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Is purine nucleoside phosphorylase an example of a morpheein?

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Abstract

Purine nucleoside phosphorylase (PNP) is a ubiquitous enzyme of the nucleoside salvage pathway and it is characterized by non-Michaelis kinetics. Kinetic data in many cases (for some substrates or some concentration range of a co-substrate) are best described by the double hyperbolic equation. It was suggested that this is an indication of this enzyme being an example of a morpheein, i.e. the protein that exists as an equilibrium of the quaternary structure isoforms. In this paper we summarize our already published data for calf spleen and *E. coli* PNPs, as well as some new experiments conducted for the latter enzyme, which show the influence of various parameters on the activity and oligomeric structure in the solution of both proteins. We have been using a variety of methods including enzyme kinetics, steady-state emission spectroscopy, and size exclusion chromatography, as well as analytical ultracentrifugation and circular dichroism spectroscopy. Taken together the results let us conclude that calf spleen and *E. coli* PNPs are not morpheeins and in solution under a variety of conditions they exist, respectively, as a stable trimer and hexamer (a trimer of dimers).

1. Introduction

Recently a concept of morpheeins has been introduced [1]. The term 'morpheein' ('mor-phee'-in') is derived from the classic pronunciation of the word 'protein' ('pro-tee'-in') and from the verb 'to morph' meaning to change shape. A morpheein is a homooligomeric protein ('homo' in terms of aminoacid sequence of subunits) which can exist in more than one quaternary structure of different functionality. The multiplicity of each structure is dictated by different conformations of the monomeric unit. Porphobilinogen synthase (PBGS) was claimed to be an example of the phenomenon [1]. The predominant oligomeric form of this enzyme, as shown by determination of many crystal structures, is that of a homooctamer, but a rare human PBGS

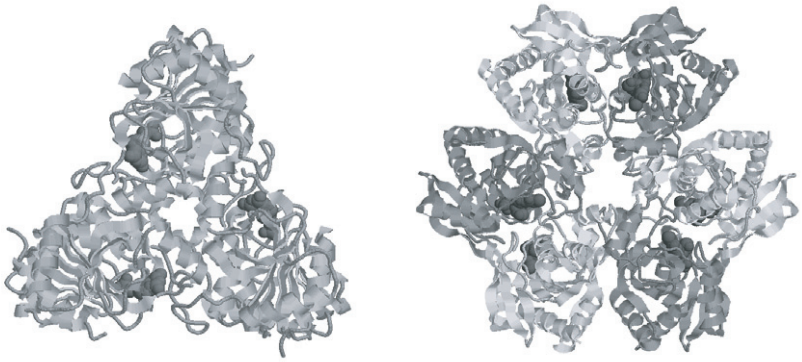

Figure 1. The crystal structures of trimeric calf spleen (left panel) and hexameric *E. coli* (right panel) PNPs (PDP codes: 1LV8 [5] and 1K9S [8], respectively). The ligands complexed at the active sites are shown in the space-filling presentation (multisubstrate analogue inhibitor 2,6-diamino-(S)-9-[2-(phosphonomethoxy)propyl]purine for calf spleen PNP, and 6-methylformycin A/N7-methylformycin A and phosphate/sulfate for *E. coli* enzyme).

allele, F12L, reveals the presence of a hexamer [2]. The structural transition between hexamer and octamer seems to proceed through the equilibrium containing two different dimeric forms of the enzyme and is facilitated by the interactions of ligands at the enzyme active site [3].

The concept of a morpheein was postulated to be the more general phenomenon of the enzyme activity regulation process. Several other enzymes described in the literature were selected to be possible examples of morpheeins, among them purine nucleoside phosphorylase (PNP, EC.2.4.1) [1]. Since for almost 20 years we have been studying enzymes, homotrimers and homohexamers belonging to the two main subfamilies of the PNP family intensively by a variety of experimental techniques, we decided to analyse in more detail the possibility of their potential morpheein behaviour.

Purine nucleoside phosphorylase, the ubiquitous enzyme of the nucleoside salvage pathway, catalyses the reversible phosphorolytic cleavage of the glycosidic bond of purine nucleosides, as follows:

β-nucleoside + orthophosphate $\leftrightarrow$ purine base + α-D-pentose-1-phosphate.

