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^{31}P NMR studies of *O*-acetylserine sulfhydrylase-B from *Salmonella typhimurium* ☆

Rong Guan [a], Susan A. Nimmo [a], Klaus D. Schnackerz [b], Paul F. Cook [a,*]

[a] Department of Chemistry and Biochemistry, University of Oklahoma, 620 Parrington Oval, Norman, OK 73019, USA
[b] Theodor-Boveri-Institut für Biowissenschaften, Physiologische Chemie I, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany

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ABSTRACT

O-Acetylserine sulfhydrylase (OASS) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the conversion of *O*-acetylserine and bisulfide to L-cysteine and acetate in bacteria and higher plants. Enteric bacteria have two isozymes of OASS, A and B, produced under aerobic and anaerobic growth conditions, respectively, with different substrate specificities. The ^{31}P chemical shift of the internal and external Schiff bases of PLP in OASS-B are further downfield compared to OASS-A, suggesting a tighter binding of the cofactor in the B-isozyme. The chemical shift of the internal Schiff base (ISB) of OASS-B is 6.2 ppm, the highest value reported for the ISB of a PLP-dependent enzyme. Considering the similarity in the binding sites of the PLP cofactor for both isozymes, torsional strain of the C5–C5′ bond (O4′–C5′–C5–C4) of the Schiff base is proposed to contribute to the further downfield shift. The chemical shift of the lanthionine external Schiff base (ESB) of OASS-B is 6.0 ppm, upfield from that of unliganded OASS-B, while that of serine ESB is 6.3 ppm. Changes in chemical shift suggest the torsional strain of PLP changes as the reaction proceeds.

The apoenzyme of OASS-B was prepared using hydroxylamine as the resolving reagent. Apoenzyme was reconstituted to holoenzyme by addition of PLP. Reconstitution is pseudo-first order and exhibits a final maximum recovery of 81.4%. The apoenzyme shows no visible absorbance, while the reconstituted enzyme has a UV–visible spectrum that is nearly identical to that of the holoenzyme. Steady-state fluorescence spectra gave tryptophan emission of the apoenzyme that is 3.3-fold higher than the emission of either the native or reconstituted enzyme, suggesting that PLP is a potent quencher of tryptophan emission.

Introduction

O-Acetylserine sulfhydrylase (OASS)[1] is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the final reaction of the cysteine biosynthetic pathway in bacteria and plants, the conversion of *O*-acetylserine (OAS) and bisulfide to L-cysteine and acetate [2]. In enteric bacteria, there are two isoenzymes of OASS, A and B. The B-isozyme of *O*-acetylserine sulfhydrylase (OASS-B) is expressed in *Salmonella typhimurium* under anaerobic growth conditions [3] and it appears to be less substrate selective than the A-isozyme for both the amino acid and nucleophilic substrates [4]. OASS-B adheres to a Bi Bi ping-pong kinetic mechanism on the basis of initial velocity studies [4]. The proposed chemical mechanism of OASS-B is similar to that observed for the A-isozyme [5]. The substrate, OAS, binds with its α-amine unprotonated to facilitate a nucleophilic attack on C4′ of the internal Schiff base (ISB) to generate the OAS external Schiff base (ESB). The lysine (K41) that participates in ISB linkage, acts as a general base to deprotonate C_α in the β-elimination reaction, giving the α-aminoacrylate ESB and acetate. The B-isozyme has a turn-over number 12.5-fold higher than the A-isozyme and an approximately 10-fold lower value of K_{OAS} [4,6].

The three-dimensional structures of OASS-A and -B from *S. typhimurium* have been solved to 2.2 and 2.3 Å, respectively, in the absence of ligands as an open form [7]. As expected, these two homodimeric structures exhibit an overall fold very similar to each other, given the 40% identity in amino acid sequences of A- and B-isozyme [6,8]. The main difference between the two isozymes is the more hydrophilic active site of the B-isozyme with two ionizable residues, C280 and D281, replacing residues P299 and S300, respectively, in the A-isozyme [6], Fig. 1. Aspartate-281 is above the *re* face of the cofactor and is within hydrogen-bonding distance of Y286, while C280 is located 3.4 Å from the pyridine nitrogen of the internal Schiff base. The higher temperature factors of OASS-B relative to OASS-A, also suggests OASS-B has a higher degree of conformational flexibility along the reaction pathway,

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* Corresponding author. Fax: +1 405 325 7182.
E-mail address: pcook@ou.edu (P.F. Cook).

[1] *Abbreviations used:* OASS, *O*-acetylserine sulfhydrylase; OAS, *O*-acetyl-L-serine; OASS-B, B-isozyme of *O*-acetylserine sulfhydrylase; ISB, internal Schiff base; ESB, external Schiff base; PLP, pyridoxal 5′-phosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); DTT, dithiothreitol; TNB, 5-thio-2-nitrobenzoate; D_2O, deuterium oxide.

