Dominant role of local dipoles in stabilizing uncompensated charges on a sulfate sequestered in a periplasmic active transport protein

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Abstract

Electrostatic interactions are among the key factors determining the structure and function of proteins. Here we report experimental results that illuminate the functional importance of local dipoles to these interactions. The refined 1.7-Å X-ray structure of the liganded form of the sulfate-binding protein, a primary sulfate active transport receptor of *Salmonella typhimurium*, shows that the sulfate dianion is completely buried and bound by hydrogen bonds (mostly main-chain peptide NH groups) and van der Waals forces. The sulfate is also closely linked, via one of these peptide units, to a His residue. It is also adjacent to the N-termini of three α -helices, of which the two shortest have their C-termini "capped" by Arg residues. Site-directed mutagenesis of the recombinant *Escherichia coli* sulfate receptor had no effect on sulfate-binding activity when an Asn residue was substituted for the positively charged His and the two Arg (changed singly and together) residues. These results, combined with other observations, further solidify the idea that stabilization of uncompensated charges in a protein is a highly localized process that involves a collection of local dipoles, including those of peptide units confined to the first turns of helices. The contribution of helix macrodipoles appears insignificant.

Keywords: charge stabilization; electrostatics; helix termini; local dipoles; mutagenesis; peptide dipoles

The structure of the sulfate-bound form of the *Salmonella typhimurium* sulfate-binding protein (SBP), a primary periplasmic receptor of active transport, refined at resolutions of 2.0 Å (Pflugrath & Quicho, 1985, 1988) and 1.7 Å (J.S. Sack & F.A. Quicho, submitted), has provided us with an excellent and well-defined system to study electrostatic interactions, especially in relation to charged ligand binding. The structure consists of two domains, with a similar α/β folding motif, separated by a deep cleft. The sulfate is completely dehydrated and sequestered deep in the cleft with its charges uncompensated. All four oxygens of the bound sulfate accept a total of seven strong hydrogen bonds from five NH groups of main-chain peptide units, 1 NH of a Trp side chain, and 1 OH of a Ser residue (Fig. 1) (Pflugrath & Quicho, 1985, 1988). The sulfate further makes 40 van der Waals contacts (3.08–4.0 Å) with several residues (J.S. Sack & F.A. Quicho, submitted). Short loops between elements of the secondary structure and amino-terminal ends of

α -helices deploy many of the residues making hydrogen-bond, van der Waals, and dipolar interactions with the sulfate.

The five main-chain NH and one OH groups donating hydrogen bonds to the sulfate are further coupled to hydrogen-bond arrays (Pflugrath & Quicho, 1985, 1988). Because three of the five peptide NH donor groups are found in the first turn of three helices, they initiate arrays of hydrogen-bonded peptide units within these helices (Figs. 1, 2; kinemages). The two other main-chain peptide units are coupled to positively charged residues, one to Arg 134, which is in turn salt-linked with Asp 68, and the other to His 42, which is within 7.7 Å of the sulfate (Fig. 1). These hydrogen-bond arrays and the proximity of His 42 to the sulfate are thought to contribute to ligand fixation and charge compensation.

Since the time that the α -helix "macro-dipole model" was proposed (Wada, 1976; Hol et al., 1978), it has been assumed (even to the present) in an untold number of cases that the macrodipole plays a significant role in electrostatic interactions. It has been predicted, on the basis of this model, that the helix N- and C-termini possess half

Fig. 1. Stereoscopic view of the hydrogen bonds and hydrogen-bonding arrays directly associated in the sulfate-binding protein (SBP)-sulfate interactions. Only the first two turns of the helices α -II, α -IV, and α -VI are depicted; complete helices are shown in Figure 2. The sulfate oxygens accept seven hydrogen bonds from the main-chain NH groups of residues 11, 45, 131, 132, and 173, the $O\gamma H$ of Ser 130, and the $N\epsilon H$ of Trp 192. Further details of the hydrogen-bonding interactions are found in Pflugrath and Quiocho (1985, 1988). There are 40 van der Waals contacts (3.1–4.0 Å) between SBP and the sulfate (J.S. Sack & F.A. Quiocho, submitted). The model (adapted from Quiocho et al., 1987) and the one shown in Figure 2 are from the structure refinement of the *Salmonella typhimurium* SBP at 1.7 Å resolution (J.S. Sack & F.A. Quiocho, submitted).

positive charge and half negative charge, respectively, that are separated by the length of the helix (Hol, 1985; Hol et al., 1978). Such a role in SBP, although less favored (Pflugrath & Quiocho, 1985; Quiocho et al., 1987), also seemed possible because the N-termini of three helices— α -II (residues 44–54), α -IV (residues 131–148), and α -VI (residues 172–182)—are close to the sulfate (Figs. 1, 2). Interestingly, the C-terminal ends of the 3-turn α -II and α -VI helices are capped by positively charged Arg 77 and Arg 102, respectively (Fig. 2). A survey of refined protein

structures revealed that peptide units of helix termini are often involved in hydrogen bonding with polar (some charged) groups (Presta & Rose, 1988).

