as to how the proteins can discriminate between the charged methylated $m^7G$ base and the unmodified guanosine base. The $m^7G$ base is stacked between aromatic protein side chains and hydrogen bonded to acidic protein residues (Fig. 23.2.4.5). One long-held hypothesis is that IF-4E, with dual tryptophan residues, binds specifically to the positively charged form of the base through a charge-transfer complex (Ueda, Iyo, Doi, Inoue & Ishida, 1991). The formation of a charge-transfer complex is evident in small-molecule studies and spectroscopic studies with IF-4E (Ueda, Iyo, Doi, Inoue, Ishida *et al.*, 1991). However, VP39 performs the same discrimination with the much less electronegative phenylalanine and tyrosine side chains (Hodel *et al.*, 1997). So far, no charge-transfer complex has been observed in VP39.

The recognition of charged methylated bases is important not only in mRNA processing, but also in the repair and recognition of DNA damaged by alkylating carcinogens. The mechanism by which the charged $m^7G$ base is recognized is probably similar to how other positively charged bases, such as 3-methyladenosine, O2-methylcytosine and O2-methylthymidine, are recognized. In fact, the *E. coli* DNA repair enzyme, AlkA, will catalyse the glycolysis of all of these bases (Lindahl, 1982). The structure of AlkA is known, but only in the absence of a substrate (Labahn *et al.*, 1996). In this structure, a number of solvent-exposed tryptophan residues are found at the putative active site. This observation suggests that AlkA may recognize positively charged bases through an aromatic 'sandwich', much like that found in IF-4E and VP39.

### 23.2.5. Phosphate and sulfate

Novel features of molecular recognition and electrostatic interactions of these two tetrahedral oxyanions have emerged from our crystallographic and functional studies of the phosphate-binding protein (PBP) and sulfate-binding protein (SBP), which serve as extremely specific initial receptors for ATP-binding cassette (ABC)-type active transport or permease in bacterial cells. The complexes of these proteins have $K_d$ values in the low $\mu M$ range. Although phosphate and sulfate are structurally similar, at physiological pH PBP and SBP exhibit no overlap in specificity (Medveczky & Rosenberg, 1971; Pardee, 1966; Jacobson & Quiocho, 1988). This stringent specificity prevents one tetrahedral oxyanion nutrient from becoming an inhibitor of transport for the other. The specificity of the PBP-dependent phosphate transport system is also shared by other phosphate transport systems in eukaryotic cells and across brush borders and into mitochondria.

As described below, discrimination between anions is based solely on the protonation state of the ligand. Sulfate, a conjugate base of a strong acid, is completely ionized at pH values above 3, whereas phosphate, a conjugate base of a weak acid, remains protonated up to pH 13.

The structure of the PBP–phosphate complex was initially determined at 1.7 Å resolution (Luecke & Quiocho, 1990). The resolution has been pushed to an ultra high resolution of 0.98 Å, the first reported for a protein with a molecular weight as high as 34 kDa with a bound ligand (Wang *et al.*, 1997). The bound phosphate is completely desolvated and sequestered in the protein cleft between two domains. It makes 12 hydrogen bonds with the proteins (11 with donor groups and one with an acceptor group), as well as one salt link to an Arg that is in turn salt-linked to an Asp residue (Fig. 23.2.5.1). The distances of the 12 hydrogen bonds between phosphate and PBP obtained from the ultra high resolution structure range from 2.432 to 2.906 Å (Wang *et al.*, 1997). The Asp56 carboxylate, the lone acceptor group, plays two key roles in conferring the exquisite specificity of PBP. It recognizes, by way of the hydrogen bond, a proton on the phosphate and presumably