The PNP enzymes are divided into two classes: homotrimers occurring mainly in mammals and homohexamers found in various microorganisms [4]. The crystallography approach revealed that although both trimeric [5, 6] and hexameric [7, 8] phosphorylases have one complete active site per monomer (figure 1), each active site contains the residue/s (one for the trimer and two for the hexamer) from the neighbouring monomer. Furthermore, homohexamers are trimers of dimers (figure 1) and the exchange of active site residues takes place only within the dimers [7, 8]. In the case of trimeric phosphorylases, the exchange is not mutual.

PNPs are characterized by complex, non-Michaelis kinetics. Such phenomena may be caused by various reasons, among them equilibrium of different oligomeric forms of the enzyme (morpheein is the particular case of it), cooperativity between the active sites or their heterogeneity, inclusion of the catalytic steps, random binding of substrates or substrate acting as a modifier [9].

There have been conflicting reports regarding the coexistence of various oligomeric species of PNP (which might differ in specific activity) and the catalytic activity of phosphorylase monomer. Jensen and Nygaard [10] showed that only the hexameric forms of *E. coli* and

S. typhimurium PNP were enzymatically active. By contrast, the analytical ultracentrifugation studies by Nixon *et al* [11] indicated that *E. coli* PNP may exist as an equilibrium mixture of at least two oligomeric forms (trimer and hexamer or tetramer and octamer). Moreover, Ropp and Traut [12] reported that dilution of the trimeric PNP from calf spleen leads to dissociation of the protein into subunits with a marked increase in specific activity caused by an allosteric effect of phosphate, which acts as an allosteric regulator. Finally, Jaffe [1] suggested that PNPs are an example of a morpheein, i.e. the protein that exists as an equilibrium of the quaternary structure isoforms.

In this paper we summarize the results of our investigation regarding the effect of various factors which could induce protein dissociation into subunits or shift an equilibrium between hypothetical quaternary structure isoforms on the enzyme activity and oligomeric structure of both *E. coli* (hexameric) and calf spleen (trimeric) PNP in solution. These include the effects of dilution, temperature, ionic strength, and presence of guanidine chloride and phosphate. We recall already published data as well as some new experiments. In our studies we used enzyme kinetics, steady-state emission spectroscopy, size exclusion chromatography, analytical ultracentrifugation and circular dichroism spectroscopy.

2. Materials and methods

All reagents and materials were of the highest quality commercially available, and only those of spectral grade, checked by UV absorption and/or fluorescence emission, were employed.

Partially purified PNP from *E. coli* (∼60% pure, about 60 U mg^{-1}) was further purified as described in [13]. The PNP concentration was measured spectrophotometrically at pH 7.0 with extinction coefficient $\varepsilon^{1\%}(278\ \text{nm}) = 2.7$ [14].

Ultraviolet absorption was monitored with a Kontron (Switzerland) UVIKON 930 recording instrument and an M40 spectrophotometer (Zeiss, Germany) fitted with a thermostatically-controlled cell compartments. Steady-state fluorescence measurements were carried out on a Spex (USA) FluoroMax spectrofluorometer in 5 mm × 5 mm Suprasil cuvettes.

Enzyme activity was monitored spectrophotometrically in 50 mM phosphate buffer (pH 7.0) using 2, 5, 10 or 50 mm pathlength cuvettes. The coupled xanthine oxidase procedure with 0.5 mM inosine as a substrate [15] was used with observation wavelength at 300 nm or the direct spectrophotometric method based on changes in absorption at 260 nm of 7-methylguanosine (m^7 Guo) as a substrate [16].

Size exclusion chromatography (gel filtration) was carried out at room temperature in an FPLC system on Superose™ 12, HR 10/30, 300 mm × 10 mm column with a 280 nm UV-1 detector (Pharmacia LKB Biotechnology, Sweden). PNP was eluted with 50 mM TRIS-HCl buffer (pH 7.6) containing 0.1 M KCl at a flow rate of 0.4 ml min^{-1} and 0.2 ml fractions were collected. The molecular mass of PNP was determined based on molecular weight marker filtration (cytochrome C, 12.4 kDa; carbonic anhydrase, 29 kDa; BSA, 67 kDa; β-amylase, 200 kDa).

