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# Crystal structure of *Escherichia coli* inorganic pyrophosphatase complexed with $\text{SO}_4^{2-}$

## Ligand-induced molecular asymmetry

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**Abstract** The three-dimensional structure of inorganic pyrophosphatase from *Escherichia coli* complexed with sulfate was determined at 2.2 Å resolution using Patterson's search technique and refined to an R-factor of 19.2%. Sulfate may be regarded as a structural analog of phosphate, the product of the enzyme reaction, and as a structural analog of methyl phosphate, the irreversible inhibitor. Sulfate binds to the pyrophosphatase active site cavity as does phosphate and this diminishes molecular symmetry, converting the homohexamer structure form ( $\alpha_3$ )<sub>2</sub> into  $\alpha_3'\alpha_3''$ . The asymmetry of the molecule is manifested in displacements of protein functional groups and some parts of the polypeptide chain and reflects the interaction of subunits and their cooperation. The significance of re-arrangements for pyrophosphatase function is discussed.

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**Key words:** Inorganic pyrophosphatase; *Escherichia coli*; Crystal structure; Complex with sulfate; Cooperativity

## 1. Introduction

Soluble inorganic pyrophosphatase (EC 3.6.1.1.) (PPase) hydrolyses inorganic pyrophosphate to inorganic phosphate ( $\text{P}_i$ ), thus providing an equilibrium shift in many key biosynthetic processes in the cell toward synthesis. PPases have been purified and characterised from different sources—from bacteria to mammals. Prokaryotic PPases are homohexamers, whereas eukaryotic PPases are homodimers. PPases from *Saccharomyces cerevisiae* and *Escherichia coli* are the best-studied enzymes.

The structural solution of *S. cerevisiae* PPase in 1984 marked the beginning of X-ray investigations of PPases [1]. The three-dimensional structure of its complex with  $\text{Mn}^{2+}$  and  $\text{P}_i$  was solved in 1991 [2] and was refined by the authors in 1996 [3]. Later this complex was described once again [4]. In 1994 we published the structural analysis of *E. coli* PPase at 2.5 Å resolution [5] and refined it to 2.2 Å [6]. Simultaneously and independently, the 2.7 Å structure of *E. coli* PPase was described [7]. Comparison of the results of two parallel investigations reveals that PPase crystallizes in two different forms depending on the precipitant used. In the presence of  $\text{NH}_4\text{Cl}$ ,

crystals contained one subunit per asymmetric unit with parameters  $110.4 \times 110.4 \times 76.8$  Å [6]. The use of  $(\text{NH}_4)_2\text{SO}_4$  resulted in the crystal form containing two monomers per asymmetric unit with cell dimensions  $110.4 \times 110.4 \times 153.0$  Å [7]. The reason for these differences is discussed in this work. We have shown that crystallization of PPase in the presence of  $(\text{NH}_4)_2\text{SO}_4$  resulted in the enzyme complexed with sulfate. The sulfate binding to the PPase active site induces a trimer asymmetry within a hexamer similar to that caused by interaction of PPase with methyl phosphate.

## 2. Experimental

### 2.1. Crystallization

Recombinant *E. coli* PPase was prepared as described previously [5]. The enzyme was crystallized in hanging drops (20 µl) of 0.1 M Tris-HCl buffer (pH 7.5), 35%  $(\text{NH}_4)_2\text{SO}_4$  and 4 mg/ml protein against a solution of 0.1 M Tris-HCl buffer (pH 7.5), 45%  $(\text{NH}_4)_2\text{SO}_4$ . Within 2 weeks rhombohedral crystals of size  $0.4 \times 0.4 \times 0.6$  mm appeared. The crystals belong to space group R32 with unit cell constants  $a = b = 110.4$  Å and  $c = 154.8$  Å. The asymmetric unit contains two subunits.

### 2.2. X-ray data collection

X-ray diffraction data were measured on an image plate system (MAR research, Hamburg) on the beamline X11 in the EMBL outstation c/o DESY with  $\lambda = 1.009$  Å. Total number of measured reflections with resolution 2.196 Å is 153537 (18662 unique reflections with completeness 99.2%). Data were processed with Denzo [8].