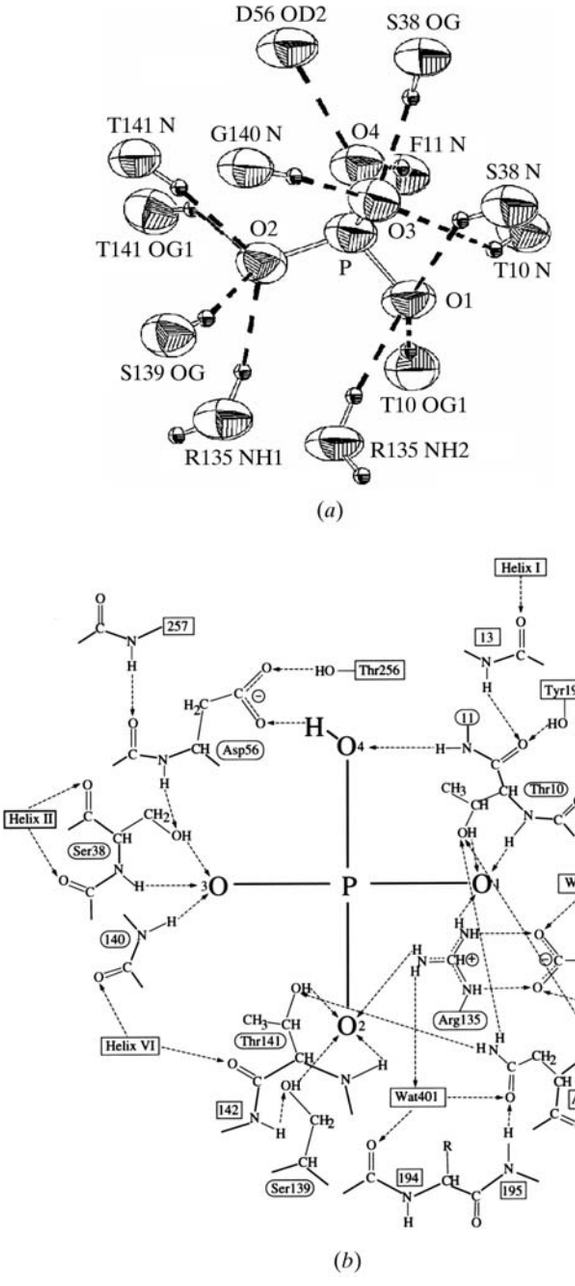


Fig. 23.2.5.1. 12 hydrogen-bonding interactions between the phosphate-binding protein (PBP) and phosphate. (*a*) Displacement ellipsoids of the atoms involved in the interactions from the 0.98 Å atomic structure (Wang *et al.*, 1997). (*b*) Schematic diagram of the interactions, including additional hydrogen bonds.

disallows, by charge repulsion, the binding of a fully ionized sulfate dianion (Luecke & Quiocho, 1990).

The SBP binding-site cleft is also tailor-made for sulfate (Pflugrath & Quiocho, 1985). In keeping with the stringent specificity of SBP for fully ionized tetrahedral oxyanions (Pardee, 1966; Jacobson & Quiocho, 1988), the bound sulfate, which is also completely dehydrated and buried, is held in place by seven hydrogen bonds made entirely with donor groups from uncharged polar residues of the protein (Fig. 23.2.5.2) (Pflugrath & Quiocho, 1985). The absence of a hydrogen-bond acceptor group accounts for the inability of SBP to bind phosphate. Interestingly, the absence of a salt link and the formation of five fewer hydrogen bonds with the

