LIGAND AND GENETIC STUDIES OF THE SULFATE-BINDING PROTEIN
AND
CHARACTERIZATION OF THE CALCIUM-BINDING SITE IN THE
GALACTOSE-BINDING PROTEIN

by

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ABSTRACT

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by

Bruce Lee Jacobson

The effect of pH on the binding affinities of the conjugate bases of four different tetrahedral oxyacids to the periplasmic sulfate-binding protein from Salmonella typhimurium has been determined. In light of the highly refined 2 Å structure of the complex of the sulfate-binding protein with sulfate, and considering the protonation state and net charge of the various oxyacids, the pH dependence of chromate binding and the extremely low affinity of phosphate are attributable mainly to a lack of hydrogen bond acceptors in the binding site. These studies demonstrate that the binding site of the sulfate-binding protein is stringently designed to tightly bind tetrahedral, fully ionized, oxyacid dianions.

Based on the refined 2.0 Å structure of the periplasmic sulfate-binding protein from Salmonella typhimurium, twelve site-directed mutants in the E. coli periplasmic sulfate-binding protein were designed to test specific hypotheses regarding protein-ligand complex stabilization and binding mechanism.

Mutants at position 42 (H42N, H42G, H42D) demonstrate that the fidelity of the H-bond array to His 42 is more important in stabilizing the protein–SO$_4^{2−}$ complex than
the proposed positive charge. The structural and chemical differences between serine and cysteine were examined by the series of mutants S130G, S130A, S130C. Mutations in the interdomain salt-bridges formed in the closed conformation of the protein were shown to affect the sulfate on- and off-rates. The necessity for protein conformational change in sulfate binding and release was tested by introducing a disulfide between the two domains. These studies further illuminate binding protein specificity, complex stability, and flexibility.

The relative affinities for various metals which bind to the calcium-binding site of the *E. coli* periplasmic D-galactose-binding protein in solution have been determined. In order of affinity the metals are: $\text{Ca}^{2+} \approx \text{Tb}^{3+} \approx \text{Pb}^{2+} > \text{Cd}^{2+} > \text{Sr}^{2+} > \text{Mg}^{2+} >> \text{Mn}^{2+} > \text{Ba}^{2+}$. The results of these solution studies support the hypothesis that for a given metal-binding loop, the ligands provided by the protein, and the cation hydration energy, size, and charge are major factors contributing to binding affinity.
Dedication

This thesis is dedicated to my wife Anita, whose love has been and always will be my foundation and support.
Acknowledgements

I am sincerely grateful to Professor Florante A. Quiocho for his support and patience, but most importantly, for his extraordinary talent for clear, concise scientific reasoning, which I shall strive to emulate. I am sincerely grateful to Polly Vermersch who taught me that with perseverance and the proper controls cloning does work, and for providing the pKK-ABP expression system. Thanks to the members of Dr. J. S. Olson’s lab, and particularly Dr. Doug Lemon, for their time, knowledge and stopped-flow apparatus. I am indebted to Jing Jiu He for his help with the SBP expression system, for providing the SBP mutants S130C, H42N, and H42G, and for sharing his data on the SBP mutants S130A, and S130G. I am grateful to Dr. P. Evans of the MRC at Cambridge, England for providing the SBP gene. I would also like to thank M. N. Vyas and N. K. Vyas for their contributions to the GBP project. I would like to thank all the members of QCI for sharing their knowledge and experience with me.
Portions of Chapters 2, 4, 5, and 6 have been published previously:


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CHAPTER 1

BINDING PROTEINS: FUNCTION AND STRUCTURE

1. Overview

1.1. Transport and Chemotaxis

Survival in the highly competitive world of microscopic organisms requires efficient utilization of available resources. To meet this challenge, bacteria have evolved systems to find and sequester nutrients at extremely low concentrations. Chemotaxis, the ability to detect and move up or down a chemical gradient, and high-affinity transport are two such systems.

1.2. Gram-negative Membrane

Gram-negative bacteria are protected from their environment by a complex system of three layers: an outer membrane, a cell wall, and an inner membrane (Nikaido & Vaara, 1987; Park, 1987; Cronan et al., 1987). The outer membrane is composed of phospholipids, matrix proteins, lipid polysaccharides and is covalently attached to the peptidoglycan cell wall via lipoproteins. Specific and non-specific pores in the outer membrane allow entry of certain small molecules, but are impervious to most others including many antibiotics (Ames, 1986). The inner membrane, similar to the cytoplasmic membrane of single membrane systems, is composed of phospholipids and
contains many proteins including the membrane-bound components of chemotaxis and transport. Approximately two dozen types of binding proteins are found in the periplasmic space between the inner membrane and the cell wall, and are often called periplasmic binding proteins.

Periplasmic binding proteins serve as initial, high-affinity receptors for the osmotic shock-sensitive, high affinity transport systems for various ligands including sugars, amino acids, inorganic anions and other nutrients as well as initial receptors for the chemotactic response to galactose, maltose, ribose, and oligopeptides (Ames, 1986; Wilson & Smith, 1978; Adler, 1975). The central role of binding proteins in these two processes is shown schematically in Figure 1. As initial receptors, binding proteins bind their respective ligand with high affinity but are also capable of easily releasing it to the membrane-bound components of the system which are responsible for transport into the cell. Thus, binding proteins enable the transport system to operate efficiently at low concentrations; $K_M$ for maltose transport is 1 μM in the presence of binding protein (Ames, 1986), and > 25 mM in the absence of binding protein (Shuman, 1982).

1.3. Reasons for Study

Because each periplasmic binding protein binds a single ligand with high specificity and high affinity ($K_d$s $\approx$ 0.5 μM (Miller et al., 1983)), these proteins offer excellent systems for the study of a variety of protein-ligand interactions. Furthermore, well-refined, high resolution X-ray structures of seven of these proteins, including
Figure 1. The central role of binding proteins in chemotaxis and transport.
sulfate-binding protein (SBP) at 2.0 Å (Pflugrath & Quiocho, 1985, 1988), L-arabinose-binding (ABP) protein at 1.7 Å (Quiocho & Vyas, 1984), d-galactose-binding protein (GBP) at 1.9 Å (Vyas et al., 1988), leucine/isoleucine/valine-binding protein (LIVBP) at 2.4 Å (Sack et al., 1989), the leucine-specific binding protein (LsBP) at 2.4 Å (Sack et al., 1989), phosphate-binding protein (PBP) at 1.7 Å (Luecke, personal communication) and the maltose-binding protein (MBP) at 1.7 Å (Spurlino et al., In preparation) have led to detailed study and a new understanding of protein-sugar and electrostatic interactions at the atomic level.

1.4. Structural Theme and Proposed Binding Model

Crystallographic studies of periplasmic binding proteins have established a structural theme which has been consistently maintained by each of the seven structures solved so far (Sack et al., 1989; Luecke, personal communication); all are ellipsoidal and composed of two domains connected by a flexible hinge region, with the ligand bound in the cleft between the two domains (see Figure 2). Previous studies suggest that ligand binding occurs via a hinge-bending motion in which the two domains move relative to each other about the flexible hinge region, but preserve their individual structure (Newcomer et al., 1981; Newcomer et al., 1981; Sack et al., 1989).

As discussed above, transport and chemotaxis both require binding protein interaction with membrane bound protein components. For such a system to work efficiently the membrane-bound components must be able to distinguish between the liganded and
Figure 2. Periplasmic binding protein structures. Arabinose-binding protein (pink), Galactose-binding protein (orange), Maltose-binding protein (purple), Leucine/Isoleucine/Valine-binding protein and Leucine-specific binding protein (gold), Sulfate-binding protein (blue), Phosphate-binding protein (yellow). Note the features common to this family of proteins: 1) Two domains connected by a flexible hinge region; 2) A distinct cleft between the domains; 3) The ligand, represented as a small circle for each protein except for LIV/LsBP which was crystallized without bound ligand, is bound between the two domains, in the cleft. Photograph courtesy of Dr. F. A. Quiocho.
unliganded binding protein. The proposed hinge-bending model with structurally dis­tinct open (unliganded) and closed (liganded) forms fulfills this requirement nicely. It has been shown by mutant studies that binding proteins such as MBP and GBP which are involved in both chemotaxis and transport have distinct recognition sites in each domain (John Spurlino, personal communication) for each of these processes which interact independently with the respective membrane-bound components (Duplay et al., 1987; Scholle et al., 1987).
PART I.

LIGAND AND GENETIC STUDIES OF THE SULFATE-BINDING PROTEIN
CHAPTER 2

THE IMPORTANCE OF THE PROTON

2. Introduction

2.1. General Information

The sulfate-binding protein from *Salmonella typhimurium* (SBP), first purified and characterized by Pardee (Pardee, 1966), is a typical example of a periplasmic binding protein. SBP binds sulfate, chromate, and selenate with $K_d$s of 0.12 μM, 0.3 μM, 5.0 μM respectively (Jacobson & Quiocho, 1988). The amino acid sequence determined by chemical methods (Isihara & Hogg, 1980) consists of 310 amino acids with a calculated molecular weight of 34,100. One very interesting feature of the amino acid sequence is the conspicuous absence of sulfur containing residues: there are no cysteines or methionines in the protein. This is an excellent example of conservation of resources, since it would be wasteful for a bacterial cell starved for sulfur to require it for its sulfate transport system.

2.2. X-ray Structure

The X-ray structure of sulfate-binding protein, containing 308 ordered amino acids and the bound sulfate has been refined at 2.0 Å (Pflugrath & Quiocho, 1985, 1988). On the basis of this structure, several corrections were made to the chemically determined
sequence. Similar to other binding proteins, SBP is ellipsoidal—composed of two similar domains connected by a flexible hinge region with a cleft between them (see Figure 3). The sulfate is bound in this cleft primarily by seven hydrogen bonds, five of which come from peptide nitrogens. The sulfate dianion is completely inaccessible to the solvent. Surprisingly, there are no positively-charged residues within van der Waals radius of the bound sulfate which could serve to neutralize the sulfate’s negative charge. Pflugrath & Quiocho (1985) previously proposed that hydrogen bonds, by virtue of their directionality, convey geometric specificity to the SBP binding site, and that local dipoles and hydrogen bond arrays radiating from the binding site could serve to neutralize the charge of the bound sulfate. Recent evidence from other binding proteins demonstrates that this mode of stabilization may be more prevalent than heretofore believed (Quiocho et al., 1987). In addition, there are two salt bridges between the two domains which form at the lips of the cleft in the closed conformation (Glu 15 to Arg 174, and Asp 68 to Arg 134) which stabilize the closed conformation.

Previous ligand binding studies of SBP in solution by Pardee (Pardee, 1966) have shown that: 1) sulfate binding to SBP is pH insensitive over the range of pH 5.2 to pH 8.3; 2) chromate, which quenches SBP tryptophan fluorescence, can quantitatively and reversibly displace sulfate bound to SBP; 3) selenate inhibits sulfate binding; and 4) phosphate does not bind to SBP.

The purpose of this portion of this thesis was to extend these studies to determine the dissociation constants, and off-rates for these oxyanions and to investigate the effect
Figure 3. Sulfate-binding protein structure. The $\alpha$-carbon backbone is shown in red. The sulfate (blue) is bound in the cleft between the two domains.
3. Materials and Methods

3.1. Growth and Purification of SBP From *Salmonella typhimurium*

*Salmonella typhimurium* strain LT-2 was streaked from stab culture to glucose minimal plates (Vogel-Bonner salts, 0.2% glucose, 15 grams / liter Bacto-agar) and grown for 48 hours at 37°C. Eight 12 ml cultures of glucose minimal media (Vogel-Bonner salts, 0.2% glucose) were inoculated with single colonies of LT-2, and grown overnight at 37°C in a shaking water bath. Four grew well and were used to inoculate six 1 liter cultures of glucose minimal media. These cultures were grown for seven hours at 37°C in a shaking air incubator then used to inoculate the 180 liters of growth media (Vogel-Bonner salts (MgCl₂ replacing MgSO₄, 0.2% glucose, 150 μM L-djenkolic acid, a known derepressor of SBP synthesis) in the fermentor at the University of Texas Medical School. The culture was grown for 21 hours with high aeration. Harvest using a Sharples centrifuge yielded 289 grams of wet paste. The cells were resuspended in 1 liter of 1 mM phosphate buffer pH 7.8 and ran through the French press three times @6000-7000 psi, then centrifuged 90 minutes at 12,000 x g at 4°C. The supernatant was collected and 595 grams of ammonium sulfate (50%) was added with stirring over one hour with the pH maintained at 7.8 with NH₄OH. Solution was centrifuged 1 hour at 7000 x g and 4°C. 764 grams of ammonium sulfate (saturating) were added to the supernatant, and stirred overnight at 4°C. This slurry was centrifuged 1 hour at 12000 x g and 4°C. The pellets were combined, resuspended in 1 mM phosphate buffer pH 7.8,
and dialyzed extensively versus 1 mM phosphate buffer pH 7.8. The crude extract was loaded on a DEAE-52 column, and eluted with a 1.5 L x 1.5 L 0-50 mM KCl gradient followed by a 1.5 L x 1.5 L 50-200 mM KCl gradient. Fractions containing SBP were detected using PAGE. Fractions 90-130, 131-165, and 166-210 were combined separately. The purest fractions (131-165) were concentrated to from 250 ml to 75 ml and loaded onto a DEAE-53 column. Elution was with a 1.0 L x 1.0 L, 0-50 mM KCl gradient. Fractions 134-144 were shown by PAGE to contain pure SBP and were pooled with a yield of 195 mg. The remainder of the good fractions of the first column were pooled and ran on a DEAE-53 column with a yield of another 282 mg. Azide was added to a final concentration of 0.02% and the protein was stored at 4°C until use.

3.2. Resin Assay

The resin used for this assay was AG1-X8 (Cl⁻ form), an analytical grade anion exchange resin purchased from Bio-Rad. The radioisotope $^{35}$SO$_4^{2-}$ was purchased from New England Nuclear. Scintillation counting was performed with either a Beckman LS 3801, or a Beckman LS 1801 Liquid Scintillation Counter. Assays were performed in 20 mM Tris pH 8.1 buffer.

This assay, a slightly modified form of that first applied to SBP by Pardee (Pardee et al., 1966), is based on the ability of binding proteins to successfully compete with the resin for the anionic ligand. Because this is an equilibrium assay and the ligand is used in excess, it is not necessary that the protein be ligand free. However, in order to quanti-
of pH on their binding to SBP. This work demonstrates the exceptional substrate specificity of the SBP binding site and the narrow limits within which the protein can accommodate changes in the ionization state of the ligands.
state the decrease in specific activity caused by bound ligand, and to avoid competition from secondary ligands, the protein must be saturated with the ligand being studied.

The assay was performed as follows. 0.25 g of AG1-X8 resin were weighed into a 1.5 ml Eppendorf tube. For each experiment two tubes were used for blanks and five for each protein concentration. Into the blank tubes was pipetted 1.1 ml of 0.04 mM ligand (approximately $1.5 \times 10^5$ cpm). Into the protein tubes was pipetted 1.1 ml of 0.04 mM ligand (approximately $1.5 \times 10^5$ cpm) containing the proper protein concentration. The tubes were incubated for five minutes with intermittent mixing, then centrifuged in a micro centrifuge for two minutes $18000 \times g$. Two 80 µl aliquots were removed from each tube to separate scintillation bags containing 10 ml of scintillation fluid, sealed, and counted. The $^{35}$S was counted in the 0-1000 window.

Dissociation constants ($K_d$'s) were calculated using a Pascal program written specifically for this assay. The program is included as Appendix I. The theory behind this calculation is discussed below.

In the blank tube the equilibrium condition is described by the expression below.

\[
\text{Ligand + Resin} \xrightarrow{} \text{Ligand-Resin}
\]

Whereas in the protein tube the equilibrium is described by the following expression.

\[
\text{Protein-Ligand} \xrightarrow{} \text{Protein + Ligand + Resin} \xrightarrow{} \text{Ligand-Resin}
\]
Assuming the free ligand concentration remains the same in both tubes, the counts from the blank tube correspond to free ligand, and the counts in the protein tube correspond to free ligand plus bound ligand. Thus the three parameters necessary to calculate the $K_d$ can be easily obtained.

\[
\begin{align*}
\text{CPM from Blank} & \rightarrow [\text{Ligand}_{\text{Free}}] \\
\text{Protein CPM} - \text{Blank CPM} & \rightarrow [\text{Protein-Ligand}] \\
\text{Total Protein} - \text{Protein-Ligand} & \rightarrow [\text{Free}_{\text{Protein}}]
\end{align*}
\]

The $K_d$ is then

\[
\frac{[\text{Ligand}_{\text{Free}}][\text{Protein}_{\text{Free}}]}{[\text{Protein-Ligand}]}
\]

3.3. pH Studies

In preparation for the pH experiments SBP was dialyzed vs. 1 mM Tris-HCl pH 7.0 or 1 mM Tris-HCl, 10 µM Na$_2$CrO$_4$ pH 7.0. For most of the experiments, protein concentrations were determined spectrophotometrically using an extinction coefficient of 1.2 mg$^{-1}$cm$^{-1}$ml at 280 nm (Pardee, 1966). In cases where bound chromate would interfere with this method, protein concentration was determined using the Bio-Rad colorimetric assay with SBP of known concentration as a standard. SBP was then diluted approximately thirty times into the appropriate buffer — 40 mM Bis-Tris-HCl (pH 5.0 to pH 7.0) and 40 mM Tris-HCl pH 7.0 to pH 9.6). Sulfate binding activity was
assayed by the method of Pardee et al., (1966) as discussed above. Dissociation constants for chromate, and phosphate binding were determined using partition constants attained from competition experiments based on the quenching of tryptophan fluorescence, and the sulfate dissociation constants obtained as above. Dissociation constants for selenate were determined similarly except chromate was the primary ligand and selenate the displacing ligand.