Temperature-dependent kinetic data were analysed according to the Arrhenius equation in the logarithmic form [17]: $\ln k = \text{const} - E_a/RT$, where E_a is the apparent activation energy, T the absolute temperature and k the reaction rate constant equivalent to the specific activity of the enzyme.

3. Results and discussion

3.1. Effect of dilution, phosphate and ionic strength

The molecular mass (M) of *E. coli* PNP in the concentration range 6.2×10^{-4}–62 mg ml^{-1} was examined by size exclusion chromatography (the sensitivity of detection does not allow us to

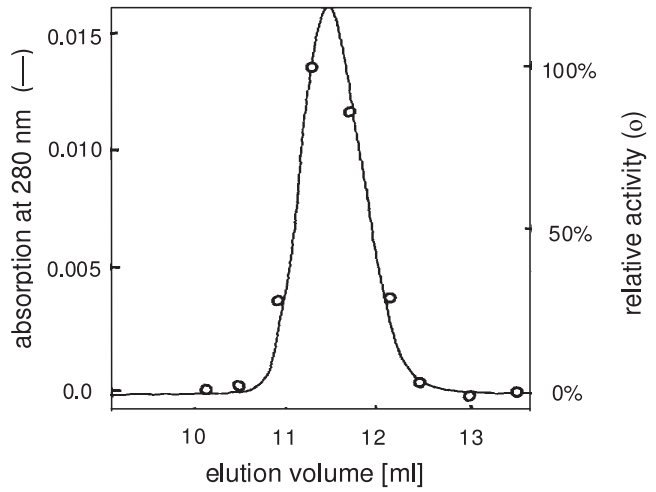

Figure 2. Correlation between relative enzyme activity (o) and concentration of *E. coli* PNP in the chromatographic profile of the protein eluted from the size exclusion chromatography column (—). The column was equilibrated with 50 mM TRIS-HCl buffer (pH 7.6) containing 0.1 M KCl, and 0.2 ml fractions were collected. The concentration of PNP subjected to gel filtration was 1.4 mg ml^{-1}. The PNP activity of chromatographic fractions was measured versus 0.5 mM inosine in 50 mM phosphate buffer pH 7.0 at 25 °C.

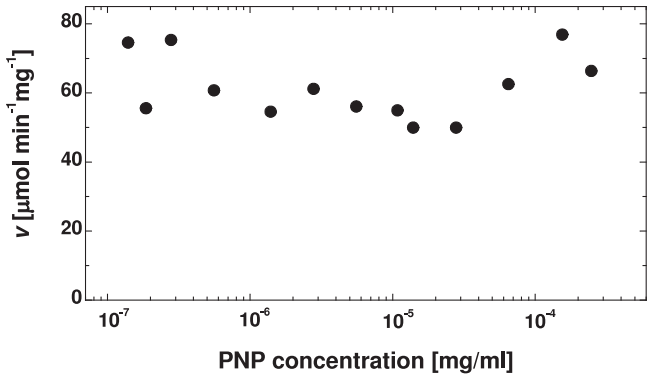

Figure 3. Effect of dilution on *E. coli* PNP specific activity (v). The enzyme was diluted into 50 mM phosphate buffer pH 7.0 freshly before activity measurement. Activity was measured in 50 mM phosphate buffer pH 7.0 versus 0.5 mM inosine at 25 °C.

test lower enzyme concentrations). The elution profile of PNP was always a single symmetric peak. Moreover, the shape and position of the peak were independent of protein concentration, as well as preincubation with phosphate (8 mM), and overlapped well with the enzyme activity profile (figure 2). The average value of M determined on the basis of gel filtration experiments is 135 ± 28 kDa, and this corresponds to an M of hexameric PNP (155 kDa, as calculated from the aminoacid sequence [18]).

We have also found that the specific activity of *E. coli* PNP was not affected by further dilution to 1.4×10^{-7} mg ml^{-1} (figure 3). This suggests that dilution of PNP does not lead to dissociation into subunits of different catalytic properties from those of the hexamer.

