

Oligomeric Structure of Mammalian Purine Nucleoside Phosphorylase in Solution Determined by Analytical Ultracentrifugation

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The influence of phosphate, ionic strength, temperature and enzyme concentration on the oligomeric structure of calf spleen purine nucleoside phosphorylase (PNP) in solution was studied by analytical ultracentrifugation methods. Sedimentation equilibrium analysis used to directly determine the enzyme molecular mass revealed a trimeric molecule with $M_r = (90.6 \pm 2.1)$ kDa, regardless the conditions investigated: protein concentration in the range 0.02–1.0 mg/ml, presence of up to 100 mM phosphate and up to 200 mM NaCl, temperature in the range 4–25 °C. The sedimentation coefficient (6.04 ± 0.02) S, together with the diffusion coefficient (6.15 ± 0.11) 10^{-7} cm²/s, both values obtained from the classic sedimentation velocity method at 1.0 mg/ml PNP concentration in 20 mM Hepes, pH 7.0, yielded a molecular mass of (90.2 ± 1.6) kDa as expected for the trimeric enzyme molecule. Moreover, as shown by active enzyme sedimentation, calf spleen PNP remained trimeric even at low protein concentrations (1 µg/ml). Hence in solution, similar like in the crystalline state, calf spleen PNP is a homotrimer and previous suggestions for dissociation of this enzyme into more active monomers, upon dilution of the enzyme or addition of phosphate, are incorrect.

Key words: Purine Nucleoside Phosphorylase (PNP), Oligomeric State, Analytical Ultracentrifugation

Introduction

Purine nucleoside phosphorylase (PNP, EC.2.4.2.1), an ubiquitous enzyme of the salvage pathway, catalyzes the reversible phosphorolysis of the glycosidic bonds of purine ribonucleosides and 2'-deoxyribonucleosides. PNPs can be divided into two classes, low molecular mass enzymes, which usually have a molecular mass of about 90 kDa, and high molecular mass class enzymes with molecular mass of about 150 kDa (see Bzowska *et al.*, 2000). Low molecular mass PNPs are specific for 6-oxopurine nucleosides [or rather N(1)-H purine nucleosides, according to recent findings (Bzowska *et al.*, 2004)] and are found both in higher organisms and in procaryotes, while high molecular mass enzymes are found mainly in lower organisms and have broader substrate specificity, accepting both 6-amino and 6-oxopurine nucleosides, and many analogues (Bzowska *et al.*, 2000).

PNPs from various organisms and inhibitors of some PNPs have broad potential medical and

practical applications. Phosphorylases from many sources are widely used in coupled assays for phosphate-liberating enzymes, while less specific *E. coli* PNP may be employed in the enzymatic synthesis of nucleosides (Bzowska *et al.*, 2000). The crucial role of PNP in T-cell proliferation has been demonstrated in patients with inherited PNP deficiency. Therefore human PNP is a target for inhibitor development. Such compounds should be useful in treating T-cell related autoimmune diseases and T-cell cancers (Bzowska *et al.*, 2000; Bantia *et al.*, 2002, 2003). The differences in substrate specificity between low and high molecular mass PNPs is profited from in anticancer suicide gene therapy in which nontoxic nucleoside prodrugs are cleaved in tumor cells transfected with the gene for *E. coli* to cytotoxic purine analogs (Bennett *et al.*, 2003). Selective inhibitors of PNPs from parasites that lack *de novo* synthesis of nucleosides (e.g. from *Plasmodium falciparum* and *Schistosoma mansoni* causing malaria and schistosomiasis, respectively) are considered potential

antiparasitic agents (Shi *et al.*, 2004; Freitas da Silveira *et al.*, 2004).

The crystal structures of PNPs from several sources have been determined revealing homotrimeric enzymes in the low molecular mass class (Bzowska *et al.*, 2000, 2004; Fedorov *et al.*, 2001). However, there was a suggestion in the literature (Ropp and Traut, 1991) for dissociation in solution of trimeric calf spleen phosphorylase into active monomers with specific activity 50-fold higher than that of the trimer. The monomeric state was reported to be triggered by either very low enzyme concentration or by the presence of high concentration of phosphate which is one of the substrates, hence expected to bind in the enzyme active site. However, in the case of triggering dissociation into monomers, phosphate was claimed to bind to a distinct regulatory binding site.