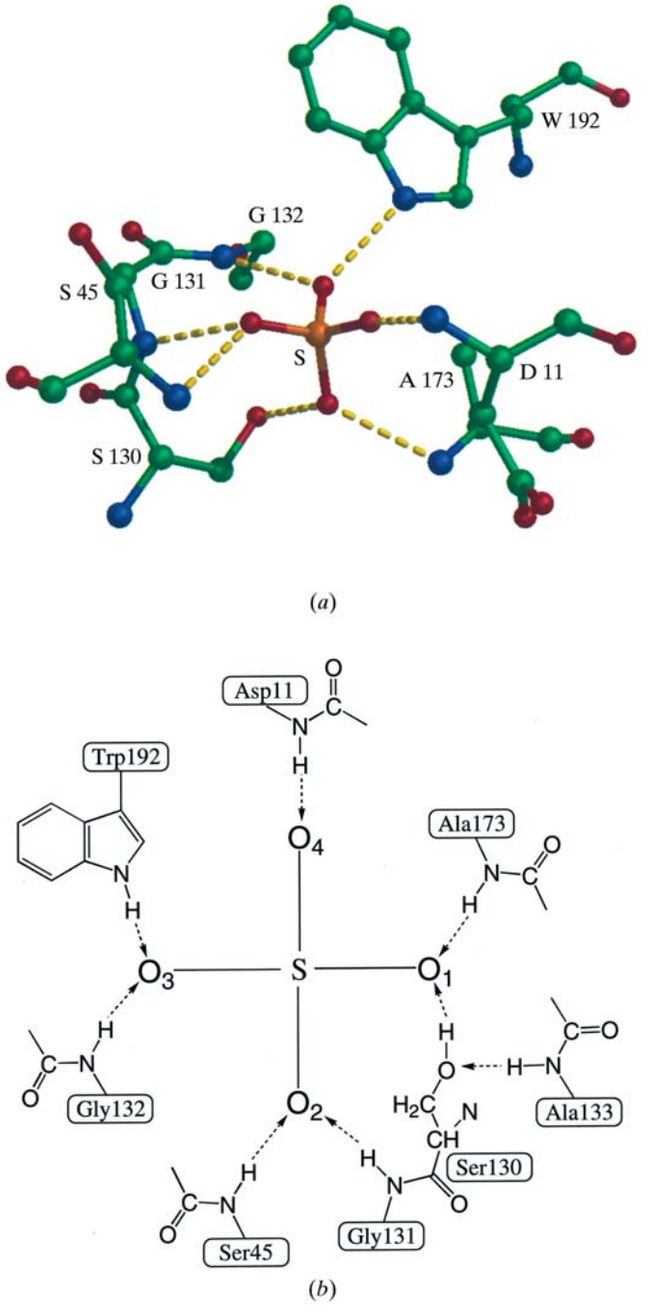


Fig. 23.2.5.2. Seven hydrogen-bonding interactions between the sulfate-binding protein (SBP) and sulfate. (*a*) Interactions based on the 1.7 Å structure (J. Sack & F. A. Quiocho, unpublished data). (*b*) Schematic diagram of the interaction.

bound sulfate (Fig. 23.2.5.2*b*) than with the bound phosphate (Fig. 23.2.5.1*b*) do not make the affinity of the SBP–sulfate complex any weaker than that of the PBP–phosphate complex. In fact, the sulfate binds 10–20 times more tightly to SBP (Pardee, 1966; Jacobson & Quiocho, 1988). Also, the hydration energies of both anions are likely to be similar.

The ability of PBP and SBP to differentiate each oxyanion ligand through the presence or absence of proton(s) is an extremely high level of sophistication in molecular recognition. The importance of complete hydrogen bonding in recognition of buried ligands is powerfully demonstrated in PBP and SBP. As the sulfate is fully ionized (*i.e.* possesses no hydrogen at physiological pH), repulsion occurs at Asp56 of PBP specifically for this dianion. On the other hand, SBP is unable to bind phosphate because it contains no hydrogen-bond acceptor in the binding site. Significantly, despite the potential for a large number of matched hydrogen-bonding pairs, a single mismatched hydrogen bond (*e.g.* a fully ionized sulfate providing no proton for interaction with Asp56 of PBP and no acceptor group in SBP for a phosphate proton) represents a binding energy barrier of 6–7 kcal $mol^{-1}$ (1 kcal $mol^{-1}$ = 4.184 kJ $mol^{-1}$).