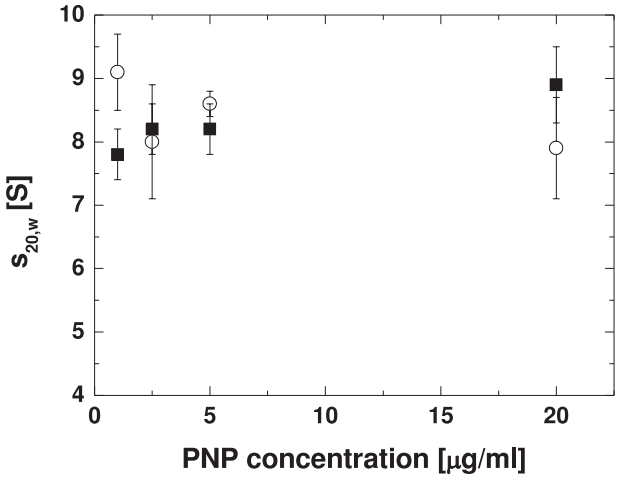

Figure 4. Sedimentation coefficients of *E. coli* PNP as a function of protein concentration obtained from active enzyme sedimentation experiments in 50 mM phosphate buffer pH 7.0. Nucleoside concentrations were 150 or 300 μM in the case of 7-methylguanosine (m^7 Guo) (○) and 300 μM in the case of its 6-thiocounterpart (2-amino-6-mercapto-7-methylpurine ribonucleoside, MESG) (■). Centrifugation was performed at 40 000 rpm and 20 or 25 °C. Data from [19].

These findings were confirmed by analytical ultracentrifugation experiments [19], conducted for a broad *E. coli* PNP concentration range and under various experimental conditions. Similar studies were also done for trimeric calf spleen PNP [20].

The sedimentation velocity experiments for *E. coli* PNP gave symmetrical apparent sedimentation coefficient distributions ($g(s^0_{20,w})$), regardless of protein concentration (0.02–1.4 mg ml^{-1}), presence of phosphate (P_i up to 50 mM) and ionic strength (NaCl up to 0.5 M), indicating a single molecular species in solution [19]. Table 1 contains the values of sedimentation coefficients of *E. coli* PNP, determined as the maxima of $g(s^0_{20,w})$ and the molecular masses of the sedimenting particle, calculated using the Svedberg equation. The $s^0_{20,w}$ values and molecular masses are independent of experimental conditions and are consistent with the single species being a PNP hexamer. Moreover, as shown by active enzyme centrifugation [19], the values of sedimentation coefficients of *E. coli* PNP show no dependence on the protein concentration in the range 1–20 μg ml^{-1} (figure 4) and are consistent with the sedimentation of a single particle not smaller then the hexamer ($\bar{s}_{20,w} = 8.3 \pm 0.4$ S). Furthermore, sedimentation equilibrium studies confirm, that the enzyme does not exist as a mixture of active and inactive (and thus invisible with active enzyme centrifugation) various oligomeric forms. The data obtained under all applied conditions (phosphate up to 10 mM, KCl up to 100 mM, temperature 4 or 20 °C, enzyme concentrations between 0.18 and 1.7 mg ml^{-1}) correspond to a single-species model with molecular mass of 150 ± 10 kDa indicating the hexameric form of *E. coli* PNP. Moreover, the self-association model led us to obtaining a stoichiometry equal to 6 with a very high equilibrium constant towards hexameric oligomer and a monomer molecular mass (∼25 kDa) consistent with the mass calculated from the aminoacid sequence of PNP.

For calf spleen PNP, sedimentation equilibrium analysis under a broad spectrum of conditions (protein concentration in the range 0.02–1.4 mg ml^{-1}, presence of phosphate up to 100 mM and NaCl up to 200 mM, temperature in the range 4–25 °C), revealed a single species with molecular mass 90.6 ± 2.1 kDa, which corresponds to the homotrimeric

Table 1. The sedimentation coefficients and molecular masses of *E. coli* PNP obtained from sedimentation velocity experiments at 20 °C, pH 7.0 and 60 000 rpm. Various buffer conditions and protein concentrations were used. Data from [19].

