$ was only released late in reoxidation. The yields of superoxide varied significantly, from 0.6 mol at pH 7.2 to 1.3 mol at pH 9.2. This observation, together with the fact that the value of $k_e$ decreases with pH, would suggest that, at lower pH, increased stability of the enzyme-oxygen adduct permits the existence of a competing pathway, $\text{D}(2) \rightarrow \text{D}(0)$, with the release of hydrogen peroxide. At all pH values studied, identical amounts of superoxide were trapped in the fast and slow phases of reoxidation (Table 3.1). The postulated pathway of reoxidation is also supported by the observations...
that levels of $O_2$ adequate to remove only 2 electrons from fully reduced xanthine oxidase did not yield any superoxide (Fig. 3.7) and that the yield of superoxide did not increase significantly in going from 2- to 6-electron reduced enzyme (Fig. 3.5).

Hille and Hassey (90), in a paper published simultaneously with this work, have reported also using cytochrome c to study the production of $O_2^-$ during the reoxidation of 2-, 4-, and 6-electron reduced enzyme at pH 8.5. They have reported results consistent with those reported here. They found that approximately 2 moles of $O_2^-$ per mole of flavin are produced during complete reoxidation of enzyme at any of these three reduction levels. These authors see no diminution of the amount of $O_2^-$ produced as the overall reduction level of the enzyme is changed from 6 to 4 to 2 electrons, because they use substoichiometric amounts of xanthine to reduce the enzyme. Since xanthine is a 2-electron donor, essentially none of the 3- or 1-electron reduced enzyme will be produced and the full amount of expected superoxide will be observed (cf. Fig. 3.5). In general, though, the results of Hille and Hassey are in good agreement with mine and with Scheme 1.

As previously discussed by Olson et al. (46), Scheme 1 explains observation concerning the reduction of cytochrome c during catalysis. Fridovich (79) has reported that the superoxide-mediated reduction of cytochrome c increases whenever the concentration of xanthine is lowered or the concentration of oxygen is raised. He also reported a far higher level of superoxide production at pH 10.0 than at pH 7.0. Then the concentration of oxygen is high and that of xanthine is low, the enzyme cycles mainly between 2-electron-reduced and oxidized states (46). As indicated in Scheme 1, oxidation of XC(1) and Xf(2) occurs
largely by 1-electron transfer steps so that O$_2^-$ is the principal product in agreement with the results of Fridovich (79). On the other hand, when the concentration of O$_2$ is low and that of xanthine is high, the enzyme is largely reduced and little superoxide is produced in the oxidative steps. The dependence of superoxide production on pH is also explainable in terms of the results presented here. First, there is less production of O$_2^-$ at pH 7 during the oxidation of reduced enzyme than at pH 10 (Table 3.1). Second, the overall oxidation rate increases with increasing pH so that even at high concentrations of xanthine the fraction of the enzyme which is in the 1- or 2-electron-reduced state increases with pH at a fixed oxygen concentration. As a result, at a high pH more O$_2^-$ is expected to be produced at pH 10 than at pH 7 in agreement with the results of Fridovich (79).

The rates of both phases of oxidation vary with pH. Double reciprocal plots of the rates of the fast phase suggest that the pH effect arises from uncompetitive inhibition by protons. I have observed that the flavin radicals produced by partial reduction of xanthine oxidase exhibit 5.3 linewidths typical of the neutral radical at low pH (91) and of the anion radical at pH 9.9. Neutral and anionic radicals have been correlated with dehydrogenase and oxidase activities (91). Thus, the inhibition of the electron transfer and peroxide release step in Scheme 2 is probably asserted through changes in the state of protonation at the flavin redox system. This suggestion is consistent with the proposal by Hassey and Herrlich (92) that oxidase activity is conferred upon flavin by protein stabilization of the $\overset{1}{F}a$-4$\overset{2}{a}$ anionic locus which is believed to favor formation of the $\overset{1}{F}a$ peroxide adduct (the $\overset{1}{F}a$ position is sometimes referred to as C$^{10a}$ in the flavin literature). Sub-
sequent breakdown of this adduct to H₂O₂ and oxidized flavin is presumed to be promoted when the negative charge is localized at the N¹ position. Lowering the pH will lead to protonation of N¹ with the attendant reduction in the rate of electron transfer to the oxygen atoms and the subsequent release of peroxide. Alternatively, reaction of reduced free flavin yields the C⁴a peroxide adduct (93, 94). If this adduct is formed during the reoxidation of xanthine oxidase too, then breakdown of the product would be promoted by deprotonation of the N⁵ position, with results undistinguishable from those of the previous case. A third possibility is that the original reaction produces the C⁴a adduct, which then rearranges to form the C¹a adduct (95). Whichever the actual reaction pathway, however, protonation of either N¹ or N⁵ will lead to the observed inhibition of product release at low pH.
CHAPTER IV

The Room Temperature Potentiometry of Xanthine Oxidase:

pH-dependent Redox Behavior of the Flavin, Polybienc

and Iron-Sulfur Centers

A theme central to understanding the reaction mechanism of enzymes containing multiple redox centers is the elucidation of the fate of the electrons within the protein itself as they travel from the reducing to the oxidizing substrate. This involves, in part, the characterization of the potentiometric behavior of the redox centers, which by establishing the electron affinities of the different groups provides information about the fate of the electrons within the enzyme. Such studies may also yield clues about structure and mechanistic details not related to internal electron transport.

Xanthine oxidase is amenable to this type of approach. Several workers have measured the potentials of the different groups relative to each other (46), and relative to the Standard Hydrogen Electrode under a variety of conditions (76, 77). However, all of these determinations have been made by poising the system at a given overall reduction state at room temperature, and then determining the electron distribution among the centers by taking EPR spectra at 25-120 °C. There are, however, potentially severe complications attendant to this technique. These problems stem from the well-known dependence of equilibrium constants on temperature. First, as recently discussed by Hainer and Olson (76), the midpoint potentials of redox centers are inherently temperature dependent, and a large change in temperature is therefore expected to lead to substantial changes in the electron affinities of the various compo-
ments of the system. Second, the ionization constants of protonated groups are also temperature dependent. Williams-Smith et al. have measured, with the use of indicator dyes, the pH changes of a variety of buffer solutions upon freezing and have reported considerable changes for some of them (e.g. phosphate, pyrophosphate, Tris) (96). Changes in pH with temperature can also occur, of course, for the ionizable protons present in the redox centers and undermine the reliability of the conclusions obtained from analysis of the dependence of the potentials on pH.

All these complications can produce misleading results, since the electron distributions measured at low temperature are not necessarily the same as those present at room temperature, where the system's potential was measured and where other mechanistic data are usually recorded.

In order to determine the electron affinities of the redox centers of xanthine oxidase without the above complications, I have undertaken an investigation of the potentiometric behavior of this enzyme at room temperature at a variety of pH values ranging from 6.15-9.9. For this purpose I used circular dichroism to follow the changes in the Fe/S clusters, and room temperature EPR to follow the flavin and molybdenum centers.