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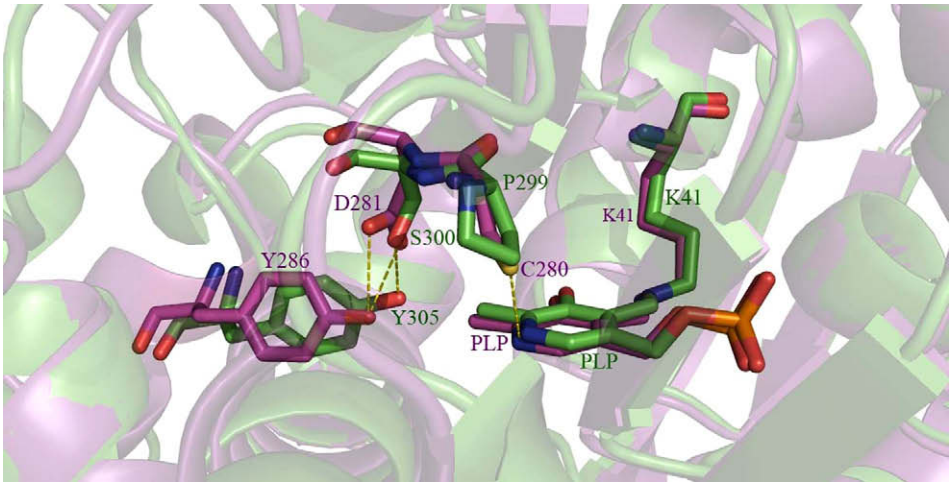

Fig. 1. Superposition of the active sites of OASS-A (green) and OASS-B (purple). The dashed lines represent hydrogen bonds. This figure was created using Pymol from DeLano Scientific LLC. The structures used in this figure have accession number of 1OAS for OASS-A and 2JC3 for OASS-B in the Protein Data Bank. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

consistent with the faster rate of B-isozyme compared to the A-isozyme [6].

The PLP cofactor provides a valuable probe of protein dynamics and catalysis for PLP-dependent enzymes, due to its localization at the enzyme active site and the sensitivity to changes in enzyme structure along the reaction pathway. Considering the tight link between electronic and conformational states, it is not surprising that ^{31}P NMR methods have been exploited for PLP-dependent enzyme to reveal the conformational landscape of catalytic actions by focusing on the binding modes of the phosphate group on PLP [9–14].

Resolution of the PLP cofactor and its reconstitution with the apoenzyme has been used to study the binding site of PLP for several PLP-dependent enzymes, including the A-isozyme of OASS [15–19]. Preparation of apo OASS-A by resolution of PLP was shown to be crucial for the studies of enzyme stability and dynamics [20–23]. A method for resolving PLP from the active site of OASS-B would thus be useful. The method used for OASS-A (5 M guanidinium hydrochloride in the presence of OAS) [19] was tried first for OASS-B on the basis of the structural similarity of the two isozymes. However, OASS-B is not as stable as OASS-A in the presence of guanidinium hydrochloride and was irreversibly denatured, even at lower concentrations than were used for OASS-A. As a result, other methods were tested in order to identify an effective resolution of PLP from OASS-B.

In this paper, ^{31}P NMR studies of OASS-B were carried out to probe conformational dynamics of OASS-B. Data are discussed in terms of the overall mechanism of the enzyme in comparison to the A-isozyme. In addition, we report a method for resolution of PLP from OASS-B, using hydroxylamine as the resolving reagent. The properties of the apo OASS-B and its reconstitution with the PLP cofactor have also been studied.

Materials and methods

Chemicals

L-Serine, L-cysteine and hydroxylamine were from Sigma. Deuterium oxide (99.9 atom % D) was from Cambridge Isotope Laboratories, Inc. Hepes and Mes were from Research Organics, Inc. All other chemicals and reagents were obtained from commercial sources, were reagent grade, and were used without purification.

Enzyme

Recombinant OASS-B from *S. typhimurium* was expressed and purified following the protocol of Chattopadhyay et al. [6]. The OASS-B used for NMR experiments was purified with a slightly modified protocol. In this modified protocol, PLP was not added to the crude enzyme (supernatant) before it was loaded onto the Ni–NTA column, to avoid PLP other than that in the active site bound to enzyme. The purified OASS-B was stored in 5 mM Hepes, pH 8.0, at −80 °C.

^{31}P NMR spectroscopy

The Fourier transform ^{31}P NMR spectrum of OASS-B without ligand bound was collected at 121.5 MHz (Varian 300 MHz) and 160.9 MHz (Varian 400 MHz), respectively, using a 5 mm multinuclear probe head with broadband ^{1}H decoupling. ^{31}P NMR spectra of OASS-B in presence of L-cysteine and L-serine were collected using the Varian 400 MHz instrument, also using a 5 mm probe head. All NMR samples were maintained at 21.0 °C in a 5 mm NMR tube, with 10% D_2O in the solution as a field/frequency lock. A spectral width of 3004.8 Hz was acquired in 12,288 data points with pulse angles of 30° and 45° for the 300 MHz and 400 MHz instruments, respectively. The acquisition time was 1.6 s and a relaxation delay of 3.0 s was applied. The exponential line broadening used prior to Fourier transformation was 10.0 Hz. Positive chemical shifts in ppm are downfield changes with respect to 85% H_3PO_4.

