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Catalytic Properties of Monomeric Species of Brain Pyridoxine-5'-phosphate Oxidase

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The structural stability of brain pyrydoxine-5'-phosphate (PNP) oxidase and the catalytic properties of the monomeric species were investigated. The unfolding of brain pyridoxine-5'-phosphate (PNP) oxidase by guanidine hydrochloride (GuHCl) was monitored by means of fluorescence and circular dichroism spectroscopy. Reversible dissociation of the dimeric enzyme into subunits was attained by the addition of 2 M GuHCl. The perturbation of the secondary structure under the denaturation condition resulted in the release of the cofactor FMN. Separation of the processes of refolding and reassociation of the monomeric species was achieved by the immobilization method. Dimeric PNP oxidase was immobilized by the covalent attachment to Affi-gel 15 without any significant loss of its catalytic activity. Matrix-bound monomeric species were obtained from the reversible refolding processes. The matrix bound-monomer was found to be catalytically active, possessing only a slightly decreased specific activity when compared to the refolded dimeric enzyme. In addition, limited chymotrypsin digestion of the oxidase yields two fragments of 12 and 16 kDa with a concomitant increase of catalytic activity. The catalytically active fragment was isolated by ion exchange chromatography and analyzed for association of two subunits using the FPLC gel filtration analysis. The retention time indicated that the catalytic fragment of 16 kDa behaves as a compact monomer. Taken together, these results are consistent with the hypothesis that the native quaternary structure of PNP oxidase is not a prerequisite for catalytic function, but it could play a role in the regulation.

Keywords: Brain PNP-oxidase, Catalytic fragment, Immobilization method, Monomeric species, Reversible unfolding

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Introduction

Pyridoxine-5'-phosphate oxidase (PNP oxidase: EC 1.4.3.5) catalyzes the terminal step in the biosynthesis of pyridoxal-5'-phosphate (PLP): the oxidation of both pyridoxine-5'-phosphate (PNP) and pyridoxamine-5'-phosphate (PMP) to PLP. The enzyme activity was detected in rabbit liver (Kazarinoff and McCormick, 1975; Choi, *et al.*, 1983), pig brain (Churchich, 1984; Choi, *et al.*, 1987), yeast (Tsuge *et al.*, 1983) and bacteria (Henderson, 1965; Notheis *et al.*, 1995). The enzyme isolated from mammalian tissues is a dimmer composed of two identical subunits of about 28 kDa each. The flavin mononucleotide (FMN) group acts as a coenzyme and is absolutely required for catalytic activity (Wada and Snell, 1961). Kinetic studies published by Choi *et al.* (1983) established that the oxidase can function via either a binary or ternary complex mechanism, depending upon the nature of the substrate. Recently Ngo *et al.* (1998) reported that no PNP oxidase activity was detected in liver and neutrally derived tumor cells. This result suggested that tumor tissue utilizes a different pathway in the synthesis of PLP apart from the pathway utilized by normal tissues. The absence of oxidase activity, and its relationship to other metabolic processes occurring in abnormal cells, remains to be explained.

Since PNP oxidase contributes to the formation of PLP, it has been suggested that some regulatory mechanisms have to exist for the adequate formation of PLP in cells. Results from several laboratories have shown that the purified oxidase exhibits product inhibition and the Ki for the PLP range from 1 to 5 μM (Kwok and Churchich, 1980). Therefore, the inhibitory effect exerted by PLP may be one of the mechanisms contributing to the regulation of the concentration of cofactor in brain and liver tissue. Choi *et al.* (1987) demonstrated the modulation of a Schiffs base between the ε-amino group of a specific lysyl residue of the protein and the carbonyl group of PLP. Another plausible mechanism is that there are some regulatory molecules modulating the catalytic function of enzymes involved in the production of

PLP (Takeuchi *et al.*, 1985). We investigated the regulation of the catalytic activity of brain PNP oxidase by tryptophan metabolites (Kwon, *et al.*, 1991), and demonstrated that the enzyme is activated by the reversible binding of tryptophan metabolites to regulatory domain that are distinct from the catalytic domain.

Despite these studies on the mechanisms of catalysis and the regulation of the oxidase, relatively little information has been reported on the structure and the stability of the enzyme. Recently we have shown that the refolding process of PNP oxidase is accelerated by GroEL, and the structural rearrangements of the catalytic domain is the last step to take place in the process (Kwon and Churchich, 1999). In an attempt to further increase our understanding of the structural relation to the catalytic function, we decided to investigate catalytic properties of the monomeric species of the enzyme. In this present investigation, two different approaches, enzyme immobilization method and limited proteolysis, were used to demonstrate our hypothesis that the native quaternary structure of PNP oxidase is not a prerequisite for catalytic function, but it could play a role in the regulation.

