FOR THE RECORD

Molecular architecture of *E. coli* purine nucleoside phosphorylase studied by analytical ultracentrifugation and CD spectroscopy

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Abstract

Purine nucleoside phosphorylase (PNP) is a key enzyme of the nucleoside salvage pathway and is characterized by complex kinetics. It was suggested that this is due to coexistence of various oligomeric forms that differ in specific activity. In this work, the molecular architecture of *Escherichia coli* PNP in solution was studied by analytical ultracentrifugation and CD spectroscopy. Sedimentation equilibrium analysis revealed a homohexameric molecule with molecular mass 150 ± 10 kDa, regardless of the conditions investigated—protein concentration, 0.18–1.7 mg/mL; presence of up to 10 mM phosphate and up to 100 mM KCl; temperature, 4–20°C. The parameters obtained from the self-associating model also describe the hexameric form. Sedimentation velocity experiments conducted for broad protein concentration range (1 µg/mL–1.3 mg/mL) with boundary (classical) and band (active enzyme) approaches gave $s_{20,w}^0 = 7.7 \pm 0.3$ and 8.3 ± 0.4 S, respectively. The molecular mass of the sedimenting particle (146 ± 30 kDa), calculated using the Svedberg equation, corresponds to the mass of the hexamer. Relative values of the CD signal at 220 nm and the catalytic activity of PNP as a function of GdnHCl concentration were found to be correlated. The transition from the native state to the random coil is a single-step process. The sedimentation coefficient determined at 1 M GdnHCl (at which the enzyme is still fully active) is 7.7 S, showing that also under these conditions the hexamer is the only catalytically active form. Hence, in solution similar to the crystal, *E. coli* PNP is a hexameric molecule and previous suggestions for coexistence of two oligomeric forms are incorrect.

Keywords: purine nucleoside phosphorylase (PNP); oligomeric state; analytical ultracentrifugation; CD spectroscopy; active enzyme sedimentation

The ubiquitous purine nucleoside phosphorylase (PNP, purine nucleoside:ortho-phosphate ribosyl transferase, EC 2.4.2.1.), a key enzyme of the anabolic and catabolic pathways of purine nucleosides, catalyzes the reversible

phosphorolytic cleavage of the glycosidic bond of purine nucleosides and their analogs, as follows:

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Inhibitors of PNP are potential immunosuppressive drugs that could be used against the host versus graft reaction in organ transplantation and in T-cell tumors (Stoeckler 1984). PNP inhibitors could also prevent intracellular degradation of some (nucleoside-based) anti-tumor

and anti-viral agents (Montgomery et al. 1993). Recently, *Escherichia coli* PNP has been shown to be a promising candidate for tumor-directed gene therapy (Parker et al. 1997; Deharvenge et al. 2004).

There are two main structural classes of PNPs: homotrimers found mainly in mammals and homohexamers (trimers of dimers) occurring in various micro-organisms (Bzowska et al. 2000). In either class, the phosphorylases contain one complete active site per PNP monomer. In the case of the PNP hexamer, however, the minimal functional unit seems to be a dimer, because each active site contains two residues contributed by the neighboring monomer (Koellner et al. 1998, 2002).

There have been conflicting reports concerning the catalytic activity of monomeric PNP and a possible role of coexistence of various oligomeric species (which might differ in specific activity) in complex kinetic characteristics observed for PNPs from various sources (Bzowska et al. 2000). Jensen and Nygaard (1975) showed that only the hexameric forms of *E. coli* and *S. typhimurium* PNP were enzymatically active. In contrast, the analytical ultracentrifugation studies by Nixon et al. (1998) suggested that *E. coli* PNP exists as an equilibrium mixture of at least two oligomeric forms. Moreover, Ropp and Traut (1991) 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 and that phosphate acts as an allosteric regulator of the process. However, recent studies of calf spleen PNP have unequivocally shown that the only active enzyme form is a homotrimer, regardless of the conditions investigated, e.g., the presence of phosphate (Bzowska 2002; Behlke et al. 2005).