Presumably, based on the macrodipole idea, capping by Arg residues compensates for the helix macrodipoles' partial negative charge, as well as transforms the macrodipoles into large monopoles with a greater ability to attract negatively charged ligands at the helix N-termini, as for example in the case of SBP. Theoretical analysis indicates that the strength of the macrodipole field is largely

Fig. 2. Stereoscopic view of the three SBP helices (α -II, α -IV, and α -VI), whose N-termini are close to the bound sulfate. Note that the first turn of each helix donates one peptide NH hydrogen bond to the sulfate oxygens (see Fig. 1) and that α -II and α -VI, which consist of three turns, are the shortest of the three helices. Also depicted are Arg 77 and Arg 102 capping the C-terminal ends of α -II and α -VI, respectively. Arg 77 $N\eta 1H$ donates a hydrogen bond to the C-terminus peptide carbonyl oxygen of residue 52 of α -II (N to O distance = 2.94 Å). Both $N\eta 1H$ and $N\eta 2H$ of Arg 102 donate H bonds to the α -VI C-terminus carbonyl oxygen of residue 180, with distances of 3.47 Å and 2.91 Å, respectively (Fig. 2). The loop (residues 183–186) (also drawn) following the last turn of α -VI deploys backbone carbonyl oxygens (from residues 184, 185, and 186) that make four additional hydrogen bonds with the guanidinium group of Arg 102.

derived from the first or last two turns of a helix, with marginal but still definite contribution from two additional turns (Hol et al., 1978).

To our knowledge, there are no experimental studies of proteins that probe the helix-macrodipole effect by charged capping residues on electrostatic interactions associated with ionic ligand binding such as the one in SBP. Herein we report results of site-directed mutagenesis and ligand-binding measurements to experimentally test whether helix capping with Arg 77 and Arg 102 of SBP, as well as the close coupling of His 42, have any influence on the sulfate-dipole interactions.

Results and discussion

Neutralization of positively charged residues close to the sulfate or capping helix C-termini

His 42, Arg 77, and Arg 102 residues are barely accessible to solvent; the accessible surface areas, as defined by Lee and Richards (1971), of 14 Å² for His 42 and Arg 77, and 20 Å² for Arg 102 represent only 3.6, 3.1, and 4.5%, respectively, of the accessible areas of the corresponding free residues. Using an expression system of a nearly identical *Escherichia coli* SBP (Jacobson et al., 1991), site-directed mutagenesis was performed, mutant proteins were purified to homogeneity, and sulfate-binding activity was determined accurately. The results shown in Table 1 are illuminating.

Neutralizing His 42 by an Asn substitution caused a miniscule increase in the sulfate dissociation constant (Table 1). This rules out any effective contribution of the close proximity and coupling via a peptide unit of the positively charged His residue to sulfate binding and charge compensation. The finding strongly suggests that the effectiveness of dipoles is limited to those closest to the sulfate. Assuming a normal pK of His 42, the mutagenesis result is consistent with previous data showing that dianion binding to SBP from both *S. typhimurium* (Pardee, 1966; Jacobson & Quijcho, 1988) and *E. coli* (J.J. He & F.A. Quijcho, unpubl.) is insensitive to a wide range of pH changes (from pH 4.5 to 10). Presumably, the protonated, positively charged form of His 42 at acidic pH

range does not enhance dianion binding. None of the groups donating hydrogen bonds to the sulfate oxygens are capable of ionizing below pH 13.

As is evident in the data shown in Table 1, neutralizing the Arg residues (singly and together) that cap the two 3-turn helices imparts no unfavorable effects on the charge-dipole interactions occurring at the corresponding N-termini. These results were unexpected in light of the relatively short length of the two helices and of the prediction (Hol et al., 1978) that the third and fourth turns of the helix backbone further augment (about 25%) the macrodipole field strength. (The locations of the helix-capping Arg residues relative to the helix N-termini are roughly similar to the location of the backbone NH groups of the fourth helix turn.) The simplest conclusion we can draw from these results is that the effect of the helices on either the binding of the sulfate or the capping by the positively charged Arg residues is confined to a length so short (i.e., almost entirely the first and last turns, with amide and carbonyl partial charges, respectively) as to make the macrodipoles of little consequence on both electrostatic interactions (further discussed below). Although neutralization of the charged capping residues does not have any influence, the mutagenesis experiments do not address the direct effects of helix termini dipoles. Even with an excellent system like that found in SBP, it is extremely difficult to design experiments that probe directly at the effects of peptide backbone dipoles confined to the first turn of a helix.