### 2.3. Refinement

The refined atomic model of the apoenzyme [6] with excluded water molecules was used for both rotational and translational searches with AMoRe [9] using 12–3.5 Å data. Two solutions with the highest peaks were related by two-fold axis parallel to  $x$  with  $z = 0.21$ . The refinement was carried out using the CCP4 suite (CCP4, 1994) and REFMAC [10] with all data within resolution shell 12–2.2 Å, except for 5% of reflections taken out to calculate the free R-factor. From the start, atomic coordinates and individual constrained B-factors were refined using alternating manual rebuilding with program 'O' [11]. No non-crystallographic restraints were implemented. When the R-factor dropped to 22%, solvent molecules were gradually included in the model if peaks with height greater than  $3\sigma$  in the difference electron density map were at a distance of 2.4–4.0 Å from protein atoms. Final R and R-free factors are 19.2 and 26.6%, respectively.

### 2.4. Inhibition of enzyme by methyl phosphate

The reaction of  $1 \cdot 10^{-4}$  mM PPase with 0.5–50 mM methyl phosphate was studied as in [12]. The pseudo-first-order rate constants of

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inactivation ( $k^{app}$ ) were obtained using the 'Enzfitter Biosoft' program.

### 3. Results and discussion

#### 3.1. Some structural features of the apoenzyme

The crystals of apoPPase were grown in the presence of  $\text{NH}_4\text{Cl}$  as a precipitant. The three-dimensional structure at 2.2 Å resolution and the secondary structure elements were described earlier [6]. To understand the structure re-alignment produced by specific ligand binding, it is necessary to take into consideration the set of intramolecular hydrogen bonds and ionic interactions stabilizing a native protein conformation. About 80 H-bonds are formed between peptide groups. In addition, 86 polar amino acids are capable of being hydrogen donors and/or acceptors, as well as forming ion pairs. Actually, there are several tens of interactions of amino acid groups with one another or with peptide linkages. Undoubt-

edly, 150  $\text{H}_2\text{O}$  molecules located in the apoenzyme structure also participate in forming the hydrogen bond network. Enumerating many of these interactions is beyond the scope of this article, but a full picture may be analysed with atomic coordinates deposited in the Protein Data Bank (ID code 1IGP). The existence of the branched interaction system not only stabilises the spatial structure, but also ensures structural reorganization by means of breakage of some linkages and the formation of others.

These re-arrangements of H-bonds can proceed due to the existence of some mobile segments of the polypeptide chain. As follows from Fig. 1, the average B-factor value for the apoenzyme is 25 Å<sup>2</sup>, while 3 or 4 regions are much more mobile. Primary among these are regions Ser-63–Asp-67, Lys-94–Asp-102 and Glu-145–Glu-153. All these polypeptide segments are located in irregular structure regions and have few contacts with the molecule body. These sequences include groups of the active site, Asp-65, Asp-67, Asp-97 and Asp-