In this work, we investigated the oligomeric structure of PNP from *E. coli* using analytical ultracentrifugation and circular dichroism spectroscopy. We found that hexameric PNP is predominant and stable under a variety of experimental conditions.

Results and Discussion

Sedimentation velocity (boundary sedimentation)

Sedimentation velocity experiments were performed to examine the effect of ionic strength, protein concentration, and the presence of phosphate (P_i) on the oligomeric state of PNP in solution. PNP at high (~ 1.3 mg/mL) and low (~ 0.03 mg/mL) concentration was centrifuged in Tris or Hepes buffer (pH 7.0) without or with 0.5 M NaCl and 50 mM phosphate.

Figure 1 shows the apparent sedimentation coefficient distributions ($g(s_{20,w}^0)$) for two PNP concentrations (0.03 and 1.3 mg/mL) (Fig. 1A) and for the latter concentration, additionally in the presence of 0.5 M NaCl or/and 50 mM P_i (Fig. 1B). All of the distributions in Figure 1 are

Figure 1. Sedimentation coefficient distributions ($g(s_{20,w}^0)$) obtained for *E. coli* PNP. Sedimentation velocity experiments were carried out in (A) 50 mM Tris-HCl (pH 7.0) for 1.3 mg/mL (---) and 0.03 mg/mL (—) PNP and in 50 mM phosphate buffer (pH 7.0) with 1M GdnHCl for 0.6 mg/mL (---) and 0.03 mg/mL (○) PNP and (B) for 1.3 mg/mL PNP in 50 mM Tris-HCl (pH 7.0) without (---) and with (—) 0.5 M NaCl, with 50 mM sodium phosphate (○), or with 0.5 M NaCl and 50 mM sodium phosphate (---). Centrifugation was at 60,000 rpm and 20°C.

symmetrical, indicating a single molecular species in solution. Similar results were obtained also in the other experimental conditions mentioned above, leading to the average sedimentation coefficient: $\bar{s}_{20,w}^0 = 7.7 \pm 0.3$ S. The molecular mass of the sedimenting particle (146 ± 30 kDa), calculated using the Svedberg equation, is consistent with the single species being a PNP hexamer (predicted molecular weight of 155,000 Da using the monomer molecular mass of 25.8 kDa [Hershfield et al. 1991]). We conclude that hexameric PNP does not dissociate upon ~ 50 -fold dilution, at high ionic strength, or in the presence of phosphate.

The crystal structure of *E. coli* PNP suggested that the assembly of homohexameric PNP could involve association of three dimers (Koellner et al. 2002). Hence, it is possible that under destabilizing conditions PNP can dissociate into dimers that might be catalytically active. To verify this hypothesis, we have examined the effect of guanidine hydrochloride (GdnHCl) on enzymatic activity, circular dichroism (CD) spectra, and sedimentation coefficient of *E. coli* PNP.

Figure 2A compares CD spectra for PNP (1.75 mg/mL) under the native and the denaturing conditions (0–4 M GdnHCl). Relative values of CD signal at 220 nm and PNP activity toward 0.5 mM m^7 Guo as a function of GdnHCl concentration are compared in Figure 2B. The correlation between these two parameters is evident—the enzyme activity and secondary structure

Figure 2. (A) CD spectra obtained for 1.75 mg/mL PNP in 50 mM phosphate buffer (pH 7.0) without (—) and with 1M (---), 2M (▲), and 4M (○) guanidine hydrochloride (GdnHCl) and for 4 M GdnHCl without PNP (*). (B) Influence of a denaturant (GdnHCl) on enzymatic activity and secondary structure of *E. coli* PNP. Relative values of CD signal at 220 nm (□) and enzymatic activity of PNP with 0.5 mM $m^7\text{Guo}$ as a nucleoside substrate (●) are presented as a function of GdnHCl concentration.

are lost as the concentration of GdnHCl increases. The transition from the native state of PNP to the random coil is a single-step cooperative process. Figure 2B shows that at 2 M GdnHCl, only ~30% of the enzyme activity remains, whereas PNP is ~98% active at 1 M of GdnHCl. Thus, we have chosen the latter concentration of GdnHCl as the highest concentration of the destabilizing factor at which the enzyme is almost fully active, and we have used these conditions to determine the sedimentation coefficient of PNP.