Results and Discussion

Fe/S Centers:

Xanthine oxidase has a rather intense CD spectrum which strongly
FIGURE 4.1

Potentiometric Titrations of the Fe/S Centers in Xanthine Oxidase. The symbols represent the fractional change in the intensity of the CD signal observed as a function of measured electrode potential. The lines represent best fits of the theoretical signal change expected for the system, calculated as described in the text. A, pH 6.15; B, pH 7.2; C, pH 7.7; D, pH 8.3; E, pH 9.2; F, pH 9.9. The buffer systems used are given in Chapter 2.
resembles that of spinach ferredoxin and other common two-iron two-sulfur proteins (81). Furthermore, the intensity of this spectrum is approximately twice that of these latter proteins and is believed to arise from both iron-sulfur centers in the enzyme (48), with the flavin and molybdenum centers making little or no contribution (34). These circumstances make circular dichroism spectroscopy an excellent tool for investigating the redox behavior of these centers.

A series of potentiometric titrations was performed at different pH values in the range 6.15-9.9. These experiments were carried out using an experimental mixture composed of the enzyme and a variety of mediator dyes to insure equilibration between the protein and the potentiometric electrode. The dyes show no significant dichroism in either their oxidized or reduced forms, and thus do not contribute to the observed spectral changes. These compounds, however, possess significant optical absorbance and therefore compete with the enzyme for the circularly polarized light, thereby decreasing the signal-to-noise ratio. Fortunately, the CD of the enzyme is rather large and the problem is not catastrophic.

At all pH values studied the CD spectra change systematically with changes in potential (see for example Fig. 4.1), with the apparent midpoint for the changes decreasing from -210 mV at pH 6.2 to -297 mV at pH 9.9. However, the change in potential required to produce complete reduction of the Fe/S centers is more than 200 mV, and when the CD data are analyzed via semilogarithmic plots, the resulting linear plots exhibit slopes appropriate to a value of ½ for n, the Nernst coefficient. This type of behavior is characteristic of a system with two n=1 centers with midpoint potentials which are different but still close enough
that the individual titration curves are not resolved. The respective
spectral contributions of the individual Fe/S centers in xanthine ox-
dase have not been determined before, but from the observation that the
1 intensity is twice that of spinach ferredoxin (43), one can speculate
that both centers have similar contributions to the 1 spectrum. The
equation for a system composed of two n = 1 centers was fitted to the
data acquired at the different pHs with a computer program in which the
values for the midpoint potentials of the two centers and their relative
spectral contributions were allowed to vary. The best fits at the dif-
ferent pH values were obtained using very similar spectral weights. The
fractional values of these weights fall in the range 0.46 to 0.53 and
show no systematic variation with pH. Plots of the theoretical curves
expected for a system with identical spectral contributions and with the
midpoint potentials which gave the best fits are shown as the solid
lines in Fig. 4.1 as fits to the data obtained at pH values 6.3 and 9.9.
It is evident from these plots that the assumption of similar spectral
weights explains the data well. The midpoint potentials of the two Fe/S
centers obtained at the different pH values are given in Table 4.1. Al-
though my data do not allow me to establish which center titrates first,
I have assigned Fe/S II as the more positive of the two iron-sulfur
centers because Barber and Siegel (77), using low temperature EPR, find
that Fe/S II is the iron-sulfur center with the most positive potential.
This result lends some support to my assignment, particularly since Fe/S I
shows similar characteristics under both sets of conditions (see below).

The variation of the midpoint potential with changes in pH, as
shown in Fig. 4.6a, is strikingly similar for both Fe/S centers. The
values for these potentials are separated by a nearly constant differ-
ence of 90-100 mV (see Table 4.1), and show a moderate dependence on pH, both of them becoming about 100 mV more negative as the pH is increased from 6.15 to 9.9. This type of behavior is compatible with a system in which there is one dissociable proton, the pH, of which is a function of the oxidation state. The pH dependence of the midpoint potential of such a system is given by:

$$E_m = E_o + (R/T/2F)\log\left[\frac{(pK_R - pH)}{(1 + 10^{-pK_R - pH})}\right]$$

where $E_m$ represents the observed midpoint potential, $E_o$ the pH independent midpoint potential, and $pK_o$ and $pK_R$ represent the pH values for the oxidized and the reduced forms of the center respectively.

Equation 4.1 was fitted to the experimentally determined values for the midpoint potentials of both iron-sulfur centers. The best fits were obtained with the following values: for Fe/S I; $E_o = -3.4$ mV, $pK_o = 6.4$, $pK_R = 8.1$; for Fe/S II; $E_o = -249$ mV, $pK_o = 6.4$, $pK_R = 8.0$.

A comparison of the theoretical curves expected for this type of system with my results is shown in Fig. 4.6a.

Barber and Siegal (77), have used the same protonation scheme for analyzing the pH dependence of the data they acquired by low temperature EPR and obtained values of $E_o = -363$ mV, $pK_o = 7.0$, and $pK_R = 8.5$ for Fe/S I and $E_o = -297$ mV, $pK_o = 8.2$, and $pK_R = 9.0$, for Fe/S II. A comparison of these latter values with those obtained by my room temperature measurements shows that the behavior of both centers is affected by the change in temperature, but that Fe/S II is the most affected of the two centers. While there is only a 20 mV difference between the potential measured at room temperature for Fe/S I and that obtained at low
TABLE 4.1

Midpoint Potentials of the Prosthetic Groups of Xanthine Oxidase.
The values for the midpoint potentials at the different pH values were obtained by nonlinear fitting of the Nernst equation that describes each one of the systems as described in the text. Since no EPR data were taken at pH 6.15, the values in parenthesis represent extrapolations of the pH behavior of the flavin and molybdenum groups to that pH value. The numbers were obtained using the equations describing pH dependence of potentials given in the text. All the values are given in millivolts.

<table>
<thead>
<tr>
<th>pH</th>
<th>Fe/S I</th>
<th>Fe/S II</th>
<th>FAD/FADH</th>
<th>FADH/FADH₂</th>
<th>Mo(VI)/Mo(V)</th>
<th>Mo(V)/Mo(IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rapid</td>
<td>Slow</td>
<td>Rapid</td>
<td>Slow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.15</td>
<td>-261</td>
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<td>(-203)</td>
<td>(-168)</td>
<td>(-239)</td>
<td>(-321)</td>
</tr>
<tr>
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<td>7.7</td>
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<td>-217</td>
<td>-301</td>
<td>-237</td>
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<td>-342</td>
</tr>
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<td>8.3</td>
<td>-332</td>
<td>-224</td>
<td>-319</td>
<td>-238</td>
<td>-360</td>
<td>-366</td>
</tr>
</tbody>
</table>
temperature (77), the corresponding difference for Fe/S II is 50 mV.
This difference in sensitivity is also exhibited by the values of $pK_0$ and $pK_a$, with the values associated with Fe/S II again showing the greatest sensitivity. $pK_0$ for Fe/S II shows a difference of 1.8 units between the values obtained at low temperature and room temperature, respectively. Comparison of my results with those of Cammack et al. (76), also shows this difference in behavior. While there is only a small difference between the potentials obtained by these authors for Fe/S I at pH 8.2 and low temperature, and the value reported here (-336 mV vs -332 mV, cf. Table 4.1); there is a significant difference between the corresponding potentials for Fe/S II (-255 mV vs -224 mV, cf. Table 4.1).