Fluorescence measurements were performed at 20°C on an SLM/Aminco model 4800 spectrofluorometer. The sample was excited at \( \lambda = 295 \text{ nm} \) and spectra were taken from 310 nm to 350 nm. Primary ligand (sulfate or chromate) was added to a final concentration of 5 or 10 \( \mu \text{M} \) in a total volume of 3 ml. Displacing ligand (chromate or selenate) additions not exceeding 1% of the total volume were made with a 10 \( \mu \text{l} \) Hamilton syringe. The spectra were corrected for chromate absorbance (chromate titrations) and then integrated using the software supplied with the instrument.

Determination of the dissociation constants for the phosphates required extreme experimental conditions. Two samples of SBP were dialyzed vs. 1 M potassium phosphate buffer at pH 6.0 and 8.5 to replace bound sulfate with phosphate. Displacement of phosphate by chromate or sulfate was performed in 1 M phosphate buffer.

3.4. Off-rate Studies  Off-rate experiments were done on a Gibson-Durrum stopped-flow, rapid mixing apparatus equipped for fluorescence measurements. All experiments were done with 2 \( \mu \text{M} \) protein in 20 mM Tris pH 8.1, at 20° C, except some preliminary
studies which were done in 20 mM ammediol pH 7.6. Excitation wavelength was 285 nm, and emission was monitored at wavelengths greater than 350 nm.

Shown in Figure 4 is the increased quenching caused by \( \text{CrO}_4^{2-} \) binding. This is due to energy transfer from the excited tryptophan to \( \text{CrO}_4^{2-} \). Because sulfate and chromate cause different amounts of quenching it is possible to follow the displacement reactions in the stopped flow. The binding process with two competing ligands can be described by the following expression (Miller et al., 1980):

\[
\begin{align*}
&\text{PX} \xrightarrow{k_1} X + P + Y \xrightarrow{k_3} \text{PY} \\
&\text{Rate equation:} \\
&\frac{d(PX)}{dt} = PX \left[ \frac{k_1X(k_2 - k_4)}{k_1X + k_3Y + k_4} - k_2 \right] + \frac{k_1Xk_4P_0}{k_1X + k_3Y + k_4}
\end{align*}
\]  

The solution of this equation is:

\[
k_{obs} = k_2 - \frac{k_1X(k_2 - k_4)}{k_1X + k_3Y + k_4}
\]  

This expression can be simplified to:
Figure 4. Chromate binding to SBP. The addition of 20 μM chromate to 1 μM *S. typhimurium* SBP results in a significant decrease in fluorescence intensity due to radiationless energy transfer from excited tryptophan to chromate. Excitation wavelength was 290 nm.
When $X >> Y$ Eq. (4) simplifies to $k_{obs} = k_2$, and when $X << Y$ Eq. (4) simplifies to $k_{obs} = k_4$. $k_{obs}$ was calculated from the delta fluorescence data using the exponential fitting program ULTIMA2. $k_{obs}$ vs. $[\text{CrO}_4^{-}]/[\text{SO}_4^{2-}]$ was then plotted and fit to equation (4) using the fitting program CURFIT (Bevington, 1969).

4. Results and Discussion

4.1. Introduction

Previous ligand binding studies of SBP in solution by Pardee (Pardee, 1966) have shown that: 1) sulfate binding to SBP is pH insensitive over the range of pH 5.2 to pH 8.3; 2) chromate, which quenches SBP tryptophan fluorescence, can quantitatively and reversibly displace sulfate bound to SBP; 3) selenate inhibits sulfate binding; and 4) phosphate does not bind to SBP.

The purpose of this portion of this thesis was to extend these studies to determine the dissociation constants, and off-rates for these oxyanions and to investigate the effect of pH on their binding to SBP. This work demonstrates the exceptional substrate specificity of the SBP binding site and the narrow limits within which the protein can accommodate changes in the ionization state of the ligands.
4.2. The Sulfate-binding Site of SBP

As knowledge of the molecular geometry of the ligand-binding site of SBP was crucial to interpretation of the results of these studies, a brief description of the tertiary structure of the binding site based on the well-refined 2 Å structure (Pflugrath & Quiocho, 1985; 1988) is provided. The sulfate, which is completely sequestered deep in the cleft between the two lobes of the bilobate protein, is held tightly in place mainly by a total of seven strong hydrogen bonds, donated by five main-chain peptide NH groups, a serine hydroxyl, and the NH group of a tryptophan (Figure 5, and Table 1). While there are no counter-charges near the sulfate dianion, it has been proposed that the charges on the sulfate are stabilized by dipole interactions, especially those through hydrogen bonds formed with polarized peptide units (Pflugrath & Quiocho, 1985; Quiocho et al., 1987).

4.3. Sulfate and Selenate

The binding affinities of each of the four oxyanions which bind to SBP are shown in Figure 6 and Table 2. In view of the atomic interactions between sulfate and SBP, and the ligand characteristics outlined in Table 3, it is not surprising that sulfate and selenate binding affinities are pH independent over a wide pH range. As the conjugate bases of strong acids of the type MO₄²⁻ these ligands exist exclusively as dianions above pH 4. Moreover, there are no groups associated with the sulfate that have pKₐ's below 13.
Figure 5. The sulfate-binding site of SBP. View of the hydrogen bonds (thin lines) between the sulfate-binding protein and the sulfate as determined by extensive structure refinement at 2.0 Å (Pflugrath & Quiocho, 1986; 1988). Carbon atoms are gray, nitrogens are blue, oxygens red and sulfur is in yellow. Note that the protein provides only hydrogen bond donors, five of which come from main chain NH groups.
Table 1.  
*Hydrogen Bonds Between SBP and Bound Sulfate*

<table>
<thead>
<tr>
<th>Protein Atoms (Donor)</th>
<th>Sulfate Atoms (Acceptor)</th>
<th>Distance (Angstroms)</th>
<th>Angle°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser 130 OG</td>
<td>O-1</td>
<td>2.76</td>
<td>167</td>
</tr>
<tr>
<td>Ala 173 N</td>
<td>O-1</td>
<td>2.70</td>
<td>151</td>
</tr>
<tr>
<td>Ser 45 N</td>
<td>O-2</td>
<td>2.84</td>
<td>169</td>
</tr>
<tr>
<td>Gly 131 N</td>
<td>O-2</td>
<td>2.83</td>
<td>170</td>
</tr>
<tr>
<td>Gly 132 N</td>
<td>O-3</td>
<td>2.77</td>
<td>149</td>
</tr>
<tr>
<td>Trp 192 NE1</td>
<td>O-3</td>
<td>2.82</td>
<td>157</td>
</tr>
<tr>
<td>Asp 11 N</td>
<td>O-4</td>
<td>2.67</td>
<td>152</td>
</tr>
</tbody>
</table>

From Pflugrath & Quiocho, 1985
Figure 6. The pH dependence of the binding affinity for SBP—oxyanion complexes. The pH dependence of binding of sulfate (■), selenate (▲), and chromate (◇) to *S. typhimurium* sulfate-binding protein. The errors in the sulfate $K_d$s are the standard deviations of ten independent measurements at each pH. Others are estimated as ± 10% of the $K_d$ value.
Table 2.

*Affinities of the Binding of Tetrahedral Oxyacids to the Sulfate-binding Protein*

<table>
<thead>
<tr>
<th>Species</th>
<th>pH</th>
<th>$K_d$ (µM)</th>
<th>$\Delta G$ (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO$_4^-$</td>
<td>5.0 to 8.1</td>
<td>0.12</td>
<td>-9.3</td>
</tr>
<tr>
<td>SeO$_4^-$</td>
<td>5.0 to 8.0</td>
<td>5.0</td>
<td>-7.1</td>
</tr>
<tr>
<td>CrO$_4^-$</td>
<td>6.7 to 8.1</td>
<td>0.3</td>
<td>-8.7</td>
</tr>
<tr>
<td>HCrO$_4^-$</td>
<td>5.0</td>
<td>3.1</td>
<td>-7.4</td>
</tr>
<tr>
<td>HPO$_4^-$</td>
<td>8.5</td>
<td>$\sim 6 \times 10^4$</td>
<td>$\sim -1.6$</td>
</tr>
<tr>
<td>H$_2$PO$_4^-$</td>
<td>6.0</td>
<td>$\sim 1 \times 10^6$</td>
<td>$\sim 0$</td>
</tr>
</tbody>
</table>
Table 3.

Relevant Properties of Tetrahedral Oxyacids

<table>
<thead>
<tr>
<th>Oxyacid</th>
<th>Acid Dissociation Constants(^a)</th>
<th>M—O</th>
<th>Molar Volume(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pK_1) (pK_2) (pK_3)</td>
<td>(Å)</td>
<td>(cm(^3) mole(^{-1}))</td>
</tr>
<tr>
<td>Sulfate</td>
<td>(-3) 1.92 –</td>
<td>1.49(^c)</td>
<td>39</td>
</tr>
<tr>
<td>Selenate</td>
<td>(-3) 1.92 –</td>
<td>1.65(^d)</td>
<td>41</td>
</tr>
<tr>
<td>Chromate</td>
<td>0.74 6.49 –</td>
<td>1.60(^e)</td>
<td>41</td>
</tr>
<tr>
<td>Phosphate</td>
<td>2.12 7.21 12.67</td>
<td>1.54(^e)</td>
<td>39</td>
</tr>
</tbody>
</table>

\(^a\) Weast (1987).
\(^b\) Hückel, (1950).
\(^c\) Pauling (1960).
\(^d\) Zingaro & Cooper (1974).
\(^e\) Miller (1936).
What is perhaps surprising is that selenate affinity is 40-fold lower than sulfate (see Table 2). This difference, which was also observed in preliminary experiments done by Pardee (Pardee et al., 1966), remains unexplained. One possible explanation is the Se-O bond length, which is given in Table 3 as 1.65 Å (the value from the crystal structure of sodium selenate). The S-O bonds of the sulfate bound to SBP were stretched to 1.7 Å with a molecular modeling program to mimic an Se-O bond. Comparison of this model complex with the determined SBP-sulfate structure indicates little deformation of binding site geometry; the means of the seven hydrogen bond distances and angles in the model SBP-selenate complex are 2.67(0.15) Å and 153(10)°, respectively, which are to be compared with 2.76(0.07) Å and 159(9)° as determined in the SBP-sulfate complex (Pflugrath & Quiocho, 1985). Such differences could be compensated for by minor changes in the local conformation and/or in the relative orientation of the two domains which provide the hydrogen bonding groups.

Molecular modeling is, of course, only one way of looking at such a problem. SBP's adaptability to small variances in ligand size is confirmed by experiments with chromate (Cr-O = 1.6 Å). Above pH 7, the fully ionized chromate is an analogue of sulfate, and binds to SBP with near identical affinity though the Cr-O bond length is 0.1 Å longer than the S-O bond length of sulfate. Obviously there is sufficient inherent freedom of motion in the protein structure to accommodate such minute geometric variations. However, it is very likely not without cost. Chromate and selenate affinities may demonstrate the the cost of steric interactions in the binding site. Steric hindrance has
been implicated as the determining factor responsible for the 10,000-fold decrease in affinity seen in the S130C mutant to be discussed below.

4.4. Phosphate

The ligand specificity of SBP is dramatically demonstrated by the protein’s inability to bind either monobasic or dibasic phosphate to a significant extent. Although phosphate binding to SBP had not been previously described, phosphate binding has now been indirectly observed. Competitive displacement of phosphate by sulfate in 1 M potassium phosphate buffer resulted in an increase in total fluorescence and a blue shift of 5 nm, which did not occur in 1 M Tris buffer pH 9.6 (see Figures 7 & 8). Displacement of the phosphate by chromate resulted in fluorescence quenching similar to that seen in sulfate–chromate competition studies (see Figure 9). This strong quenching indicates that phosphate is binding to the tryptophan containing, sulfate specific site. Thus the extent of phosphate displacement by sulfate or chromate could be easily followed by fluorescence spectroscopy and the analysis was identical to the titrations described above.

Dibasic phosphate (HPO$_4^{2-}$) binds to SBP with an affinity at least 4 orders of magnitude lower than sulfate (SO$_4^{2-}$) or chromate (CrO$_4^{2-}$). What property of phosphate could allow SBP to distinguish it from sulfate or chromate so effectively? The longer P–O bond length (1.54 Å vs. 1.49 Å for sulfate) is not responsible for this difference (see above discussion). Nor can this difference be attributed to deformation of the geometry
Figure 7. Displacement of phosphate bound to SBP by sulfate. The fluorescence spectrum of 1 μM *S. typhimurium* SBP in 1 M potassium phosphate buffer pH 9.6 increases and exhibits a blue shift of 6 nm upon addition of 100 μM sulfate. Note the similarities to the treated SBP spectra (see Figure 16 and 17). Excitation wavelength was 290 nm.
Figure 8. SBP in 1 M Tris buffer. The fluorescence spectrum of 1 μM SBP in 1 M buffer pH 9.6 does not change upon the addition of 100 μM sulfate, indicating that under these conditions the protein is still saturated with sulfate. Excitation wavelength was 290 nm.
Figure 9. Displacement of phosphate bound to SBP by chromate. Similar to sulfate-bound SBP (see Figure 4) the fluorescence intensity of 1 μM *S. typhimurium* SBP in 1 M potassium phosphate buffer pH 9.6 decreases significantly upon addition of 20 μM chromate. Excitation wavelength was 290 nm.
around the phosphorus due to protonation. Neutron and X-ray diffraction studies have shown that protonated phosphates remain tetrahedral and P–O bond lengths of the protonated oxygens deviate from those for the unprotonated oxygens by less than 0.1 Å (Bacon & Pease, 1955; Smith et al., 1955).

Phosphate does differ from sulfate in one very important respect — protonation. The deleterious effect of a protonated oxyacid on binding can be explained by the X-ray structure of the SBP-sulfate complex. The binding site contains only protein groups able to donate hydrogen bonds to oxygen atoms of the dianions; there are no residues, especially close to the sulfate oxygens, which could accept an ‘active’ hydrogen from the protonated phosphate and chromate. Such a proton could also destabilize the SBP-ligand complex by interfering with the formation of the requisite hydrogen bonds shown in Figure 5.

The oxygen atom of the γ-OH of Ser130 could be proposed as a potential hydrogen bond acceptor, but the use of the γ-hydroxyl oxygen as an acceptor would be unsatisfactory as it does not solve the basic problem of an unpaired hydrogen bond donor group buried in the binding site. Donation of a hydrogen bond from a protonated oxyacid to the oxygen of the γ-OH of Ser130 would require rotation of the hydroxyl proton away from the oxyacids. Rotation of the hydroxyl proton could disrupt the H-bond donated from NH 133. Concomitantly and more importantly, the now ‘active’ hydroxyl proton must be stabilized, but the SBP structure indicates no hydrogen bond acceptor for this proton either.
4.5. Chromate

Chromate binding studies indicate protonation can not be the sole ligand characteristic which affects binding to SBP. As shown in Figure 6, chromate binding shows a singly ionizing system which approximately follows the pH titration curve for the species \( \text{HCrO}_4^- \) to \( \text{CrO}_4^{2-} \). At high pH's chromate is in the dianionic \( \text{CrO}_4^{2-} \) form and its affinity for SBP in this form is not significantly different from that of sulfate. However, below pH 6.0, where significant amounts of chromate exist in the \( \text{HCrO}_4^- \) form, its affinity for SBP is about 10-fold less than in its unprotonated form, but not \( >10^4 \)-fold as seen between \( \text{SO}_4^{2-} \) and \( \text{HPO}_4^{2-} \). Though protonated chromate is geometrically similar to dibasic phosphate, its net charge is only \((-1)\) as opposed to \((-2)\) for the dibasic phosphate. This decreased charge could represent a significant reduction in the energy required to stabilize the SBP-\( \text{HCrO}_4^- \) complex. Hence at low pH, chromate represents a trade-off between an unfavorable proton and a favorable reduction in net charge. The resultant increase in \( K_d \) (decreased affinity) indicates that the effect of a proton dominates slightly. Interestingly, a similar decrease in affinity is also observed in going from \( \text{HPO}_4^{2-} \) to \( \text{H}_2\text{PO}_4^- \).

The pH dependence of chromate is not due to the formation of dichromate at low pH. Although the chromate–dichromate equilibrium tends toward dichromate at low pH, the low chromate concentrations used in these experiments strongly favor the chromate species (Cotton & Wilkinson, 1980); thus, the reduced affinity of SBP for
chromate at low pH (see also Figure 6) can not be attributed to a decrease in chromate concentration due to dichromate formation. The formation of pyrophosphate from orthophosphate does not occur under the conditions of these experiments (Cotton & Wilkinson, 1980).