Materials and Methods

Purification of PNP oxidase The method used in the purification of sheep brain PNP oxidase was based on the method originally applied to the purification of the enzyme from pig brain (Churchich, 1984). Enzymatic activity was measured at 25°C in 0.1 M Tris-HCl (pH 8.3) by monitoring the formation of pyridoxal-5'-P, which has an absorption maximum at 415 nm in the Tris buffer. The assay mixture consisted of pyridoxine-5'-P (20 μM) in a total volume of 1 ml.

Enzymatic Assays The enzymatic activity of PNP oxidase was measured at pH 8.4 in 0.1 M Tris-HCl (25°C). The rate of formation of PLP was measured by following the increase in absorbance at 414 nm. At this wavelength, the Schiff base formed between Tris and pyridoxal-5'-P has an extinction coefficient of 5900 $M^{-1}cm^{-1}$. Initial rate measurements were carried out by monitoring the change in absorbance at 415 nm for at least 3 min. A molecular weight of 56,000 for the PNP oxidase with a specific activity of 0.024 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ was used in the calculations of molar enzyme concentrations. One unit of specific activity is defined as the amount of protein that catalyzes the formation of 1 μmol of pyridoxal-5'-P/min at 25°C. Absorption was recorded in a Shimadzu UV 160 spectrophotometer. The concentration of enzyme was determined by the Bradford method.

Unfolding of PNP oxidase PNP-oxidase at a concentration of 2 mg/ml was treated with a denaturation buffer, 2 M guanidine hydrochloride (GuHCl) in a 10 mM potassium phosphate buffer (pH 7.4) for 30 min at 25°C. For refolding experiments, the denatured enzyme was diluted to a final concentration of 40 mM GuHCl with the renaturing buffer and incubated for 1 hr at 25°C. The renaturation buffer consisted of 10 mM potassium phosphate (pH 7.4) with 10 μM FMN. The refolding process was monitored by measuring the formation of PLP using either the fluorometric method, or the spectrophotometric method.

Circular Dichroism Spectra CD spectra were recorded on a Jasco (J-40A) spectropolarimeter using a cell of 0.2 cm path-length. The spectrometer was routinely calibrated with the asymmetric compounds (+) 10-camphorsulfonic acid (CSA). Protein concentrations ranged from 0.035 to 0.1 mg/ml and spectral data were acquired over the range 250-200 nm. Ellipticity [θ], expressed in terms of mean residue ellipticity, was calculated with the aid of the following equation:

$$[\theta] = MRW/10 \cdot l \cdot c$$

where MRW is the mean residue weight, l, path length, and c, concentration in g/ml.

Fluorescence Spectroscopy Emission spectra were recorded in a Perkin-Elmer LS-50B spectrofluorometer. The excitation and emission bandwidth were 5 nm. The spectra were recorded at a constant temperature (25°C). Care was exercised to prevent photochemical modification of FMN by exposing the samples to illumination for a short period of time (2 min).

Gel filtration Analysis HPLC was performed on an LKB instrument (2152 LKB, 2150 HPLC Pump). The column used in all HPLC analyses was a TSK 3000SW column (600 × 7.5 mm) with pre-column (100 mm × 7.5 mm). The mobile phase was 10 mM potassium phosphate buffer (pH 7.0) for the analysis of native proteins, and 10 mM phosphate buffer (pH 7.0) containing 2 M GuHCl for the analysis of dissociated PNP oxidase. The flow rate was 0.2 ml/min and the pressure was 1.1 Mpa. The calibration of molecular mass values of the fractions separated by the TSK 3000 column was achieved by the construction of standard curves using proteins of known molecular mass.

FPLC analysis was carried out at 4°C using a Superose 12 column (480 mm × 16mm) (Particle size 20-40 μm) at a flow rate of 0.2 ml/min in a Pharmacia instrument. The mobile phase was 50 mM potassium phosphate (pH 7.5). Bovine serum albumin (66 kDa), ovalbumin (45 kDa) and chymotrypsinogen (25 kDa) were used as standards of known molecular mass.