The behavior of the data is most simply explained by the presence of a dissociable proton at each of the iron sulfur centers, with the $pK_a$ of this proton increasing from 6.4 to 6.0 during the reduction of the center. This proton may be bound to the Fe/S center itself, or may bind to a nearby group which can interact electrostatically with the cluster. I believe the latter possibility is the likeliest one because we find that the CD spectrum of the enzyme shows no changes with pH variation over the range investigated. This is consistent with observations that EPR spectra recorded at liquid helium temperatures show no changes in lineshape with variations in pH (unpublished results). The type of pH dependence observed in the individual Fe/S centers of xanthine oxidase has been observed previously by Fee et al. (97) who found a slight pH dependence of the potential of parsley ferrodoxin which they attributed to a change in $pK_a$ of the Fe/S center in that protein. Stonbaugh (93) has also reported a similar, small pH dependence for
Fe/S proteins obtained from a variety of microorganisms. He reported the pH dependence of these proteins to fall in the range -2 to -24 mV/pH unit. The values of -22 and -24 mV/pH unit that I observe for Fe/S I and Fe/S II respectively fall comfortably within this range.

In contrast with the results of Barbor and Siegel (77), I observe the protonation behavior of the two centers to be identical. It is only the intrinsic potentials of the groups which distinguish one Fe/S center from another. This situation is consistent with the proposal that the two centers in the enzyme have essentially identical structures. This is further enhanced by my finding that the CD properties of the two centers are remarkably similar. Alternatively, the data are also consistent with the Fe/S centers being sufficiently close that both of them are affected by the protonation and deprotonation of a single ionizable group.

As I noted above, the best fits to the potentiometric data are obtained when identical CD intensities are used for the two centers. This conclusion is supported by a comparison of the difference spectrum obtained during the last third of the titration with that obtained during the first third. The changes obtained in the two cases differ only in detail and not in the overall structure (Fig. 4.2). In marked contrast with this situation, the EPR (48) and optical (46) spectra for these two centers have been reported to be widely different.

A competing explanation of the behavior of the CD potentiometric data is provided by a scheme in which the two Fe/S centers have similar initial potentials, but are linked allosterically so that reduction of either center reduces the potential of the other by 100 mV. In this case, it would be impossible to separate the spectral components because at all times during the titration both centers would contribute in un-
FIGURE 4.2

Circular Dichroism Difference Spectra Associated with the Reduction of the Iron-Sulfur Centers in Xanthine Oxidase. The spectra shown in this figure were obtained by subtracting the spectrum obtained at 31.3% reduction from the spectrum of fully oxidized enzyme (solid line) and by subtracting the spectrum of fully reduced enzyme from that of 64.4% reduced enzyme. The degree of reduction was taken directly from the change in the intensity of the circular dichroism spectrum.
varying proportions to the spectral changes. This situation would yield results similar to mine, but I consider this possibility as unlikely because other workers find the Fe/S centers to behave independently when observed by low temperature EPR (76, 77).

PAD:

The potentiometric behavior of the flavin in xanthine oxidase was followed by E.R at a variety of pH values ranging from 7.2-9.9. No E.R titrations were carried out at pH 6.2 because, at this low value, the enzyme has a pronounced tendency to form bubbles which get trapped in the narrow lumen of the E.R flat cell and prevent quantitation of the observable species. At all the pH values investigated, an observable amount of flavin semiquinone appears and subsequently disappears as the system potential is lowered (Fig. 4.3). Both the maximum amount of semiquinone produced (see Table 4.1), and the potential at which it occurs change with pH. The quantity diminishes, and the potential yielding the maximum signal becomes more negative as the pH is increased. The mid-point potentials for reduction of FAD to FMNH and for reduction of FMNH to FMNH2 at the various pH settings were obtained as described above. The values obtained are given in Table 4.1 and illustrated in Fig. 4.3b.

Over the whole range investigated, the potential associated with reduction of fully oxidized flavin to flavin semiquinone remains higher than that associated with the reduction of the semiquinone form to fully reduced flavin. This result is readily apparent, for the maximum amount of flavin semiquinone never reaches as much as 33% of the total flavin present, as would be the case if the values of the potentials were equal.
FIGURE 4.3

Potentiometric Titrations of the Flavin in Xanthine Oxidase. The symbols represent the integrated EPR signal intensity of the flavin semiquinone. The lines represent best fits of the theoretical signal change expected for the system, calculated as described in the text. A, pH 7.2; B, pH 7.7; C, pH 8.3; D, pH 9.2; E, pH 9.9. The buffer systems used are given in Chapter 2.
It is also apparent that the potentiometric behavior of the flavin shows considerable pH dependence, with each of the flavin couples exhibiting a characteristic dependence on the pH (see Fig. 4.6b). The value of the potential associated with the formation of the flavin semiquinone, $(E_{n1})$, shows a change of approximately -60 mV/pH unit over the range investigated. The value of the midpoint potential associated with the addition of the second electron, $(E_{n2})$, on the other hand, shows a much less pronounced pH dependence. This behavior is consistent with a system in which reduction takes place in two 1-electron steps, each of which is accompanied by the addition of 1 dissociable proton. The pH dependence of the two midpoint potentials associated with such a system is given by:

$$E_{n1} = E_1 + \frac{RT}{nF} \log \left(1 + 10^{-pH - pK_{1}}\right)$$

$$E_{n2} = E_2 + \frac{RT}{nF} \log \left\{ \frac{1 + 10^{-pH - pK_{2}}}{1 + 10^{-pH - pK_{1}}} \right\}$$

(4.2)

where $E_1$ and $E_2$ represent the pH independent midpoint potentials for the FAD/FADH$^+$ and the FADH$^+$/FADH$^+\_2$ couples respectively, and $pK_1$ and $pK_2$ represent the $pK_a$ values associated with the protons acquired during the first and second electron additions respectively. These equations were fitted to the experimentally obtained values for $E_{n1}$ and $E_{n2}$. The following values were obtained for the parameters which were allowed to vary freely: $E_1 = -393$ mV, $E_2 = -240$ mV, $pK_1 = 9.5$, $pK_2 = 7.4$. The theoretical curves generated using these values for the parameters in Equation 4.2, are compared to the data in Fig. 4.6b.

A comparison of the $pK_a$ values obtained for the flavin, with the
corresponding values obtained by potentiometry of free flavin by Draper and Ingraham (99), shows that both values for xanthine oxidase are close to 1 pH unit higher than for free flavin. This is presumably due to interaction with either an anionic group nearby, or perhaps to the flavin site being partially hydrophobic.

As in the case of the Fe/S centers the potentiometric behavior of the FAD is also affected by temperature. Barber and Siegel (77) have found a similar protonation sequence at low temperature. The values reported by these authors for the midpoint potentials and \( pK_a \) are:

\[
E_1 = -410 \text{ mV}, \quad E_2 = -235 \text{ mV}, \quad pK_{A1} = 8.8, \quad pK_{A2} = 6.7.
\]

A comparison of the two sets of values shows that the change in temperature has a moderate effect in the \( pK_a \) values of the two ionizable groups in the reduced flavin. Both values are lowered by 0.7 pH units in the transition from room temperature to liquid nitrogen temperature. The pH independent midpoint potentials, on the other hand, show very little temperature effect, the difference between the two sets amounting to no more than about 10 mV.