### 23.2.5.1. *Dominant role of local dipoles in stabilization of isolated charges*

A novel finding of further paramount importance and wide implication is how the isolated charges of the protein-bound phosphate and sulfate are stabilized. No counter-charged residues or cations are associated with the sulfate completely buried in SBP. Although a salt link involving Arg135 is formed with the phosphate bound to PBP, it is shared with an Asp residue (Fig. 23.2.5.1*b*). Moreover, site-directed mutagenesis studies indicate that phosphate binding is quite insensitive to modulation of the salt link (Yao *et al.*, 1996). These findings are a powerful demonstration of how a protein is able to stabilize the charges by means other than salt links. Experimental and computational studies indicate that local dipoles, including the hydrogen-bonding groups and the backbone NH groups from the first turn of helices, immediately surrounding the sulfate and phosphate are responsible for charge stabilization (Pflugrath & Quiocho, 1985; Quiocho *et al.*, 1987; Åqvist *et al.*, 1991; He & Quiocho, 1993; Yao *et al.*, 1996; Ledvina *et al.*, 1996). Helix macrodipoles play little or no role in charge stabilization of the anions. The same principle of charge stabilization by local dipoles also applies for the following buried uncompensated ionic groups: Arg151 of the arabinose-binding protein (Quiocho *et al.*, 1987), the zwitterionic leucine ligand bound to the leucine/isoleucine/valine-binding protein (Quiocho *et al.*, 1987), the potassium in the pore of the potassium channel (Doyle *et al.*, 1998) and Arg56 of synaptobrevin-II in a SNARE complex (Sutton *et al.*, 1998).

### 23.2.5.2. *Short hydrogen bonds*

The ultra high resolution refined structure of the PBP–phosphate complex is the first to show structurally the formation of an extremely short hydrogen bond (2.432 Å) between the Asp56 carboxylate of PBP and phosphate. Although this short hydrogen bond is within the proposed range of low-barrier hydrogen bonds with estimated energies of 12–24 kcal $mol^{-1}$ (Hibbert & Emsley, 1990), its contribution to phosphate binding affinity has been assessed to be no better than that of a normal hydrogen bond (Wang *et al.*, 1997). Thus, a unique role for short hydrogen bonds in biological systems, such as in enzyme catalysis (Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994), remains controversial.

### 23.2.5.3. *Non-complementary negative electrostatic surface potential of protein sites specific for anions*

The presence of an uncompensated negatively charged Asp56 is unusual for an anion-binding site, as observed in PBP. In fact, a related discovery of profound ramification is that the binding-cleft region of PBP has an intense negative electrostatic surface potential (Fig. 23.2.5.3*a*) (Ledvina *et al.*, 1996). Non-complementarity between the surface potential of a binding region and an anion ligand is not unique to PBP. We have reported similar findings for SBP, a DNA-binding protein, and, even more dramatically, for the redox protein flavodoxin (Fig. 23.2.5.3*b*) (Ledvina *et al.*, 1996). Evidently, for proteins such as these, which rely on hydrogen-

