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A Nonconservative Serine to Cysteine Mutation in the Sulfate-Binding Protein, a Transport Receptor

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Serine 130 is one of seven residues that form a total of seven hydrogen bonds with the sulfate completely sequestered deep in the cleft between the two lobes of the bilobate sulfate-binding protein from *Salmonella typhimurium*. This residue has been replaced with Cys, Ala, and Gly by site-directed mutagenesis in an *Escherichia coli* expression system. Replacement with the isosteric Cys caused a 3200-fold decrease in the sulfate-binding activity relative to the wild-type activity, whereas replacement with Ala and Gly resulted in only 100- and 15-fold decreases, respectively. The effect of the Cys substitution is attributed largely to steric effect, whereas the Gly substitution more nearly reflects the loss of one hydrogen bond to the bound sulfate with a strength of only 1.6 kilocalories per mole.

BECAUSE OXYGEN AND SULFUR possess similar chemical properties and belong to the 6A chemical group of the periodic elements, interconversion of serine and cysteine is considered to be the most conservative between any pair of amino acid residues in proteins. Indeed it is this consideration that motivated investigators to undertake the conversion of serine-type proteinases (such as trypsin and subtilisin) to cysteine-type proteinases (such as papain) as far back as 25 years ago by chemical means (*1*, *2*) and more recently by recombinant technology (*3*).

This consideration prompted us to investigate the effect of a Ser to Cys mutation at the oxydianion-binding site of the sulfate-binding protein (SBP). The sulfate-binding protein belongs to a large class of proteins found in the periplasmic space of Gram-negative bacteria. These monomeric proteins, collectively called "binding proteins," serve as initial high-affinity receptors for the active transport systems for various carbohydrates, amino acids, oligopeptides, and oxyanions (*4*). The SBP from *S. typhimurium* and *E. coli* has identical 310 amino residues, giving a molecular weight of ~34,700 daltons (*5*, *6*), and one high-affinity ligand-binding site (*7*, *8*). The ligand-binding site is exquisitely designed to bind fully ionized tetrahedral oxydianions (such as sulfate, selenate, and chromate) but not the weak acid phosphate (*7*, *8*).

The crystal structure of the liganded form of the *S. typhimurium* SBP that has been well refined at 2.0 Å resolution (*9*) and at 1.7 Å resolution (*10*) has revealed the atomic interactions between the protein and sulfate. The sulfate dianion, which is completely desolvated and sequestered ~7.5 Å deep in the cleft between the two lobes of the bilobate SBP, is held in place by seven hydrogen bonds formed with five NH groups of peptide units, one NH of Trp^{192} side chain, and one OH of Ser^{130} (Fig. 1). The sequence of Ser^{130} OH, Gly^{131} NH, and Gly^{132} NH forms a tripod for docking the O1, O2, and O3 sulfate oxygens, respectively. All of the hydrogen-bond donor groups to the sulfate are also buried. Serine 130, which precedes the first turn of a helix, is semi-enclosed in the wall of one domain facing the cleft.

The parameters of the hydrogen bond (OH···O) between Ser^{130} γ-OH and the sulfate oxygen derived from the 1.7 Å refined structure (*10*) are 2.65 Å for the O···O distance and 159.5° for the angle. The hydrogen-bond distance is the shortest of the seven hydrogen bonds (overall mean distance = 2.78 Å) between SBP and sulfate (*9*, *10*). As can be seen in Fig. 1, Ser^{130} γ-OH is involved in cooperative hydrogen bonding by further accepting an NH from the third peptide unit (Ala^{133}) of the first turn of helix IV (N···O distance = 2.95 Å).