Experiment conditions	PNP concentration (mg ml^{-1})	$s^0_{20,w}$ (S)	M[a] (kDa)
50 mM TRIS	0.03	7.7 ± 0.4	161
50 mM TRIS + 0.5 M NaCl	0.03	7.8 ± 0.3	149
50 mM TRIS + 50 mM P_i [b]	0.03	7.8 ± 0.2	149
50 mM TRIS + 0.5 M NaCl + 50 mM P_i	0.015	7.8 ± 0.2	115
50 mM TRIS	1.3	7.7 ± 0.4	177
50 mM TRIS + 0.5 M NaCl	1.4	7.7 ± 0.4	153
50 mM TRIS + 50 mM P_i	1.3	7.7 ± 0.3	172
50 mM TRIS + 0.5 M NaCl + 50 mM P_i	1	7.7 ± 0.2	139
50 mM P_i + 1 M GdnHCl	0.63	7.7 ± 0.3	
50 mM P_i + 1 M GdnHCl	0.03	7.7 ± 0.3	

[a] The estimated accuracy was 10%.
[b] P_i is the abbreviation for orthophosphate.

molecule [20]. Furthermore, active enzyme sedimentation experiments confirmed that calf spleen PNP remained trimeric even at low protein concentrations (1 μg ml^{-1}) [20].

3.2. Effect of destabilizing factors (temperature, GdnHCl)

The crystal structure of *E. coli* PNP is a trimer of dimers [7, 8] (figure 1), suggesting that the smallest operating unit of the enzyme could be a dimer. Hence, it is possible that destabilizing conditions can cause dissociation of PNP into catalytically active dimers. To verify this hypothesis, the effect of guanidine hydrochloride (GdnHCl) on enzymatic activity and circular dichroism (CD) spectra, as well as the effect of temperature on enzymatic activity and fluorescence spectra of *E. coli* PNP, was examined.

As stated above, analytical ultracentrifugation experiments showed that calf spleen and *E. coli* PNP are stable trimer and hexamer, respectively, at temperature in the range 4–25 °C. Thus we checked if high temperature can destabilize *E. coli* PNP hexamer and induce dissociation.

Increasing the temperature from 14 to 45 °C led to an increase of activity with a linear Arrhenius plot (figure 5), indicating that the protein does not undergo any conformational change. A rapid drop in the reaction rate due to protein denaturation was observed above 50 °C. The calculated activation energy was 11.5 ± 0.6 kcal mol^{-1}, typical for enzymatic reactions [21].

On the other hand, the fluorescence intensity of *E. coli* PNP at 304 nm (excitation at 276 nm) decreased linearly with increase in temperature in the range 14–52 °C (figure 6), with no effect on location and shape of the emission and excitation spectra, as is typical for tyrosine proteins [22]. This points to a temperature-dependent non-radiative deexcitation process and confirms the observation that the enzyme does not dissociate into subunits with an increase in temperature.

Application of denaturing conditions also does not cause decomposition of the hexamer into functional units. The process of protein unfolding (CD spectra) and loss of the PNP activity at the presence of GdnHCl (0–4 M) were monitored simultaneously [19]. It was found that the values of CD signal at 220 nm and enzyme activity as a function of GdnHCl concentration are correlated and that the transition from the native state of PNP to the random coil is a single-step

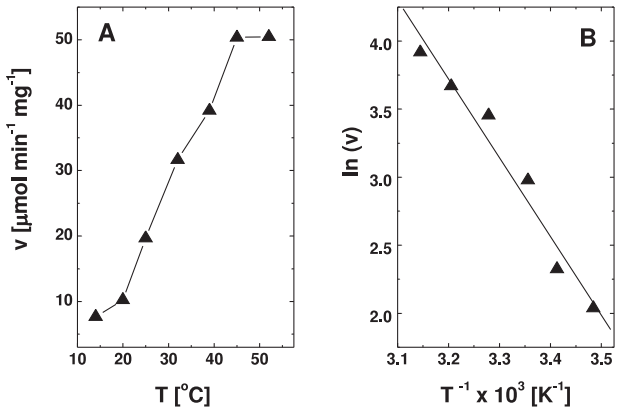

Figure 5. (A) The temperature dependence of *E. coli* PNP activity (v) versus 400 μM m[7] Guo and phosphate, measured in 50 mM phosphate buffer pH 7.0. (B) An Arrhenius plot of the same data in the range 14–45 °C; the solid line represents a linear fit of the data.