Nevertheless, in contrast to the lack of effects of the mutations described above (Table 1), we previously found that replacing Ser 130, whose dipolar γ -OH makes a direct hydrogen bond with the sulfate (Fig. 1), with Gly and Ala reduced the sulfate-binding activity by 15- and 100-fold, respectively (He & Quijcho, 1991). This underscores the importance of the local environment on charge stabilization.

Presently we have no direct results to eliminate the possibility that other positively charged residues re-cap the helices following replacement of the Arg residues by Asn. Because polar uncharged residues (e.g., Asn, Gln, etc.) also cap and often make hydrogen bonds with helix termini (Presta & Rose, 1988), re-capping by other positively charged residues is not necessary and, indeed, may not be possible in SBP due to steric hindrance by the interposing Asn replacement and other nearby residues. Moreover, examination of the wild-type SBP structure indicates that drastic conformational changes in the mutant proteins will be required in order to bring the nearest positively charged residues close to the helix C-termini. Lys 228 and Lys 232, the closest to the α -II C-terminus, are not only located about 12 Å from the C-terminus but also are salt-linked to Glu 231 and Glu 57, respectively. Although Lys 207 NZ is 4.6 Å from the C-terminus of α -VI, the presence of Phe 207 between the Lys residue and the helix C-terminus prevents closer contact.

Table 1. Sulfate-binding activities of wild-type and asparagine mutants of the sulfate-binding protein

Protein	$K_d \times 10^7$ (M) ^a
Wild type	2.48 (0.08)
His-42 to Asn	2.99 (0.06)
Arg-77 to Asn	2.23 (0.06)
Arg-102 to Asn	2.24 (0.07)
Arg-77 and Arg-102 to Asn	2.43 (0.08)

^a Each K_d value is the average of four independent determinations at pH 7.2 with standard deviation in parentheses.

Charge stabilization by a constellation of local dipoles

Taken together, our mutagenesis results lead to the conclusion that the mechanism by which isolated charges are stabilized in a protein environment is of a highly localized nature. The mechanism proposed previously assumes that the collection of dipoles immediately surrounding isolated ionic groups is responsible for charge stabilization (Pflugrath & Quiocho, 1985; Quiocho et al., 1987; Åqvist et al., 1991; others cited therein). These concentrated first-shell dipoles are likely also to influence the properties substantially (e.g., pK , catalytic properties, ligand affinity, transport, or movement) of ions and charged groups. Interestingly, we have observed that the collections of local dipoles associated with electrostatic interactions in proteins are heavily populated by peptide units (many from the first or last turns of helices) and hydroxyl side chains, of which several are involved in hydrogen-bonding interactions with the ionic groups (Quiocho et al., 1987, unpubl.; Luecke & Quiocho, 1990). Other than the SBP-sulfate complex, several other excellent examples portraying the extensive involvement of local dipoles in charge stabilization include the following interactions seen in well-refined structures: a ligand-binding site Arg residue in the L-arabinose-binding protein complexed with sugars (Quiocho et al., 1987), an Arg residue in the xylose isomerase (Henrick et al., 1989), a leucine zwitterion complexed with one domain of the leucine transport protein (Quiocho et al., 1987; Sack et al., 1989), a persulfide anion with rhodanese (Wierenga, 1986), a sulfate group of hirugen inhibitor bound to thrombin (Skrzypczak-Jankun et al., 1991), a phosphate nutrient with a phosphate transport receptor (Luecke & Quiocho, 1990), and many phosphoryl groups of ligands (e.g., FMN complexed with flavodoxin [Smith et al., 1980], FAD with glutathione reductase [Karplus & Schulz, 1987], and DNA with DNase I [Suck et al., 1988]). It is important to note that, of these examples, no helix terminus is linked with the electrostatic interactions associated with the arabinose-binding protein, xylose isomerase, thrombin, and DNase I, and only one is linked with the flavodoxin (see also Quiocho et al., 1987).

Functional roles of dipoles localized at helix termini

Our results, combined with other observations, raise a question as to which single feature of helices—termini peptide dipoles or macrodipoles—is of prime importance in electrostatic interactions in proteins. Whereas macrodipoles have been assumed to play a major role in countless cases on the basis of the observations of the proximity of ionic groups to helix termini (predominantly negatively charged groups to N-termini [Hol et al., 1978; Hol, 1985; Presta & Rose, 1988; examples above]), nevertheless, the collections of local dipoles (including those confined to the first or last turns of helices) could as well be assumed

to be playing a dominant role instead (dipoles can also include isolated ordered water molecules and solvent, if the site is accessible).