4.6. Off-rates

The difference in chromate binding affinity seen with decreasing pH was attributed to destabilization of the SBP-HCrO$_4^-$ complex (an off-rate effect) in the above discussion. However, this could merely be the effect of decreasing the true concentration (or activity) of the CrO$_4^{2-}$ species (an on-rate effect). The relative magnitudes of the sulfate, chromate and selenate off-rates, as well as the pH dependence of the chromate off-rate and the pH independence of the sulfate and selenate off-rates shown in Figure 10 demonstrate that the affinities of sulfate, chromate and selenate are due to differences in their off-rates. Note that the difference in the off-rates (3 s$^{-1}$ for sulfate vs. 14 s$^{-1}$ for chromate at pH 9, and 106 s$^{-1}$ for chromate at pH 5), and selenate (100 s$^{-1}$) agree with the differences in affinity (0.12 μM for sulfate vs. 0.3 μM for chromate at pH 9, 3.1 μM for chromate at pH 5 and selenate 5.0 μM). These results prove conclusively that the HCrO$_4^-$ species binds to SBP, and that its decreased affinity is indeed due to the deleterious effect of a non-hydrogen-bonded proton in the binding site.

The unfavorable effect of protonated oxyacids, and that sulfate and selenate exist exclusively as fully ionized dianions above pH 4, and that their affinities are pH
Figure 10. The pH dependence of the off-rate for SBP—oxyanion complexes. Note the pH independence if the sulfate (■) and selenate (▲) off-rates. In contrast the chromate (◆) off-rate exhibits a pH dependence attributable to the $pK_2$ (6.6) of chromic acid. The relative magnitudes of the off-rates for these oxyanions indicate that off-rate is the determining factor of relative binding affinity.
insensitive, completely rule out the possibility of HSO$_4^-$ or HSeO$_4^-$ forming a stable complex with SBP over the range of pH investigated.

4.7. Protonation

The computed binding energies listed in Table 2 allow the effect of charge and protonation state on oxyacid binding to SBP to be quantitated. The similar differences in binding energies between CrO$_4^{2-}$ (−8.7 kcal/mole) and HPO$_4^{2-}$ (−1.6 kcal/mole) and between HCrO$_4^-$ (−7.4 kcal/mole) and H$_2$PO$_4^-$ (− 0 kcal/mole), which amount to ΔΔG ≈ 7 kcal/mole in both cases, represent the unfavorable effect of an ‘active’ proton in the binding site. Comparing this difference with those between HCrO$_4^-$ and CrO$_4^{2-}$ (1.3 kcal/mole) and H$_2$PO$_4^-$ and HPO$_4^{2-}$ (− 1.6 kcal/mole), attributed to a trade-off between a deleterious proton and an advantageous decrease in charge, shows the gain in stabilization seen with a decreased charge is approximately −5.7 kcal/mole. To our knowledge this is the first quantitative estimate of the effect of an nonhydrogen-bonded ‘active’ proton and ligand charge on binding affinity.
CHAPTER 3

SITE-SPECIFIC MUTAGENESIS OF THE SULFATE-BINDING PROTEIN

5. Introduction

Although a sulfate-binding protein from *E. coli* has never been described, an *E. coli* gene for a sulfate-binding protein has been sequenced by Hellinga (Hellinga & Evans, 1985). This sequence, identified as encoding a sulfate-binding protein by computer database search, predicts a seventeen residue leader peptide in addition to the 310 amino acid final product. The leader peptide is believed responsible for directing the protein to the periplasmic space and is cleaved after the protein enters the periplasm. Appendix II contains a comparison of the amino-acid sequence predicted by the *E. coli* DNA sequence with the corrected version of the chemically determined *S. typhimurium* SBP sequence (Pflugrath & Quiocio, 1988), as well as that predicted by the sequence of the *E. coli* gene determined as part of this thesis. Figure 11 shows the 20 differences in the *E. coli* sequence mapped onto the *S. typhimurium* structure. Note that these differences are for the most part confined to the perimeter of the protein where they would not be expected to interfere with binding activity. Furthermore, the majority of the mutations are of a conservative nature (e.g. phe ⇒ tyr).
Figure 11. Differences in *E. coli* sequence mapped to *S. typhimurium* structure. The α-carbon backbone is shown in red. The sulfate (blue) is bound in the cleft between the two domains. Amino acids in the *S. typhimurium* structure which differ in the *E. coli* protein are highlighted in yellow.
LB has been studied extensively since it was first described in 1966 (see introduction to Chapter 2). The depth of the knowledge gained in these past twenty-four years shows the potential of a small but expanding number of proteins whose function can be studied in detail, the rational design of ligands and substrates, and the elucidation of their specific mechanism.

The roles of many of the proteins of the ligase system in the processes of conjugation remain unclear as do the roles of the proteins of the recombination system. (Myers and Nester [15]). This is not unexpected in view of the complex machinery involved. The nature of the systems is unknown, and the roles of the proteins are only beginning to be understood. The first step in unraveling the intricate relationship between the proteins of the systems and the functions of their substrates is the determination of their three-dimensional structures.
SBP has been studied extensively since it was first described in 1966 (see Introduction to Chapter 2). The depth of the knowledge gained in these past twenty-four years allows SBP to be included with a small but growing number of proteins whose function can be discussed at the atomic level. Though the structure is known in great detail, the proposed mechanisms for binding and SBP-SO	extsubscript{4}^{2-} complex stabilization require experimental confirmation.

The purpose of this portion of this thesis was to further understand the relationship between structure and function in SBP. Conventional biochemical techniques and site-specific mutagenesis were used to address specific questions regarding protein-ligand complex stabilization, and binding mechanism. Based on the refined 2.0 Å structure of \textit{S. typhimurium} SBP, twelve site-directed mutants were designed to test specific hypotheses regarding protein-ligand complex stabilization, and binding mechanism. The residues His 42, Ser 130, Glu 15, and Asp 68 were chosen because of their proposed roles in sulfate binding. A disulfide was also introduced between the domains to test the hinge-bending model.

6. Materials and Methods

6.1. Cell Strains

\textit{E. coli JM101}: supE, thi, Δ(lac-proAB), [F’, traD36, proAB, lacI\textsuperscript{Q}ZΔM15]. This strain was created by Joachim Messing (Yanisch-Perron \textit{et al.}, 1985) for use with the pUC plasmids, and the M13 derived bacteriophage. JM101 was used for growth and
maintenance of the original *sbp* containing plasmid pHE1005 kindly provided by P. Evans, the *sbp* containing M13-mp8 derivative, and the pKK223-3 derived expression system.

**E. coli BW313:** HfrKL16 PO/45 [lysA(61-62)], *dut1, ung1, thi1, relA1*. This is an *ung* strain obtained from Kunkel (Kunkel *et al.*, 1987) which can be used to generate uracil containing DNA for use in site-specific mutagenesis.

**E. coli RZ1032:** As BW313, but Zbd-279::Tn10, *supE44*. This strain carries an amber suppressor which allows it to be used as a host for M13 vectors (Kunkel *et al.*, 1987). This strain was used to generate the uracil containing templates.

### 6.2. Site-specific Mutagenesis

The oligonucleotidenucleotide primers used for site-directed mutagenesis and their sources are listed in Table 4. In addition to the oligonucleotide primers five other oligonucleotides spaced approximately every 200 base-pairs on the *sbp* gene were synthesized for use as sequencing primers.

Site-specific mutagenesis was performed using the method devised by Kunkel (Kunkel *et al.*, 1987). Certain aspects of the procedure were very sensitive for the SBP system. Therefore, detailed protocols for each stage of the mutagenesis are provided.
Table 4.

*Oligonucleotides for Site-directed Mutagenesis*

<table>
<thead>
<tr>
<th>Old Residue</th>
<th>New Residue</th>
<th>Old Codon</th>
<th>New Codon</th>
<th>Oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>SER 130</td>
<td>GLY 130</td>
<td>AGC</td>
<td>GGC</td>
<td>5'ACCAGAGCCTTTCCGGATTAa</td>
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<tr>
<td>CYS 130</td>
<td>AGC</td>
<td>TGC</td>
<td></td>
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<tr>
<td>GLU 15</td>
<td>GLN 15</td>
<td>GAA</td>
<td>CAA</td>
<td>5'CGTACAATTGGCGCGTGAa</td>
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<tr>
<td>ASP 68</td>
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<td>GAC</td>
<td>AAC</td>
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<tr>
<td>GLY 68</td>
<td>GLY 68</td>
<td>GAC</td>
<td>GGC</td>
<td>5'CGCAATTTGCAGCCATAGCCAGb</td>
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<tr>
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<tr>
<td>SER 129</td>
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<td>TGC</td>
<td>5'GCCACCGCATTTCGGATb</td>
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<tr>
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<td>GLY 42</td>
<td>CAC</td>
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<td></td>
<td>5'GCCACCAGCCTCCGACTGACG3a</td>
</tr>
<tr>
<td>ASP 42</td>
<td>CAC</td>
<td>GAC</td>
<td></td>
<td>5'GCCACCAGCTCTGACTGACG3b</td>
</tr>
</tbody>
</table>

*a* From K. S. Matthews' Lab at Rice University. Oligonucleotides were deblocked overnight, dried and resuspended in TE prior to use.

*b* From Dr T. Caskey's Lab at Baylor College of Medicine. No treatment was necessary.
6.3. Purification of Single-Stranded DNA.

Two methods were used for the purification of single-stranded DNA. The choice of method depended on whether the DNA was to be used for sequencing or mutagenesis reactions.

For mutagenesis, 50 ml of LB was inoculated with 1 ml of RZ1032 and 10 μl of phage supernatant. The culture was grown at 37° C for four hours. Longer growth times invariably resulted in spontaneous deletion of significant portions of the SBP gene. Cells were removed by centrifugation (8000 x g, 10 minutes 4° C). 10 ml of the supernatant were removed and 2.4 ml of 20% PEG were added and mixed well. The solution was cooled for 10 minutes on ice, then centrifuged (8000 x g, 10 minutes 4° C). The supernatant was decanted and the pellet centrifuged again briefly to remove residual PEG solution which clings to the sides of the centrifuge tube. The additional spin allows this PEG to be easily removed with a Pipetman. The pellet was resuspended in 200 μl TE, phenol extracted twice, ether extracted twice and ethanol precipitated. The ethanol pellet was dried, and resuspended in 200 μl TE.

For sequencing, 2 ml of LB were inoculated with 50 μl JM101 (previously grown overnight in LB) and a single phage plaque picked from a culture plate. Culture was grown at 37° for four hours. 1 ml of culture was removed to a 1.5 ml Eppendorf tube. Cells were removed by centrifugation in a microfuge (18000 x g, 5 minutes). Supernatant was removed to a new tube, and 240 μl of 20% PEG added. The solution was mixed well, cooled on ice for 10 minutes, then centrifuged for 10 minutes in a microfuge
(18000 x g). The supernatant was removed and the pellet centrifuged briefly to remove residual PEG. The pellet was resuspended in 100 µl TE, phenol extracted twice, ether extracted twice, and ethanol precipitated. The ethanol pellet was dried and resuspended in 20 µl TE. This prep yielded sufficient DNA for three sequencing reactions.

6.4. Mutagenesis Reaction

In a sterile Eppendorf tube 2 µl kinase buffer pH 7.5, 2 µl 0.1 M dithiothreitol, 2 µl 10 mM ATP, 9 ng oligonucleotide, 1 µl kinase, were combined and water added to 20 µl. The reaction was incubated at 37° C for 1 hour. 1.2 µl 20X SSC and 2 µl (1 µg) U-containing single-stranded template were added to the phosphorylated oligonucleotide. The mixture was heated to 75°, and cooled slowly to room temperature. To the annealing mix was added 10 µl 10X polymerase buffer (0.33 M Tris-Acetate pH 7.9, 0.66 M potassium acetate, 0.10 M magnesium acetate, 1 mg/ml bovine serum albumin), 10 µl 0.1 M dithiothreitol, 10 µl 10 mM ATP, 1 µl T4 DNA polymerase (2.5 units), and 1 µl (2 units) T4 DNA ligase. The reaction was incubated 5' on ice, 5' at 15° C, 1 hour at 37° C. Alternatively, the synthesis reaction could be incubated at 15° C overnight. The latter of these methods was more consistently succesful. The entire reaction was used to transform 400 µl of competent JM101 prepared by the method of Kushner (1978). Candidate plaques were screened by sequencing. Mutation efficiency was typically 30-50%. DNA shown by sequencing to contain a mutant SBP gene was used to transform JM101 for plaque purification and resequencing. Confirmed mutants were transformed and plated. The entire SBP gene was sequenced for each mutant to ensure that additional
unplanned mutations had not occurred. The sequencing strategy is shown in Figure 12.

Replicative (double-stranded) forms of phage shown to contain a mutant SBP gene were purified using the WL lysis method described below. Fifty ml cultures of LB were inoculated with 1 ml JM101 and one plaque picked from a culture plate. Cultures were grown for four hours, and the cells pelleted by centrifugation (8000 x g, 10 minutes). Cells were resuspended in 800 µl WL lysis buffer (25 mM Tris-HCl pH 7.6 10 mM EDTA). 240 µl of 10 mg/ml lysozyme were added and the solution incubated on ice for seven minutes. The solution was divided in two, and 750 µl of Brij lysis mix (1% Brij 58, 0.4% sodium deoxycholate, 0.625 M EDTA, 0.05 M Tris HCl pH 8) and 50 µl of 5M NaCl were added to each tube. Tubes were spun in a microfuge for 15' at 18000 x g. Supernatant was removed to four new tubes and to each was added 125 µl of 50% PEG and 125 µl of 5 M NaCl. Tubes were mixed well, cooled on ice for 10 minutes and centrifuged in a microfuge for 10 minutes. Pellets were resuspended in 200 µl of TE total, phenol extracted twice, ether extracted twice, ethanol precipitated, dried and resuspended in 100 µl of TE.

High level expression of *E. coli* SBP and mutants was accomplished by creating a hybrid protein consisting of the control region and the leader peptide from the arabinose-binding protein coupled to SBP and inserting it into the expression vector pKK223-3 from Pharmacia (a Tac system (Brosius & Holy, 1984)). The construction of this system is shown schematically in Figure 13. This novel approach was necessary because SBP produced by a similar system which contained the control and leader
Figure 12. Sequencing Strategy. Five oligonucleotides were used for sequencing the SBP gene. The numbers in small type identify the oligonucleotide primer by name. The arrows indicate the approximate region of the SBP gene which can be sequenced using the respective primer.
Figure 13. Creation of the SBP Expression System. The SBP gene was originally received from P. Evans as an insert in pUC9. The SBP and CDH (CDP-diglyceride hydrolase) were subcloned into M13mp9. The Nco I site, which allows transfer of the SBP gene (minus the promoter and leader regions) to the ABP expression system, was made by site-specific mutagenesis. The cloning of SBP into the ABP expression system was accomplished with the assistance of Jing Jiu He in our lab.
Introduction of an Nco I site via Site-specific Mutagenesis

Clone Nco I - Pst I fragment into Nco I - Pst I cleaved pKK-ABP
sequences from the SBP gene was not efficiently transported into the periplasm. This was shown by electrophoretic comparison of the supernatant from osmotically-shocked cells with cells subjected to total lysis with SDS. Conveniently, ABP and SBP have identical leader cleavage recognition sites (Met-Ala). Electrophoretic comparison of SBP prepared with this hybrid system with *Salmonella* SBP showed them to be identical indicating that the ABP leader region is efficiently cleaved.

Mutant SBP genes created by site-specific mutagenesis were subcloned into this expression system as shown in Figure 14. An efficient method for screening expression candidates was developed based on the Pharmacia PhastSystem electrophoresis system. Eight two ml cultures from single candidate colonies were grown for six hours in LB containing 30 μg / ml AMP. One ml of each culture was removed to a 1.5 ml eppendorf tube. Cells were pelleted by centrifugation for one minute in a microfuge (18000 x g), and resuspended in 100 μl TE. Cells were lysed by addition of 100 μl of SDS loading buffer. Note that the cells were NOT boiled; this mild SDS lysis frees mainly periplasmic proteins. Presence of SBP in lysates was determined by SDS-PAGE.

Candidates that showed SBP production in the SDS lysis test were further confirmed by a small osmotic shock prep. Another two ml culture was grown for six hours in LB containing 30 μg / ml AMP. One ml of each culture was removed to a 1.5 ml eppendorf tube. Cells were pelleted by centrifugation for one minute in a microfuge (18000 x g), and resuspended in 10 μl 200 mM Tris-HCl, pH 8. 10 μl of 200 mM Tris-HCl, pH 8 + 1 M sucrose and 0.3 μl 0.1 M EDTA, pH 8 were added and the solution
Figure 14. Subcloning into the SBP expression system. SBP mutants, made in M13, were transferred to the pKK-ABP expression system by subcloning the Nco I-Pst I fragment into Nco I-Pst I cleaved pKK-ABP.
Creation of a Mutant SBP via Site-specific Mutagenesis

Clone Nco I - Pst I fragment into Nco I - Pst I cleaved pKK-ABP

M13-Nco

M13-Nco (Mutant)

pKK-SBP (Mutant)
incubated at room temperature for 10 minutes. 0.6 μl 10 mg / ml lysozyme made fresh in water were added followed by immediate addition of 20 μl distilled water. The solution was mixed gently and incubated for 30 minutes. Add 0.8 μl 1 M MgSO4 to stabilize the spheroplasts. Spin at 18000 x g in microfuge for 10 minutes. The supernatant was tested by SDS-PAGE and antibody reaction for the presence of SBP.