Sedimentation velocity experiments in the presence of 1M GdnHCl were performed for two PNP concentrations (0.63 and 0.03 mg/mL) in 50 mM P_i buffer (pH 7.0). In both cases, sedimentation coefficient distributions ($g(s^0_{20,w})$) are almost symmetrical and their maxima overlap (Fig. 1A). The values of sedimentation coefficient (7.7 ± 0.3 S) agree with those obtained for PNP without the addition of the denaturing factor. This result indicates that even under destabilizing conditions of 1 M GdnHCl, the PNP hexamer remains stable at low protein concentration and is the only catalytically active form of *E. coli* PNP.

Active enzyme centrifugation (band sedimentation)

In order to study the sedimentation behavior of PNP at very low protein concentrations (1–20 $\mu\text{g/mL}$), we performed the active enzyme sedimentation experiments (Cohen et al. 1967; Cohen and Mire 1971; Kemper and

Everse 1973). Only catalytically active forms of an enzyme can be examined with this method, since the observation is based on either the appearance of the product of the reaction or on the disappearance of the substrate. Representative radial absorption distributions of the reaction mixture generated as the enzyme-band sediments inside the centrifuge cell are shown in Figure 3 (for MESG as a nucleoside substrate) and in Figure 4 (for $m^7\text{Guo}$). The absorption change (increase in Fig. 3 or decrease in Fig. 4) is a single-step profile, indicating that there is only one active oligomeric form of the enzyme in solution (Cohen and Claverie 1975).

To directly compare the band and the boundary sedimentation coefficients, i.e., values obtained from the active enzyme method with those from the classical sedimentation velocity studies, a band sedimentation experiment for high PNP concentration (3.4 mg/mL) was performed. In this case, the enzyme was layered on a buffered solution without substrates and the enzyme concentration profiles were measured using protein absorption at 280 nm (Fig. 5).

Table 1 contains the values of sedimentation coefficients of *E. coli* PNP obtained at 1–20 $\mu\text{g/mL}$ concentration range with the active enzyme centrifugation method. Sedimentation parameters could not be determined for PNP concentrations below 1 $\mu\text{g/mL}$, due to low absorption changes obtained with such small amounts of the enzyme.

Sedimentation coefficients were calculated using the midpoint method for determination of the location of the enzyme band center (r) (see Materials and Methods). Representative plots of $\ln(r)$ versus time are shown for MESG substrate (Fig. 3, inset) and for $m^7\text{Guo}$ (Fig. 4, inset). In all cases, the plots are linear and the values

Figure 3. Active enzyme centrifugation experiment for 20 $\mu\text{g/mL}$ PNP and the substrate MESG. Centrifugation was performed at 40,000 rpm and 20°C. Absorbance distribution, corresponding to the concentration of the product of phosphorolysis of MESG, observed while the enzyme layer moves along the cuvette filled with the reaction mixture containing both substrates (300 μM MESG and 50 mM phosphate) was measured at 360 nm. Radial absorption profiles were obtained in 5-min intervals. Inset shows the plot of $\ln(r)$ vs. time (r is the position of the midpoint of the sedimenting boundary) (■) and the linear fit of these data (—).

Figure 4. Active enzyme centrifugation experiment for 20 µg/mL PNP and substrate m⁷Guo. Centrifugation was performed at 40,000 rpm and 25°C. Absorbance distribution, corresponding to the concentration of the product of phosphorolysis of m⁷Guo, observed while the enzyme layer moves along the cuvette filled with the reaction mixture containing both substrates (300 µM m⁷Guo and 50 mM phosphate) was measured at 260 nm. Radial absorption profiles were obtained in 7.5-min intervals. The inset shows plot of $\ln(r)$ vs. time (r is the position of the midpoint of the sedimenting boundary) (■) and the linear fit of these data (—).

of $s_{20,w}$ are independent of either PNP concentration or nucleoside substrate (Table 1). This result confirms the existence of only one active oligomeric form of the enzyme at very low protein concentration.