We have recently cloned and sequenced the gene for the SBP and have developed a suitable expression system in *E. coli* for the overproduction of mutant proteins (*6*). Besides identical amino acid content, the *E. coli* SBP is extremely similar in antigenecity, ligand specificity, and equilibrium and kinetics of ligand binding to the *S. typhimurium* SBP (*6*). There are only 18 residue differences between the two SBPs, and based on the structure of the *S. typhimurium* SBP-sulfate complex, these are located at or near the protein surface, nowhere within a radius of 19 Å to the bound sulfate (*6*). Most importantly, these residue changes do not affect the ligand-binding activity of the *E. coli* SBP. Indeed, all of the residues important to sulfate binding are absolutely pre-

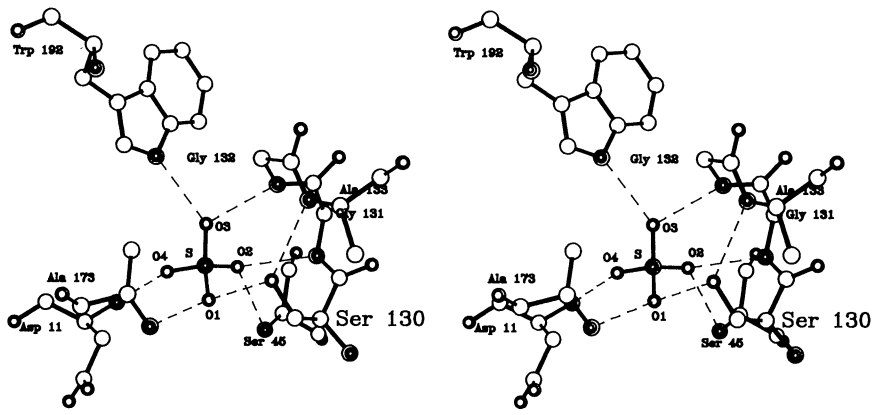

Fig. 1. Stereoscopic view of the hydrogen-bonding interactions between SBP and sulfate. Carbons are depicted as single circles, oxygens as double circles, and nitrogens as triple circles. [These interactions are those observed in the 1.7 Å crystallographic refinement of the *S. typhimurium* SBP-sulfate complex structure (*10*).]

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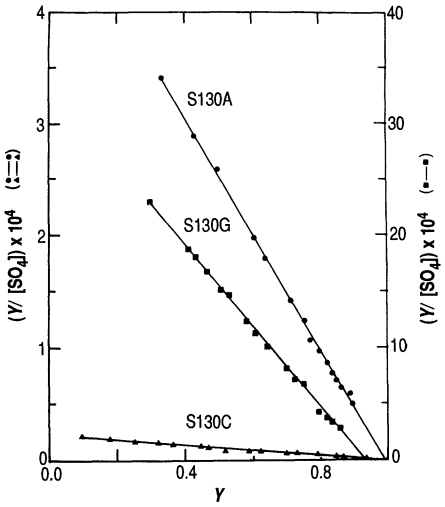

Fig. 2. Scatchard plots of the sulfate binding to SBP mutants $Ser^{130}Gly$ (■), $Ser^{130}Ala$ (●), and $Ser^{130}Cys$ (▲). The left ordinate is for the Ala^{130} and Cys^{130} mutants, and the right ordinate is for the Gly^{130} mutant.

served in the *E. coli* sequence, including those in and around the ligand-binding site cleft and those at the interface between the two domains. We report results of the site-directed mutagenesis replacement of Ser^{130} to Cys, Ala, and Gly in *E. coli* SBP (*11*).

All of the mutant proteins were purified to homogeneity (*12*). Thiol determination of the Cys^{130} SBP by 5,5′-dithiobis[2-nitrobenzoic acid] reaction indicated one Cys residue per molecule. Typical Scatchard plots of the equilibrium sulfate binding data obtained for the Cys^{130}, Ala^{130}, and Gly^{130} SBP mutants are presented in Fig. 2. Sulfate-binding measurements of the Cys^{130} SBP in the presence or absence of a reducing agent essentially gave identical dissociation constants.