6.5. Growth and Purification of *E. coli* SBP and Mutants

A small starter culture (approximately 100 ml) of JM101 containing the appropriate plasmid was inoculated from a single colony and grown overnight in Luria Broth (LB) (10 grams Bacto-Tryptone, 10 grams NaCl, 5 grams Yeast Extract, per liter) containing 30 μg / ml ampicillin. Twelve liters of LB plus 30 μg / ml ampicillin were each inoculated with 5 ml of this culture. Growth was performed overnight in a New Brunswick G-25 incubator shaker with the temperature maintained at 37° C, and the flasks agitated at a rate of 225 rpm.

The cell culture was harvested by centrifugation (20 min 6000 x g) at room temperature; the cell pellets from 12 liters of culture were resuspended in 60 ml total 200 mM Tris-HCl, pH 8. Resuspended cells were combined in a 250 ml centrifuge bottle. 60 ml of 200 mM Tris-HCl, pH 8 + 1 M sucrose and 1.5ml 0.1 M EDTA, pH 8 were added and the mixture equilibrated for 10 minutes. 2.4 ml 10 mg / ml lysozyme in water (fresh) were added followed immediately by 120 ml distilled water. The solution was mixed gently. Lysis was typically complete in 30 min at which time 4.0 ml 1 M MgSO4
was added to stabilize the spheroplasts. The shocked cells were centrifuged at 23000 x g for 60 min. The supernatant, which contained the periplasmic proteins, was removed to a clean flask and 0.02% azide added to store. At this point a small sample of the shockate was used to confirm the presence of SBP by SDS-PAGE and by antibody reaction with rabbit anti-SBP. The shockate was dialyzed vs. 1 mM potassium phosphate pH 7.6, 0.02% azide. Further purification was accomplished by column chromatography through DEAE-53. The dialyzed shockate was loaded onto a DEAE-53 column. SBP was eluted with a 250 ml x 250 ml 0-50 mM KCl gradient. Often the SBP was found in the flow-through. Fractions containing SBP were identified by electrophoresis and antibody reaction, combined, concentrated with an Amicon ultrafiltration system, and dialyzed vs 10 mM Tris pH 7.2, 0.02% NaN₃. Dithiothreitol (100 µM) was added to all buffers when mutant proteins contained cysteine(s). Final purification was accomplished by HPLC through a Synchrom Q300 anion exchange column. SBP was eluted with a 0-50 mM potassium acetate gradient. A 12-liter prep typically yielded approximately 100 mg of pure protein.

6.6. Cysteine Determination The presence of cysteine in the S130C, S129C, G46C, and mutants was confirmed by reaction with 5′5′ dithionitrobenzoic acid (DTNB). Previous to this reaction fresh dithiothreitol was added to the protein to a final concentration of 1 mM to ensure the cysteine was in its maximally reduced form. Dithiothreitol was removed by passage through a P6DG acrylamide based desalting column. Collected protein was purged with nitrogen, tightly capped, and used within one hour.
A series of L-cysteine standards (10-100 μM) was prepared in assay buffer (0.1 M Tris pH 8 for native conditions and 0.1 M potassium phosphate, 6 M guanidine-HCl, 1 mM EDTA pH 8 for denaturing conditions) and used to create a calibration curve. To 1 ml of each of the cysteine standards was added 50 μl of 11 mM DTNB (prepared fresh in 0.1 M Tris pH 8). After mixing, (reaction was typically immediate) the absorbance at 412 nm was determined against a blank containing 1 ml of buffer and 50 μl DTNB. The calibration curve was calculated using the software supplied with the IBM 9420 spectrophotometer.

For native protein determinations, 50 μl of DTNB were added to 1 ml of protein (typical concentration 30 μM). After mixing the absorbance at 412 nm was measured and extent of reaction was determined from the above calibration curve.

For cysteine determination under denaturing conditions, 50 μl of DTNB was added to 900 μl of 0.1 M potassium phosphate, 6 M guanidine-HCl, 1 mM EDTA pH 8 in each of two cuvettes. After mixing, these samples were used to blank the spectrophotometer. To one cuvette, 100 μl of concentrated protein (200 μM) was added to the other 100 μl of buffer. The solutions were mixed thoroughly and the absorbance determined at 412 nm. The extent of reaction determined from the above calibration curve.

6.7. Disulfide Determination

The disulfide between Cys 46 and Cys 129 in the Cys 46/Cys 129 mutants was confirmed by the method of Iyer (Iyer & Klee, 1973). Cys 46/Cys 129 was concentrated
to 4 x 10^{-4} \text{ M} \text{ in 100 mM potassium phosphate buffer pH 8.0. One ml of concentrated }
protein was placed in the spectrophotometer light path with buffer as blank. The starting
A_{310} was noted. Reaction was started by adding 10 \mu l of 0.1 M dithiothreitol. Reaction
extent was monitored spectrophotometrically at 310 nm. The reaction was nearly
instantaneous under denaturing conditions. Under native conditions the reaction was
complete in less than a minute.

6.8. Chemical Modification 500 \mu l of 1 mg / ml G46C protein in 70 mM potassium
phosphate pH 8 and 1 \mu l 100 \mu M dithiothreitol were added to 500 \mu l of 40 mM 4'-
((iodoacetyl)amino)methylfluorescein (Fluorescein) in water. Note that the added
fluorescein was in significant excess, adequate to compensate for competition by
dithiothreitol. The reaction was allowed to proceed overnight in the dark. Fluorescein
labelled protein was purified by passage through a P6DG desalting column. Due to
interactions between the fluorescein and the acrylamide column, unlabelled protein was
also separated from labelled protein by this column. The degree of retardation and order
of elution was: unlabelled protein < labelled protein << unincorporated fluorescein.

6.9. Resin Assay

The resin assay was performed as described in the Materials and Methods section
of Chapter 2.
6.10. Fluorescence Titrations

Fluorescence titrations were performed on an SLM Aminco 4800 spectrofluorometer. The excitation wavelength was 290 nm and emission at $\lambda = 325$ for the treated proteins and $\lambda > 350$ nm for the untreated proteins was observed by using the monochromator or a $\lambda > 350$ nm pass filter, respectively. Second order light (2 x the excitation wavelength) was also filtered with a 300 nm cut-off filter. Temperature was maintained at 20° C with a circulating water bath. Protein concentration was 1 $\mu$M in all cases. The cuvette was capped with a rubber septum and purged with nitrogen immediately before use. Sulfate additions were made with a Hamilton syringe and did not exceed 5% of the total volume (2 ml). Dissociation constants were determined by Scatchard analysis. For the S130C pH studies protein was first dialyzed vs. 1 mM Tris-HCl pH 7.0, then diluted approximately 30 times into the buffer of appropriate pH (40 mM Bis-Tris-HCl (pH 5.0 to pH 7.0) and 40 mM Tris-HCl pH 7.0 to pH 9.6)
7. Results and Discussion

The purpose of the work described in this portion of this thesis was to further understanding of the relationship between structure and function in SBP. Conventional biochemical techniques and site-specific mutagenesis were used to address specific questions regarding protein-ligand complex stabilization, and binding mechanism. Based on the refined 2.0 Å structure of *S. typhimurium* SBP, twelve site-directed mutants were designed to test specific hypotheses regarding protein-ligand complex stabilization, and binding mechanism. The residues His 42, Ser 130, Glu 15, and Asp 68 were chosen because of their proposed roles in sulfate binding. A disulfide was also introduced between the domains to test the hinge-bending model. A typical autoradiograph of a sequencing gel showing the sequence of the mutated region for E15Q is provided in Figure 15. Mutants H42N, H42G, S130C, S130AS, and S130G were provided by Jing Jiu He.

7.1. Expression and Purification of SBP Mutants

High level expression of *E. coli* SBP and mutants was accomplished by creating a hybrid protein consisting of the control region and the leader peptide from the arabinose-binding protein coupled to SBP and inserting it into the expression vector pKK223-3 from Pharmacia (a Tac system (Brosius & Holy, 1984)). This novel approach was necessary because SBP produced by a similar system which contained the control and leader sequences from the SBP gene was not efficiently transported into the
Figure 15. Autoradiograph of a sequencing gel showing the mutated region of the E15Q.
periplasm. Electrophoretic comparison of SBP prepared with this hybrid system with *Salmonella* SBP showed them to be identical indicating that the ABP leader region is efficiently cleaved. A 12-liter prep of JM101 containing this expression plasmid typically yielded 100 mg of pure protein as shown by a single band on SDS-PAGE.

### 7.2. Fluorescence Titrations

As with all binding proteins SBP was purified with bound ligand. The ubiquitous nature of sulfate made it impossible to prepare sulfate-free protein by previously described methods (Miller *et al.*., 1980, 1983). However, SBP dialyzed vs. 10 μM Na$_2$CrO$_4$ in 40 mM Tris pH 8, then treated with 10 mM dithiothreitol or 1 mM sodium borohydride one hour before use, appears to be ligand-free.

The fluorescence spectrum of each of the proteins with and without sulfate before and after treatment to remove ligand are shown in Appendix III, and a typical example is shown in Figures 16 and 17. Although some of the mutants appear to have been purified sulfate-free (D68G, D68N, SBP-Dis, E15Q), there is evidence that the treated form is the true ligand-free species. All the treated proteins could be successfully titrated with sulfate while the mutants, and a guanidine-treated SBP (which yields an identical fluorescence spectra as the low affinity mutants, see Figure 18) gave anomalously greater than stoichiometric fluorescence changes at initial low concentration additions. Titrations of the treated, and untreated forms of D68G are shown in Figure 19. Furthermore the $K_d$s obtained from titration of treated protein agree well with those obtained by
Figure 16. Fluorescence spectra of untreated *Salmonella typhimurium* SBP. The addition of 10 mM sulfate to 1 μM *Salmonella typhimurium* SBP in 40 mM Tris pH 8 results in no change in the fluorescence spectrum. Excitation wavelength was 290 nm.
Figure 17. Fluorescence spectra of treated *Salmonella typhimurium* SBP. Fluorescence intensity of 1 μM DTT treated *Salmonella typhimurium* in 40 mM Tris pH 8 decreased upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
Figure 18. Guanidine-treated SBP fluorescence spectra. The addition of 10 mM sulfate to *S. typhimurium* SBP treated with 3 M guanidine to remove sulfate results in a 6 nm blue shift and a slight increase in fluorescence intensity. Spectra provided courtesy of P. Vermersch in our lab. Excitation wavelength was 290 nm.
Figure 19. Comparison of the titrations of untreated (♦) and treated D68G (■). Note that in the untreated titration the first two additions result in a greater fractional saturation (Y) than predicted by stoichiometry.
the resin assay.

The measured dissociation constants, the $\Delta G$ of binding and off-rates for sulfate for all the SBP mutants in this study are listed in Table 5. Scatchard plots of the fluorescence titrations and off-rate traces are shown in Appendix IV.

7.3. His 42

His 42 is an essential residue in the resonating hydrogen bond array from O-4 of the sulfate shown in Figure 5. According to the proposed model this array "connects" the positively charged histidine and the negatively charged sulfate, thus aiding the neutralization of the $(-2)$ charge of the $\text{SO}_4^{2-}$. His 42 is also exposed to solvent which could also play a role in stabilization of the negative charge.

The three mutations at this position (H42N, H42D, and H42G) were designed to constitute a series of related changes. Replacement of His 42 with Asn will preserve the local hydrogen bonding but remove the potential for positive charge. Replacing His 42 with Gly will remove both the charge and disrupt local hydrogen bonding. The final change of His to Asp was expected to have a profound detrimental effect on binding affinity because aspartate can not participate directly in the H-bond array and due to the placement of a negative charge at the end of the proposed H-bond array.

The results were not wholly consistent with the proposed role of His 42. Because H42N, and H42D had little effect on binding affinity, the positive charge of His 42 can
Table 5. Equilibrium Binding and Kinetic Constants for Wild-type and Mutant SBP—Sulfate Complexes. The < sign in front of several of the $K_d$s indicates the value is an upper limit due to non-ideal titration conditions (see Appendix IV). Data for S130A, and S130G mutants was provided by Jing Jiu He. Measurement of the off-rates for H42N, and H42G were not possible due to insufficient protein available at the time these experiments were done. It was not possible to determine the off-rates for S130C due to technical considerations.
Table 5.  
Equilibrium Binding and Kinetic Constants for Wild-type and Mutant SBP—Sulfate Complexes

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_d$ (μM)</th>
<th>Resin Assay</th>
<th>Fluorescence</th>
<th>$\Delta G$</th>
<th>$\Delta \Delta G$</th>
<th>$k_{off}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. typh. SBP</strong></td>
<td>0.12 (0.03)</td>
<td>&lt;0.7</td>
<td>-9.3</td>
<td>—</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>E. coli SBP</strong></td>
<td>0.16 (0.03)</td>
<td>&lt;0.6</td>
<td>-9.1</td>
<td>—</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>S130C</td>
<td>—</td>
<td>$1 \times 10^3$ ($1 \times 10^2$)</td>
<td>-4.0</td>
<td>5.1</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>S130A</td>
<td>—</td>
<td>18</td>
<td>-6.4</td>
<td>2.7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>S130G</td>
<td>—</td>
<td>3</td>
<td>-7.4</td>
<td>1.7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>H42N</td>
<td>0.22 (0.03)</td>
<td>—</td>
<td>-8.9</td>
<td>0.2</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>H42G</td>
<td>1.8 (0.1)</td>
<td>3.5</td>
<td>-7.7</td>
<td>1.4</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>H42D</td>
<td>0.26 (0.04)</td>
<td>&lt;0.3</td>
<td>-8.8</td>
<td>0.3</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>D68N</td>
<td>0.8 (0.1)</td>
<td>0.9</td>
<td>-8.2</td>
<td>1.0</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td>D68G</td>
<td>0.8 (0.1)</td>
<td>1.1</td>
<td>-8.2</td>
<td>1.0</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>E15Q</td>
<td>0.42 (0.06)</td>
<td>&lt;0.7</td>
<td>-8.6</td>
<td>0.5</td>
<td>235</td>
<td></td>
</tr>
<tr>
<td>S129C</td>
<td>0.35 (0.04)</td>
<td>&lt;0.5</td>
<td>-8.7</td>
<td>0.4</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>G46C</td>
<td>0.20 (0.05)</td>
<td>&lt;0.5</td>
<td>-8.9</td>
<td>0.2</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>S129C &amp; G46C</td>
<td>1.4(0.3)</td>
<td>1.6</td>
<td>-7.8</td>
<td>1.3</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>SBP-Dis</td>
<td>0.10 (0.01)</td>
<td>—</td>
<td>-9.4</td>
<td>-0.3</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>
be considered nonessential whereas the effect of the H42G mutation implies that the fidelity of the array is more important. Thus the role of residue 42 is not to provide a positive charge to offset the (-2) charge of the sulfate but to complete the resonating H-bond array which enables the charge of the sulfate to be dissipated to solvent. This new hypothesis requires further testing. Removal of a large side-chain such as histidine creates the potential for rearrangement of local structure. The determination of the X-ray structures of these mutants will be essential to the evaluation of the importance of His 42, particularly in the case of the H42G mutant.

7.4. Ser 130

According to the proposed model for sulfate binding to SBP, Ser 130 plays an important role in complex stabilization. Ser 130 not only donates a hydrogen bond to the bound sulfate, but also in part defines the binding-site geometry by accepting a hydrogen bond from Gly 133. The mutations at this postion were designed to determine if cysteine is really a conservative mutation for serine and the importance of the hydrogen bond from Ser 130 Oγ to Gly 133 NH to the overall stability of binding site geometry.

The replacement of Ser 130 with Cys was confirmed by reaction with DTNB (90%). Although this is the most conservative mutation possible, This mutant results in dramatically decreased sulfate binding affinity (approximately 10,000-fold lower than wild-type, see Table 5). In contrast, replacement of Ser 130 with Gly or Ala decrease
sulfate binding affinity by approximately 30 or 200-fold respectively (Jing Jiu He in our lab). The S130G and S130A mutants imply that the binding site geometry is stable in the absence of a hydrogen bond to the Gly 133 peptide NH, and that a stable, albeit weaker, SBP–sulfate complex can form in the absence of the hydrogen bond from Ser 120 Oγ. In light of these mutants, the most likely explanation for the low binding affinity of the S130C mutant is steric hindrance due to the large size of cysteine relative to serine. Serine and cysteine are compared in Figure 20.

Another interesting effect is the pH dependence of sulfate binding to S130C shown in Figure 21. Because cysteine is a stronger acid than serine (pKₐ 8.3), deprotonation of cysteine at high pHs results in reduced binding affinity due to electrostatic repulsion between the S⁻ of cysteine and the negatively charged sulfate oxygens.