Sedimentation coefficients obtained using active enzyme centrifugation are slightly higher ($\bar{s}_{20,w} = 8.3 \pm 0.4$ S) (see Table 1) than those from the classical sedimentation velocity method ($\bar{s}_{20,w}^0 = 7.7 \pm 0.3$ S) (see Fig. 1), although only the latter coefficients were corrected to values obtained in the limit of a dilute protein solution (which leads to an increase of s). Overestimation of s could be due to too high a concentration of the enzyme (Kemper and Everse 1973), but the data in Table 1 do not show any correlation between PNP concentration

Figure 5. Band centrifugation of *E. coli* PNP (3.4 mg/mL). Centrifugation was performed at 40,000 rpm and 20°C. Protein was loaded on 50 mM HEPES buffer (pH 7.0) with 150 mM KCl. Absorbance distribution of protein observed while enzyme layer moves along the cuvette was measured at 280 nm. Radial absorption profiles were obtained in 15-min intervals. The inset shows plot of $\ln(r_{\max})$ vs. time (r is the maximum of a spectrum) (■) and the fit of these data (—).

Table 1. Sedimentation coefficients of *E. coli* PNP obtained in the low protein concentration range (1–20 µg/mL) from active enzyme sedimentation experiments^a

$s_{20,w}$	λ_{obs} [nm]	Nucleoside substrate	PNP concentration [µg/mL]	T [°C]
8.9 ± 0.6	360	MESG	20	20
8.2 ± 0.4	355	MESG	5	20
8.2 ± 0.4	355	MESG	2.5	25
7.8 ± 0.4	355	MESG	1	25
7.9 ± 0.8	260	m ⁷ Guo ^b	20	25
8.6 ± 0.2	260	m ⁷ Guo	5	25
8.0 ± 0.9	260	m ⁷ Guo	2.5	25
9.1 ± 0.6	260	m ⁷ Guo	1	25
8.8 ± 0.5	280	HEPES + KCl	3400	20

^aBand sedimentation; see Figures 3–5.

^bConcentration of m⁷Guo was 300 µM.

If not otherwise indicated, nucleoside concentrations were 150 µM in the case of m⁷Guo and 300 µM in the case of MESG. Centrifugation was at 40,000 rpm. The last entry in the table shows the conditions used in and the result obtained from the band sedimentation experiment conducted with direct observation of PNP (without the substrates).

and the value of s . In addition, different values of sedimentation coefficient were obtained for the same enzyme concentration examined with both methods. Moreover, the sedimentation coefficient determined from band sedimentation for high concentration of PNP (3.4 mg/mL) was also greater ($s_{20,w} = 8.8 \pm 0.5$ S) than the values obtained from the sedimentation velocity method.

The active enzyme centrifugation method is prone to artifacts and has some limitations resulting from indirect observation of the enzyme sedimentation. To analyze the data, we used an approximate method of midpoints for calculation of sedimentation coefficient, which give precise values of s for enzymes with sedimentation coefficient above 3 S (Llewellyn and Smith 1978). The existence of an enzyme as rapid (cf. sedimentation velocity) equilibrium of two oligomeric forms complicates interpretation of results. In such cases, however, the values of s are lowered, whereas we observe reverse tendency. In our opinion, the active enzyme sedimentation is less accurate than the boundary sedimentation method, which could be associated with the time-dependent values of density and viscosity of the medium around the moving protein layer (Ralston 1993).

Overall, the results in Table 1 are consistent with the sedimentation of a single particle not smaller than the hexamer at very low PNP concentration. Furthermore, sedimentation equilibrium studies (see below) confirm that the enzyme does not dissociate into inactive subunits (which are invisible with active enzyme centrifugation).