It is interesting to note that both ionizable groups show identical temperature dependences. This dependence is, coincidentally, of a similar magnitude but opposite in sign, to the difference between the \( pK_a \) values of free and xanthine oxidase bound flavin, so that if the data for the enzyme are collected at low temperature there appears to be little difference between free and bound flavin. On the other hand, the pH independent potentials show very little change with temperature. This situation suggests that the group responsible for the observable effect is not on the flavin itself, but elsewhere on the protein.
The behavior of the flavin is best explained, as expected, by the model in which reduction of the flavin to semiquinone and then to the dihydroquinone form is accompanied by addition of one proton at each step. In agreement with Barber and Siegel (77), I find that considerably larger amounts of semiquinone are formed at the lower pH values. This pH dependence of semiquinone stability is in accordance with the work of Axelson et al. (35) and of Clson et al. (46) who suggested this phenomenon, and with the finding that the rate of the slow phase of reoxidation of xanthine oxidase increases as the pH decreases (see Chapter 3) since the rate of this phase appears to be proportional to the amount of semiquinone formed during the reaction (46).

No Centers:

The potentiometric behavior of the molybdenum centers of xanthine oxidase was followed by pH under the same conditions used for investigating the flavin. As in the case of the flavin, the amounts of the observable species ($\text{Mo}^{\text{II}}$) seen during the titration increase and then decrease as the system potential becomes more negative. This phenomenon is observed for both the "rapid" and "slow" molybdenum signals (see Figs. 4.4, 4.5). For both types of signals, the maximum intensity observed diminishes when the pH is increased and the potential at which this maximum occurs become more negative. The midpoint potentials obtained at the various pH values for both the "rapid" and "slow" molybdenums are presented in Table 4.1, and depicted in Figs. 4.6c, d.

As in the case of the other centers in xanthine oxidase, the behavior of the molybdenum centers shows considerable pH dependence. The
FIGURE 4.4

Potentiometric Titrations of the "Rapid" Molybdenum in Xanthine Oxidase.

The symbols represent the integrated EPR signal intensity of the molybdenum center. The lines represent best fits of the theoretical signal change expected for the system, calculated as described in the text.

A, pH 7.2; B, pH 7.7; C, pH 8.3; D, pH 9.2; E, pH 9.9. The buffer systems used are given in Chapter 2.
FIGURE 4.5
Potentiometric Titrations of the "Slow" Molybdenum in Xanthine Oxidase.
The symbols represent the integrated EPR signal intensity of the molybdenum center. The lines represent best fits of the theoretical signal change expected for the system, calculated as described in the text.
A, pH 7.2; B, pH 7.7; C, pH 8.3; D, pH 9.2; E, pH 9.9. The buffer systems used are given in Chapter 2.
two kinds of molybdenum centers show different types of behavior, in accordance with their different chemical structures. The potential associated with addition of the first electron to rapid molybdenum shows a ~60 mV/pH unit dependence while the potential associated with addition of the second electron shows a pH dependence of only ~30 mV/pH unit.

This is the type of behavior expected for a species which in the oxidized form has a group which can be protonated. Addition of an electron to the center promotes the protonation of a second group the charge of which is in turn offset by addition of a second electron. The net effect is that of one proton addition accompanying each 1-electron reduction step. In contrast with the flavin case, protonation of the group can precede reduction in the rapid molybdenum center:

\[
\text{Mo(VI)} + \text{H}^+ \xrightarrow{\Delta G_1} \text{Mo(VI)}\text{H}^+ \xrightarrow{\Delta G_2} \text{Mo(V)}\text{H}^+ + \text{H}^+ \xrightarrow{\Delta G_2} \text{Mo(V)}\text{H}_2 \xrightarrow{\Delta G_2} \text{Mo(V)}\text{H}_2^+
\]

The pH dependence of the observable potentials for a system of this type is then:

\[
\begin{align*}
\beta_{\text{m1}} &= \Delta G_1 - (R/T) \log [ (1 + 10^{(\Delta G_1 - \Delta G_2 )/T}) / (1 + 10^{(\Delta G_2 - \Delta G_2 )/T}) ] \\
\beta_{\text{m2}} &= \Delta G_2 - (R/T) \log [ 1 + 10^{(\Delta G_1 - \Delta G_2 )/T} ]
\end{align*}
\]

These equations were fitted to the experimentially obtained potentials associated with the reduction of the rapid molybdenum. The resulting theoretical curves are compared with the data in Fig. 4.6c. The following values were obtained for the disposable parameters: \( \Delta G_1 = -309 \text{ mV}, \)

\( \Delta G_2 = 7.9, \beta_{\text{m2}} = -301 \text{ mV}, \beta_{\text{m2}} = 3.4. \)

The pH dependence of the potentials associated with reduction of
the slow molybdenum is somewhat different from that of the rapid molybdenum (see Fig 4.6). While the value associated with addition of the first electron shows a dependence similar to that found with rapid molybdenum, the value associated with addition of the second electron in slow molybdenum behaves differently from its rapid counterpart. In the low pH region, it shows a dependence of -60 mV/pH unit which becomes smaller as the pH value increases, until the potential becomes independent of pH at about pH 9. This type of behavior is consistent with a system in which, as in the case of the rapid molybdenum, addition of two electrons is accompanied by addition of two protons, and one of the protons can bind to the oxidized form of the center. However, in this case addition of two electrons is required before the second proton can bind:

\[
\text{Mo(VI)} + H^+ \rightarrow \text{Mo(VII)}^+ \rightarrow \text{Mo(IV)}^2+ + H^+ \rightarrow \text{Mo(IV)}H^+
\]

The pH dependence of the observable potentials for a system of this type is:

\[
\varphi_{\pm 1} = -1 - (\Delta \gamma / \Delta \kappa) \log [1 + 10^{(\Delta \kappa - \Delta \kappa_1)}] \\
\varphi_{\pm 2} = \varphi_2 + (\Delta \gamma / \Delta \kappa) \log [1 + 10^{(\Delta \kappa_2 - \Delta \kappa_1)}]
\]

These equations were fitted to the experimentally obtained potentials associated with the reduction of the slow molybdenum. The resulting theoretical curves are compared with the data in Fig. 4.6a. The following values were obtained: \( \varphi_1 = -320 \text{ mV}, \varphi_1 = 7.5, \varphi_2 = 9.5, \varphi_2 = -477 \text{ mV}. \) Comparison of the protonation behavior of the two types of molybdenum.
FIGURE 4.6
Dependence on pH of the Midpoint Potentials of the Various Redox Centers in Xanthine Oxidase. The symbols represent the values for midpoint potentials obtained at different pH values from titrations such as those depicted in Figs. 4.1-4.5. The curves represent best fits of the theoretical values expected for the different systems as a function of pH, calculated as described in the text. A, Iron-sulfur centers; the squares represent the potentials associated with Fe/S I, while the triangles represent those associated with Fe/S II. B, FAD; C, Rapid Molybdenum; D, Slow Molybdenum. In B, C, and D the squares represent addition of the first electron and the triangles represent addition of the second electron.
Slow Molybdenum

Midpoint Potential, mV

pH

$E_1$

$E_2$
centers during reduction shows that addition of the first electron occurs under similar circumstances; however, there is a marked difference in the behavior observed during addition of the second electron for the two types of centers. The rapid and slow molybdenum centers have been proposed to differ by a thiolate group present in the rapid form which is replaced by an oxo group in the slow molybdenum (32, 56). The large difference in behavior can then be explained if the second electron accomplishes a reduction of these groups in their respective parent centers.

I cannot compare the values of my parameters to those obtained by Barber and Siegel (77) in the case of the molybdenum centers, because they found protonation behavior different from that which I observe for both types of Ni centers. However, comparison of the behavior of the midpoint potentials with changes in pH at room temperature (Fig. 4.6c, d), with the behavior observed at low temperature (77) makes it apparent that temperature changes have a marked effect on the electrochemical properties of the centers. Furthermore, this effect in the slow molybdenum is very different from that in the rapid molybdenum. This observation is, of course, consistent with the different chemical natures of the two molybdenum centers, and the change in protonation behavior at the two temperatures presumably arises from changes in pK_{a} which are far more drastic than those observed for either the flavin or the iron-sulfur centers of the enzyme.