7.5. Interdomain Salt Linkages

The interdomain salt-bridges between Glu 15 and Arg 174, and Asp 68 to Arg 134 shown in Figure 22 have been proposed as another way in which the SBP–sulfate complex is stabilized. Changing the acidic, negatively charged amino acids glutamate and aspartate to their corresponding neutral amides, or glycine was expected to decrease binding affinity due to destabilization of the closed form of SBP. As per prediction, replacing the acidic amino acid glutamate with glutamine results in approximately a three-fold decrease in binding affinity, and replacing Asp 68 with either Asn or Gly results in an approximately seven-fold decrease in affinity. The greater effect of the Asn
Figure 20. Serine vs. Cysteine. Cysteine is structurally distinct from serine. The $\text{C}_\beta-\text{S}$ bond is 0.41 Å longer than the $\text{C}_\beta-\text{O}$ bond, and the $\text{S}-\text{H}$ bond is 0.34 Å longer than the $\text{O}-\text{H}$ bond. Additionally the $\text{C}_\beta-\text{S}-\text{H}$ angle is 13° more acute than the $\text{C}_\beta-\text{O}-\text{H}$ angle. Structures are based on neutron diffraction studies of small molecules (Frey et al., 1973; Kerr & Ashmore, 1973).
Figure 21. pH dependence of sulfate binding affinity to S130C. The stability of the S130C—SO$_4^{2-}$ complex exhibits a pH dependence attributable to the p$_{Ka}$ of the $-$SH group of cysteine. The decrease in affinity with deprotonation of cysteine is consistent with charge repulsion between $-$S$^-$ and SO$_4^{2-}$. Note that the p$_{Ka}$ for cysteine appears to be abnormal (perhaps a full pH unit higher) due to the interactions with the bound sulfate.
Figure 22. Interdomain salt-bridges in SBP. The C$_\alpha$ backbone of *S. typhimurium* SBP is shown in red, the bound sulfate in blue, and the salt-bridges formed between Asp 68 and Arg 134 (left), and between Glu 15 and Arg 174 (right) in yellow. Note that the basic residues are in one domain (bottom) and the acidic residues are in the other domain (top).
68 mutations is possibly due to the better geometry of this salt-bridge (see Figure 22).

The binding affinities alone imply that the salt-bridges are only moderately important in stabilizing the SBP structure. However, the off-rates for these mutants dramatically demonstrate the true effect of these mutations. The spectacular 100-fold decrease in off-rates of these mutants clearly proves these salt-bridges are an important factor in the stabilization of the closed form of SBP. Furthermore, because the magnitude of the off-rate increase is considerably greater than the change in affinity, a compensating increase in on-rate is implied.

Additionally, the decreased off-rates allow calculation of the contribution of the salt-bridges to the overall lability of the SBP–sulfate complex, since an increase in rate implies a decrease in activation energy. If the effect of the salt-bridge mutants is due to a lowering of the activation energy for opening the cleft, and the Arrhenius factor is assumed the same for mutant and wild-type SBP the change in activation energy is described by $\Delta E_A = -RT \ln (k_{sbp} / k_{mutant})$. Using an average off-rate calculated from all three mutants the decrease in activation energy due to the removal of a salt-bridge is estimated to be approximately 2.3 kcal/mole. This is a reasonable value for an exposed salt-bridge.

7.6. Introduction of Cysteines for Chemical Modification and Disulfide Bond Formation
Disulfide bonds have been introduced into proteins to improve their stability such as with lysozyme (Perry & Wetzel, 1984, 1986), dihydrofolate reductase (Villafranca et al., 1987), and subtilisin (Pantoliano et al., 1987, 1988; Katz & Kossiakoff, 1986), or in the case of lysozyme (Matsumura & Matthews, 1989) and the λ repressor (Sauer et al., 1986), to modulate their activity. Similar to the lysozyme case we believed that a disulfide connecting the two domains of SBP could be used to modulate its activity by serving as a reversible "lock". Because native SBP contains no sulfur containing residues it is particularly amenable to the introduction of cysteines via site-specific mutagenesis. Ser 129 and Gly 46 were chosen for the disulfide site because the geometry based on Cα to Cα distance and dihedral angles is acceptable within the rules outlined by Jane Richardson (Richardson, 1981), and by their location in the cleft (see Figure 23). For comparison, the location of the proposed disulfide is shown relative to the positions of the interdomain salt-bridges in Figure 24. A bonus from this mutagenesis was that the initial single mutants (G46C and S129C) contain unique cysteines for chemical modification. The disulfide containing and chemically modified proteins were used to address the validity of the hinge-bending model (for the most recent review see Sack et al., 1989) for sulfate binding.

The various cysteine mutants differed in extent and ease of reaction with DTNB. The reaction with Cys 46 occurs readily in the native form as well as in the denatured form (80% in the native form, 100% in the denatured form). Furthermore, it is difficult to keep this mutant in the reduced form. These observations imply that Cys 46 is readily
Figure 23. Location of proposed disulfide. The Cα backbone of *S. typhimurium* SBP is shown in red, the bound sulfate in blue, and the proposed disulfide in gold. In this view, the anticipated pathway for sulfate binding and release is perpendicular to the plane of the photograph, either forward out of the figure or backward into the figure.
Figure 14. The relative locations of the intramolecular salt-bridges and the proposed disulfide. The Cα backbone of S. typhimurium XBP is shown in red, the bound adenine in blue.
Figure 24. The relative locations of the interdomain salt-bridges and the proposed disulfide. The $C_\alpha$ backbone of *S. typhimurium* SBP is shown in red, the bound sulfate in blue, the salt-bridges formed between Asp 68 and Arg 134 (left), and between Glu 15 and Arg 174 (right) in yellow, and the proposed disulfide in gold.
accessible to solvent. In contrast, the Cys 129 is easily maintained in the reduced form, and reacts only slightly with DTNB in the native form (25%). The denatured protein reaction with DTNB goes virtually to completion (95%). These results are consistent with Cys 129 being relatively buried and protected from solvent.

Placing single cysteines in the cleft has created unique sites for chemical modification. Both the Cys 46 and Cys 129 mutants have been reacted with 5-((2-iodoacetyl)amino)ethyl)amino)napthalene-1sulfonic acid (1,5 IEADANS), and 4'-((iodoacetyl)amino)methylfluorescein (fluorescein) in hope that these fluorescent probes could be used to distinguish between an open and a closed form of the protein. The Cys 129 was too unreactive for efficient modification. The Cys 46 mutant reacted well with fluorescein. Unfortunately, no change in fluorescein fluorescence could be detected upon addition of sulfate. However, chromate binding does decrease fluorescein fluorescence (Figure 25). This quenching is most likely due to a decrease in energy transfer from tryptophan to fluorescein due to energy transfer to chromate.

The creation of a disulfide bond between the two domains was spectacularly successful! It should be noted that the formation of this disulfide is strong evidence for the similarity of the S. typhimurium and the E. coli SBP structures. The presence of the disulfide was confirmed by reaction with dithiothreitol (100 % in the oxidized form), resistance to DTNB labelling ( < 15 % reaction), and stopped-flow studies in the presence and absence of dithiothreitol. The most exciting result is the kinetics of the binding reaction. The off-rate for wild-type E. coli SBP is $5 \text{s}^{-1}$. In contrast to the wild-type,
Figure 25. Fluorescein fluorescence spectra of Cys46-fluorescein. The fluorescence intensity of 1 μM fluorescein labelled Cys46 is decreased significantly by the addition of 10 μM chromate. Excitation wavelength was 290 nm.
off-rates for the disulfide form of SBP are independent of the ligand (sulfate and chromate have identical off-rates), independent of the ligand concentration (first order) and very slow, with a rate of 0.12 s\(^{-1}\).

Stopped-flow experiments clearly show two forms of SBP-Dis. Before dithiothreitol addition the majority of the protein is in the oxidized form (81 % in initial stopped-flow experiments). Addition of 10 mM dithiothreitol shifts the equilibrium quantitatively to the reduced form. The equilibrium could be shifted quantitatively to the oxidized disulfide form by bubbling air through the protein solution. Off-rates could be determined for both forms and are shown in Table 5.
CONCLUDING REMARKS

8. Specificity of the Sulfate-binding Protein

Chapter 2 examined the different binding affinities of several oxyacids to SBP and the effect of pH on those affinities. SBP is uniquely suited to such studies since the lack of ionizable groups in the binding site allows the pH dependence or independence of binding affinity to be attributed solely to ligand characteristics. The specificity and pH dependence of oxyacid binding to SBP can not be attributed to a single ligand characteristic: state of protonation and net charge both affect binding affinity. Knowledge of the atomic interactions between SBP and sulfate and the data presented here show that the sulfate-binding protein is designed to tightly bind tetrahedral, fully ionized, oxyacid dianions, and stabilize their negative charges. Furthermore, this work not only demonstrates the extraordinary specificity of SBP, but also the power of hydrogen bonds, and the untoward effect of an unpaired donatable proton. These are basic concepts in protein structure and function, and thus have important implications in protein folding, and ligand specificity and affinity.
9. Serine vs. Cysteine

Serine differs from cysteine in size (serine is considerably smaller than cysteine), and in its pKₐ (8.5 for cysteine, ~ 13 for serine). The mutations at position 130 clearly demonstrate these two fundamental chemical differences between serine and cysteine, and the fallacy of the common misconception that serine to cysteine and vice versa are conservative mutations. The replacement of serine by cysteine in the binding site of SBP resulted in a profound detrimental effect on binding affinity, and a pH dependence that could only be attributed to deleterious steric interactions and electrostatic repulsion between the cysteine and the sulfate.

10. Hinge-bending Model

There is a significant body of evidence which implies that the cleft between the two domains of binding proteins must open for ligand binding and release to occur. It is believed that when unliganded, the open and closed forms of the binding proteins are in rapid equilibrium. Ligand binding results in stabilization of the closed form and subsequent shifting of this equilibrium predominantly to the closed form. The evidence for this hinge-bending model has been published elsewhere (Newcomer et al., 1981; Newcomer et al., 1981; Miller et al., 1983; for the most recent review see Sack et al., 1989) and will only be summarized here.

The structures of four different states of binding proteins have been solved: cleft closed with bound ligand, cleft closed ligand-free, cleft open with bound ligand, and
cleft open ligand-free. One powerful piece of evidence comes from looking at the structures of binding proteins with bound ligand. The ligand is bound deep in the cleft, inaccessible to solvent and there is simply no room for entrance or exit from the binding site without some sort of conformational change. Further support for the open conformation comes from the structures of LIVBP and LsBP. These proteins were crystallized in the open form, with the clefts separated by as much as 18 Å. Initial binding to one domain, followed by cleft closure has been postulated and is supported by the fact that in ABP, GBP, and SBP the majority of the hydrogen bonds are provided by one domain, and by the observation that leucine soaked into LIVBP crystals binds specifically to one domain. Evidence for displacement of bound water molecules is provided by the structure of sugar-free ABP which crystallized in the closed conformation with non-specifically bound waters in the binding site (N. K. Vyas, unpublished data). Evidence of a conformational change was furnished by low-angle X-ray scattering experiments which indicate that the radius of gyration of ABP decreases upon ligand binding (Newcomer et al., 1981).

The purpose of creating a disulfide bond connecting the domains of SBP was to further demonstrate that the cleft must open for ligand to enter the binding site. The disulfide which forms between Cys 46 and Cys 129 severely restricts access of ligand to the binding site by decreasing the conformational freedom of the two domains relative to one another. Opening the disulfide with dithiothreitol returns freedom of motion to the domains and the protein returns to wild-type kinetics. The behavior of this mutant
clearly demonstrates the need for hinge-bending motion for proper function of SBP.

11. Oxyanion-binding Proteins: A Special Case?

The pursuit of "sulfate-free" SBP (a.k.a. the Holy Grail) has been a frustrating distraction from more productive experiments. Time, and time again, purportedly sulfate-free SBP was brought to the stopped-flow. Time, and time again, protein which clearly still had sulfate bound to it was brought back to the lab, stuck into the refrigerator, and forgotten. Then, a novel method of preparing ligand-free SBP was developed. Protein treated in this way could be titrated with sulfate to an endpoint of 1 mole of sulfate per mole of protein, and more importantly, exhibited a fluorescence increase for sulfate, and a fluorescence decrease for chromate. Clearly this time the protein was sulfate-free; on-rates were just an experiment away. The stopped-flow, however, had other plans. As predicted, the fluorescence did increase upon sulfate binding, and did decrease upon chromate binding. Unfortunately, kinetically both reactions looked like displacements. Previously, the displacement of sulfate by chromate was observed and no reaction occurred when the protein was mixed with sulfate. But now, both ligands exhibit displacement kinetics. They have to be displacing something. Or, perhaps there is another explanation.

Displacement kinetics occur when the rate limiting step is the displacement of a bound ligand. The observed rate then approaches the off-rate of the bound ligand as the concentration of the displacing ligand is increased. In the case of sulfate-bound SBP the
observed rate of reaction with chromate tends toward the sulfate off-rate. Perhaps, in
the case of the sulfate-free proteins, the limiting step is the opening of the protein, and
release of non-specifically bound water molecules or Cl⁻ ions. This would be funda­
mentally different than the sugar binding proteins and the histidine-binding protein for
which on-rates could be measured implying the rate of opening and closing were not
rate limiting.

There are significant differences between SBP (and PBP) and the sugar-binding
proteins. The oxyanion binding proteins bind spherically symmetric, highly-charged
ligands (protonation and deprotonation of phosphate can be considered fast even on this
time scale), whereas sugars are uncharged and highly asymmetric. The implication for
reaction rate is that the binding of a highly charged, symmetric molecule is expected to
be near diffusion limited whereas the binding of sugars due to orientation requirements
are much slower (~ 1 x 10⁷ M⁻¹ s⁻¹ (Miller et al., 1983)). The oxyanion-binding pro­
teins also have interdomain salt-bridges which form in the closed conformation of the
protein. From mutants which remove these salt-bridges comes the strongest evidence
that oxyanion-binding proteins are a special case.

As per prediction, mutations which destroy the salt-bridges result in decreased
binding affinity. However, the off-rates for these mutants dramatically demonstrate the
true effect of these mutations. The spectacular 100-fold decrease in off-rates of these
mutants clearly proves these salt-bridges are an important factor in the stabilization of
the closed form of SBP. Furthermore, because the magnitude of the off-rate increase is
considerably greater than the change in affinity a compensating increase in on-rate is implied by the relation $K_d = \frac{k_{off}}{k_{on}}$. This is extraordinary; the salt-bridges can only form in the closed conformation. For these mutations to affect the on-rates, the on-rates must be dependent on the rate of opening the closed form of the protein.

The functional requirements for a protein which binds oxyanions are fundamentally different than those for a protein which binds sugars. Oxyanions are small, rapidly diffusing, symmetric, highly charged molecules. The basic problem is formation of a stable complex with a molecule whose prevailing tendency is to diffuse away rapidly. Oxyanion-binding proteins sacrifice freedom of motion (formation of salt-bridges) in exchange for complex stability.

The above hypothesized model for SBP function has implications for binding protein-dependent transport. Because it is the interaction between the membrane components and the binding protein which elicits the chemotactic signal, chemotaxis requires the membrane components be able to distinguish between liganded and unliganded binding protein. Transport, which also requires interaction between membrane-bound components and binding protein, do not have this strict requirement of distinguishing bound and unbound forms. Because ligand is transferred from binding protein to membrane-bound transport components, the process of active transport can be elicited by this transfer rather than interaction between the binding protein and membrane bound components.
It is an unfortunate fact that at this time no methodology exists for experimentally proving or disproving that unliganded SBP is in a closed form a significant amount of the time. The indirect evidence presented above is intended to provoke thought and inspire interest in developing new experimental methods (e.g. the introduction of salt-bridges into ABP) which will address this question and further our understanding of binding protein function.

12. A Comment About Mutagenesis

Understanding the subtle, often unappreciated, differences between amino acids such as serine and cysteine, or the full role of the salt-bridges in SBP activity is a necessary prelude to "rational" protein design. There is now great potential for expanding our understanding of protein architecture. Mutagenesis, coupled with the ability to express high levels of protein suitable for crystallization allow one to pose precise structural questions which could not be answered by scanning the Protein Data Bank. Furthermore, the actual crystallization and structure determination of a mutant protein for which the wild-type structure is known accurately is now at the level of an undergraduate project. There is concern, and it is not unjustified as mutant proteins are generated with relative ease, that the time spent determining mutant structures will significantly interfere with the determination of new structures. For this reason, control should be exercised. A residue should not be changed to each of the nineteen other amino acids just because oligonucleotides are cheap. Mutagenesis must serve the same purpose as any other scientific experiment: a method to test a specific hypothesis.
PART II.

CHARACTERIZATION OF THE CALCIUM-BINDING SITE OF THE D-GALACTOSE-BINDING PROTEIN
13. Introduction

Galactose-binding protein (309 residues, MW ~32,000) is another periplasmic binding protein isolated from *Escherichia coli*. GBP serves as the initial receptor for the osmotic shock sensitive, high affinity transport system for galactose, and glucose, and is an essential component of the chemotactic response to these sugars.

The three-dimensional structure of GBP with bound glucose was solved by Vyas (1988) and is similar to the structure of other binding proteins (see Figure 26). During refinement of the structure, a novel Ca$^{2+}$ binding site was discovered. The structure of the GBP calcium-binding site has been described in detail elsewhere (Vyas et al., 1987), and will only be summarized here. The Ca$^{2+}$ is bound in a nine-residue loop (residues 134-142) in the C-terminal domain. This loop, flanked by a reverse-turn and a β-strand, provides five oxygen ligands from every second residue. Glu 205 provides the last two oxygen ligands. There are no water molecules directly coordinated to the calcium. The net charge of the coordinating ligands is −3. The coordination around the calcium is nearly pentagonally bipyramidal and superimposable with the EF-hand loop of parvalbumin with an r.m.s. difference of 0.60 Å (Vyas et al., 1987)). There are other marked
Figure 26. Galactose-binding protein structure. The α-carbon backbone is shown in red. The glucose (yellow) is bound in the cleft between the two domains. The bound calcium is shown as a blue sphere.
similarities in both calcium sites of GBP and the EF-hand loop (Vyas et al., 1987). However, because of the novel geometry of the binding site, and the fact that only five of the ligands are provided by the nine residue loop as opposed to seven by the eleven residue loop in the EF hand, this structure has been termed the "lock-washer".