Sedimentation equilibrium

Sedimentation equilibrium studies were performed to directly determine the molecular mass and the oligomeric

state of PNP. Nixon et al.'s (1998) ultracentrifugation study suggested that *E. coli* PNP exists in solution as an equilibrium mixture of either trimer and hexamer or tetramer and octamer. Those models have been controversial in the light of crystallographic data—the crystal structure of *E. coli* PNP shows an apparent trimer of dimers (Koellner et al. 1998, 2002).

We performed sedimentation equilibrium experiments at various conditions, including those applied by Nixon et al. (1998). Experiments were conducted in HEPES (pH 7.0) (with or without P_i) and in Tris (pH 7.5), and covered the range of enzyme concentrations between 0.18 and 1.7 mg/mL. In all cases, temperature was either 4°C or 20°C.

The sedimentation equilibrium data for PNP in Tris at 20°C (Fig. 6), as well as the data obtained with other conditions (data not shown), are consistent with a single-species model. The values of molecular mass obtained from the sedimentation equilibrium data (150 ± 10 kDa) correspond to the molecular mass of the PNP hexamer (155 kDa).

The self-association model was also considered in data analysis with no improvement of the fits. Moreover, the parameters of the latter model also describe the hexameric form of the enzyme since the mass of associating species is equal to the mass of PNP monomer (~ 25 kDa), the stoichiometry of self-association is 6, and the equilibrium constant is very high ($\sim 10^{100} \text{ M}^{-5}$). These results indicate that PNP is a stable hexamer.

Figure 6. Sedimentation equilibrium experiment of *E. coli* PNP in 50 mM Tris-HCl buffer (pH 7.5) with 100 mM KCl and 0.1 mM DTT at 20°C. Centrifugation was performed at 10,200 rpm. The enzyme was loaded at 0.46 mg/mL (A), 1.08 mg/mL (B), and 1.7 mg/mL (C). Protein concentration gradient in equilibrium measured at 280 nm (shown as open circles at bottom) is shown together with the single-species model fit (line). (Top) Residuals ($A_{\text{exp}} - A_{\text{mod}}$) for this model (fitted simultaneously to all three data sets). Similar curves and residual plots were obtained for the self-association model (i.e., coexistence of monomers and N-mer oligomer). The following parameters for these two models were obtained: single-species model, $M = 150,746$ Da; self-association model, $M_{\text{monomer}} = 25,988$ Da, $N = 5.8$, $K_a = 4.0 \times 10^{87} \text{ M}^{-5}$.

Conclusion

The results of this work show unequivocally that irrespective of protein concentration, ionic strength, temperature, presence of substrates, or destabilizing factors, the only active form of *E. coli* PNP is a hexamer.

As mentioned in the introduction, all PNPs, both trimeric and hexameric, show complex kinetic characteristics, which suggests possible coexistence of different active oligomeric enzyme forms (e.g., Ropp and Traut 1991). The results of this study, together with the results of similar experiments conducted for trimeric calf spleen PNP (Behlke et al. 2005), disprove that hypothesis by showing that the only active enzyme forms are hexamer and trimer for the bacterial and the mammalian enzyme, respectively. We have previously outlined the molecular phenomena responsible for the complex kinetics observed for phosphorolytic reaction catalyzed by calf-spleen trimeric PNP (Bzowska 2002). These are (1) sequential but random, not ordered, binding of substrates; (2) unusually potent binding of some purine bases (e.g., hypoxanthine); and (3) a dual role of phosphate acting as a substrate but also as the reaction modifier that stimulates a release of the tightly bound purine base.