Such a dissociation of trimeric PNP into more active monomers, if confirmed, would account for the complex kinetic characteristic observed for phosphorylases (Bzowska *et al.*, 2000). However, crystal structures of calf spleen PNP complexed with various ligands, including phosphate, have not confirmed the presence of any additional site for binding of phosphate (Mao *et al.*, 1998). It was also shown that the specific activity of calf spleen PNP does not increase upon dilution of the enzyme, and for the enzyme in a concentration below 1 $\mu\text{g/ml}$ no monomers were detected in a filtration through membrane experiments (Bzowska, 2002). The present study was therefore undertaken to determine by classical analytical ultracentrifugation methods, most appropriate for this purpose, the influence of phosphate, ionic strength, temperature and enzyme concentration on the oligomeric structure of calf spleen PNP in solution. Experimental conditions were also extended to very low enzyme concentrations using the active enzyme sedimentation method introduced many years ago by Cohen *et al.* (1967). This approach enables the correlation of oligomeric state with specific activity for a highly diluted enzyme.

Materials and Methods

Materials

Calf spleen PNP (20–34 units/mg) and xanthine oxidase from buttermilk (~1 unit/mg) suspensions in 3.2 M and 2.3 M ammonium sulphate, respectively, inosine, 7-methylguanosine, and Hepes [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfo-

nic acid)] were purchased from Sigma. The commercial calf spleen PNP was desalted as described earlier (Bzowska, 2002). For each preparation the phosphate concentration was determined using a method involving phosphomolybdate complexes reduced by ascorbic acid (Ames, 1966) and the PNP concentration was measured spectrophotometrically and calculated using the enzyme extinction coefficient of 9.6 cm^{-1} at 280 nm for a 1% solution (Bzowska, 2002).

Analytical ultracentrifugation

Molecular mass studies of PNP were carried out in a XL-A type analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA) equipped with UV absorption optics. Three methods were employed: sedimentation velocity, sedimentation equilibrium and active enzyme sedimentation.

The sedimentation equilibrium was analyzed using externally loaded six-channel centerpieces with 12 mm pathlength, usually filled with 75 μl of solution. This type of cells allows the analysis of three solvent-solution pairs in one run. The sedimentation equilibrium was reached after 2 h overspeed at 22,000 rpm followed by an equilibrium speed of 18,000 rpm at 10 °C and 25 °C for 24–30 h. The radial absorbance of each compartment was scanned at three different wavelengths between 230 and 300 nm.

Molecular mass calculations were done by simultaneous fitting the sets of three radial absorbance distribution curves described by:

$$A_r = A_{r,m} \exp[MF(r-r_m)]$$

with

$$F = [(1 - \rho\nu)\omega^2]/2RT$$

using the program POLYMOLE (Behlke *et al.*, 1997). In these equations ρ is the solvent density, ν is the partial specific volume, ω is the angular velocity, R is the gas constant and T is the absolute temperature. A_r is the radial absorbance and $A_{r,m}$ is the corresponding value at the meniscus position. The partial specific volume $\nu = 0.736 \text{ ml/g}$ was calculated from the amino acid composition of the protein and the density increments of the individual amino acids.

Sedimentation velocity experiments were done in a standard double sector cell filled with about 300 μl solution and run at 50,000 rpm at 20 °C. From the time-dependent radial concentration profiles recorded either at 280 nm or at 285 nm,

sedimentation and diffusion coefficients were simultaneously determined using the program LAMM (Behlke and Ristau, 1997). The combination of both parameters with the partial specific volume (see above) allows to calculate the molecular mass from the Svedberg equation.

In addition to the conventional sedimentation velocity runs, active enzyme sedimentation experiments were carried out using the Vinograd-type double sector centerpiece (Kemper and Everse, 1973). A thin layer of PNP solution in 20 mM Hepes, pH 7.0 was applied on the solution of 200 μ M 7-methylguanosine (m^7 Guo) – a substrate of PNP – in 100 mM phosphate buffer, pH 7.0. The density of the substrate solution was increased by addition of 200 mM NaCl in order to form a sharp enzyme boundary on the top of substrate solution and to make sure that, before the desired ultracentrifugation speed was reached, the enzyme is present only at meniscus (Kemper and Everse, 1973). The phosphorolysis of m^7 Guo is almost nonreversible and the apparent K_m is about 13 μ M (Kulikowska *et al.*, 1986). The initial substrate concentration of 200 μ M, *i.e.* about 15 K_m , was used to fulfill the condition that every enzyme molecule in the band reacts with the substrate with the same velocity. The phosphorolysis was followed by the direct spectrophotometric assay based on differences in extinction coefficients between m^7 Guo and the corresponding purine base 7-methylguanine. The moving boundary of PNP through the solution of m^7 Guo was recorded at 239 nm ($\Delta\epsilon = +1300 \text{ M}^{-1} \text{ cm}^{-1}$) and 260 nm ($\Delta\epsilon = -4600 \text{ M}^{-1} \text{ cm}^{-1}$) (Kulikowska *et al.*, 1986) and used to obtain the sedimentation coefficients of PNP at very low enzyme concentrations (1–20 μ g/ml).