Calcium-binding sites pose some of the most perplexing structure-function relationship questions in the field. The vast majority of these sites conform to the EF-hand motif proposed by Kretsisger (Kretsinger and Nockolds, 1973), yet their affinities and specificities vary considerably (Levine and Williams, 1982). Understanding the physical properties of calcium binding loops has been difficult because in most proteins containing EF-hand type sites the loops are paired (Kretsisger, 1987). The 1.9 Å, highly refined structure (Vyas et al., 1988) of a protein with a lone calcium-binding site, free from cooperative effects often seen in other systems, allows direct correlation of the basic biochemical properties of affinity and specificity with structural properties such as binding site flexibility.

The purpose of these studies was four-fold: to confirm the existence of the metal-binding site revealed by X-ray crystallography; to determine its stoichiometry, affinity, and specificity; to correlate biochemical properties with structure; and to investigate the effect of sugar binding on the geometry of the metal binding site.
14. Materials and Methods

14.1. Chemicals

Metals of the highest possible purity, calcium (II) chloride (99.99% pure), terbium (III) chloride hexahydrate (99.999% pure), magnesium (II) chloride (99.995% pure), strontium (II) chloride (99.995% pure), cadmium (II) chloride (99.99% pure), lead (II) chloride (99.999% pure), barium (II) chloride (99.999% pure), were obtained from Aldrich and used without further purification. DEAE-cellulose was obtained from Whatman. P-6DG and Bio-Rad Protein Assay were obtained from Bio-Rad. $^{45}\text{CaCl}_2$ was purchased from Dupont-New England Nuclear. Pipes and Sephadex G-25/50 were obtained from Sigma.

14.1.1. Metal Solutions

Concentrated stocks (100 mM for all metals with the exception of PbCl$_2$) which was 10 mM) were prepared in deionized, glass distilled water. Due to the hygroscopic character of many of the metals used, concentrations were confirmed by EDTA titration. Metal stock solutions were stored for up to a week in plastic bottles at 4° C.

14.2. Protein Purification and Preparation

GBP was purified by Bill Black as previously described (Parsons and Hogg, 1974) from E. coli B/r ara39, and LA5709 containing the plasmid pVB2-wt kindly provided by Dr. M. Manson (Scholle et al., 1987).
For titrimetric experiments and sugar-binding studies, calcium- and sugar-free GBP was used. Like many binding proteins, purified GBP contains bound ligand. This finding led to the development of a mild and efficient method for removing bound ligand (Miller et al., 1980, 1983). By including EGTA with the solution originally found to remove bound glucose it was possible to simultaneously remove bound calcium and bound glucose without loss of sugar-binding activity. The procedure is as follows. GBP was treated with 3 M guanidine-HCl, 5 mM EGTA, 5 mM EDTA in 50 mM Tris-HCl, pH 8.2. Guanidine-HCl was removed by dialysis against 5 mM EDTA, 50 mM Tris-HCl, pH 8.2, followed by dialysis against 5 mM EDTA, 10 mM Tris-HCl, pH 7.4. Prior to metal-binding studies, EDTA and Tris-HCl were removed by passage through a Sephadex G-25/50 column equilibrated with 20 mM Pipes, pH 7.0. It was later discovered that the Sephadex column (poly-glucose) released enough glucose to resaturate GBP. To alleviate this problem a P-6DG (acrylamide based) column was used to remove Tris and EDTA.

14.3. Equilibrium Dialysis

500 μl aliquots of GBP (approximately 0.17 mg / ml) in 20 mM Pipes pH 7.0 were dialyzed vs. 100 ml of the same buffer containing 0.1 μM to 10 μM CaCl₂. 10 μl aliquots of ⁴⁵CaCl₂ were added to each graduated cylinder. After dialysis for ≥ 48 hours 100 μl aliquots from the buffer and from the bag were taken and counted. Another 100 μl aliquot was taken from the bag and used to determine protein concentration using the
Bio-Rad protein assay and GBP of known concentration (as determined by absorbance at 280 nm) as a standard.

14.4. Competition Studies

14.4.1. Fluorescence Spectroscopy

Fluorescence spectroscopy was performed on an SLM-Aminco model 4800 spectrofluorometer. The temperature was maintained at 20° C with a circulating water bath. An excitation wavelength of 290 nm yielded (see excitation spectrum, Figure 27) efficient energy transfer while shifting the second order light beyond the terbium emission maximum (545 nm). Interestingly, at a slit width of 4 nm or below the terbium emission peak split into two peaks located at 542 nm and 548 nm (see Figure 28).

Tb$^{3+}$ additions not exceeding 15 μl total were made with a Hamilton syringe to a starting volume of 3 ml of calcium- and sugar-free protein ([GBP] = 5 μM). Spectra were taken from 530 nm to 570 nm with a slit width of 4 nm. To minimize noise, ten measurements were taken at each wavelength and averaged internally by the instrument. The extent of Tb$^{3+}$ binding was determined by calculating the area under the major Tb$^{3+}$ emission peak (546 nm - 554 nm) using the software supplied with the instrument.

14.4.2. Equilibrium Dialysis

The binding affinities of other metals in solution were determined by their ability to displace bound terbium, similar to the method described by Luk (1971), or their ability
Figure 27. The excitation spectra of Tb$^{3+}$-loaded GBP. The excitation spectrum of 1 µM GBP previously equilibrated with 6 µM TbCl$_3$ indicates that tryptophan residues are primarily responsible for energy transfer to terbium.
Figure 28. The effect of slit width on Tb$^{3+}$ fluorescence spectra of Tb$^{3+}$-loaded GBP. The emission spectrum of 1 μM GBP previously equilibrated with 6 μM TbCl$_3$ at a slit width of 8 nm produce a single peak. The terbium fluorescence doublet can be resolved by reducing the slit width to 4 nm. Excitation wavelength was 290 nm.
to compete with $^{45}$Ca in an equilibrium dialysis experiment similar to the method described by Newcomer (Newcomer et al., 1979).

14.5. Off-rate Determination

The off-rate for $^{3+}$ from the calcium-binding site of GBP was determined by monitoring the decrease in $^{3+}$ fluorescence after addition of a 200-fold excess of $^{2+}$ to 1 μM, 5 μM $^{3+}$. Excitation wavelength was 290 nm and emission was monitored at 548 nm. Correction for photo-oxidation was made by running a similar time course in which the calcium addition was replaced by a buffer addition.

15. Results and Discussion

15.1. Introduction

Calcium-binding sites pose some of the most perplexing structure-function relationship questions in the field. The vast majority of these sites conform to the EF-hand motif proposed by Kretsinger (Kretsinger and Nockolds, 1973), yet their affinities and specificities vary considerably (Levine and Williams, 1982). Understanding the physical properties of calcium binding loops has been difficult because in most proteins containing EF-hand type sites the loops are paired (Kretsinger, 1987). The 1.9 Å, highly refined structure (Vyas et al., 1988) of a protein with a lone calcium-binding site, free from cooperative effects often seen in other systems, allows direct correlation of the basic biochemical properties of affinity and specificity with structural properties such as
The purpose of these studies was four-fold: to confirm the existence of the metal-binding site revealed by X-ray crystallography; to determine its stoichiometry, affinity, and specificity; to correlate biochemical properties with structure; and to investigate the effect of sugar binding on the geometry of the metal binding site.

15.2. Terbium as a Probe for Calcium-binding Sites

Terbium has been used extensively to investigate calcium binding sites (Gross et al., 1987; Horrocks and Collier, 1981; Horrocks and Sudnick, 1981; Luk, 1971; Nelson et al., 1977). This popularity is due to several characteristics of terbium which not only render it useful in determining binding stoichiometry, but make it sensitive to the nature of the binding-site as well. Proteins with high-affinity calcium-binding sites bind terbium stoichiometrically and specifically without significant structural changes (Horrocks, 1982). Additionally, protein bound terbium experiences considerable enhancement of its characteristic green fluorescence due to Förster dipole-dipole energy transfer from aromatic residues near the binding site (Horrocks and Sudnick, 1981). The intensity of the terbium fluorescence is dependent on the efficiency of this energy transfer which is in turn dependent on the distance from the donor (tryptophan) to the acceptor (terbium), the geometry of the tryptophan and the terbium, the overlap integral for tryptophan fluorescence and terbium absorbance, and the refractive index of the medium (Cantor and Schimmel, 1980).
15.3. Affinity and Specificity of the Calcium-binding Site

There are many instances in which the metal bound in proteins could be displaced stoichiometrically by terbium titration (Luk, 1971; Gross et al., 1987). This was not the case with GBP; attempts to titrate native GBP with terbium yielded no change in terbium fluorescence. However, if GBP was incubated with 20-fold excess terbium, displacement of calcium with an accompanying increase in terbium fluorescence was observed over time, suggesting a slow calcium off-rate was the cause of this difficulty. Indeed, calcium- and sugar-free GBP could be titrated with Tb$^{3+}$ to an endpoint of 1 mole of Tb$^{3+}$ added per mole of GBP. Additionally, the off-rate for terbium, estimated from the observed rate of terbium displacement by a 500-fold excess of calcium, is $3 \times 10^{-3}$ sec$^{-1}$ (see Figure 29). The predicted on-rate for terbium based on the above off-rate and the $K_d$ below is $1.2 \times 10^3$ M$^{-1}$ sec$^{-1}$. The off-rate for calcium could not be determined directly because high concentrations of terbium can cause protein aggregation (Gross et al., 1987). Scatchard plots (Figure 30) of the results of the terbium titration and the calcium equilibrium dialysis experiment indicate $K_d$ values of 2.6 µM for Tb$^{3+}$ binding and of 4.7 µM for calcium binding.

Previously attempts have been made to correlate binding affinity with differences in cation size (Chao et al., 1984). Our experiments were designed to test the cation size hypothesis for a single, independent binding site. We found that the size criteria alone is unable to predict the relative affinities of the metal cations used in this study because it
Figure 29. Displacement of Tb$^{3+}$ from the calcium-binding site of GBP by calcium.
Terbium bound to GBP (1 μM GBP, 5 μM TbCl$_3$) was displaced by addition of 2.5 mM CaCl$_2$. Excitation $\lambda = 290$, emission $\lambda = 546$. The off-rate calculated from this experiment is $3 \times 10^{-3}$ s$^{-1}$. 
Figure 30. Skatchard plot of calcium (■) and terbium (□) binding to GBP. Calcium data was obtained by equilibrium dialysis whereas terbium data is from a titration of metal-free protein. Solid lines indicate least squares fits of the data, which yield $K_d$ values of 4.7 μM for calcium and 2.6 μM for terbium.
ignores the fundamental importance of hydration energy in coordination chemistry as stressed by Cotton and Wilkinson (1988), "The process of forming [complexes] in aqueous solution... is really one of displacing one set of ligands, which happen to be water molecules, with another set.'', When solvation is considered the binding energy of the protein-cation complex can be described as:

\[
\Delta G = \Delta G_{SS} + \Delta G_{PP} + \Delta G_{LL} + \Delta G_{PS} + \Delta G_{PL} + \Delta G_{PLS}
\]

where S represents a molecule of the solvent, P, a protein molecule, and L, a ligand molecule. In dilute solution, the contributions of the LL, and PP interactions are negligible. Furthermore, GBP is fundamentally different from many other calcium binding loops (e.g. calmodulin) in that it does not undergo a significant conformational change upon cation binding (see discussion below), thus the SS interactions, which represent the hydrophobic effect, remain essentially constant. Thus, equation (1) simplifies to:

\[
\Delta G = \Delta G_{PS} + \Delta G_{LS} + \Delta G_{PL} + \Delta G_{PLS}
\]

\(\Delta G_{PS}\) is a property of the protein and constant for a given set of experimental conditions, and similarly, \(\Delta G_{LS}\), the cation hydration energy, is characteristic for a given cation. Furthermore, the locations of crystallographically determined water molecules near the binding site are identical in both the Ca\(^{2+}\) and Cd\(^{2+}\) bound structures indicating that at least for the cadmium case \(\Delta G_{PLS}\) does not vary greatly with the nature of the metal bound. Note these are ordered, bound, water molecules near the binding site,
there are no waters directly involved in cation binding. Thus, differences between the relative cation-binding affinities predicted by hydration energy alone and the experimentally determined order reflect the contributions of the $\Delta G_{PL}$ term.

Below are three series of relative binding affinities: that predicted by hydration energy alone, that predicted by size relative to calcium, and the experimentally determined order. It is quite clear that neither hydration energy alone, or size alone can account for the experimental data. However, if the experimental results (see Table 6) are compared carefully with the predictions some very useful information about the calcium-binding site of GBP emerges.

**Binding Order:**

**Experimental:**  
Tb$^{3+} \approx$ Ca$^{2+} = \text{Pb}^{2+} > \text{Cd}^{2+} > \text{Sr}^{2+} > \text{Mg}^{2+} > \text{Ba}^{2+}$

**Predicted:**

**Hydration Energy:**  
Ba$^{2+} > \text{Pb}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+} > \text{Cd}^{2+} > \text{Mg}^{2+} > \text{Tb}^{3+}$

**Cation Size:**  
Ca$^{2+} > \text{Cd}^{2+} > \text{Tb}^{3+} > \text{Sr}^{2+} > \text{Pb}^{2+} > \text{Mg}^{2+} > \text{Ba}^{2+}$

**15.3.1. Barium, Lead, and Strontium**

The most striking difference between the relative affinities predicted by hydration energy alone and the experimental results is that barium and terbium are interchanged. Barium is the least strongly hydrated of the metals studied, and therefore predicted to
Table 6. Properties of Metals Which Bind to GBP. Dissociation constants \( (K_d) \) were determined by competition with calcium or terbium as described in the text, using independently determined values for calcium (4.7 \( \mu \)M) and terbium (2.6 \( \mu \)M). Values in parentheses indicate standard deviations.
### Table 6.

*Properties of Metals Which Bind to GBP*

<table>
<thead>
<tr>
<th>Metal</th>
<th>$K_d$ (μM)</th>
<th>Dialysis</th>
<th>Fluorescence</th>
<th>$\Delta G_{\text{hyd}}^a$ (kJ / mole)</th>
<th>Ionic Radius$^b$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$</td>
<td>190(20)</td>
<td>460(30)</td>
<td>-1838</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>1000(100)</td>
<td>-</td>
<td>-1770</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Tb$^{3+}$</td>
<td>5(1)</td>
<td>-</td>
<td>-3409</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>60(20)</td>
<td>60(5)</td>
<td>-1736</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>-</td>
<td>2.0(0.2)</td>
<td>-1515</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Sr$^{2+}$</td>
<td>130(30)</td>
<td>110(5)</td>
<td>-1386</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>Pb$^{2+}$</td>
<td>5(1)</td>
<td>-</td>
<td>-1434</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>2000(200)</td>
<td>-</td>
<td>-1258</td>
<td>1.34</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ (Marcus, 1985)

$^b$ (Weast, 1987)

$^c$ From small molecule studies (Lonsdale *et al.*, 1985)
bind tightly to the calcium-binding site. The best explanation for its low binding affinity is that its large unhydrated ionic radius ($r_i = 1.34 \, \text{Å}$) and the Ba–O bond length of 2.76 Å (Lonsdale et al., 1985) are beyond the size limit of the binding site irrespective of any advantage provided by its low hydration energy. This size limit is more clearly defined by Pb$^{2+}$ and Sr$^{2+}$ which compete efficiently for the binding site in spite of their relative size. Thus, there appears to a critical cationic radii between 1.20 Å (Pb$^{2+}$) and 1.34 Å (Ba$^{2+}$) above which binding affinity decreases significantly, presumably due to steric constraints. Below this limit, cation hydration energy is an important factor in determining binding affinity.

### 15.3.2. Terbium

Hydration energy alone also fails to properly predict the tight binding affinity of terbium. This is because hydration energy, which describes the stability of metal–water complex, reflects the greater stability imparted to a complex when the charge of the central metal is increased. The stronger interactions of terbium with its ligands no doubt also occur in the protein, leaving size as the principal determining factor in terbium binding affinity to the GBP site. It should be noted that the net charge of the metal-free GBP site is $-3$, adequate to stabilize the added charge of terbium.

### 15.3.3. Cadmium

Differences in calcium- and cadmium- binding affinities have been attributed to cadmium's preference for 6-fold coordination (Szebenyi and Moffat, 1986). To further
probe this possibility, cadmium binding to GBP has been studied in solution and in crystals (Vyas et al., 1989). Coordination of the two metals, and positions of surrounding protein residues are identical within the error of the experiment. This is consistent with NMR experiments of other proteins which predict identical environments for bound calcium and cadmium (Cavé et al., 1979; Vogel et al., 1985). The lower stability of the GBP–Cd$^{2+}$ complex in solution, can be attributed in part to hydration energy. However, the difference between calcium’s and cadmium’s hydration energies is much greater than the difference in their binding energy implying that other factors stabilize, rather than destabilize the GBP–Cd$^{2+}$ complex. In light of the close structural similarities of the two complexes, and the above discussion of the calcium- and cadmium- binding affinities to GBP in solution, it is unlikely that cadmium’s preference for six-fold coordination is responsible for its lower binding affinity.

15.3.4. Magnesium

Previous investigations of other systems (Chao et al., 1984) have attributed the low binding affinity of magnesium solely to its small size relative to calcium. However, if the GBP site discriminated against cations smaller than calcium in the same manner as it does against those that are larger, then magnesium – because of its small size and high hydration energy – would be expected to be the worst competitor for the binding site. The data do not support this prediction. Rather, magnesium competes well with calcium and terbium. Thus, it seems that the small size of magnesium contributes positively to
complex stability offsetting the adverse effect of its high hydration energy. This is consistent with the observed relative affinities of Group IIA elements in their complexes with hydroxycarboxylic acids, polycarboxylic acids, and polyaminocarboxylic acids: Mg < Ca > Sr > Ba (Cotton and Wilkinson, 1988).