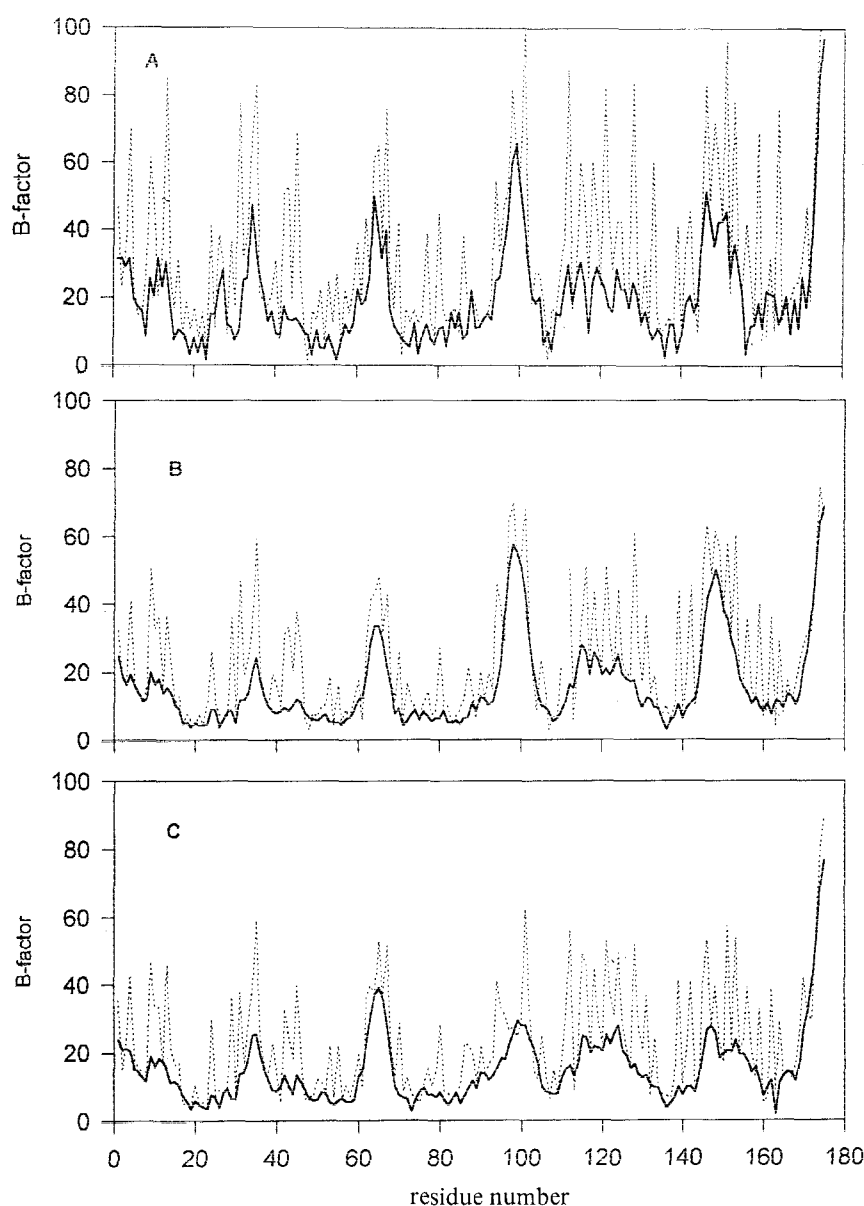


Fig. 1. Plot of variation of average B-factors with the residue number for the main chain (solid lines) and for the side-groups (dotted lines). A: apoenzyme. B:  $\alpha'$ -subunit. C:  $\alpha''$ -subunit.

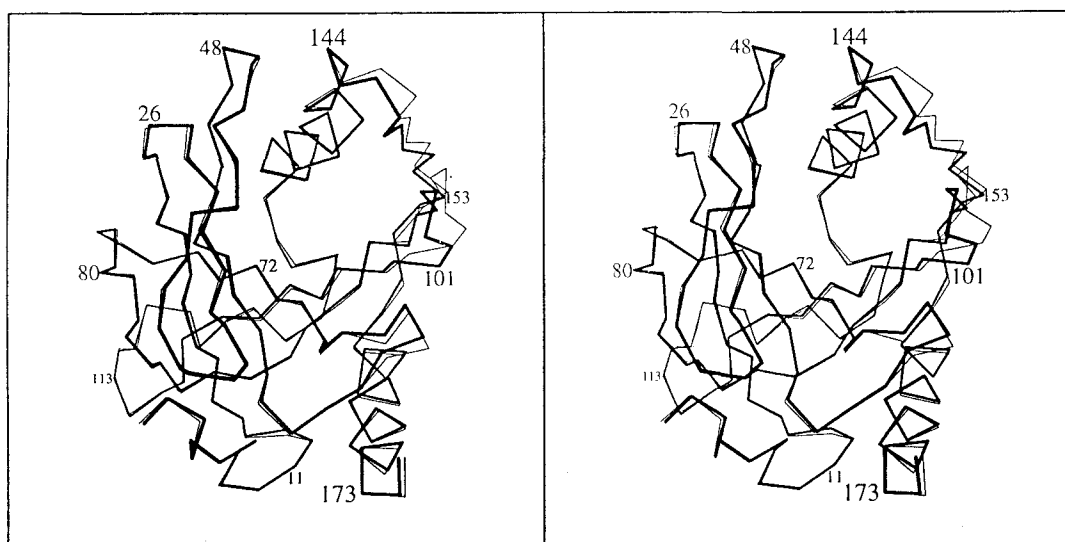


Fig. 2. Superposition of polypeptide chain courses of  $\alpha'$ -subunit of PPase-sulfate complex (black color) and of apoPPase (grey color).

102. Such a principle of active site organization and its evolutionary stability assumes that the mobility of these regions is essential for enzyme function. This mobility generates an active conformation for substrate binding and for release of the products of enzymatic reaction. All such segments include Gly-residues, namely 66, 100 and 147, and two of them (100 and 147) are conservative in all the known PPases and Gly-66 in the known prokaryotic PPases. It will be shown later that these polypeptide chain parts respond to ligand binding at the active site by changing the position of a large number of functional groups.

### 3.2. The structure of the sulfate-enzyme complex

The sulfate complex was obtained by PPase crystallization in the presence of ammonia sulfate. X-ray analysis at 2.2 Å showed that each subunit contains one sulfate ion bound at the active site. Comparison of the sulfate position with the position of phosphate in *S. cerevisiae* PPase complex  $\text{Mn}_2(\text{P}_i\text{Mn})_2\text{PPase}$  [3] reveals that the sulfate binding site coincides with the site of that phosphate, which is probably the departing group in pyrophosphate bond hydrolysis. Both sulfate and  $\text{P}_i$  are surrounded by basic amino acids (Arg-43, Lys-142, Lys-29 and Tyr-141).