Optical Absorption Characteristics:

Optical stoichiometric titrations at different pH values performed
in the absence of mediator dyes provide an appropriate tool for corroborating the observed redox characteristics of xanthine oxidase and insure that the observed potentials are not artifacts of the potentiometric experiments. Both the flavin and the Fe/S centers absorb at 450 nm while only the Fe/S centers contribute to the absorption at 550 nm. While the Mo center does not absorb in the visible region, the values for its midpoint potentials relative to the other redox groups have a substantial effect on the shape of the titration curves (46). Accordingly, I performed a series of reductive titrations of xanthine oxidase with dithionite at different pH values in the range 6.2-9.9. At pH 6.2 the fractional absorbance change at 450 nm leads that at 550 nm throughout the titration (Figs. 4.7 and 4.8). In particular the absorption change at 550 nm shows a substantial lag at the beginning of the titration. As the pH is raised the absorbance change at 450 nm becomes steeper, while the early part of the change at 550 nm occurs faster than the change at 450 nm, so that there is a crossover which occurs progressively later in the titration as the pH is increased (see Fig. 4.8b-d). The data of pH 8.3 (Fig. 4.8c) are comparable to the results obtained at pH 8.2 by Olson et al. (45); the latter experiments were performed in 0.1 M pyrophosphate buffer, while my data were obtained in 0.1 M Bicine.

Since the midpoint potentials express the electron affinities of the different centers of xanthine oxidase, they can be used to predict the electron distribution expected within the enzyme for a given level of reduction. Knowledge of this distribution, in conjunction with the spectral characteristics of the different species involved in the process, can then be used to predict the absorbance changes associated with
FIGURE 4.7

Optical Absorbance Changes Observed During Stoichiometric Titrations of Xanthine Oxidase. The spectra were collected as described in Chapter 2. A, pH 6.15; B, pH 9.9.
FIGURE 4.6

Optical Stoichiometric Reductive Titrations of Xanthine Oxidase. The symbols represent fractional absorbance changes at two different wavelengths with respect to the oxidized enzyme. The squares stand for data taken at 450 nm, while the triangles represent data acquired at 550 nm. The solid lines represent theoretical absorbance curves predicted using the potentials listed in Table 4.1 and calculated as described in the text assuming no extinction changes for the molybdenum center. The broken lines represent the theoretical changes obtained in the same manner assuming extinction changes to be associated with reduction of molybdenum as described in the text. The data were taken as described in Chapter 2. A, pH 6.2; B, pH 7.2; C, pH 8.3; D, pH 9.2; E, pH 9.9. The buffer systems used are given in Chapter 2.
this level of reduction.

A consideration of the spectroscopic properties of xanthine oxidase at a given pH presents a set of ten possible chromophores, encompassing the different known oxidation states of the 4 redox centers in the enzyme. Furthermore, the situation becomes more complicated when one notes that more than one protonation state may exist at each level of reduction of each center. The spectroscopic contributions of all these species at the different wavelengths have not been elucidated. However, with the use of some approximations, enough information is available to attempt a description of the spectroscopic behavior of the enzyme during a reductive titration. First, analysis of the data as absorbance changes makes the resolution of the absolute spectra associated with the different species unnecessary. Second, the different molybdenum species are assumed to make little or no spectral contributions in the optical region. Thus, investigation of the reductive behavior of the enzyme at 450 and 550 nm calls only for knowledge of the extinction changes associated with the flavin and iron-sulfur centers. Third, examination of the spectra of fully oxidized and fully reduced enzyme reveals that at the wavelengths of interest there is no pH dependence of the spectral characteristics of the reduced and oxidized states of the flavin and the iron-sulfur centers in the pH range investigated. Fourth, while the spectral characteristics of the semiquinone forms of the flavin in xanthine oxidase have not been unequivocally established, the contribution of the flavin to the spectrum of oxidized enzyme is very much that of a typical flavoprotein (34). Therefore, it is reasonable to assume that the spectral characteristics of other forms of the flavin in this enzyme will also be similar to those of other flavoproteins.
The absorbance changes of xanthine oxidase as a function of overall reduction state (Fig. 4.8) have been investigated numerically with the above simplifying assumptions. The expected values for the extinction changes at a given reduction level were obtained by establishing the electron distribution expected among the various redox centers in the manner described by Olson et al. (46), using the redox potentials in Table 4.1. The spectral contributions of the different species present were then calculated employing the selected spectral weights and summed to obtain the expected absorbance change for the reduction level. The changes in extinction coefficient associated with the two stages of reduction in the flavin are summarized in Table 4.2. The total extinction changes for the iron-sulfur centers are 14,400 M\(^{-1}\) cm\(^{-1}\) and 6,900 M\(^{-1}\) cm\(^{-1}\) at 450 and 550 nm respectively (46). However, the fractional contributions of the individual Fe/S centers have not been unequivocally established using data obtained exclusively at room temperature. Olson et al. (46) have reported a set of values for these extinction coefficients, but they relied on low temperature ESR for an estimate of the degree of reduction of Fe/S I and Fe/S II. Insofar as one of our objectives was to independently assess the assignments of Olson et al. (46), we allowed the relative contributions of the individual Fe/S centers to vary until a best fit was obtained to the data obtained at 450 and 550 nm. The theoretical lines obtained for the different pH values with values for the fractional extinction contributions that gave the best fits are compared to the data in Fig. 4.8 where it is seen that the predicted absorbance changes give a reasonable fit to the experimental results. More specifically, the trend toward a higher crossover point for the titration curves at 450 and 550 nm with increasing pH is suc-
TABLE 4.2

Extinction Coefficients for the Different Species of Flavin Used to Simulate Absorbance Changes of Xanthine Oxidase During Stoichiometric Reductive Titrations.

<table>
<thead>
<tr>
<th>Species</th>
<th>Change in Extinction Coefficient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADH₂ FAD</td>
<td>12,200</td>
<td>(34)</td>
</tr>
<tr>
<td>FADH (red) FAD</td>
<td>4,300</td>
<td>(101)</td>
</tr>
<tr>
<td>FADH (blue) FAD</td>
<td>4,200</td>
<td>(102)</td>
</tr>
</tbody>
</table>
cesfully predicted. Also predicted are the presence of a lag at 550 nm
during titrations at pH 6.2, as well as the opposite phenomenon observed
at higher pH, where about 90% of the absorbance changes observed take
place during addition of the first four electrons.

The contributions to the total extinction change of the enzyme by
Fe/S I determined by the above method were 12,000 ± 500 M\(^{-1}\) cm\(^{-1}\) at
450 nm and 5400 ± 200 M\(^{-1}\) cm\(^{-1}\) at 550 nm. The corresponding values for
Fe/S II were 2400 ± 500 M\(^{-1}\) cm\(^{-1}\) and 1500 ± 200 M\(^{-1}\) cm\(^{-1}\) respectively.