Preparation of immobilized PNP-oxidase Affi-gel 15 was washed with 0.1 M NaCl, water and the linking buffer (30 mM Kpi, pH 7). A 1 : 1 slurry of the gel was then prepared in a cold linking buffer containing 2 mg protein/ml activated gel. The mixture was then stirred slowly at 4°C for several hours. The gel slurry was washed with 0.5 M ethanolamine/HCl (pH 7) to block excess linkage sites. Non-covalently bound PNP-oxidase was removed by suspending the gel in 10 volume of a washing buffer, 30 mM potassium phosphate pH 7.4 and 0.5 M NaCl at 4°C. After stirring for several minutes, the gel was sediment by centrifugation at a low speed. The procedure was repeated several times until no protein was detected in the suspending buffer solution.

For preparation of matrix-bound monomeric species, dissociation of the matrix-bound dimer PNP oxidase was achieved by incubating the gel with the denaturation buffer. The resin slurry was stirred slowly for 1 hour at 4°C, and then the dissociated subunit was removed by washing with the same denaturation

buffer several times. The matrix-bound monomer was then allowed to reconstitute by using the same washing procedure as used in the immobilization described previously, except the washing buffer contained an additional 10 μM FMN. The denaturant was removed simultaneously in this step. The gel was stored in a minimum volume of the same buffer at 4°C. Caution was exercised to ensure a low density of linkage sites in order to facilitate attachment of the enzyme through a single subunit.

Chymotryptic Digestion and Isolation of Active Fragment Pyridoxine-5'-P oxidase (2 mg/ml) was incubated with chymotrypsin (0.1 mg/ml) in 100 mM sodium phosphate (pH 7) at 25°C for 1 hour. Aliquots withdrawn from the incubation mixture at several time intervals were allowed to react with the trypsin inhibitor (0.2 mg/ml) and used for enzymatic assays and SDS-polyacrylamide gel electrophoresis.

For ion-exchange chromatography, a TSK DEAE-5pw column fitted to an HPLC instrument (Phamacia LKB Biotechnology, Inc.) was used for the purification of the peptide obtained after chymotrypsin digestion of the PNP oxidase. The peptide applied to the column were eluted using a linear gradient from the 0 to 100% solvent system B (solvent system A: 5 mM sodium phosphate (pH 7.4); solvent system B: 0.2 M NaCl in solvent system A) at 25°C. Fraction collected from the column were monitored at 280 nm, assayed for catalytic activity, and used for determination of the FMN content by means of fluorescence spectroscopy in the presence of 2 M GuHCl. Excitation was at 450 nm, and emission was at 530 nm.

Materials Sephadex G-100 and AH-Sepharose 4 B were purchased from Pharmacia LKB Biotechnology Inc. Protein standards for electrophoresis experiments were obtained from Bethesda Research Laboratories. Pyridoxine-5'-P was prepared by reduction of pyridoxal-5'-P with $NaBH_4$.

Results

Stability of PNP-oxidase Denaturation of PNP oxidase by GuHCl was monitored by fluorescence spectroscopy. Binding of FMN to the oxidase results in a complete quenching of the fluorescence emitted by FMN over the spectral range 450-550 nm. Upon addition of GuHCl to a final concentration of 2 M, the fluorescence of FMN is dramatically increased, indicating that unfolding of the protein brings about release of the cofactor (Fig. 1). These results are interpreted to mean that the microenvironment surrounding the cofactor at the catalytic site is perturbed under the condition. Therefore, fluorophore FMN can be used as a probe of conformational changes at the catalytic site induced by a function of denaturant concentration. The fluorescence emission of the cofactor FMN was fully recovered from the quenching, which suggests that the active site is exposed to the solvent upon unfolding. Released FMN resulting from the denaturation was also characterized by a shift in the band position of the absorption spectrum. The absorption peaks of bound FMN are centered at around 372 and 445 nm, whereas the absorption bands of free FMN show maximums at 384 and 448 nm.

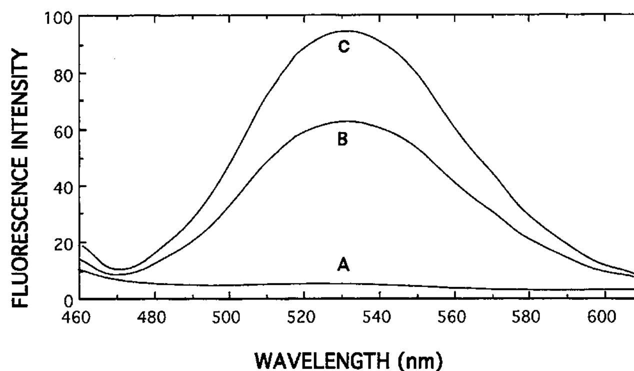

Fig. 1. Fluorescence emission spectra of the PNP oxidase in the absence and presence of GuHCl. Emission spectra of the PNP oxidase were recorded in the absence (A) and presence of 1 M (B) and 2 M GuHCl (C). The excitation wavelength 380 nm.