We heavily favor the helix termini or peptide dipoles as nearly the sole source of the helix effect. There are examples of charge-dipole interactions in proteins, as well as in neutral ionophores, that lack the participation of helices and consequently must rely exclusively on local dipoles (see above; Quiocho et al., 1987). Indeed, the participation of local dipoles, including those in helix termini, is more prevalent than helix macrodipoles. Although the SBP-sulfate complex shows the extreme case of a large concentration of helix termini, there are enough local dipoles, including nine NHs of peptide units from the first turns of the three helices, near the sulfate to stabilize the dianion charges. These NHs need not all participate in direct interaction with the sulfate. (The close proximity of several helix N-termini to the binding site is not unique to the SBPs. In fact, it is a common feature of all the structures [at least a dozen] of periplasmic-binding proteins involved in active transport, including those that bind uncharged carbohydrate ligands [Quiocho, 1991].) Because the close coupling (via a main-chain peptide unit) of the positively charged His 42 to the sulfate contributes negligibly to the electrostatic interaction (Table 1), the peptide units at the second turn of a helix, whose NH groups are located at distances to the sulfate roughly similar to the location of N δ 1 of His 42 (Fig. 1), are expected to contribute nothing measurable either. Thus, the dipolar groups in the binding site responsible for charge stabilization could be no farther than those adjacent to the bound sulfate. This is consistent with theoretical analysis, which indicated that the helix effect is due almost entirely to the first turn, and that helix-capping charged residues have no effect on electrostatic interactions (Åqvist et al., 1991).

It can be argued that the proximity of His 42, Arg 77, and Arg 102 to the protein surface could reduce whatever influence these residues may have on the electrostatic interactions. However, these three residues are almost totally buried, and the C-termini of the helices are located even further inside of the SBP structure. Moreover, structure stabilization effects resulting from the interactions between charged side chains and α -helix termini have been observed even though these interactions occur close to the protein surface (Nicholson et al., 1988; Sali et al., 1988). Nicholson et al. (1991), in their study of these interactions in T4 lysozyme, further concluded that "the charge-stabilization effects are attributed to the partial charges on the unsatisfied amides and carbonyls in the first and last turn of the helix," affirming our "helix terminal dipole model" based on prior work (Pflugrath & Quiocho, 1985; Quiocho et al., 1987; Åqvist et al., 1991).

Helices have a unique feature for playing a major role in electrostatic interactions: their termini, often combined with the loop preceding or following them, are a rich source of concentrated and fixed dipoles (Quiocho et al.,

1987). This role, arising from the combination of a helix terminus and a loop, is further illustrated in the electrostatic interaction associated with the Arg 102 residue capping the C-terminus of α -VI; the C-terminus and following loop (residues 183–186) provide an aggregate of seven peptide carbonyl dipoles, of which four (three from the loop) make a total of six hydrogen bonds with the guanidinium group (Fig. 2).

Because charge–dipole interactions are dominant in the formation of the complex of SBP with sulfate and, indeed, of other periplasmic transport receptors with charged nutrients (e.g., phosphate anion and amino acid zwitterions), they are likely to be of considerable importance for efficient active transport and rapid ion movements in general (Quioco et al., 1987; Pflugrath & Quioco, 1988; Luecke & Quioco, 1990; Quioco, 1991). This type of interaction is also highly desirable in enzyme reactions, contributing to catalysis by participating in the activation of functional groups and/or stabilizing charged intermediates along the catalytic pathway (Quioco et al., 1987; Quioco, 1991); see, for example, Lodi and Knowles (1993).

Materials and methods

The expression vectors and methods used in the SBP mutagenesis in *E. coli* have been described fully (Jacobson et al., 1991). The sequences of both *E. coli* and *S. typhimurium* SBP consist of 310, residues of which only 19 are nonidentical. These nonidentical residues, which are located on the surface of the three-dimensional structure of SBP, are in no way related to those residues indicated in this study, including the ones that were replaced. They also are not associated with the ligand-binding site and the three helices shown in Figures 1, 2.

In each mutagenesis, the entire SBP gene was sequenced in order to insure that no mutations other than the one designed had occurred. Methods for purifying native and mutant proteins and determining equilibrium sulfate-binding constant were as described elsewhere (Pardee, 1966; Jacobson & Quioco, 1988; Jacobson et al., 1991). All purified proteins migrate as a single band on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate and on isoelectric focusing.

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