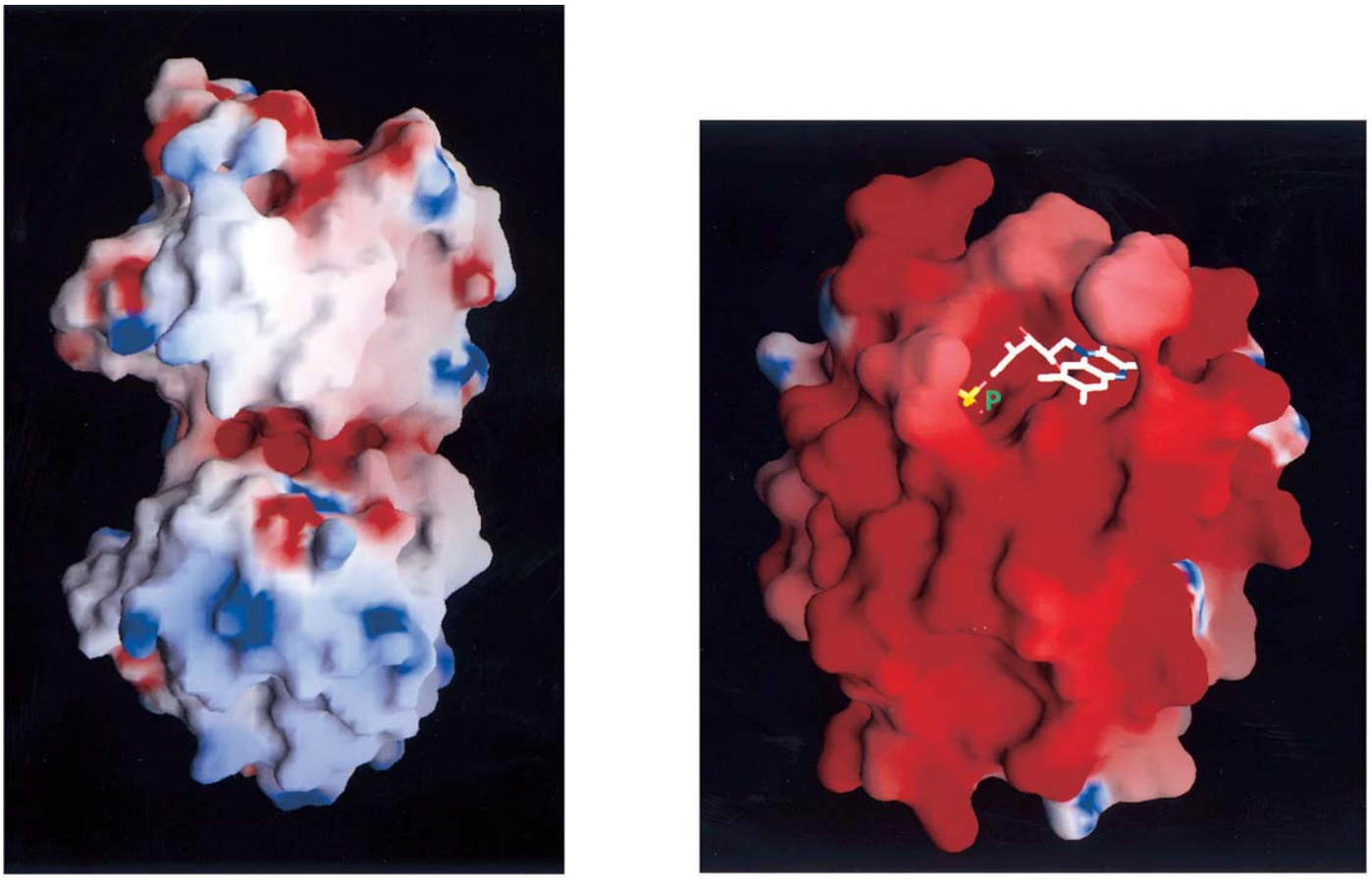


Fig. 23.2.5.3. Electrostatic surface potential of (*a*) the phosphate-binding protein and (*b*) flavodoxin. The molecular surface electrostatic potentials, calculated and displayed using *GRASP* (Nicholls *et al.*, 1991), are $-10\,kT$ (red), neutral (white) and $+10\,kT$ (blue) [see Ledvina *et al.* (1996) for more details]. (*a*) Wild-type phosphate-binding protein based on the X-ray structure of the open cleft, unliganded form (Ledvina *et al.*, 1996). The phosphate-binding site is located in the cleft (with negative surface potential) in the middle of the molecule and between the two domains. (*b*) Flavodoxin with bound flavin mononucleotide (FMN). The phosphoryl group (P) of the FMN is bound in a pocket with intense negatively charge surface potential. The surface potential was calculated without the bound flavin mononucleotide using the structure from the Protein Data Bank (PDB code: 2fox).

bonding interactions with only uncharged polar residues for anion binding and electrostatic balance, a non-complementary surface potential is not a barrier to binding. This conclusion is supported by very recent fast kinetic studies of binding of phosphate to PBP and the effect of ionic strength on binding (Ledvina *et al.*, 1998).

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