Results

Sedimentation equilibrium

The molecular mass of calf spleen PNP in 20 mM Hepes buffer, pH 7.0, was determined from the radial concentration distribution obtained at sedimentation equilibrium. These experiments covered the range of enzyme concentrations between 0.02 and 1.0 mg/ml (Fig. 1). The average molecular mass obtained, $(90.6 \pm 2.1) \text{ kDa}$, is about three-fold that of the monomer (32.093 kDa, Swiss Prot entry P55859) indicating that in the above concentration range PNP in solution is a trimeric molecule.

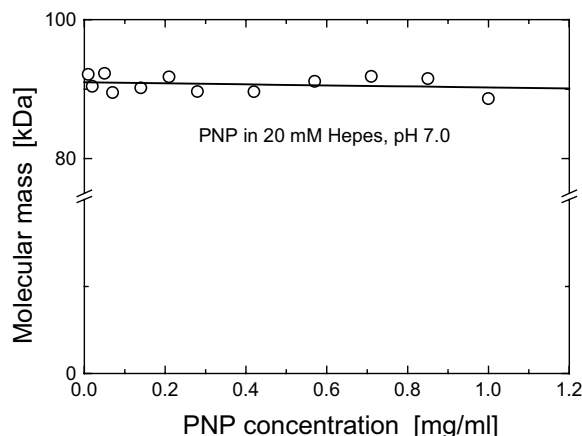


Fig. 1. Molecular mass of calf spleen PNP in 20 mM Hepes buffer at pH 7.0 and 10 °C obtained by the sedimentation equilibrium method at various enzyme concentrations.

Addition of up to 100 mM phosphate does not influence the molecular mass value significantly (Fig. 2) neither at 10 °C not at 25 °C. The stability of the enzyme is slightly lower when the phosphate concentration is raised up to 250 mM, the effect being more pronounced at 0.02 mg/ml enzyme concentration as compared with 0.5 mg/ml sample concentration (not shown), and also at 25 °C as compared with 10 °C (Fig. 2). However, there is no indication for complete dissociation of the trimeric molecule into monomers, even at 250 mM phosphate concentration. The maximum

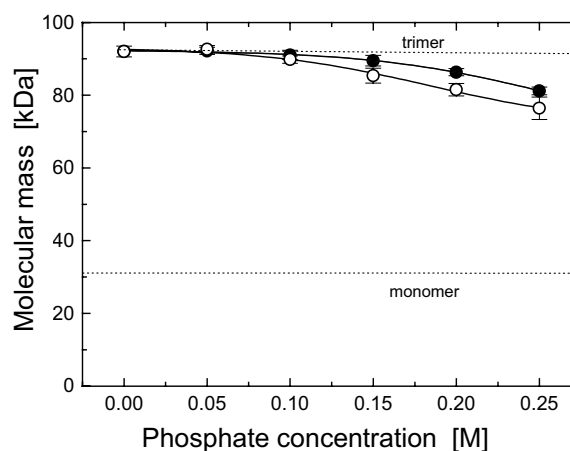


Fig. 2. Influence of phosphate on the molecular mass of calf spleen PNP (0.02 mg/ml) at 10 °C (●) and 25 °C (○) studied by the sedimentation equilibrium method.

fraction of monomers estimated from the data presented in Fig. 2 is about 20% (at 0.02 mg/ml and 25 °C). NaCl in a concentration up to 150 mM does not change the molecular mass of the enzyme (data not shown).