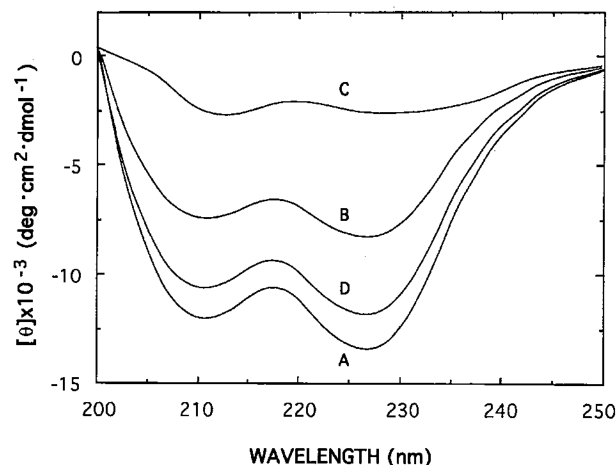

Fig. 2. Circular dichroism of the PNP oxidase in the absence and presence of GuHCl. Circular dichroism spectra of the PNP oxidase (0.1 mg/ml) recorded in 20 mM Kpi (pH 7), in the absence (A) and presence of 1 M (B) and 2 M GuHCl (C). CD spectrum (D) recorded after dilution of 2 M GuHCl-treated enzyme with the phosphate buffer. The final concentration of GuHCl is about 40 mM. All the measurements were performed after incubation of the protein with the denaturant or the diluent for 30 min at 25°C.

The effect of GuHCl on the secondary structural changes of the PNP oxidase was investigated by means of CD spectroscopy at 220 nm, a measure of the α-helix content of proteins. Figure 2 shows the circular dichroism spectra of the enzyme (100 μg/ml) recorded in the absence or presence of different concentrations of GuHCl. The secondary structure of the PNP oxidase was drastically changed in the presence of 2 M GuHCl. All measurements were performed after incubation of the protein for 30 min at 25°C. The results included in Fig. 2 show that some residual dichroism was still observed in the presence of 2 M denaturant. The ellipticity at 220 was apparently not further decreased as the GuHCl

concentration changed to 3 M. Renaturation studies were performed by diluting the denaturant to investigate reversibility of the process. Dilution of the GuHCl-treated sample with a 10 mM potassium phosphate buffer (pH 7.4) resulted in recovery of a good deal of the secondary structure, as demonstrated by the CD spectrum (Fig. 2, D) recorded 30 min after the 50 fold dilution of the sample with the buffer. This time of incubation was sufficient to achieve equilibrium. Longer incubation times (i.e. 1 hour) did not induce further changes in the CD spectrum.

Assuming a two-state model for the equilibrium foldedunfolded species, the equilibrium constant (Ke) is related to the fraction of unfolded species, represented by the following equations.

$$Ke = \frac{\alpha}{1-\alpha} = e^{-\Delta G/RT}$$

where α is determined from the mean residue ellipticity measured at 220 nm, and $[\theta]_N$ and $[\theta]_U$ are the ellipticities for the native, and unfolded conformations, respectively.

$$\alpha = \frac{[\theta]_N - [\theta]_{obs}}{[\theta]_N - [\theta]_U}$$

Since the free energy of unfolding was found to vary linearly with the GuHCl concentration, $\Delta G_{H2O} = 4$ kcal/mol was determined by extrapolating to zero the denaturing agent concentration (Kuwajima, 1995). The mid-point concentration ($G_{1/2}$) was about 1.1 M.

From the CD measurements alone, however, it is virtually impossible to determine whether or not the denaturing agent has induced dissociation of the dimeric structure. Therefore, it was necessary to use a different physical technique to access the state of association of denatured enzyme. HPLC gel filtration proved to be a suitable technique for monitoring the state of aggregation of the PNP oxidase in the presence of GuHCl. As shown by the elution patterns obtained from gel filtration experiments (Fig 3), the elution profile of the PNP oxidase (0.4 mg/ml) exhibits a symmetrical peak in the absence of GuHCl that can be attributed to species of 56 kDa. Upon dilution of the protein, the elution profile remains invariant. Thus, dimeric species exist in solution at protein concentrations below 1 μM. In contrast to the behavior of native enzyme, a significant change in the elution profile was detected in the presence of 2 M GuHCl. The retention time of the major peak increased markedly, which was interpreted to mean that the apparent molecular weight of the protein decreased from about 56 kDa to 28 kDa. The molecular weights were interpolated from the retention times of standard proteins. When the unfolded protein, exposed to 2 M GuHCl for 30 min, was applied to the gel filtration column and eluted with a 10 mM potassium phosphate buffer, it was found that its profile is practically indistinguishable from that of native oxidase. This result indicated that the dimerization process occurs with the refolding of the domain structure upon dilution of the denaturant.