15.3.5. Manganese

The low affinity for Mn\(^{2+}\) binding to the Ca\(^{2+}\)-binding site of GBP is due to fundamental chemical differences between Mn\(^{2+}\) and the other metals used in this study. Contrary to the other metals investigated, whose coordination chemistry principally involves electrons in s-orbitals, the coordination chemistry of manganese, as with all transition metals, is dominated by electrons in the d-orbitals.

Group II metals (Mg, Ca, Sr, Ba), as demonstrated by small molecule complexes, prefer oxygen ligands, whereas transition metals prefer nitrogen ligands and complex strongly to imidazole ligands in particular due to favorable overlap between the d-orbitals of the metal and the \(\pi\)-orbitals of the imidazole ring (Cotton & Wilkinson, 1988). In fact, a search of the Protein Data Bank (Bernstein et al., 1977; Abola et al., 1987) for transition metal complexes (Fe, Cu, Mn, Co, Zn) reveals that with the exception of proteins containing Fe-S complexes, all transition metal binding sites include a histidine ligand. This suggests that the selectivity of a metal-binding site can be controlled by the nature of the complexing ligands provided. An interesting experiment, with the potential of changing the specificity of the metal-binding site, would be the
replacement of Glu 205 with a histidine.

15.4. Independence of Sugar and Calcium Sites

One of the advantages of using terbium as a calcium probe is its sensitivity to environment. The dependence of transfer efficiency on the distance between the donor tryptophan residue and terbium to the sixth power makes it particularly responsive to small conformational changes about the calcium-binding site. The excitation spectrum of GBP complexed with Tb$^{3+}$ exhibits a peak at 291 nm indicating that a tryptophan residue is primarily responsible for the energy transfer. The bound cation is 5.8 Å from the center of the six-membered ring of Trp 127 and 11.4 Å from the center of the six-membered ring of Trp 133. Trp 127 is primarily responsible for energy transfer to bound terbium because of its proximity to the binding site. Contribution from Trp 133 is possible, but presumably minor because of the relative weakness of energy transfer from tryptophan to terbium exemplified by the $R_0$ value of 3.35 Å obtained by Horrocks and Sudnick (1981) for the parvalbumin system.

The possibility that sugar binding could affect the environment of the bound terbium was also explored. Neither D-galactose nor D-glucose (also a substrate) binding to sugar-free, terbium-loaded GBP causes a change in terbium fluorescence (see Figures 31 and 32), although the change in protein fluorescence (see Figures 33 and 34) is as previously described (Miller et al., 1980).
Figure 31. Tb\(^{3+}\) fluorescence spectra of Tb\(^{3+}\)-loaded GBP with galactose. The Tb\(^{3+}\) fluorescence spectrum of sugar-free Tb\(^{3+}\)-loaded GBP (1 \(\mu\text{M}\) GBP, 6 \(\mu\text{M}\) TbCl\(_3\)) is not significantly different from that of Tb\(^{3+}\)-loaded GBP with bound galactose. Excitation wavelength was 290 nm.
Figure 32. Tb\(^{3+}\) fluorescence spectra of Tb\(^{3+}\)-loaded GBP with glucose. The Tb\(^{3+}\) fluorescence spectrum of sugar-free Tb\(^{3+}\)-loaded GBP (1 µM GBP, 6 µM TbCl\(_3\)) is not significantly different from that of Tb\(^{3+}\)-loaded GBP with bound glucose. Excitation wavelength was 290 nm.
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Figure 33. Protein fluorescence spectra of Tb$^{3+}$-loaded GBP with galactose. Sugar-free Tb$^{3+}$-loaded GBP (1 μM GBP, 6 μM TbCl$_3$) exhibits an increase in fluorescence and a shift upon binding galactose. This is consistent with previous sugar-binding studies of GBP (Miller et al., 1980). Excitation wavelength was 290 nm.
Figure 34. Protein fluorescence spectra of Tb$^{3+}$-loaded GBP with glucose. Sugar-free Tb$^{3+}$-loaded GBP (1 μM GBP, 6 μM TbCl$_3$) exhibits a decrease in fluorescence and a shift upon binding glucose. This is consistent with previous sugar-binding studies of GBP (Miller et al., 1980). Excitation wavelength was 290 nm.
The sugar-binding site is approximately 30 Å from the calcium-binding site and the tryptophan nearest the bound calcium (Vyas et al., 1988). Therefore, sugar binding should not directly affect terbium fluorescence. Thus, the lack of change in terbium fluorescence upon sugar binding indicates identical local geometry of the calcium-binding site in the presence and absence of sugar. Similarly, the intrinsic protein fluorescence spectra of calcium-free and calcium-bound GBP were compared in the presence and absence of substrate to determine whether occupancy of the metal site affected sugar binding. No discernible differences were observed (Data not shown).

The above results are consistent with the proposed sugar binding mechanism. Crystallographic studies of periplasmic binding proteins have established a structural theme which has been consistently maintained by each of the seven structures solved so far (Sack et al., 1989; Luecke, personal communication); all are composed of two domains connected by a flexible hinge region, with the ligand bound in the cleft between the two domains. Previous studies suggest that ligand binding occurs via a hinge-bending motion in which the two domains move relative to each other about the flexible hinge region, but preserve their individual structure (Newcomer et al., 1981; Newcomer et al., 1981; Sack et al., 1989). According to this model, the conformational change is confined to the flexible hinge region. The two domains move toward each other, but preserve their individual structure. The calcium-binding site, located at one end of the ellipsoidal protein, well away from the cleft and the sugar-binding site, would not be expected to experience a change in environment.
16. The Function of the Calcium-binding Site in GBP

On the basis of affinity for calcium, EF hands have been classified into two groups – structural and regulatory. Structural sites (e.g. parvalbumin and the C-terminal domains of troponin C) have $K_d$ values for calcium binding of $\sim 10^{-9}$ M, and Mg$^{2+}$ is thought to compete for these sites under physiological conditions, whereas regulatory sites (e.g., calmodulin, N-terminal domains of troponin C, intestinal calcium-binding protein) have $K_d$s of $\sim 10^{-6}$ M and are not believed to bind Mg$^{2+}$ under physiological conditions (Satyshur et al, 1988, Levine and Williams, 1980).

The function of the calcium-binding site in GBP is still uncertain. It is certainly not a regulatory site similar to those in calmodulin. Regulatory sites release calcium rapidly ($k_{off} \sim 10$ sec$^{-1}$ (Forsén et al. (1986))), enabling them to respond quickly to changes in calcium concentration. In contrast to calmodulin, terbium is released slowly from the calcium site of GBP ($k_{off} = 3 \times 10^{-3}$ sec$^{-1}$). Furthermore, the GBP calcium-binding site and sugar-binding site are functionally independent. Thus, release of calcium as a signal in response to sugar binding can not be a potential function.
Observations that cation dissociates very slowly ($k_{off} = 3 \times 10^{-3} \text{ sec}^{-1}$ for the GBP-Tb$^{3+}$ complex), and that substrate binding does not perturb terbium fluorescence support a structural role for the calcium-binding site of GBP. Additionally, of the EF-hand sites, site I or the CD-hand of parvalbumin (a structural site), most closely resembles the GBP site (Vyas et al., 1987). The CD hand and the GBP site differ from other calcium-binding loops at position 9 (Gln in GBP and Glu in the CD loop) which is of sufficient length for the side chain oxygen to coordinate directly to the calcium ion. In contrast, the side chain at this position in other EF-hand loops is too short, and a bound water molecule bridges the gap between the side chain and the calcium (Herzberg et al., 1985; Kretsinger, 1987). In spite of the close similarity with the CD-hand calcium-binding loop, calcium binding to the GBP site, which lacks flanking helices, is 2 orders of magnitude weaker.

This disparity in affinity values, which at first may appear inconsistent with a structural role, is actually a consequence of the different cellular locations of GBP and parvalbumin. The affinity of the CD loop for calcium ($\sim 10^{-8} \text{ M}$) and magnesium ($\sim 10^{-3} \text{ M}$) ensures that at physiological concentrations the site will be saturated with one of these metals (Levine and Williams, 1982). Presumably, the free calcium concentrations in the periplasm is similar to extracellular concentrations observed for other systems ($\sim 10^{-3} \text{ M}$ (Levine and Williams, 1982). Therefore, GBP, despite its relatively low $K_d$, is certain to be saturated with calcium under physiological conditions.
More work is certainly needed in this area. A structural role would imply that calcium is necessary for function. However, calcium is clearly not needed for sugar binding. An intriguing possibility is that the calcium-binding site is recognized by the membrane-bound components of chemotaxis. Preliminary results indicate that of the five proteins tested (ABP, GBP, MBP, SBP, and PBP), only MBP and GBP bound calcium. Of the proteins tested, MBP and GBP are also the only ones involved in chemotaxis. A general survey of binding proteins for calcium binding would be of great interest.
REFERENCES


Cantor, C. R. & Schimmel, P. R. Biophys. Chem. 21, 448-451 (1980).


Appendix I.

Pascal Program "ResinAnal"

program ResinAnal(input, output);

{This program was written to compute the dissociation constant for binding}
{proteins based on data obtained from the resin assay of Pardee (Pardee, 1966)}
{It requires two input files: a blank file which contains most of the}
{information about assay conditions, and the CPM values for the blank,}
{and a protein file which contains the protein type and}
{the CPM values for the protein samples.}
{Examples of the two types of files are shown below}

{BLANK FILE}

{February 24, 1986}
{SO4  Ligand Type}
{0.244  Protein concentration in micromolar}
{775.6  Specific activity}
{1.15  Decay Factor}
{417}
{420}
{439  Data}

{PROTEIN FILE}

{SBP}
{1912}
{2255}
{1766}
{1827}
{2366}

const
LigInput='Ligand.dat';
ProtInput='Protein.dat';
Ligcon=3.00;
type
  String = varying [4] of char;
  Files = varying [25] of char;
  Counts = array [1..100] of real;

var
  ProteinConc : real;
  AveCpm : real;
  SpecAct : real;
  ProSpecAct : real;
  DecayFactor : real;
  FreeLigCon : real;
  FreeProtein : real;
  BoundProtein : real;
  StanDev : real;
  ErrorfreeLig : real;
  ErrorFreeprot : real;
  ErrorBoundProt : real;
  CPM : Counts;
  Ligand : String;
  Protein : String;
  DataRead:integer;
  AssayDate:Files;
  LigandFile:Files;
  Outputfile:Files;
  Proteinfile:Files;
  Outfile:Text;
  InFile:Text;

procedure AverageArray(CPM:counts; DataRead:integer;
  var AveCpm:real;
  var Standev:real);

{This procedure computes the average and}
{the standard deviation of an array of reals}

var
  Count : integer;
  SumCpm : real;
  SumSqr : real;
begin

    SumCpm:=0;

    for Count:= 1 to DataRead do
    begin
        SumCpm:=SumCpm + CPM[Count];
    end;{for}

    AveCpm:= SumCpm / DataRead;
{Computes Average CPM and...}

    for Count := 1 to DataRead do
    begin
        SumSqr:=SumSqr +
        sqr((AveCpm)-(CPM[Count]));
    end;{for}

    StanDev := sqrt(SumSqr/(DataRead-1));
{..Standard deviation }

end;{AverageArray}

procedure FreeLigand(AveCpm:real; var FreeLigCon:real;
SpecAct:real; DecayFactor:real;
ProteinConc : real;
StanDev:real;
var FreeLigError:real);

{This procedure computes the free ligand}
{concentration and the error in the calculation}

const
    DPMpermCi=2.2e9;
    SBPMolWt=34100; {grams per mole}
    SampleVol=0.00008; {liters}
LigCon=3.00; {umolar}

var

DPMpermMole:real;
uMolesLig:real;
ErroruMolesLig:real;

begin

DPMpermMole:=SpecAct*DecayFactor*DPMpermCi;
uMolesLig:=(AveCpm/DPMpermMole)*1000;
ErroruMolesLig:=StanDev/DpmPermMole*1000;
FreeLigCon:=uMolesLig/SampleVol;
FreeLigError:=ErroruMolesLig/SampleVol;

end; {FreeLigand}

procedure BondProtein(AveCpm:real; var FreeProtein:real; var BoundProtein:real; FreeLigCon:real; ErrorFreeLig:real; ProteinConc:real; StanDev:real; var ErrorFreeProt,ErrorBoundProt:real);

{This procedure computes the concentration}
{of bound protein, the concentration of free protein and the errors in the calculation}

const

DPMpermCi=2.2e9;
SBPMolWt=34100; {grams per mole}
SampleVol=0.00008; {liters}

var

DPMpermMole:real;
uMolesProtein:real;
ErroruMolesProt:real;

begin

DPMpermMole:=ProSpecAct*DecayFactor*DPMpermCi;
uMolesProtein:=(AveCpm/DPMpermMole)*1000;
ErroruMolesProt:=(StanDev/DPMpermMole)*1000;
BoundProtein:=uMolesProtein/SampleVol-FreeLigCon;
ErrorBoundProt:=ErroruMolesProt/SampleVol
FreeProtein := ProteinConc - BoundProtein;
ErrorFreeProt:= ((0.01 * ProteinConc) + ErrorBoundProt);

begin
write('LigandFile?[sys$input] '){Asks for blank input file}
if not eoln then
  readln(Ligandfile)
elseresult(LigandFile:=' sys$input';

write('Proteinfile?[sys$input] ');{Asks for protein input file}
if not eoln then
  readln(Proteinfile)
elseresult(ProteinFile:=' sys$input';

write('Outputfile?[sys$output] ');{Asks for desired output file file}
if not eoln then
  readln(Outputfile)
elseresult(OutputFile:=' sys$output';

open (Infile,LigandFile,old);
reset(Infile);
readln (Infile, AssayDate);  {Reads assay date}
readln (Infile, Ligand);    {Reads ligand}
readln (Infile, ProteinConc); {Reads Protein Concentration}
readln (Infile, SpecAct);    {Reads Specific Activity}
readln (Infile, DecayFactor); {Reads Decay Factor}
ProSpecAct:= SpecAct * LigCon/(LigCon+ProteinConc);

{The above line modifies the specific activity for dilution by bound ligand}
DataRead := 0;
while not eof (InFile) do {Reads is blank CPM data}
begin
  DataRead := DataRead + 1;
  readln (InFile, CPM[DataRead]);
end; {while not eof}
close(InFile);
AverageArray(CPM, DataRead, AveCpm, StanDev);
FreeLigand(AveCpm, FreeLigCon, SpecAct,
  DecayFactor, ProteinConc, StanDev, ErrorFreeLig);
Open(OutFile, OutputFile, new);
rewrite(OutFile);
writeln(Outfile, 'This assay was performed on ', AssayDate);
writeln(OutFile, '[Free ', Ligand, '] = ', FreeLigCon:fw, '+/-',
  ErrorFreeLig:fw, ' umolar');
Open(InFile, ProteinFile, old);
reset(Infile);
readln (InFile, Protein);
DataRead := 0;
while not eof(InFile) do {Reads in protein CPM}
begin
  DataRead := dataread + 1;
  readln(Infile, Cpm[dataread]);
end; {while not eof}
Close(Infile);
AverageArray(Cpm, DataRead, AveCpm, StanDev);
BondProtein(AveCpm, FreeProtein, BoundProtein,
  FreeLigCon, ErrorFreeLig, ProteinConc,
  StanDev, ErrorFreeProt, ErrorBoundProt);
writeln(OutFile, 'Bound', Protein, ' = ', BoundProtein:fw, '+/-',
  ErrorBoundProt:fw, ' umolar');
writeln(OutFile, 'Free', Protein, ' = ', FreeProtein:fw, '+/-',
  ErrorFreeProt:fw, ' umolar');
writeln(OutFile, 'Kd = ', FreeLigCon
  * FreeProtein
  / BoundProtein:fw, '+/-',
  (ErrorFreeLig * FreeProtein
  / BoundProtein +
  FreeLigCon * ErrorFreeProt
  / BoundProtein +
  FreeLigCon * FreeProtein
  / sqr(BoundProtein) * ErrorBoundProt):fw
  , ' umolar');
Close(OutFile);
end.
Appendix II.