There are two main characteristic features of the enzyme complexed with sulfate. Firstly, sulfate binding initiates essential reorganizations inside each subunit and, secondly, it causes molecular asymmetry, i.e. structural changes are not

the same for different trimers of the hexamer molecule. As a result, the apoenzyme with 6 equal subunits turns into the  $\alpha_3'\alpha_3''$  structure, thus conserving the three-fold axis. The changes in the sulfate-protein complex are reflected in packing of molecules in the crystal, resulting in changes in the cell unit parameters. The reasons for the differences between molecular packing in the crystal will be discussed in the next paper. The shifting of several polypeptide chain parts demonstrates the superposition of C $\alpha$ -atom coordinates of the apoenzyme and  $\alpha''$ -subunits (Fig. 2). At a root-mean-square displacement of 0.56, the maximal shifts achieve 2.3 Å and the total number of shifted C $\alpha$ -atoms reaches 27. A comparison of apoenzyme and sulfate complex active sites is given in Fig. 3. It can be seen that all 16 groups of the active site have changed their positions to a greater or lesser extent. The maximum shift is noted for  $\text{NH}_1$ - and  $\text{NH}_2$ -atoms of Arg-43 (6–7 Å). In the free enzyme, the guanidine group of this arginine residue is protruded to solvent. In the sulfate complex, the guanidinium group is found at the active site. The subunit asymmetry is generated by sulfate binding which is not similar in each subunit. It is evident from a comparison of the sulfate oxygen atom distances to the protein ligands (Table 1). The bonds between sulfate and Arg-43 in the  $\alpha'$ -subunit are shorter than in the  $\alpha''$ -subunit. In  $\alpha'$ -subunit, sulfate interacts with Lys-142 but in  $\alpha''$ -subunit this bond is absent. The H-bond with Tyr-141 is shorter in the  $\alpha''$ -subunit than in  $\alpha'$ . Conversion of the apoenzyme to the  $\alpha_3'\alpha_3''$ -structure is accompanied

Table 1  
The protein ligands of the sulfate ion in  $\alpha'$ - and  $\alpha''$ -subunits

Sulfate ion	$\alpha'$ -Subunit		$\alpha''$ -Subunit	
	Ligand	Distance (Å)	Ligand	Distance (Å)
O <sub>1</sub>	Arg-43-NH <sub>1</sub>	2.82	Arg-43-NH <sub>1</sub>	3.21
	Tyr-141-OH	2.66	Tyr-141-OH	3.00
	H <sub>2</sub> O-35	3.42	H <sub>2</sub> O-197	3.31
O <sub>2</sub>	Arg-43-NH <sub>2</sub>	2.61	Arg-43-NH <sub>2</sub>	3.25
	H <sub>2</sub> O-216	3.20		
O <sub>3</sub>	Lys-142-NZ	3.34	Lys-29-NZ	3.56
O <sub>4</sub>	Lys-29-NZ	3.13	Tyr-141-OH	3.58
	H <sub>2</sub> O-35	3.18	Lys-29-NZ	3.12
			H <sub>2</sub> O-197	3.34