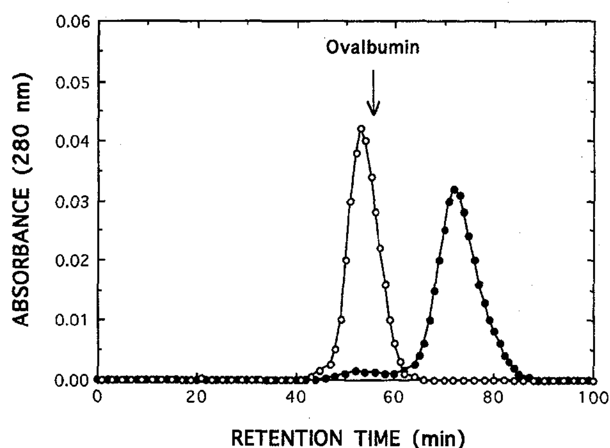

Fig. 3. Elution profile of the PNP oxidase in the presence of 2 M GuHCl. The PNP oxidase (0.1 mg/ml) was chromatographed by a LKB HPLC system fitted with a TSK 3000SW column (600 × 7.5 mm). The flow rate was set at 0.2 ml/min with a pressure of 1.1 MPa. The elution profiles of the PNP oxidase in the absence (○) and presence of 2 M GuHCl (●) are given, respectively. Ovalbumin (45 kDa) was used as a standard for molecular mass calibration, and the retention time indicated.

Conclusively, these denaturation studies showed that the compact dimeric structure of PNP oxidase is reversibly dissociated into monomers by treatment of 2 M GuHCl.

Catalytic properties of monomeric PNP oxidase The preceding results provided no information concerning the catalytic activity of monomeric species of the PNP oxidase. Denatured PNP oxidase exposed to 2 M GuHCl recovers its catalytic properties upon dilution, provided the cofactor FMN is added to the protein prior to the enzymatic assays. The separation of the dissociated monomer by gel filtration chromatography is feasible when the denaturing agent is present in the eluting solutions. As soon as GuHCl is removed by dialysis, however, the monomeric species tend to reassociate and stable dimmers are formed. Separation of the processes of refolding and reassociation could be achieved by attaching the protein subunits to a rigid matrix, which would allow refolding but prevent reassociation. This method has been extensively used to investigate the catalytic properties of oligomeric proteins immobilized on CNBr-activated sepharose (Wood, *et al.*, 1981; Kwok, *et al.*, 1987).

In the present work Affi-gel 15 was chosen as the matrix to which the PNP oxidase was covalently coupled. Experimental conditions for immobilization of the protein were specially designed to ensure attachment of the protein via one subunit only, as opposed to the attachment of both subunits. The enzymatic activity of immobilized enzyme is similar to that of native enzyme. The observed specific activity of the immobilized PNP oxidase was similar to the native enzyme activity, which suggested that the immobilization does not seem to cause a significant perturbation of the native structure.

The amount of protein bound to the gel was approximately 90% of the initial value. As outlined in "Experimental procedures", the immobilized enzyme was extensively washed with 2 M GuHCl to ensure the release of monomeric species that are not covalently attached to the matrix. Typically, the amount of protein bound to the matrix was about 50-55% of the matrix bound dimeric PNP oxidase. Thus, the dimeric enzyme was apparently dissociated, yielding matrix bound monomeric species as expected. After treatment with a renaturation buffer containing 10 μM FMN, the immobilized monomeric enzyme was assayed using PNP as a substrate at pH 8.3. The reconstituted dimeric enzyme, prepared by dialysis of the 2 M GuHCl-treated enzyme, was used as a control in the assays. The results of the enzymatic measurements are compiled in Table 1. Activity levels of the immobilized monomeric enzyme were restored to more than 90% of the values for the reconstituted dimeric enzyme in solution. This is an indication that the structural stability of the matrix bound monomer was similar to that of the native enzyme. The matrix bound monomeric enzyme was found to be quite stable when stored in a minimum volume of buffer, 30 mM potassium phosphate, pH 7.4, at 4°C. Little or no loss of activity occurred with storage times of 3-4 weeks. In conclusion, these results lend strong support to our hypothesis that monomeric PNP oxidase is catalytically competent.