While there is some variation in the values obtained at the different pH
values, this variation is not systematic and probably arises from the
uncertainty inherent in the determinations. These values are comparable
to those obtained by Olson et al. (46) who reported values of 11,600
M\(^{-1}\) cm\(^{-1}\) and 2800 M\(^{-1}\) cm\(^{-1}\) at 450 nm and 5400 M\(^{-1}\) cm\(^{-1}\) and 1500 M\(^{-1}\) cm\(^{-1}\)
at 550 nm for Fe/S I and Fe/S II respectively; comparable values for
spinach ferredoxin are 4800 M\(^{-1}\) cm\(^{-1}\) at 450 nm and 1800 M\(^{-1}\) cm\(^{-1}\) at
550 nm (103).

There is a potential contradiction between this result and the ob-
servation that the two iron-sulfur centers have very similar CD spectral
characteristics. Furthermore, the extinction change associated with re-
duction of Fe/S I has a magnitude larger than that of the absolute ex-
tinction coefficient of spinach ferredoxin. This observation led me to
consider the possibility that the two Fe/S centers have comparable chan-
ges in extinction coefficients and that the apparent discrepancy is due
to some other cause. In an attempt to resolve this question, I have
manipulated the parameters used in the fittings to make the absorbance
changes for the two centers much closer in magnitude. I find that the
only way to obtain reasonably similar spectral weights using the previous
restrictions was to assume; (i) that there is a group in the enzyme other than the molybdenum, flavin or the Fe/S centers that is consuming 20-25% of the reducing equivalents added to the titration mixture; and (ii) to invoke an unreasonably high extinction coefficient for the semiquinone form of the flavin (e.g., 10,000 M\(^{-1}\) cm\(^{-1}\) at 550 nm). These requirements are clearly unlikely since there is no extant evidence for any other reducible centers within the enzyme, and the flavin extinctions that would have to be invoked are clearly unlike those found in any other enzyme or flavin model system.

Attempts to simulate the absorbance changes obtained during a titration without altering the parameters used for the original fits, but with identical contributions by the Fe/S centers, gave very poor fits. The absorbance changes at the beginning or the titration take place much too fast, especially at 550 nm, and there is no observable lag of any kind at pH 6.2. Clearly, if the Fe/S centers and the flavin are the only contributors to the absorbance changes observed at 450 and 550 nm, the use of quite distinct absorbance spectral weights for the individual Fe/S centers is dictated by the data, and is in excellent agreement with the results originally reported (46).

An alternative way of approaching the problem is to abandon our earlier restriction and assume that one or more of the molybdenum species have absorbance bands at 450 and 550 nm and the intensities of these bands change during reduction. In such a case the seeming difference in the extinction coefficients of the Fe/S centers would arise artifically from assuming that the molybdenum center does not contribute to the absorbance changes observed during full reduction of the enzyme. This alternative assumption allows the following options:
1) all the extinction change is associated with reduction of Mo(VI) to Mo(V); 2) the absorbance change takes place during reduction of Mo(V) to Mo(IV); 3) the change is distributed between the two reduction steps. I attempted to recreate the observed absorbance changes under all three possibilities. For ease of computation, I placed the arbitrary restriction that in the third case the absorbance change was associated equally with Mo(VI) → Mo(V) and Mo(V) → Mo(IV). The fractions of the total extinction change associated with reduction of the iron-sulfur centers and the molybdenum center were adjusted manually and the goodness of the fit was estimated by eye. Comparisons of the simulated titrations were done for all the pH values investigated.

For most values of the change in extinction coefficient tried, I find the best agreement between the observed and calculated results in those cases where half of the extinction change associated with full molybdenum reduction is assigned to each of the two reduction steps (cf. section 3). I find an acceptable agreement between experimental and simulated data using the following values for the changes in extinction coefficients at 450 and 550 nm respectively: Fe/S I, 6700 and 3400 M⁻¹ cm⁻¹; Fe/S II, 4800 and 2000 M⁻¹ cm⁻¹; Mo(VI) → Mo(V) and Mo(V) → Mo(IV), 1450 and 750 M⁻¹ cm⁻¹. A comparison of some of the curves calculated using these numbers to the experimental data is shown as the broken lines in Fig. 4.8. While the fits obtained by including Mo are not exact, there is clearly an overall correlation between the observed and calculated results, with the major features of the observed changes being reproduced in the simulated data. Presumably better agreement would result if the constraints that the contributions of the two separate steps be identical and that the Mo contribute equal proportions at the two dif-
ferent wavelnths are removed. Clearly, the conjecture that part of the absorbance changes observed at 450 and 550 nm during reduction of the enzyme are due to the molybdenum center provides a viable alternative to the conclusion that Fe/S I presents a change in absorbance which is considerably larger than that shown by Fe/S II and exceeds the absolute extinction of spinach ferredoxin. This apparent inconsistency would then be a consequence of mistakingly assigning to Fe/S I the contribution of the Mo center.

Barber et al. (104) have reported the values of the potentials of the Fe/S centers in rabbit liver aldehyde oxidase are reversed with respect to their counterparts in bovine milk xanthine oxidase. Remarkably, they find that the spectral contributions of the centers in the enzyme from rabbit at 450 and 550 nm are also reversed with respect to their corresponding values in the bovine milk's protein. A very attractive explanation of this phenomenon is provided if it is assumed that molybdenum does indeed contribute to the absorbance changes observed at 450 and 550 nm. The anomalous extinction changes observed for the low potential Fe/S center (Fe/S I in xanthine oxidase and Fe/S II in aldehyde oxidase), arise then from mistakenly assigning to this center the spectral contributions of the molybdenum. Thus, the apparent switch in the relative contributions of the iron-sulfur centers in aldehyde oxidase with respect to xanthine oxidase would result from ignoring the absorbance changes in the molybdenum center during the reduction of the enzymes.

On the assumption that molybdenum does indeed contribute to the absorption spectrum of xanthine oxidase I attempted to quantitate this contribution. I simulated the spectrum of xanthine oxidase without the
molybdenum contribution by taking twice the spectrum of spinach ferredoxin and adding it to the flavin spectral contribution. The resultant spectrum (Fig. 4.9) is similar to that of xanthine oxidase, but lacks the "bump" seen at 550 nm and the intensity of the simulated spectrum diverges progressively from that of the enzyme at shorter wavelengths. The difference spectrum (see Fig. 4.9) showed a broad absorption band at about 480 nm and a smaller band at about 550 nm, as well as a much more intense band toward the ultraviolet end of the spectrum. The smaller band at 550 nm could very well be spurious and arise because spinach ferredoxin has no band at 550 nm akin to that seen in xanthine oxidase. Unlike spinach ferredoxin, adrenodoxin does have a feature at 550 nm similar to that in xanthine oxidase; however, we did not use the spectrum of this protein for the simulation because it exhibits an axial EPR signal, which is unlike the EPR signals in xanthine oxidase. However, the molybdenum spectral contributions outlined here should be treated with caution because the spectrum of allopurinol treated xanthine oxidase is not different from that of native enzyme at 550 nm, implying that reduction of Mo(VI) to Mo(IV) makes no contribution to the spectral changes at this wavelength.