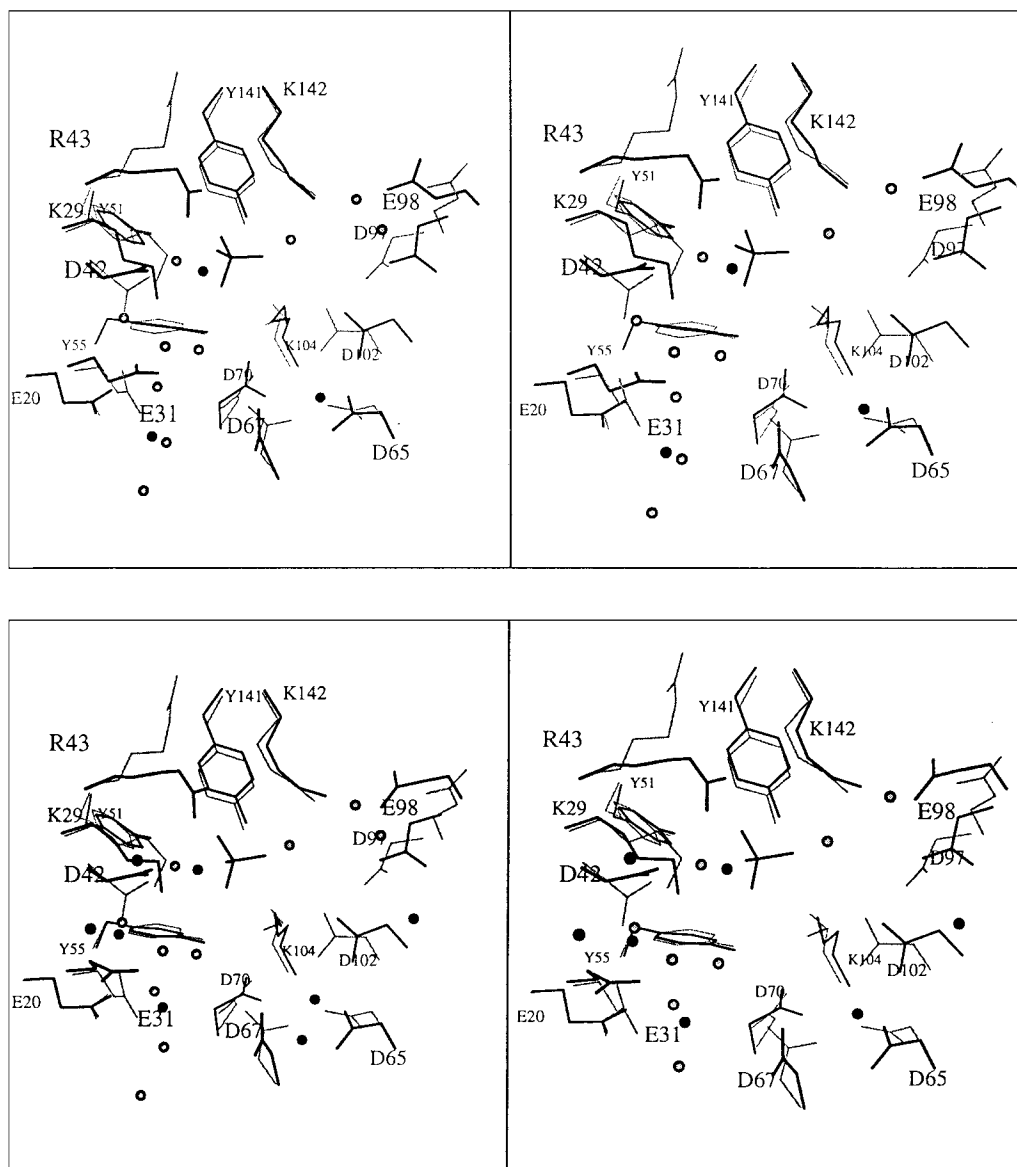


Fig. 3. Superposition of the active site groups of PPase-sulfate complex (black color) and apoPPase (grey color). Solvent molecules are shown by circles; the new molecules appearing in the complex are shown by black circles. *a*, top:  $\alpha'$ -subunit. *b*, bottom:  $\alpha''$ -subunit.

by the destruction of 9 H-bonds between the peptide groups. Simultaneously 20 and 19 new H-bonds appear in the  $\alpha'$ - and  $\alpha''$ -subunits, respectively. Even more changes occur in functional group interactions. When native subunit transforms into  $\alpha'$  and  $\alpha''$ , 17 and 14 contacts disappear but 30 and 35 new bonds are formed. The redistribution of the H-bonds and of the ionic interactions involves the groups which do not directly participate in sulfate binding. As a result of reorganization, both subunits have more determined packing than apoenzyme (Fig. 1). This is more pronounced for the  $\alpha''$ -subunit as is clear from a comparison of  $\alpha'$ - and  $\alpha''$ -subunit B-factors (Fig. 1B,C). Both structures were determined with the same average B-factor, but in the  $\alpha''$ -subunit the polypeptide chain region mobility around 98 and 146 residues diminished. The question arises about the reasons for the changes in these regions. To answer this, it is useful to examine the contacts of amino acids which are included in moving loops or are their neighbours. As has already been mentioned, Tyr-141 and Lys-