Chymotrypsin digested PNP oxidase Limited chymotryptic cleavage of pyridoxine-5'-P oxidase provided polypeptides endowed with catalytic activity (Kim and Churchich, 1989; Kwon *et al.*, 1991). In this study, this method was adopted to investigate the presence of low molecular weight species possessing catalytic activity. The enzyme (2 mg/ml) was incubated with chymotrypsin (0.1 mg/ml) in 0.1 M sodium phosphate (pH 7) at 25°C. After a 50 min incubation at 25°C, the sample (0.2 mg) was applied to a TSK-DEAE 5pw column and eluted with a linear gradient from 0 to 100% solvent system B at a flow rate of 0.1 mg/min. The separation procedure was repeated several times. The fractions endowed with catalytic activity were eluted at the end of the chromatographic pattern with a retention time of approximately 200 min. These fractions were pooled, desalted, concentrated, and subjected to a second chromatography using the same linear gradient system. The purity and the size of separated fragment were analyzed by SDS-PAGE, and it revealed a molecular mass of approximately 16 kDa, as described in a previous paper (Kwon *et al.*, 1991)

The purified fragments were applied to a gel filtration column fitted to a FPLC instrument. As shown in Fig. 4, the catalytic fragment eluted from the size exclusion chromatography displays a longer retention time than that of chymotrypsinogen (25 kDa). This result indicated that the purified fragment obtained after chymotrypsin digestion is a compact monomer, and it is catalytically active without significant changes of catalytic parameters, as shown in Table 2. It should be noted that the emission spectra of the catalytically active fragment of 16 kDa exhibits the characteristic features of the cofactor FMN when excited at 450 nm.

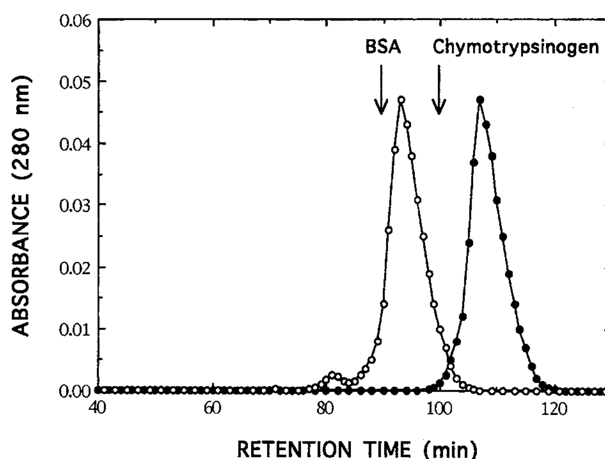

Fig. 4. FPLC elution profile of the active fragment of the PNP oxidase. After chymotryptic the digested PNP oxidase sample was separated using TSK DEAE-5pw column chromatography, the purified active fragment (0.2 mg/ml) was applied on a Superose 12 column (300 × 10 mm) attached to FPLC at a flow rate of 0.2 ml/min at 4°C (●). The elution profile of the native PNP oxidase is included (○). Standards of known molecular mass were chromatographed under similar conditions. The arrows indicate the retention times of bovine serum albumin (67 kDa) and chymotrypsinogen (25 kDa).

Discussion

The PNP oxidase isolated from brain tissues is a dimeric protein made up of two equal size subunits, but the structural requirement of the enzyme in catalysis remains to be elucidated. In this regard, it is of prime importance to elucidate the function of the two subunits in catalysis. The major question posed by the present study concerns whether or not the enzyme is able to function catalytically as a monomer.

The recovery of the oligomeric structure of an enzyme from the denatured state involves at least two steps; i.e., refolding of the individual polypeptide chains and their subsequent reassociations. However, the separation of a compact folded monomer intermediate is not easily obtained. Moreover, studies of the equilibrium properties in the dimer-monomer transition range are not suitable for answering the question if isolated monomers of the oligomeric protein possess the biological function. Therefore, the immobilization method was adopted in this study to obtain the monomeric species by means of a separation of the processes of refolding and reassociation. Recently, we investigated the unfolding processes of the PNP oxidase in the presence of GuHCl. We demonstrated that the enzyme dissociates reversibly into monomer, and the rate limiting step in the refolding process

Table 1. Activities of refolded PNP oxidase

	Activity (ΔA_{414}/mg)	
	5 min	*10 min*
Control (reconstituted dimmer)	0.367	0.684
Matrix-monomer	0.340	0.625

The enzymatic activities were measured by the increase in absorbance at 414 nm corresponding to the formation of PLP at a given incubation time. Control represents the reassociated dimeric enzyme prepared by dialysis of the 2 M GuHCl-treated enzyme; monomeric species indicates the refolded enzyme immobilized on the affi-gel 15 matrix.