Although the Ser^{130} γ-OH of SBP is only one of seven hydrogen-bond donor groups to the sulfate (Fig. 1) and the Ser to Cys mutation is considered to be the most conservative, the replacement of Ser^{130} by Cys caused a surprisingly large decrease in sulfate-binding activity, about 3200-fold (or a binding energy loss of 4.8 kcal/mol) at pH 6.0 (Table 1). In contrast, mutations of Ser^{130} to Ala and Gly weakened sulfate binding by only 2.7 and 1.6 kcal/mol, respectively (*13*). How do we best account for these results?

The far greater loss of binding activity accompanying the Ser^{130} to Cys mutation is attributable to steric effects. Cysteine is larger and has different geometries than serine. The atomic radius of sulfur is 0.4 Å larger than oxygen, and the Cys side chain has bond lengths and angles significantly different from Ser (Fig. 3) (*14*). The change in local geometry caused by the Cys substitution is likely to interfere with the formation of the cooperative hydrogen-bonding interaction seen for the γ-OH of Ser^{130} in the wild-type SBP-sulfate complex (Fig. 1). In this interaction, which is of the type NH → OH → O, the Ser OH is both a hydrogen-bond acceptor from the NH group of Ala^{133} (N to O distance of 2.95 Å) and a donor to the sulfate oxygen (O to O distance of 2.68 Å). A Cys residue in place of Ser^{130} would increase each hydrogen bond by 0.3 to 0.5 Å. Apparently, this small geometrical change is enough to cause a dramatic loss in sulfate-binding activity. As Ser^{130} precedes two successive Gly residues (131 and 132) whose NH groups also serve as hydrogen-bond donors to the sulfate (Fig. 1), the change accompanying the replacement with Cys could further adversely affect the docking of the two sulfate oxygens (O2 and O3) to the NH groups.

Steric effects are presumably not a factor in the increase in the dissociation constants arising from the mutations of Ser^{130} to Ala and Gly. The loss of sulfate-binding energy, particularly due to the Gly replacement, reflects more nearly the loss of one hydrogen bond in the SBP-sulfate complex (Table 1). Although the hydrogen bond is considered to be a very strong one, being formed in a low dielectric constant environment between a neutral OH and charged SO_4^{-2} with an O···O distance of 2.65 Å (see above), nevertheless its loss results surprisingly only a 1.6 kcal/mol decrease in binding energy. Values of the energy of hydrogen bonds have been estimated to lie in the range of −3 to −6 kcal/mol. The sevenfold further increase in K_d on mutation to Ala relative to the Gly^{130} mutant activity is likely due to the proximity of the apolar β-methyl side chain to the polar sulfate dianion.

The Cys^{130} SBP mutant has a maximum binding activity at around pH 6 (Table 1). Binding activity becomes progressively worse with increasing pH to the extent that beyond pH 9 none can be measured accurately by the fluorescence assay method. This result is indicative of a thiol to thiolate ionization, which results in charge repulsion of the sulfate dianion. In contrast, the activity of the wild-type protein is insensitive to pH from pH 5.5 to 9.5 (*7, 8*). This has been attributed to the binding of a fully ionized sulfate species and, as can be seen in Fig. 1, to the absence of ionizable groups (with pK

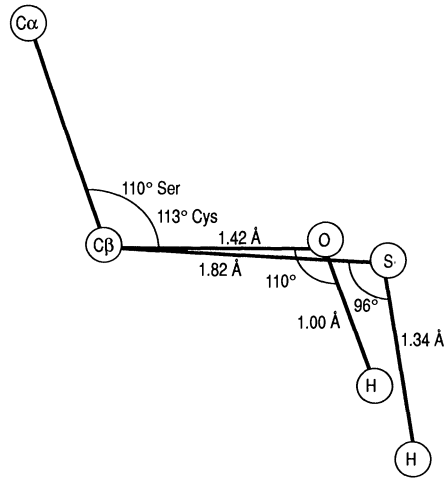

Fig. 3. Geometry of the C_β–O_γ–H bonds in serine and C_β–S_γ–H bonds in cysteine. The α- and β-carbon atoms of both amino acids are superimposed. Data obtained from (*14*).