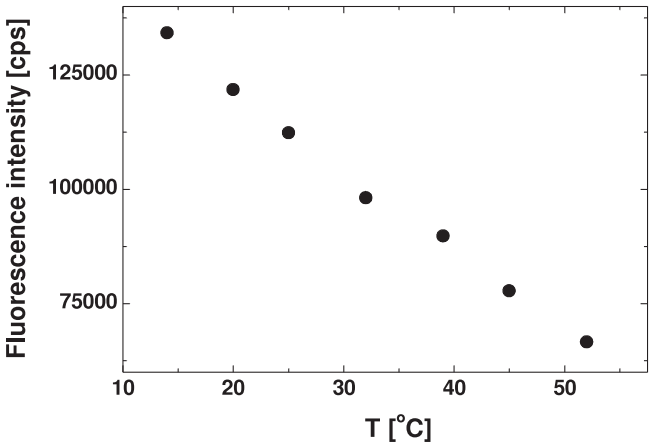

Figure 6. Effect of temperature on the fluorescence intensity at 304 nm (excitation at 276 nm) of *E. coli* PNP. The fluorescence was measured in 100 mM TRIS-HCl buffer (pH 7.6). The enzyme concentration was 43 μg ml^{-1}.

process. This shows that dissociation into subunits, if it takes place, arises directly from the loss of the secondary structure of subunits and is not an autonomous phenomenon, which may occur due to dissociation of the hexameric form into enzymatically active dimeric forms. The sedimentation coefficient determined at 1 M GdnHCl (at which the enzyme is still fully active) is 7.7 S (table 1), confirming that also under these conditions the only active form of PNP is a hexamer.

4. Conclusions

The overall results show that, irrespective of experimental conditions, the only active forms for calf spleen and *E. coli* PNPs are trimer and hexamer, respectively. None of the factors examined—temperature, dilution, ionic strength, presence of substrates (purine nucleosides and phosphate) and guanidine hydrochloride—caused a change of the oligomeric state of the

proteins examined. The complex kinetics observed for these enzymes cannot be explained by a coexistence of various oligomeric forms of different functionality, and arises from other molecular phenomena. Thus, the results described above disprove the hypothesis that PNP is an example of a morpheein and that the enzyme monomer can adopt different conformations. It is worthy of note that, though there is not any sequence homology between trimeric and hexameric phosphorylases, the monomeric units of the enzymes reveal high structural similarity [4].

The reasons for the discrepancy from the Michaelis–Menten model for trimeric calf spleen PNP have been previously elucidated [23]. The double hyperbolic kinetic characteristic of this enzyme is caused by random binding of substrates and unusually potent binding of some purine bases, the release of which is stimulated by phosphate [23].

In the case of hexameric PNPs, that structurally could be assembled as a trimer of dimers (figure 1), a complex kinetic characteristic arises from other molecular reasons. Fluorescence studies on phosphate binding to *E. coli* PNP [13] point to interaction between monomers in a dimer that leads to negative cooperativity in binding of this ligand, and two dissociation constants of 29 μM and 1.1 mM, necessary to describe the PNP/phosphate interactions. Relevant to this is the crystal structure of *E. coli* PNP [8], showing that the enzyme is a trimer of dimers containing pairs of unsymmetrical active sites of two conformations: an open one, with low substrate binding affinity and a closed one, binding substrates tightly.

The authors of the morpheein concept suggest that the high thermal stability of PBGS proteins may result from their ability to accommodate a variety of oligomeric structures [3]. However, the crystal structure of thermophilic 5′-deoxy-5′ methylthioadenosine phosphorylase (MTAP) from *Sulfolobus solfataricus*, belonging to the hexameric class of PNP, revealed the presence of the three intersubunit disulfide bonds, which stabilize the hexameric structure of the enzyme [24].