Table 2. Catalytic parameters of the FMN-fragment of PNP oxidase

	specific activity (µmol/min/mg)	Km (µM)	size (kDa)	aggregation state
Native	0.024	10	58	dimer
FMN-fragment	0.076	15	16	monomer

involves a structural rearrangement of the dimeric structure at the level of the FMN binding domain (Kwon and Churchich, 1999). The study focused on the stability and spontaneous refolding of the dimeric enzyme. The catalytic function of the refolded monomeric enzyme has not been investigated.

The immobilized subunits of the PNP oxidase was found to have a similar catalytic activity as the reconstituted dimeric enzyme. Thus, it is apparent that subunit interactions are not required for enzymatic activity, but it could play a role in the regulation. In this connection, it is noteworthy from a previous observation that regulatory molecules, such as tryptophan metabolites capable of modulating the catalytic function of the PNP oxidase, recognize structural domains of the enzyme that are distinct from the catalytic domain (Kwon *et al.*, 1991). The binding of effector molecules to the PNP oxidase could induce conformational changes in the dimeric structure that are transmitted through the subunit interface to the structural domain bearing the cofactor FMN.

Investigation of the properties of chymotrypsin digested fragment of the PNP oxidase provided a further support of our hypothesis: the dimeric structure of the PNP oxidase is not essential for catalytic activity. The result, based on a SDS-PAGE analysis, showed that the limited chymotryptic digestion of the oxidase yields two major polypeptide fragments of 16 and 12 kDa. Interestingly, this proteolysis resulted in enhancements of the catalytic power of the enzyme (Vmax/Km), as shown in Table 2. This could be directly correlated with a concomitant decrease in the inhibition exerted by product pyridoxal-5'-P, or substrate pyridoxine-5'-P (Kim and Churchich, 1989; Kwon *et al.*, 1991). In the present studies, we demonstrated that the catalytic fragment of 16 kDa does exist as a monomer. This result suggests that the other structural domains of 12 kDa must be responsible for the association of two subunits, as well as the binding of regulatory molecules. It is postulated, therefore, that the PNP oxidase consists of two structural domains, each of which imparts a particular function to the protein. In this connection, it should be noted that the chymotryptic digested enzyme was no longer susceptible to activation by effecter molecules tryptophan metabolites (Kwon *et al.*, 1991). Similar effects of limited proteolysis on the catalytic function of an enzyme have been reported. Tarelli *et al.* (1990) showed that a stable fragment obtained from limited tryptic digestion of the D-amino acid oxidase retains full catalytic activity without changes in kinetic parameters. In the case of aspartase, tryptic digestion resulted in a marked increase in enzymatic activity, which may be mediated by a conformational change away from the active site of the subunit (Lee *et al.*, 1999).

Another possible interpretation of the current observation is that removal of the regulatory domains leads to a major reorientation of the inter-subunit domains in the active fragment, which prevents the association. However, it is most likely that the catalytic fragment of the PNP oxidase conserves the native structure since it retains full catalytic activity with unaltered kinetic parameters. It is generally accepted that a structural domain of a protein retains the potential for generating the correct tertiary structure. This is consistent with an idea that protein may fold by parts followed by merging of domains. Therefore, the role of domains as folding units in brain PNP oxidase could be investigated by comparing the unfolding-folding transition of the native protein with those of the isolated domains. The isolated domain, generated by a limited proteolysis, or by a genetic engineering method, is expected to be a useful tool for the studies of the folding process as well as structural and functional studies of a protein. The elegant work of Missiakas *et al.* (1990) has shown that the two isolated domains of the phosphoglycerate kinase are able to refold in a fully reversible process like the uncleaved enzyme. Using a similar experimental approach, the role of domains as folding units in the PNP oxidase is being investigated in our laboratory.

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References

Chan, W. W. and Mosbach, K. (1976) Effects of subunit interactions on the activity of lactate dehydrogenase studied in immobilized enzyme systems. *Biochemistry* **15**, 4215-4222.

Chan, W. W., Schutt, H. and Brandt, K. (1973) Active subunits of transaldolase bound to sepharose *Eur. J. Biochem.* **40**, 533-541.