142 contact with sulfate. The Glu-145 carboxylate group formed an ionic pair with Arg-43 in the apoenzyme shifts after sulfate binding. But its movement differs in the  $\alpha'$  and  $\alpha''$ -subunits. Glu-145 OE2 shifts for 3.5 Å in the  $\alpha'$ -subunit and for 1.3 Å in the  $\alpha''$ -subunit; the contacts with Arg-43 in the  $\alpha'$ -subunit become weaker and in the  $\alpha''$ -subunit become tighter. The Glu-145 carboxylate group is hydrogen-bonded to Ser-46 in the  $\alpha'$ -subunit. This bond is absent in both the  $\alpha''$ -subunit and apoenzyme (Fig. 4). Glu-145 gets additional contacts, ensuring closer interaction with the amino acid residues of a mobile chain. So an ionic pair composed of this residue and Lys-148 (2.5 Å), a residue which was free in the apoenzyme and coordinated with Lys-146 CO in the  $\alpha'$ -subunit, forms an  $\alpha''$ -subunit. The Glu-145 apoenzyme and coordinated with Lys-146 CO in the  $\alpha'$ -subunit forms an  $\alpha''$ -subunit. The Glu-145 carbonyl group also interacts with NH-groups of Lys-148 and Gly-147. Transformation of apoenzyme to the sulfate complex is accompanied by changing  $\varphi$ -

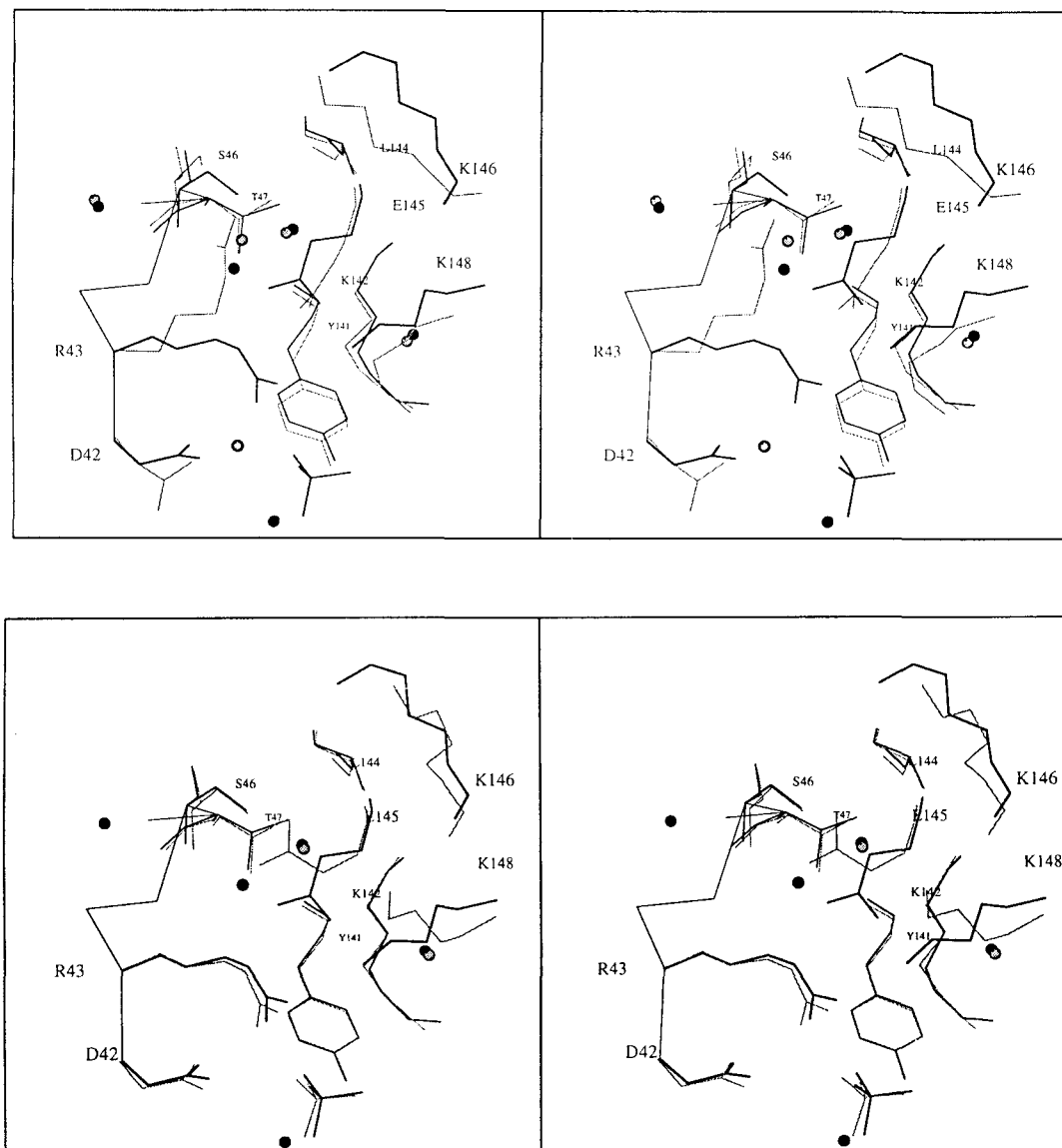


Fig. 4. Fragment of the overall superposition of  $\alpha'$ -subunit of PPase-sulfate complex (black line) and apoenzyme (a, top) or  $\alpha'$ -subunit (b, bottom). Solvent molecules are shown by spheres.