Taking into account the crystal structure of trimeric PNP, which reveals that each monomer donates one residue to the neighbouring active site, it seems unlikely that the enzyme could operate as an oligomer smaller than trimer. The significance of the trimeric structure of calf spleen PNP is confirmed by the one-third of the sites inhibition observed for tight-binding transition state inhibitors (immucillins H and G) [25]. It follows that binding of one immucillin molecule per trimer hinders binding to the other two monomers, so the monomers in the trimer are not independent. Moreover, the crystal structure of hexameric *E. coli* PNP also shows that the two monomers in the dimer are not independent and that only one of them can adopt the closed tight-binding conformation at a time. The alternate arrangement of monomers with open and closed active site conformations within the hexamer implies that communication must also occur between adjacent catalytic dimers and underlies the significance of maintaining the hexameric structure in *E. coli* PNP.

Acknowledgments

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References

[1] Jaffe E K 2005 *Trends Biochem. Sci.* **30** 490–7
[2] Breining S, Kervinen J, Stith L, Wasson A S, Fairman R, Wlodawer A, Zdanov A and Jaffe E K 2003 *Nat. Struct. Biol.* **10** 757–63
[3] Tang L, Stith L and Jaffe E K 2005 *J. Biol. Chem.* **280** 15786–93

[4] Bzowska A, Kulikowska E and Shugar D 2000 *Pharmacol. Ther.* **88** 349–425
[5] Bzowska A, Koellner G, Wielgus-Kutrowska B, Stroh A, Raszewski G, Holý A, Steiner T and Frank J 2004 *J. Mol. Biol.* **342** 1015–32
[6] Mao C, Cook W J, Zhou M, Federov A A, Almo S C and Ealick S E 1998 *Biochemistry* **37** 7135–46
[7] Koellner G, Luić M, Shugar D, Saenger W and Bzowska A 1998 *J. Mol. Biol.* **280** 153–66
[8] Koellner G, Bzowska A, Wielgus-Kutrowska B, Luić M, Steiner T and Saenger W 2002 *J. Mol. Biol.* **315** 351–71
[9] Segel I H 1975 *Enzyme Kinetics* (New York: Wiley)
[10] Jensen K F and Nygaard P 1975 *Eur. J. Biochem.* **51** 253–65
[11] Nixon A E, Hunter J L, Bonifacio G, Eccleston J F and Webb M R 1998 *Anal. Biochem.* **265** 299–307
[12] Ropp P A and Traut T W 1991 *J. Biol Chem.* **266** 7682–7
[13] Kierdaszuk B, Modrak-Wójcik A and Shugar D 1997 *Biophys. Chem.* **63** 107–18
[14] Bzowska A, Kazimierczuk Z and Seela F 1998 *Acta Biochim. Pol.* **45** 755–68
[15] Kalckar H M 1947 *J. Biol. Chem.* **167** 429–42
[16] Kulikowska E, Bzowska A, Wierzchowski J and Shugar D 1986 *Biochim. Biophys. Acta* **874** 355–63
[17] Cornish-Bowden A 1981 *Fundamentals of Enzyme Kinetics* (London: Butterworth)
[18] Hershfield M S, Chaffee S, Koro-Johnson L, Mary A, Smith A A and Short S A 1991 *Proc. Natl Acad. Sci. USA* **88** 7185–9
[19] Modrak-Wójcik A, Stępniak K, Akoev V, Żółkiewski M and Bzowska A 2006 *Protein Sci.* **15** 1794–800
[20] Behlke J, Koellner G and Bzowska A 2005 *Z. Naturf.* C **60** 927–31
[21] Laidler K J and Peterman B F 1979 *Methods Enzymol.* **63** 234–57
[22] Lakowicz J R 1999 *Principles of Fluorescence Spectroscopy* (New York: Kulwer Academic/Plenum)
[23] Bzowska A 2002 *Biochim. Biophys. Acta* **1596** 293–317
[24] Appleby T C, Mathews I I, Porcelli M, Cacciapuoti G and Ealick S E 2001 *J. Biol. Chem.* **276** 39232–42
[25] Miles R W, Tyler P C, Furneaux R H, Bagdassarian C K and Schramm V L 1998 *Biochemistry* **37** 8615–21