Table 1. Sulfate-binding activity of the wild-type and Ser^{130} replacement mutants of SBP. Each K_d value is the mean of three to five independent measurements; the number within the parentheses is the standard deviation. The K_d of the wild-type SBP was determined with an anion exchange–based equilibrium assay (*7, 8*). The K_d is similar to those determined for *S. typhimurium* SBP (*7, 8*). All mutants were assayed more conveniently by fluorescence techniques (*6–8*). Fluorescence titrations were performed on an SLM Aminco 4800 spectrofluorometer at 20°C. The protein concentration used in the assay mixture of 2 ml was 1 μM for all SBP mutants. The Gly^{130} Ala^{130} SBPs were assayed in a solution of 40 mM tris-HCl, pH 7.5. For the binding assay of Cys^{130} SBP as a function of pH, the following buffers were used: 40 mM Bistris-HCl (pH 5.0 and 6.0) and 40 mM tris-HCl (pH 7.0, 8.0, and 8.5). The cuvette containing 2 ml of protein was capped with a rubber septum and purged with nitrogen immediately before titrations. Sulfate additions were made with a Hamilton syringe and did not exceed 4% of the total volume. The excitation wavelength was set at 290 nm, and the decrease in emission at 360 nm induced by sulfate binding was monitored. Dissociation constants were determined by Scatchard analysis (see Fig. 1). The presence or absence of 100 μM dithiothreitol in the assays of Cys^{130} SBP gave essentially the same K_d values.

Protein	pH of assay	K_d (μM)	ΔG (kcal/mol)	ΔΔG (kcal/mol)
Wild type	7.5	0.17 (0.02)	−9.2	
$Ser^{130}Gly$	7.5	2.57 (0.39)	−7.6	−1.6
$Ser^{130}Ala$	7.5	16.8 (1.60)	−6.5	−2.7
$Ser^{130}Cys$	5.0	860 (60)	−4.1	−5.0
	6.0	550 (80)	−4.4	−4.8
	7.0	2640 (550)	−3.5	−5.7
	8.0	5610 (1110)	−3.0	−6.1
	8.5	8600 (1550)	−2.8	−6.4

<14) hydrogen bonding to the oxydianion in the wild-type SBP (*8*).

Although the active site Ser in proteinases has been successfully mutated to Cys, the ultimate goal or desired result has not been achieved; engineered thiol trypsin exhibits essentially no enzymatic activity with normal amide and ester substrates (*3*). The cause of the failure to achieve the desired result is not clearly understood (*15*). Small geometrical changes resulting from the mutation could have an effect far more profound than that described here. Although little publicized, the catalytic Ser or Cys residues of proteolytic enzymes are, like Ser^{130} of SBP, engaged in cooperative hydrogen bonding; they simultaneously donate to a His residue that is part of the charge relay system and accept an NH group or a solvent molecule.

The results presented here demonstrate the sensitivity of a Ser to Cys mutation. If the effect of the mutation is as deleterious as the one reported here, one wonders what the extent of the effect might be if the residue is directly involved in enzyme catalysis. In any case the use of site-directed mutagenesis to probe or quantitate structure-function relations is fraught with uncertainty. To minimize the uncertainty, it is important that many mutational changes be made. For amino acid replacements to be rationally selected and generated and for interpretation of the results to be properly made, a well-refined high-resolution structure is a prerequisite.

Finally, there seems to be a propensity of hydroxyl-containing residues, as well as peptide units, to interact with charged groups and ligands (*9, 16, 17*). An excellent example has been recently shown in the well-refined 1.7 Å resolution structure of the phosphate-binding protein in complex with phosphate; the completely dehydrated and sequestered phosphate is held in place by a total of 12 hydrogen bonds, 5 with peptide unit NH groups and 4 with hydroxyl side chains (*17*). We have further noted that ligand-gated ion channels are known to contain regions rich in hydroxyl side chains and that, like in the binding protein-phosphate complex, these hydroxyls may have properties that are particularly important in achieving the requisite specificity and speed of ion movements (*17*). The sensitivity and differential effects of mutations of Ser to Cys, Ala, and Gly at a highly specific ion-binding site as demonstrated here could be used to probe similar residues residing in these channels or in other ligand-binding sites.