Conclusions:

Examination of the data in Table 4.1 reveals that pH has a pronounced effect on the redox potentials of the prosthetic groups of xanthine oxidase. Thus all of these centers contain or are associated with groups that can be protonated at one reduction level or another. This finding is not unexpected. In 1973 Stiefel (100), based on studies of molybdenum
FIGURE 4.9

Proposed Optical Spectrum for the Oxidized Molybdenum Center of Xanthine Oxidase. The solid line represents the actual spectrum of native xanthine oxidase. The broken line represents the putative spectrum of "demolybino" xanthine oxidase calculated as described in the text. The dotted line represents the difference between the preceding spectra which is speculated to belong to the oxidized molybdenum center of the enzyme.
model compounds, had predicted that reduction of the molybdenum centers would be accompanied by protonation. It is also reasonable to expect that electron addition to the flavin will be accompanied by protonation. This is in agreement with the markedly different kinetic behaviors that the enzyme exhibits at different pH values, which has been explained in terms of the presence of ionizable groups in the flavin moiety. In particular, the increased potentiometric stability of the flavin semiquinone with respect to the Fe/S centers reported here accounts for the smaller production of superoxide during the reoxidation of the enzyme at lower pH as compared to high pH (see Chapter 3). Cammack and coworkers (76) have also reported finding different potentials depending on whether pyrophosphate or Tris buffers were used in the experiments. Williams-Smith et al. (96) suggested that this was due to a differential pH change taking place during freezing of the samples. More recently Barber and Siegel (77) have reported pH effects on the redox behavior of all the centers in xanthine oxidase. My results are in substantial agreement with those of the latter authors with respect to the sequence of electron and proton addition for the flavin and Fe/S centers. However, as discussed above, they are in clear disagreement with the protonation behavior of the molybdenum centers reported by Barber and Siegel (77). I also deduce significantly different \( pK_a \) values for the dissociable protons in the flavin and iron-sulfur centers. These discrepancies can be attributed to the difference in temperatures at which the two sets of measurements were made.

Comparison of the midpoint potentials in Table 4.1 with the results of Cammack et al. (76) and those of Barber and Siegel (77), shows that there is a pronounced temperature effect in the measured potentials of
the various redox groups of xanthine oxidase. These differences range from as little as 0 mV for Fe/S I at pH 7.2 to a maximum of 110 mV for the potential associated with addition of the first electron to "rapid" molybdenum at pH 9.9 and arise either from changes in the intrinsic redox potentials of the centers or from temperature effects on the pK_a of the ionizable groups. While there is only a moderate effect on the behavior of Fe/S I, the effect is so pronounced with the molybdenum centers that to explain the data obtained at room temperature, protonation schemes completely different from those deduced from low temperature measurements (77) are needed.

This differential effect of temperature on the different centers has an important effect on electron distributions seen in intermediate reduction states of the enzyme. At different temperatures these distributions will acquire different shapes so that comparison of data taken at different temperatures becomes complicated. Consequently, the greatest impact of this finding should be in the analysis of the kinetics of the enzyme. Since intramolecular electron transfer in xanthine oxidase can be considered instantaneous in the time scale of the kinetic techniques available to conveniently study the reactions of the enzyme (46), the thermodynamic parameters of the various redox centers control the spectroscopic changes observable during a reaction. Thus, the demonstration of the temperature sensitivity of the midpoint potentials of the various redox centers provides a compelling reason for reevaluation of data obtained by the rapid freeze technique.

In spite of these temperature and pH effects observed on the various potentials, it is interesting to find that the general scheme proposed by Olson et al. (46) for the mechanism of the enzyme holds true.
While the relative values of the potentials of the different groups are
different from those assumed by Olson et al. (46), the differences are
not so substantial as to radically alter the original mechanistic propo-
sals. Even with a revised set of potentials, the flavin is mostly re-
duced and the molybdenum is mostly oxidized at intermediate levels of
reduction. However, I do find that the enzyme is not as perfectly adap-
ted to attain maximum reduction of the flavin while maintaining maximum
oxidation of the molybdenum, as had been supposed (46), since one of the
Fe/S centers exhibits the most positive potential under all the condi-
tions investigated.

The agreement between the observed and predicted extinction chan-
ges during stoichiometric titrations, and in particular the success in
predicting trends with changes in pH, provide additional support for
the validity of the schemes proposed for the redox behavior of xanthine
oxidase. It is reassuring that the extinction coefficients which have
been used to explain the kinetic peculiarities of the enzyme (46, Chapter
3) generate acceptable simulations of the absorbance changes observed
during reductive titrations of the enzyme. However, these data are not
uniquely explained by the extinction coefficients proposed by Olson et
al. (46), and I have raised the possibility that some of the changes ob-
served in fact arise from reduction of the Mo center. I am not, however,
in a position to resolve this question and thus, there remains unresol-
ved the lack of a full agreement between the optical, CD and EPR spec-
tral properties of the enzyme. These are obviously questions which de-
serve future experimentation.
CHAPTER V
Conclusions

Xanthine oxidase belongs to a group of enzymes involved in electron transfer reactions which are capable of accepting more electrons than would seem necessary from a cursory examination of the stoichiometry of the reactions catalyzed. Xanthine oxidase, for example, catalyzes the transfer of 2 electrons from a purine substrate to molecular oxygen to form either 1 molecule of $\text{H}_2\text{O}_2$ or 2 molecules of $\text{O}_2^-$. The most probable reason for this type of makeup is control of enzymatic activity. As discussed by Palner and Olsson (78) if the rate limiting step in an electron transfer reaction is the interaction between enzyme and substrate, and electron transfer among the redox centers is so rapid that it appears to occur instantaneously, the presence of a large number of redox prosthetic groups imposes on the enzyme a "thermodynamic control" of its activities. By fine tuning the electron affinities of the different prosthetic groups the activities of the enzyme can be optimized according to the various conditions it is likely to encounter. Under these circumstances, the knowledge of the redox potentials of the different groups within the enzyme becomes of paramount importance for understanding of the kinetic properties of the protein in question.

The experiments presented in Chapter 4 have allowed the establishment at room temperature, of a set of potentials for the flavin, molybdenum and Fe/S centers of xanthine oxidase at a variety of pH values. A comparison of these midpoint potentials with values obtained at 25-120 $^\circ$C has shown that there is a significant degree of temperature dependence of these potentials.
Examination of the pH dependence of the potentials obtained in Chapter 4 has revealed the involvement of a number of protons in the oxidation-reduction reactions of the centers. The role of these protons in the enzymatic mechanism is not clear in all the cases, however, in the case of the molybdenum center it has been suggested that the oxidation of substrate is accompanied by proton transfer to some moiety in the molybdenum site (23). In the case of the flavin, changes in the ionization state of one of the sites on the molecule have a dramatic effect on the reaction rate of the reoxidation of the enzyme by O_2. This pH dependence has a substantial effect on some of the catalytic characteristics of the enzyme. Since the potentials of the individual redox groups in the protein have different pH dependences, different electron distributions among the centers will be observed at different pH values for a given overall reduction state. This means that the pH at which a reaction is carried out will have an effect on both the reaction rates and the affinities of the enzyme for its substrates. It also may have an effect on the stoichiometries observed if more than one product is possible. This prediction is borne out by the observation that in xanthine oxidase pH has, indeed, an effect on the amount of superoxide produced by the enzyme on reoxidation by O_2 (see Chapter 3).

Examination of the kinetics of reoxidation of xanthine oxidase has revealed that, as had been proposed previously (45), both superoxide and peroxide are produced in the ratios 2 : 2 per flavin. Furthermore, production of peroxide takes place almost exclusively during the fast phase of reoxidation of the enzyme, while production of superoxide is evenly divided among the two phases. These findings are in agreement with the mechanism proposed in Scheme 1 (cf. Chapter 3).
Olson et al. (46) suggested that the controlling force behind the mechanism discussed in Chapter 3, is the relative distribution of the electrode potentials of the various redox groups in xanthine oxidase. This contention is borne out both by the confirmation that these potentials do indeed occupy positions in agreement with the roles assigned to them by Olson et al. (46). However, the values of the potentials are not quite as finely tuned to providing maximal activity for the enzyme as had been believed previously (46).