and  $\psi$ -angles of glycine residues (Gly-66, Gly-100, Gly-147). The biggest differences are typical for Gly-147. Sulfate binding also markedly affects the group location around Trp-149, another amino acid residue in this polypeptide chain region. The indol ring of Trp-149 in the apoenzyme is turned to the solvent. Trp-149 in the  $\alpha'$ -subunit has both intramolecular contacts with Glu-98 and symmetric hydrogen bonds are formed between hexamers (Trp-149 NHE1...Glu-98 O, 2.8 Å) (Fig. 5). Trp-149 participation in contacts with Glu-98 ensures mutual effects between the mobile loops 145–153 and 94–101. But this is not the only bond between them. Lys-151 has two contacts with Thr-96 peptide groups, and there is also interaction between Lys-94 CO and Glu-153 NH. Thus numerous contacts between two flexible polypeptide segments ensure their coordinated movement.

### 3.3. Cooperative subunit interactions

Taking into account that the subunits of the native PPase molecule are identical in the three-dimensional structure, the

trimer asymmetry in PPase complexed with sulfate could be explained in terms of the subunit interactions in a hexameric protein molecule. These structural data confirm our hypothesis on the cooperation of subunits based on half-of-the-sites reactivity in the inactivation of PPase by phosphoric acid monoesters [12]. The inactivation kinetics are consistent with the formation of a dissociable inhibitor–enzyme complex, followed by irreversible inactivation. It is important that the maximal inactivation of PPase by methyl phosphate reaches only 50% and that maximal incorporation of the tightly bound inhibitor corresponds to 3 moles per hexamer. The dependence of the PPase inactivation rate on methyl phosphate concentration is rather complicated and reflects the interaction of subunits (Fig. 6). The enzyme loses only 50% of activity at both low (< 10 mM) and high (> 20 mM) methyl inhibitor concentration. It is clear from these results that not only the first trimer affects the neighbouring trimer, but the opposite influence is also possible. Attention was drawn to the fact that methyl-phosphate-produced trimer asymmetry in the