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11. The expression vectors and methods used in the mutagenesis in *E. coli* have been described (*6*). Mutations of $Ser^{130} \rightarrow$ Gly, $Ser^{130} \rightarrow$ Ala, and $Ser^{130} \rightarrow$ Cys and were directed by oligonucleotides 5′-CGCCACCACCGCTTTTC-3′, 5′-CGCCACCAGCGCTTTTC-3′, and 5′-CGCCACCACAGCTTTTC-3′, respectively, where the bold underlined base indicates mismatches. In each mutagenesis, the entire SBP gene was sequenced to ensure that the desired mutation was attained.
12. Proteins were purified from a 12-liter growth of appropriate mutant *E. coli* cells with the use of the method previously described for the wild-type SBP (*5, 6*). Purification of SBP with this method is achieved mainly by the use of DEAE-53 ion exchange chromatography. The Ala^{130} and Gly^{130} SBP mutants were further purified by high-performance liquid chromatography (HPLC) with a preparative Synchrom Q300 anion exchange column. In the purification of the Ser^{130}Cys mutant, β-mercaptoethanol was added to all solutions to a final concentration of 1 mM. Moreover, after chromatography on a DEAE column, the mutant protein was further purified by isoelectric focusing (ampholyte pH 6 to 8) with a Bio-Rad Rotofor Preparative IEF Cell. Protein purity was determined by SDS-polyacrylamide gel electrophoresis and isoelectric focusing with Pharmacia's PhastGel. Protein concentration was determined spectrophotometrically by using an extinction coefficient of 1.2 ml mg^{-1} cm^{-1} (*7*).
13. Note that the binding activity of Gly^{130} and Ala^{130} SBP mutants was measured at pH 7.5, the pH at which the wild-type protein activity is normally measured (*7, 8*). The Cys^{130} SBP mutant has maximum activity at about pH 6.0 (see Table 1).
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Crystal Structure of Defensin HNP-3, an Amphiphilic Dimer: Mechanisms of Membrane Permeabilization

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Defensins (molecular weight 3500 to 4000) act in the mammalian immune response by permeabilizing the plasma membranes of a broad spectrum of target organisms, including bacteria, fungi, and enveloped viruses. The high-resolution crystal structure of defensin HNP-3 (1.9 angstrom resolution, *R* factor 0.19) reveals a dimeric β sheet that has an architecture very different from other lytic peptides. The dimeric assembly suggests mechanisms by which defensins might bind to and permeabilize the lipid bilayer.

NEUTROPHILS CONSTITUTE 50 TO 70% of the total white blood cells in humans. They play a vital role in the immune response by ingesting invading microorganisms, which are then destroyed by one of two general mechanisms. The "oxygen-dependent" mechanism results from the production of superoxide, which is converted to potent oxidants termed "reactive oxygen intermediates" (*1*). The other, "oxygen-independent," defense mechanism occurs when the microbicidal-cytotoxic proteins of cytoplasmic granules are discharged into the phagocytic vacuole (*2*).

Defensins account for ~30% of the total protein in human azurophil granules (*3*). They are small (molecular weight of 3500 to 4000), cationic, disulfide cross-linked proteins that show in vitro activity against Gram-negative and Gram-positive bacteria (*3, 4*), fungi (*5*), mammalian cells (*6*), and enveloped viruses (*7*). The work of Lehrer and colleagues shows that defensins permeabilize both the inner and outer membranes of *Escherichia coli*, and that inner-membrane permeabilization is coincident with cell death (*8*). A membrane potential is apparently required for defensin action, since cells are killed only when metabolically active and they are protected by membrane-depolarizing agents such as carbonylcyanide M-chlo-

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