In the case of hexameric PNPs that structurally could be assembled as a trimer of dimers, complex kinetic characteristic arises from other molecular reasons. First, it is an interaction between monomers in a dimer that leads to negative cooperativity in the binding of ground state ligands—phosphate (Kierdaszuk et al. 1997) and nucleosides, the latter in the presence of phosphate (Koellner et al. 2002). Two conformations of active sites in every dimer are possible: an open one, with low substrate binding affinity, and a closed one, binding substrates tightly (Koellner et al. 2002). Moreover, the crystal structure has shown that two monomers in the catalytic 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. It seems that if one binding pocket is closed, it determines whether the remaining five may be (stably) closed or not. This mechanism underlies the significance of maintaining the hexameric structure in *E. coli* PNP. Indeed, the results described in this study show that the only active and stable form of this enzyme is a hexamer.

Materials and methods

HEPES, NaOH, KOH, NaCl, KCl, and Tris were from Fisher Chemicals; mono- and dibasic-sodium phosphates were from Research Organics. Inosine (Ino), N(7)-methylguanosine ($m^7\text{Guo}$), and xanthine oxidase from buttermilk (28 U/mg) were products of Sigma Chemical Co. 2-Amino-6-mercapto-7-methylpurine ribonucleoside (MESG) was obtained from Molecular

Probes as a part of EnzChek Phosphate Assay Kit. Guanidine chloride (GdnHCl) was purchased from ROTH.

Partially purified PNP from *E. coli* (~60% pure, about 60 U/mg), a gift of Dr. G. Koszalka (Wellcome Research Labs) was further purified to apparent homogeneity as described by Bzowska et al. (1998), concentrated, and stored at -80°C . Before the experiments, the sample of PNP was thawed, diluted, and dialyzed against an appropriate buffer. Protein concentrations were determined from absorbance measurements with $\epsilon^{1\%}(278) = 2.7$ (Bzowska et al. 1998).

Enzyme activity was monitored spectrophotometrically at $\sim 30^{\circ}\text{C}$ in 50 mM phosphate buffer (pH 7.0), by the coupled xanthine oxidase procedure with 0.5 mM Ino as substrate (Kalckar 1947) or by following the changes in absorption at 260 nm of m^7Guo as substrate (Kulikowska et al. 1986).

Ultraviolet absorption was monitored with a Shimadzu UV-VIS Recording Spectrophotometer (UV-2401PC) at room temperature. Circular dichroism (CD) spectra were measured with a Jasco J-720 spectrometer using a 0.01-cm cylindrical cell. Analytical ultracentrifugation was carried out using a Beckman Optima XL-I analytical ultracentrifuge with four-position AN-Ti rotor and UV absorption detection system.

Sedimentation velocity experiments

Protein solution (340 or 350 μL) and a reference buffer (350 or 380 μL) were loaded into the right and the left sector, respectively, of a double-sector 1.2-cm cell with an aluminum centerpiece and quartz windows. After equilibration at 3000 rpm at 20°C , the rotor was accelerated to a desired speed (40,000 or 60,000 rpm) and radial absorption scans of protein-concentration profile in the cell was measured (at 3- or 3.5-min intervals). Enzyme activity was not affected by centrifugation, as determined by activity measurements before and after experiments. The sedimentation velocity data were analyzed with a time-derivative method (Stafford 1992). Apparent sedimentation coefficient distributions $g(s_{20,w}^0)$ and sedimentation coefficient $s_{20,w}^0$ (as the maximum of $g(s_{20,w}^0)$) were calculated using the DCDT+ software (<http://www.jphilo.mailway.com/>). The program corrects observed sedimentation coefficients to values corresponding to the viscosity and density of water in the limit of a dilute protein solution. Molecular mass was calculated from Svedberg equation,

$M = \frac{s_{20,w}^0 RT}{D(1-\bar{v}\rho)}$, where $\bar{v}$ is partial specific volume of PNP and ρ is the density of sedimentation buffer. The diffusion coefficient (D) is related to the standard deviation (σ) of Gaussian functions fitted to sedimentation coefficient distributions $g(s_{20,w}^0)$ by the following relationship (Stafford 1997): $D = \frac{(\sigma r_m \omega^2 t)^2}{2t}$, where t is the sedimentation time, ω is the angular velocity of the rotor, and r_m the radius of the meniscus. Partial specific volume of PNP (from amino acid composition) as well as densities and viscosities of buffers were calculated using Sednterp program (<http://www.rasmb.bbri.org/rasmb/>).