Sequence Comparison of SBP from

*E. coli* and *S. typhimurium*

SBP had not previously been observed in *E. coli*. The match between the *E. coli* protein and the *Salmonella typhimurium* protein, for which the structure is known, is quite good. The amino acid sequence predicted from the DNA sequence from Hellinga (1985) is compared with the chemically modified sequence as corrected by the X-ray structure (Pflugrath & Quiocho, 1988), and amino acid sequence predicted from my own sequencing of the *E. coli* gene in Figure 35. For the most part the differences between the *S. typhimurium* sequence and the *E. coli* sequence are conservative (e.g. phe $\Rightarrow$ tyr). The locations of differences in the protein have been examined using molecular graphics and the majority lie along the perimeter of the protein where they should affect neither structure nor activity. The major difference, which is the result of a sequencing mistake in the pHE1005 sequence, is explained in Figure 36.
Figure 35. Comparison of three SBP sequences. Top line: Amino acid sequence predicted from DNA sequence determined by Hellinga (Hellinga & Evans, 1985). Middle Line: Sequence predicted from DNA sequence determined for this thesis. Bottom line: Chemically determined amino acid sequence as corrected by the refined 2.0 angstrom SBP structure (Pflugrath & Quiocho, 1988). *** Indicates a place where the sequences differ.
101 ..............................................................+..............................................................
VAL ARG LYS GLY ASN PRO LYS GLN ILE HIS ASP TRP ASN ASP LEU ILE LYS PRO GLY VAL
VAL ARG LYS GLY ASN PRO LYS GLN ILE HIS ASP TRP ASN ASP LEU ILE LYS PRO GLY VAL
VAL ARG LYS GLY ASN PRO LYS GLN ILE HIS ASP TRP ASN ASP LEU ILE LYS PRO GLY VAL

121 ..............................................................................
SER VAL ILE THR PRO ASN PRO LYS SER SER GLY GLY ALA ALA LEU GLU LEU PRO GLY SER
SER VAL ILE THR PRO ASN PRO LYS SER SER GLY GLY ALA ARG TRP ASN TYR LEU ALA ALA
SER VAL ILE THR PRO ASN PRO LYS SER SER GLY GLY ALA ARG TRP ASN TYR LEU ALA ALA

161 .............................................................................
ALA LEU TYR LYS ASN GLU GLU VAL LEU ASP SER GLY ALA ARG GRY SER THR ASN THR PHE
ALA LEU TYR LYS ASN GLU GLU VAL LEU ASP SER GLY ALA ARG GRY SER THR ASN THR PHE
ALA LEU PHE LYS ASN VAL GLU VAL LEU ASP SER GLY ALA ARG GRY SER THR ASN THR PHE

181 .............................................................................
VAL GLU PRO GLY ILE GLY ASP VAL LEU ILE ALA TRP GLU ASN GLU ALA LEU LEU ALA ALA
VAL GLU PRO GLY ILE GLY ASP VAL LEU ILE ALA TRP GLU ASN GLU ALA LEU LEU ALA ALA
VAL GLU ARG GLY ILE GLY ASP VAL LEU ILE ALA TRP GLU ASN GLU ALA LEU LEU ALA ALA
VAL GLU ARG GLY ILE GLY ASP VAL LEU ILE ALA TRP GLU ASN GLU ALA LEU LEU ALA THR
201................................................................................+............................................................................................... .
ASN GLU LEU GLY LYS ASP LYS PHE GLU ILE VAL THR PRO SER GLU SER ILE LEU ALA GLU
ASN GLU LEU GLY LYS ASP LYS PHE GLU ILE VAL THR PRO SER GLU SER ILE LEU ALA GLU
ASN GLU LEU GLY LYS ASP LYS PHE GLU ILE VAL THR PRO SER GLU SER ILE LEU ALA GLU

221....................................................................................+ .............................................................. *** .............................. .
PRO THR VAL SER VAL VAL ASP LYS VAL VAL GLU LYS GLY THR LYS GLU VAL ALA GLU
PRO THR VAL SER VAL VAL ASP LYS VAL VAL GLU LYS GLY THR LYS GLU VAL ALA GLU
PRO THR VAL SER VAL VAL ASP LYS VAL VAL GLU LYS GLY THR LYS GLU VAL ALA GLU

241................................................................................+.................................................................................. *** ......... .
ALA TYR LEU LYS TYR LEU TYR SER PRO GLU GLY GLN GLU ILE ALA ALA LYS ASN TYR TYR
ALA TYR LEU LYS TYR LEU TYR SER PRO GLU GLY GLN GLU ILE ALA ALA LYS ASN TYR TYR
ALA TYR LEU LYS TYR LEU TYR SER PRO GLU GLY GLN GLU ILE ALA ALA LYS ASN PHE TYR

261...........................................*** .................................... + .............. ******** .................................................................. .
ARG PRO ARG ASP ALA GLU VAL ALA LYS TYR GLU ASN ALA PHE PRO LYS LEU LYS LEU
ARG PRO ARG ASP ALA GLU VAL ALA LYS TYR GLU ASN ALA PHE PRO LYS LEU LYS LEU
ARG PRO ARG ASP ALA ASP VAL ALA LYS TYR ASP ASP ALA PHE PRO LYS LEU LYS LEU

281...............................................................................
+ ...... *** ................................................................................. ***
PHE THR ILE ASP GLU GLU PHE GLY GLY TRP THR LYS ALA GLN LYS GLU HIS PHE ALA ASN
PHE THR ILE ASP GLU GLU PHE GLY GLY TRP ALA LYS ALA GLN LYS GLU HIS PHE ALA ASN
PHE THR ILE ASP GLU GLU PHE GLY GLY TRP ALA LYS ALA GLN LYS GLU HIS PHE ALA ASP
301.................................................................................................+
GLY GLY THR PHE ASP GLN ILE SER LYS ARG
GLY GLY THR PHE ASP GLN ILE SER LYS ARG
GLY GLY THR PHE ASP GLN ILE SER
Figure 36. Explanation of the pHE1005 Frameshift Error residues 134-141, which are not involved in ligand binding. The area of interest from plasmid pHE1005 is approximately from bases 1850-1890 as shown (a). The $ marks where changes can be made to correct the frame shift error bringing the Hellinga sequence into agreement with that determined for this thesis. If the (-) at 1851 is omitted and a G added between bases 1876 and 1877 the sequence shown in (b) would result. This agrees perfectly with the X-Ray structure and my own sequencing.
Sequence
1850
Residues

(a) $ GC- GCG CTG GAA CTA CCT GGC AGC CTG GGC TAC GCG CTG $

ALA ALA LEU GLU LEU PRO GLY SER LEU GLY TYR ALA LEU

(b) $ GCG CGC TGG AAC TAC CTG GCA GCC TGG GGC TAC GCG CTG $

ALA ARG TRP ASN TYR LEU ALA ALA TRP GLY TYR ALA LEU
Appendix III.

Fluorescence Spectra of Treated and Untreated Proteins
Figure 37. Fluorescence spectra of untreated *Salmonella typhimurium* SBP. The addition of 10 mM sulfate to 1 µM *Salmonella typhimurium* SBP in 40 mM Tris pH 8 results in no change in the fluorescence spectrum. Excitation wavelength was 290 nm.
Figure 38. Fluorescence spectra of untreated *E. coli* SBP. The addition of 10 mM sulfate to 1 μM *E. coli* SBP in 40 mM Tris pH 8 results in no change in the fluorescence spectrum. Excitation wavelength was 290 nm.
Figure 39. Fluorescence spectra of untreated S130C. The addition of 10 mM sulfate to 1 μM S130C in 40 mM Tris pH 8 results in a 6 nm blue shift and a slight increase in fluorescence intensity. Excitation wavelength was 290 nm.
Figure 40. Fluorescence spectra of untreated H42D. The addition of 10 mM sulfate to 1 μM H42D in 40 mM Tris pH 8 results in no change in the fluorescence spectrum. Excitation wavelength was 290 nm.
Figure 41. Fluorescence spectra of untreated D68N. The addition of 10 mM sulfate to 1 μM D68N in 40 mM Tris pH 8 results in a 6 nm blue shift and a slight increase in fluorescence intensity. Excitation wavelength was 290 nm.
Figure 42. Fluorescence spectra of untreated D68G. The addition of 10 mM sulfate to 1 μM D68G in 40 mM Tris pH 8 results in a 6 nm blue shift and a slight increase in fluorescence intensity. Excitation wavelength was 290 nm.
Figure 43. Fluorescence spectra of untreated E15Q. The addition of 10 mM sulfate to 1 μM E15Q in 40 mM Tris pH 8 results in a 3 nm blue shift and a slight increase in fluorescence intensity. Excitation wavelength was 290 nm.
Figure 44. Fluorescence spectra of untreated S129C. The addition of 10 mM sulfate to 1 μM S129C in 40 mM Tris pH 8 results in no change in the fluorescence spectrum. Excitation wavelength was 290 nm.
Figure 45. Fluorescence spectra of untreated G46C. The addition of 10 mM sulfate to 1 
μM G42C in 40 mM Tris pH 8 results in no change in the fluorescence spectrum. Excita-
tion wavelength was 290 nm.
Figure 46. Fluorescence spectra of untreated S129C & G46C. The addition of 10 mM sulfate to 1 μM S129C & G46C in 40 mM Tris pH 8, 10 mM dithiothreitol results in a 6 nm blue shift and a slight decrease in fluorescence intensity. Excitation wavelength was 290 nm.
Figure 47. Fluorescence spectra of untreated SBP-Dis. The addition of 10 mM sulfate to 1 µM SBP-Dis in 40 mM Tris pH 8 results in a 3 nm blue shift and a slight increase in fluorescence intensity. Excitation wavelength was 290 nm.
WAVELENGTH [nm]

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10 mM Sulfate

Untreated
Figure 48. Fluorescence spectra of treated *Salmonella typhimurium* SBP. Fluorescence intensity of 1 μM DTT treated *Salmonella typhimurium* in 40 mM Tris pH 8 decreased upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
Figure 49. Fluorescence spectra of treated *E. coli*. Fluorescence intensity of 1 μM DTT treated *E. coli* in 40 mM Tris pH 8 decreased upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
Figure 50. Fluorescence spectra of treated S130C. Fluorescence intensity of 1 μM DTT treated S130C in 40 mM Tris pH 8 was unchanged by the addition of 10 μM chromate. Addition of 1 mM sulfate yielded decreased fluorescence and a slight blue shift. Excitation wavelength was 290 nm.
Figure 51. Fluorescence spectra of treated H42G. Fluorescence intensity of 1 μM DTT treated H42G in 40 mM Tris pH 8 decreased and blue-shifted slightly upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
The graph shows the relative fluorescence plotted against wavelength in nanometers (nm). There are three distinct curves:

1. A curve labeled "10 mM Sulfate".
2. A curve labeled "10 μM Chromate".
3. A curve labeled "Treated".

The y-axis represents relative fluorescence, ranging from 0 to 9000, while the x-axis represents wavelength, ranging from 300 to 400 nm.
Figure 52. Fluorescence spectra of treated H42D. Fluorescence intensity of 1 μM DTT treated H42D in 40 mM Tris pH 8 decreased and blue-shifted slightly upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
Figure 53. Fluorescence spectra of treated D68N. Fluorescence intensity of 1 μM DTT treated D68N in 40 mM Tris pH 8 decreased and blue-shifted slightly upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
Figure 54. Fluorescence spectra of treated D68G. Fluorescence intensity of 1 μM DTT treated D68G in 40 mM Tris pH 8 decreased and blue-shifted slightly upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
Figure 55. Fluorescence spectra of treated E15Q. Fluorescence intensity of 1 μM DTT treated E15Q in 40 mM Tris pH 8 and blue-shifted slightly decreased upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
Figure 56. Fluorescence spectra of treated S129C. Fluorescence intensity of 1 μM DTT treated S129C in 40 mM Tris pH 8 decreased and blue-shifted slightly upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
Figure 57. Fluorescence spectra of treated G46C. Fluorescence intensity of 1 μM DTT treated S46C in 40 mM Tris pH 8 decreased and blue-shifted slightly upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
A graph showing the relative fluorescence of solutions at different wavelengths. The x-axis represents WAVELENGTH [nm] ranging from 300 to 400 nm, and the y-axis represents RELATIVE FLUORESCENCE ranging from 0 to 10000. Three curves are plotted:

- 10 mM Sulfate
- 10 μM Chromate
- Treated
Figure 58. Fluorescence spectra of treated S129C & G46C. Fluorescence intensity of 1 μM DTT treated S129C & G46C in 40 mM Tris pH 8 decreased and blue-shifted slightly upon the addition of 10 μM chromate. Addition of 1 mM sulfate to the chromate bound protein increased fluorescence intensity to higher than that of the ligand-free form. Excitation wavelength was 290 nm.
Appendix IV.

Scatchard Plots of Fluorescence Titrations and Stopped-Flow Traces from Displacement Studies
Figure 59. Scatchard plot of sulfate binding to *Salmonella typhimurium* SBP. Data is from two independent titrations of 1 μM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 0.7 μM. Because of the high affinity of this protein for sulfate, binding is essentially stoichiometric and the concentration of free sulfate in these titrations is not well-defined. The calculated $K_d$ should be considered an upper limit to the actual $K_d$. 
Figure 60. Scatchard plot of sulfate binding to *E. coli* SBP. Data is from two independent titrations of 1 μM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 0.6 μM. Because of the high affinity of this protein for sulfate, binding is essentially stoichiometric and the concentration of free sulfate in these titrations is not well-defined. The calculated $K_d$ should be considered an upper limit to the actual $K_d$. 
Figure 61. Scatchard plot of sulfate binding to S130C. Data is from two independent titrations of 1 µM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 1.0 mM.
Figure 62. Scatchard plot of sulfate binding to H42G. Data is from two independent titrations of 1 μM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 3.5 μM.
Figure 63. Scatchard plot of sulfate binding to H42D. Data is from two independent titrations of 1 μM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 0.3 μM. Because of the high affinity of this protein for sulfate, binding is essentially stoichiometric and the concentration of free sulfate in these titrations is not well-defined. The calculated $K_d$ should be considered an upper limit to the actual $K_d$. 
Figure 64. Scatchard plot of sulfate binding to D68N. Data is from two independent titrations of 1 μM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 0.9 μM.
Figure 65. Scatchard plot of sulfate binding to D68G. Data is from two independent titrations of 1 μM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 1.1 μM.
Figure 66. Scatchard plot of sulfate binding to E15Q. Data is from two independent titrations of 1 μM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 0.7 μM. Because of the high affinity of this protein for sulfate, binding is essentially stoichiometric and the concentration of free sulfate in these titrations is not well-defined. The calculated $K_d$ should be considered an upper limit to the actual $K_d$. 
Figure 67. Scatchard plot of sulfate binding to S129C. Data is from two independent titrations of 1 \( \mu \text{M} \) protein. A least squares fit of all data (solid line) indicates a \( K_d \) of 0.5 \( \mu \text{M} \). Because of the high affinity of this protein for sulfate, binding is essentially stoichiometric and the concentration of free sulfate in these titrations is not well-defined. The calculated \( K_d \) should be considered an upper limit to the actual \( K_d \).
Figure 68. Scatchard plot of sulfate binding to G46C. Data is from two independent
titrations of 1 μM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 0.5
μM. Because of the high affinity of this protein for sulfate, binding is essentially
stoichiometric and the concentration of free sulfate in these titrations is not well-defined.
The calculated $K_d$ should be considered an upper limit to the actual $K_d$. 
Figure 69. Scatchard plot of sulfate binding to S129C & G46C. Data is from two independent titrations of 1 μM protein. A least squares fit of all data (solid line) indicates a $K_d$ of 1.6 μM.
Figure 70. Displacement of sulfate bound to *Salmonella typhimurium* SBP. 1 μM *Salmonella typhimurium* SBP in 40 Tris pH 8 plus 10 mM Na$_2$SO$_4$ mixed with 1 mM Na$_2$CrO$_4$ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this protein calculated from this experiment is 3 s$^{-1}$. 
Figure 71. Displacement of sulfate bound to *E. coli* SBP. 1 µM *E. coli* SBP in 40 Tris pH 8 plus 10 mM Na₂SO₄ mixed with 1 mM Na₂CrO₄ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this protein calculated from this experiment is 5 s⁻¹.
Figure 72. Displacement of sulfate bound to H42D. 1 μM H42D in 40 Tris pH 8 plus 10 mM Na₂SO₄ mixed with 1 mM Na₂CrO₄ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this mutant calculated from this experiment is 6 s⁻¹.
Figure 73. Displacement of sulfate bound to D68N. 1 μM D68N in 40 Tris pH 8 plus 10 mM Na₂SO₄ mixed with 1 mM Na₂CrO₄ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this mutant calculated from this experiment is 360 s⁻¹.
Figure 74. Displacement of sulfate bound to D68G. 1 μM D68G in 40 Tris pH 8 plus 10 mM Na₂SO₄ mixed with 1 mM Na₂CrO₄ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this mutant calculated from this experiment is 211 s⁻¹.
Figure 75. Displacement of sulfate bound to E15Q. 1 μM E15Q in 40 Tris pH 8 plus 10 mM Na$_2$SO$_4$ mixed with 1 mM Na$_2$CrO$_4$ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this mutant calculated from this experiment is 235 s$^{-1}$. 

Figure 76. Displacement of sulfate bound to S129C. 1 µM S129C in 40 Tris pH 8 plus 10 mM Na₂SO₄ mixed with 1 mM Na₂CrO₄ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this mutant calculated from this experiment is 13 s⁻¹.
Figure 77. Displacement of sulfate bound to G46C. 1 μM G46C in 40 Tris pH 8 plus 10 mM Na₂SO₄ mixed with 1 mM Na₂CrO₄ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this mutant calculated from this experiment is 11 s⁻¹.
Figure 78. Displacement of sulfate bound to S129C & G46C. 1 μM S129C & G46C in 40 Tris pH 8 plus 10 mM Na₂SO₄ mixed with 1 mM Na₂CrO₄ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this mutant calculated from the fast phase of this experiment is 26 s⁻¹.
Figure 79. Displacement of sulfate bound to SBP-Dis. 1 µM SBP-Dis in 40 Tris pH 8 plus 10 mM Na₂SO₄ mixed with 1 mM Na₂CrO₄ results in significant fluorescence quenching due to energy transfer from excited tryptophan to chromate. The off-rate for sulfate from this mutant calculated from the slow phase of this experiment is 0.12 s⁻¹.