The fact that the potentials of the redox groups of xanthine oxidase are pH dependent has an important effect on the proportions of $\text{H}_2\text{O}_2$ and $\text{O}_2^-$ produced during reoxidation of the enzyme. As discussed in Chapter 3, the amounts of $\text{O}_2^-$ observed vary from a low of 0.6 eq./flavin at low pH to a high of 1.8 eq./flavin at high pH. This is presumably a result of the increased stability of the flavin semiquinone at low pH reported in Chapter 4. The greater availability of this species permits some of the freshly generated $\text{O}_2^-$ to acquire a second electron and become $\text{H}_2\text{O}_2$.

Another feature of the reoxidation of xanthine oxidase which is pH dependent is the rate of the fast phase. This rate is affected by protonation of the flavin-$\text{H}_2\text{O}_2$ complex at the nitrogen closest to the site of attachment of the peroxide (either $N^1$ or $N^5$). This protonation inhibits the release of $\text{H}_2\text{O}_2$, which should be aided by the presence of a negative charge in the vicinal nitrogen. At all pH values, however, the reaction is relatively fast and the rate constants obtained are comparable to those for free flavin.

The rapid reaction of $\text{O}_2^-$ with reduced flavin presents an interesting problem, since at first glance the interaction of reduced flavin with triplet oxygen involves a spin forbidden reaction. Bruce (105)
has suggested that a triplet \( \text{O}_2 \) molecule interacts with the highest occupied molecular orbital (HOMO) in the flavin to generate a flavin radical and a superoxide anion. He points out that the central ring of 1,5-dihydralloxazine and the attached \(^1\text{N}^1\) function constitute simultaneously a 1,4-dihydropyrazine and an enamine. In the butterfly configuration the pyrazine ring will possess \(^1\text{N}^1\)-\(^5\text{N}^5\) orbital splitting, while in the planar conformation it will be antiaromatic. Thus, in either the planar or bent conformations the reduced flavins should easily enter into radical reactions. A one-electron transfer to \( \text{O}_2 \) overcomes the problem of spin forbiddenness, and the resultant products can further react to complete the flavin oxidation.

The presence of substantial concentrations of \( \text{O}_2^- \) in solution has been observed during the reoxidation of free flavin (105), which lends some support to the proposal by Bruce (105).

Once formed, the superoxide-flavin radical pair can further react to form a Fl-COOH adduct. It has been proposed that the -COOH moiety is attached to either the \( \text{C}^{1a} \) (92), of the \( \text{C}^{4a} \) position of the flavin (93, 94).

In support of the first proposal (\( \text{C}^{4a} \)) Yamazaki and Yamano (107) have reported producing \( \text{C}^{1a} \) adducts of 5-alkylflavins that can be trapped by \( \cdot \text{O}_2 \), isolated and subjected to mass spectral analysis.

Alternatively, extended Hückel calculations by Crf and Dolphin show that the HOMO electron density distribution, the \( \pi \)-charge distribution and the total charge distribution favor attack at the \( \text{C}^{4a} \) position. Furthermore, it has been shown that the \( \text{N}^{10} \)-2',6'-dimethyl phenyl derivative of flavin shows a rate of reaction with \( \text{O}_2 \) comparable to that of the \( \text{N}^{10} \)-methyl derivative. Therefore, it is clear that steric
availability of the $^{1a}$ of the reduced flavin is not essential for the reaction with oxygen (105).

Kemal and Bruce (109), reasoning that $N$-alkyl substitution would greatly inhibit decay of the flavin-peroxide adduct, blocked the $N^5$ position of the flavin with either $-C_3$ or $-CH_2CH_2$. Incubation of these flavin derivatives with $H_2O_2$ yields stable adducts at the $^{4a}$ position. The ethyl derivative can be obtained in 85-95% purity. Comparison of the UV-visible spectrum of this adduct with that of the intermediate formed during the reaction of a reduced flavoxygenase with oxygen has convinced these authors that the position of oxygen attack is indeed the $^{4a}$ site.

In light of the available evidence the following scheme can be proposed for the reaction of oxygen with the reduced flavin site of xanthine oxidase: an oxygen molecule reacts with a fully reduced flavin by way of an outer sphere 1-electron transfer to form an $FAH^{...}O_2^-$ complex which is held together weakly, perhaps by electrostatic interactions. The flavin then undergoes electrophilic attack at either the $^{1a}$ or $^{4a}$ positions to yield an $FAH-H_2O_2$ adduct. Breakdown of this adduct is promoted by the presence of a negative charge in the vicinal nitrogen. The $pH$ dependence of the rapid phase of reoxidation then arises from this feature. In the case of 2-electron reduced enzyme, however, after the $FAH^{...}O_2^-$ complex is formed; Fe/S II, with a higher electron affinity, is in a position to sequester the remaining electron from the flavin, forcing the release of $O_2^-$. At lower $pH$ values this electron transfer to the Fe/S II center is not as favorable and therefore, less $O_2^-$ is formed. This reaction sequence, which is depicted in Figure 5.1, is in agreement with Scheme 1 and explains the major features of the reoxidation reac-
FIGURE 5.1

Proposed Mechanism for the Reaction of Molecular Oxygen with the Reduced Flavin of Xanthine Oxidase to Produce Hydrogen Peroxide and Superoxide.
tion.

The steps outlined above (see Fig. 5.1) have been investigated by several workers using model systems. Nassey et al. (110) have studied the oxidation of tetraacetyl-riboflavin and were able to explain the kinetic data with a model in which oxygen and flavin rapidly form a dissociable complex which then decays to a superoxide-flavin semiquinone pair. They estimated this decay to have a rate constant \( k_3 \) no larger than 10 s\(^{-1}\). Nanni and coworkers (111) have reported that superoxide radical and flavin semiquinones react to produce a flavin-oxygen adduct.

Eberlein and Bruice (112) have suggested, after studying the oxidation of several flavin model systems, that the rate of this reaction approaches the diffusion rate of the reactants toward each other. Finally, Kemal et al. (115), have reported a value of \( 3.2 \times 10^{-4} \) s\(^{-1}\) for the rate constant of the decay of the \( O_2^\bullet \) adduct of 10-methyl flavin (\( k_3 \)).

In summary, the results presented in this work have provided evidence for significant temperature and pH effects on all the midpoint potentials of the redox groups of xanthine oxidase, and validated the mechanism which had been proposed previously for the reoxidation of the enzyme by showing that it successfully predicts the kinetics of production of \( H_2C_2 \) and \( C_2^- \). In particular, they showed that 2 moles of \( H_2C_2 \) and 2 moles of \( O_2^- \) are produced in the overall reoxidation, and that superoxide is produced only by the last two electrons.

These results point out several areas for future research: the kinetic data taken at low temperature will have to be reevaluated in the light of the marked temperature effects on the protein. A thorough investigation of the details of internal structures and electron transfer mechanisms should be carried out. Finally, an integration of
the knowledge rendered by the study of the oxidative and reductive kinetics of the enzyme into an overall mechanistic scheme should be undertaken.
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