Sedimentation equilibrium experiments

PNP solutions (110 or 120 μL) and a reference buffer (120 or 130 μL) were placed in 1.2-cm six-channel cells with quartz windows. The samples were equilibrated at a desired speed at 4° or 20°C for ~ 20 –60 h, and then the cells were scanned at 280 nm. Enzyme activity decreased no more than 15% during centrifugation, as determined by activity measurements before and after experiments. The experiments were performed simultaneously for three concentrations of PNP (~ 0.18 , ~ 0.36 , and

~ 0.73 mg/mL) in 50 mM HEPES-KOH (pH 7.0) with (0.24 and 10 mM) and without phosphate at 4°C (at 12,000 rpm) and 20°C (1 h of overspeed at 15,000–17,000 rpm, then at 8600–11,000 rpm for ~ 20 h). Additional experiments were carried out (at 7200, 10,200, and 12,000 rpm) in the same conditions as described by Nixon et al. (1998)—in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, and 0.1 mM DTT at 4° and 20°C for 0.46, 1.08, and 1.7 mg/mL PNP. The data were analyzed using the software supplied with instrument (Beckman) by fitting a single species model or a self-association model.

Active enzyme centrifugation

Active enzyme sedimentation experiments were carried out using a 1.2-cm cell with the Vinograd-type band-forming centerpiece and quartz windows. The sample and reference sectors were filled, respectively, with 350 and 400 μL solution containing 0.2 M NaCl and both substrates of the PNP catalyzed reaction—50 mM phosphate buffer (pH 7.0) and MESG (300 μM) or m^7Guo (150 or 300 μM). Concentrations of substrates were much higher than their K_m values, which is a necessary condition for the correct active enzyme centrifugation experiment. PNP solutions (20 μL) in 50 mM phosphate buffer (pH 7.0) were loaded into the sample well. Experiments were carried out for 20, 5, 2.5 and 1 $\mu\text{g/mL}$ protein concentrations and for both nucleoside substrates (MESG and m^7Guo). After equilibration at 3000 rpm at 20° or 25°C , the rotor was accelerated to 40,000 rpm, and movement of the enzyme band was followed by absorption changes of reaction mixture due to the appearance of products in place of substrates. The cells were scanned at 280 nm for m^7Guo and at 355 or 360 nm for MESG (in 1- or 1.75-min intervals). MESG is slightly unstable in alkaline solutions (Webb 1992), but no more than 20% of MESG molecules undergoes decomposition during the time necessary for the active enzyme centrifugation experiment (~ 4 –5 h).

For the comparison of the band and boundary sedimentation methods, an experiment with high PNP concentration (3.4 mg/mL) was performed. In this case, the enzyme was layered on a buffered solution without substrates (50 mM HEPES/KOH at pH 7.0 and 0.15 M KCl). After equilibration at 3000 rpm at 20°C , the rotor was accelerated to 40,000 rpm, and then the cells were scanned at 280 nm (in 3-min intervals).

Sedimentation coefficients (s) were calculated using the mid-point method (Kemper and Everse 1973) for determination of the location of the enzyme band center (r). The plot of the $\ln r$ versus time should yield a straight line with the slope equal to ω^2 . The sedimentation coefficients (s) obtained with this approach were corrected to the viscosity and density of water at 20°C , as follows: $s_{20,w} = s \frac{(1-\bar{v}\rho)_{20,w}}{(1-\bar{v}\rho)_{T,b}} \cdot \frac{\eta_{T,b}}{\eta_{20,w}}$. The index T,b designates the values of partial specific volume of PNP ($\bar{v}$), density (ρ), and viscosity (η) of buffer at experimental temperature; 20,w indicates standard conditions (water at 20°C).

For the high PNP concentration experiment (3.4 mg/mL), the maxima of recorded scans were used to determine the location of the center of the enzyme band, and then the sedimentation coefficient was calculated as described above.

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