Sedimentation velocity

The sedimentation velocity method has been used to confirm the trimeric quaternary structure of the calf spleen PNP in solution. From the time-dependent moving boundaries at 50,000 rpm a sedimentation coefficient of (6.04 ± 0.02) S was obtained for PNP at 1.0 mg/ml concentration in 20 mM Hepes, pH 7.0 (Fig. 3). Using the program LAMM (Behlke and Ristau, 1997), the diffusion coefficient $(6.15 \pm 0.11) \cdot 10^{-7} \text{ cm}^2/\text{s}$ was determined, which together with the above sedimentation coefficient and partial specific volume of 0.736 ml/g yields a molecular mass of (90.2 ± 1.6) kDa as expected for the trimeric enzyme molecule.

Active enzyme sedimentation

In order to study the sedimentation behavior at very low PNP concentrations the analytical active enzyme sedimentation method was employed (Cohen *et al.*, 1967) which permits determination of hydrodynamic properties of very dilute enzyme samples ($\sim 1 \mu\text{g}/\text{ml}$). The method is based on an

enzymatic activity of a protein under study. The sedimentation of the enzyme through a solution containing all substrates necessary to observe the enzyme-catalyzed reaction is followed indirectly *via* observation of the change in absorption due to disappearance of substrates and formation of products. In our case m^7Guo and phosphate were used as substrates. From the moving enzyme boundary visualized by an absorption change due to the PNP catalyzed conversion of m^7Guo to 7-methylguanine a sedimentation coefficient of (6.1 ± 0.1) S was determined for $8.7 \mu\text{g}/\text{ml}$ PNP in 110 mM phosphate buffer, pH 7.0, with addition of 200 mM NaCl. Further dilution of the enzyme to about $1.0 \mu\text{g}/\text{ml}$, which is the lower possible concentration detected with this method, gave similar results. Hence, even in a very low concentration (and in the presence of phosphate) calf spleen PNP remains trimeric in solution.

Discussion

Sedimentation equilibrium and sedimentation velocity experiments (with the classic detection based on protein absorbance and with detection based on the enzyme activity) yield a molecular mass expected for the trimeric enzyme molecule. The presence of phosphate up to 100 mM and dilution of the enzyme even to $1 \mu\text{g}/\text{ml}$ does not affect the trimeric state of the protein. Hence, the suggestion of Ropp and Traut (1991) that calf spleen PNP dissociates into monomers with a specific activity much higher than that of the trimer, can not be confirmed by ultracentrifugation methods employed here, as could not be confirmed by previously employed specific activity measurements and filtration through membrane studies (Bzowska, 2002). X-Ray diffraction revealed homotrimeric enzyme in a crystal, with active sites located at interfaces of two monomers and neighbouring subunit contributing the side chain of Phe159 to the active site pocket (Mao *et al.*, 1998; Luić *et al.*, 2001). Hence, also on the basis of the active site geometry, dissociation of the enzyme into active monomers seems to be very unlikely. The final conclusion from the present and earlier studies involving various methods is that a trimer is a biologically active form of calf spleen PNP and that unusual catalytic properties of this enzyme are not caused by dissociation into monomers upon enzyme dilution or presence of phosphate molecules.

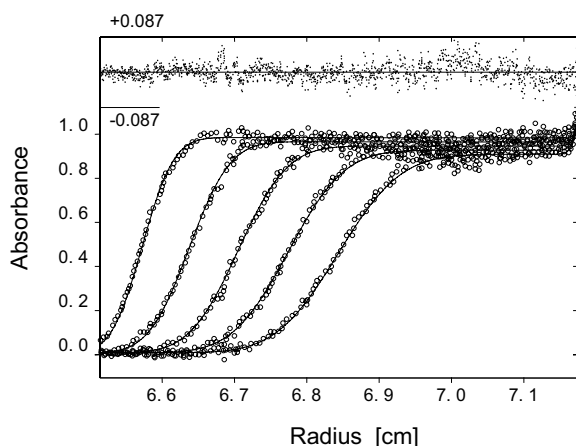


Fig. 3. Time-dependent moving boundaries (symbols \circ) of PNP ($c = 1 \text{ mg}/\text{ml}$, *i.e.* $31.2 \mu\text{M}$ as monomer) in 20 mM Hepes buffer, pH 7.0, at 20 °C. Radius is the radial distance from the center of rotation. The curves were fitted simultaneously to determine the sedimentation and diffusion coefficient using the program LAMM (Behlke and Ristau, 1997).

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