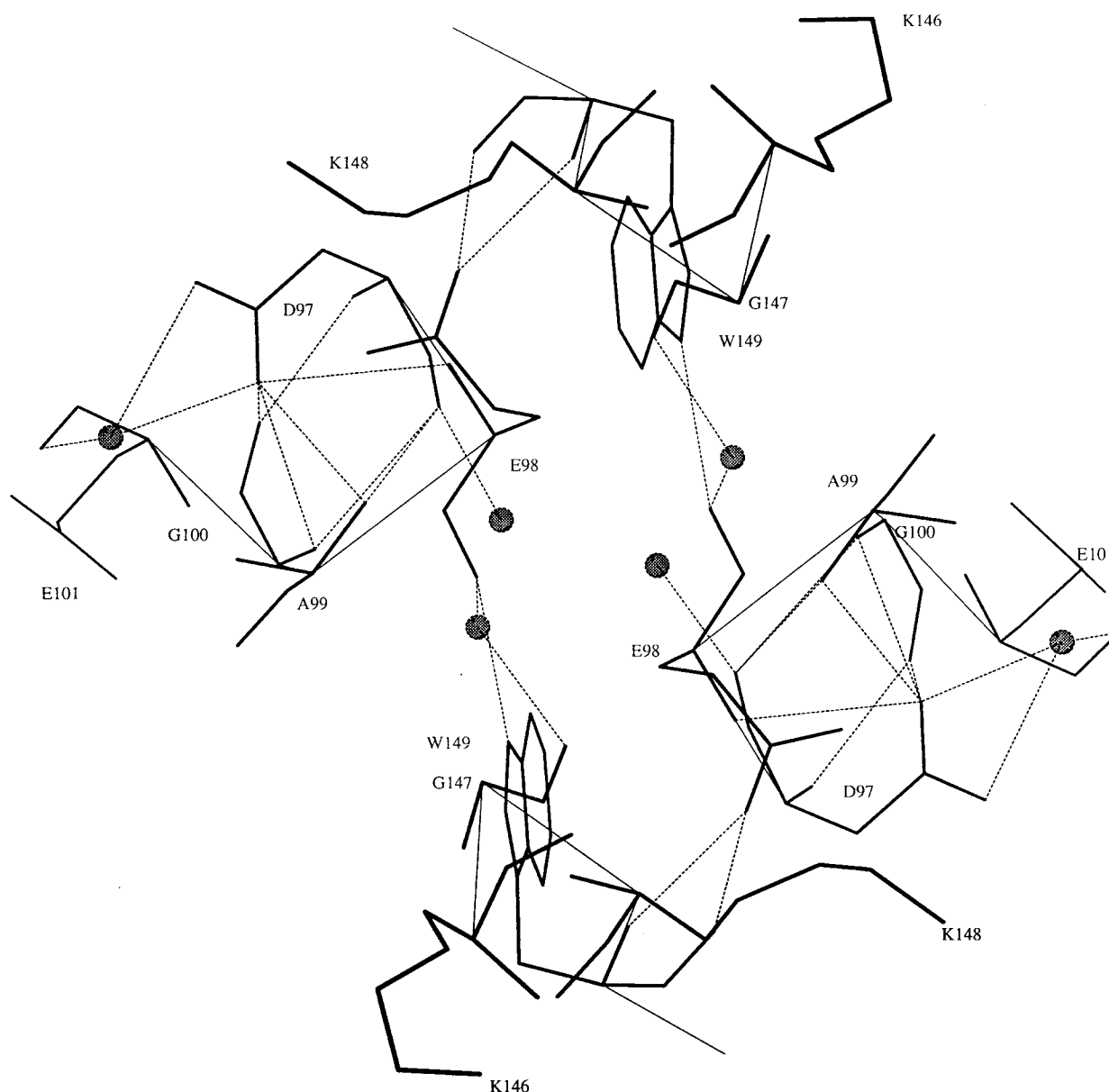


Fig. 5. Contact between two  $\alpha'$ -subunits from different hexamers. The dotted lines show inter- and intramolecular H-bonds; the spheres are solvent molecules.

PPase molecule occurred in water solutions with low protein concentrations. This fact enabled us to exclude the appearance of crystal asymmetry due to interaction between neighbouring hexamers. Similar facts were established for the *S. cerevisiae* PPase reaction with phosphoric acid monoesters [13,14]. The fundamental significance of cooperative subunit interactions readily follows from the fact that the irreversible inactivation of PPase by phosphoric acid monoesters is inherent only in native oligomers [15,16]. Disruption of intersubunit contacts in the native enzyme makes tight binding of reagent impossible. Thus, cooperative subunit interactions in PPase lead to the appearance of oligomeric enzyme properties which are absent in the individual subunit. This may be regarded as one of the pathways for enzymatic activity regulation of constitutive PPases in vivo.

Some comments on these results are in order. Interesting

data were published but not discussed by some authors [17]. Addition of substrate analog to crystallization set-ups which normally produced PPase apoform produced an asymmetric form, in spite of the analog concentration being several times less than the protein one. This means that in the experiment the enzyme not only changes its conformation but also turns into the initial state rather slowly, which should be taken into account at the kinetic scheme formulation. The subunit asymmetry in the *E. coli* PPase molecule also occurs at  $Mg^{2+}$  binding to the enzyme. This question will be the subject of our next paper.

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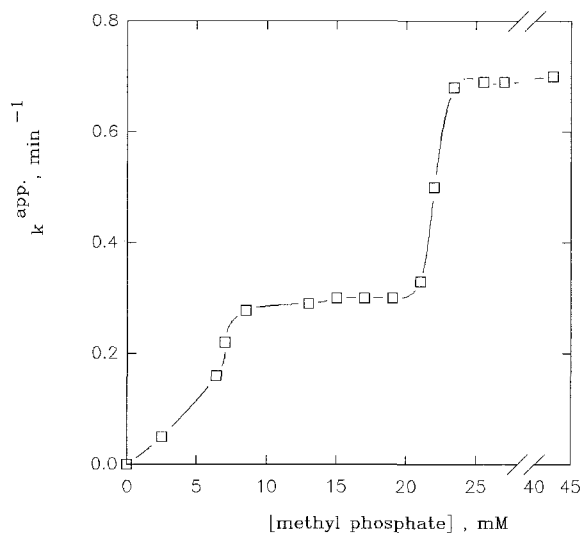


Fig. 6. Inhibition of *E. coli* PPase by methyl phosphate.

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