Choi, J. D., Bowers-Komro, D. M., Davis, M. D., Edmondson, D. E. and McCormick, D. B. (1983) Kinetic properties of pyridoxamine (pyridoxine)-5'-phosphate oxidase from rabbit liver, *J. Biol. Chem.* **258**, 840-845.

Choi, S. Y., Churchich, J. E., Zaiden, E. and Kwok. F. (1987) Brain pyridoxine-5'-phosphate oxidase: modulation of its

catalytic activity by reaction with pyridoxal 5'-phosphate and analogs. *J. Biol. Chem.* **262,** 12013-12017.

Churchich, J. E. (1984) Brain pyridoxine-5'-phosphate oxidase: a dimeric enzyme containing one FMN site, *Eur. J. Biochem.* **138,** 327-332.

Henderson, H. M. (1965) Growth of Escherichia coli NCTC 5928 in relation to enzymes in vitamin B6 metabolism. *Nature* **207**, 195-196.

Kazarinoff, M. N. and McCormick, D. B. (1975) Rabbit liver pyridoxamine (pyridoxine) 5'-phosphate oxidase, *J. Biol. Chem.* **250**, 3436-3442.

Kim, Y. T. and Churchich, J. E. (1989) Activation of a Flavoprotein by proteolysis. *J. Biol. Chem.* **264**, 15751-15753.

Kuwajima, K. (1995) Circular dichroism. *Methods. Mol. Biol.* **40**, 1041-1052.

Kwok, F. and Churchich, J. E. (1980) Interaction between pyridoxal kinase and pyridoxine-5'-P oxidase. *J. Biol. Chem.* **255**, 882-887.

Kwok, F., Scholz, G. and Churchich, J. E. (1987) Brain pyridoxal kinase dissociation of the dimeric structure and catalytic activity of the monomeric species. *Eur. J. Biochem.* **168**, 577-583.

Kwon, O. S. and Churchich, J. E. (1999) Refolding of pyridoxine-5'-P oxidase assisted by GroEL. *Biochimie* **81**, 1057-1064.

Kwon, O. S. Kwok, F. and Churchich, J. E. (1991) Catalytic and regulatory properties of native and chymotrypsin treated pyridoxine-5'-phosphate oxidase. *J. Biol. Chem.* **266**, 22136-22140.

Lee, M. S., Choi, K. J., Kwon, S. J., Kang, I. S., Ha, J. H., Kim, S. S., Han, M. S. and Yoon, M. Y. (1999) Properties of trypsin-mediated activation of aspartase from *Hafnia alvei. J. Biochem. Mol. Biol.* **32**, 573-578.

Merrill, A. H., Kazarinoff, M. N., Tsuge, H., Horiike, K. and McCormick, D. B. (1979) Pyridoxamine (pyridoxine)-5'-P oxidase from rabbit liver, *Methods Enzymol.* **62**, 568-574.

Ngo, E. O., LePage, G. R., Thanassi, J. W., Meisler, N. and Nutter, L. M. (1998) Absence of pyridoxine-5'-phosphate oxidase (PNPO) activity in neoplastic cells: isolation, characterization, and expression of PNPO cDNA. *Biochemistry* **37**, 7741-7748.

Notheis, C., Drewke, C. and Leistner, E. (1995) Purification and characterization of the pyridoxol-5'-phosphate: oxygen oxidoreductase (deaminating) from *Escherichia coli. Biochim. Biophys. Acta* **1247**, 265-271.

Takeuchi, F., Tsubouchi R. and Shibata, Y. (1985) Effect of tryptophan metabolites on the activities of rat liver pyridoxal kinase and pyridoxamine 5'-phosphate oxidase *in vitro*. *Biochem. J.* **227**, 537-544.

Tarelli, G. T., Vanoni, M. A., Negri, A. and Curti, B. (1990) Characterization of a fully active N-terminal 37 kDa polypeptide obtained by limited tryptic cleavage of pig kidney D-amino acid oxidase. *J. Biol. Chem.* **265**, 21242-21246.

Tsuge, H., Itoh, K., Akatsuka, F., Okada, T. and Ohashi, K. (1983) Inactivation of pyridoxamine-5'-P oxidase by aliphatic primary amines, *Biochem. Int.* **6**, 743-749.

Wada, H. and Snell, E. E. (1961) The enzymatic oxidation of pyridoxine and pyrioxamine phosphates, *J. Biol. Chem.* **236**, 2089-2095.

Wood, D. C., Jurgensen, S. R., Geesin, J. S. and Harrison, J. H. (1981) Subunit interactions in mitochondrial malate dehydrogenase: kinetics and mechanism of reassociation. *J. Biol. Chem.* **256**, 2377-2382.