The application of higher frequency instruments leads to higher line width, because the chemical shift anisotropy becomes the dominant relaxation mechanism [24]. The line width of ^{31}P NMR spectra obtained with the 400 MHz spectrometer can be adjusted to those obtained with a 300 MHz instrument, using the following equation derived from the frequency-dependent model in reference [24].

$$\frac{(LW_{300}LW_{d-d})}{(LW_{400}LW_{d-d})} = \frac{9}{16} \quad (1)$$

In Eq. (1), LW_{300} and LW_{400} represent line widths (Hz) measured with the 300 MHz and 400 MHz instruments, respectively, LW_{d-d} (Hz) is the value of the field independent contribution given by dipole–dipole mechanisms. In this study, LW_{d-d} is calculated to be 9.5 Hz using the line width data (LW_{300} and LW_{400}) of OASS-B with

out ligand bound. The line widths of the ^{31}P NMR spectra of OASS-B in presence of L-cysteine and L-serine were corrected to those at 300 MHz for comparison to the data obtained with OASS-A.

Enzyme assays

OASS-B activity was monitored as described by Tai et al. [4] using OAS and TNB as substrates. The disappearance of TNB was measured at 412 nm using a Beckman DU 640 spectrophotometer; the ε_{412} for TNB is 14,150 M^{-1} cm^{-1} [25]. All assays were performed in 100 mM Hepes, pH 7.0, with 0.2 mM OAS and 0.05 mM TNB at room temperature.

Resolution of PLP from OASS-B

OASS-B in 5 mM Hepes, pH 8.0 was dialyzed overnight against resolving buffer containing 500 mM phosphate, pH 7.6, 1 mM hydroxylamine and 0.2 mM DTT. The resolved OASS-B was dialyzed against 100 mM phosphate, pH 7.0 with 0.1 mM DTT. The apoenzyme obtained was stored frozen at −80 °C.

Reconstitution of PLP with apo OASS-B

A solution in 100 mM phosphate, pH 7.0, containing apo-OASS-B (2 μM), 0.1 mM DTT, and PLP in a 3-fold molar excess of enzyme was prepared. The reconstitution experiments were carried out at 4 °C. Aliquots of the reconstitution mixture were removed with time and the enzyme activity was assayed at 25 °C using the TNB assay. The control made use of the reconstitution mixture in the absence of PLP. The control gave a constant rate that was 1.3% of the native holoenzyme used to make apo-OASS-B. The reconstituted OASS-B used in the UV–visible and fluorescence spectra was prepared by combining apo OASS-B with PLP in a 1:1 M ratio. Although the reconstitution was slower, the amount of reconstituted enzyme was the same, within error, as that obtained with PLP in excess.

UV–visible spectral studies

Absorbance spectra of apo OASS-B and reconstituted OASS-B were obtained as for OASS-A [10]. All of the spectra were collected at 25 °C using a Hewlett Packard 8452A photodiode array spectrophotometer. Spectra of the native holoenzyme and reconstituted holoenzyme were obtained in 100 mM Hepes, pH 6.5 and 7.0, respectively. The spectrum of apoenzyme was collected in 100 mM phosphate, pH 7.0. The blank consisted of all components minus enzyme.

Steady-state fluorescence measurement

Fluorescence spectra of apo OASS-B and reconstituted OASS-B were recorded using a Shimadzu RF-5301 PC spectrofluorometer.

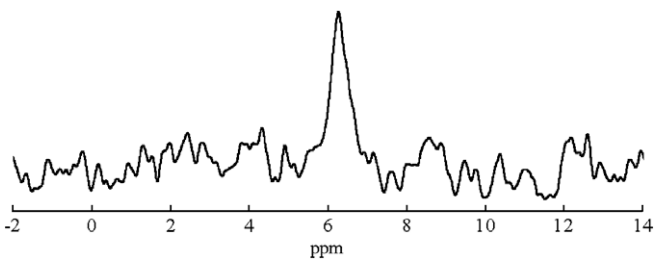

Fig. 2. ^{31}P NMR spectrum for OASS-B without ligand bound. A chemical shift of 6.2 ppm with 19.4 Hz line width is comparable to those of OASS-A (5.2 ppm, 20.5 Hz).

Table 1
^{31}P NMR-chemical shifts and line widths of OASS-B.

Ligand	OASS-A		OASS-B	
	δ, ppm	Line width, Hz	δ, ppm	Line width, Hz
None	5.2[a]	20.5[a]	6.2	19.4
L-Cysteine[c]	5.3[b]	32.5[b]	6.0	21.0[d]
L-Serine[c]	5.2, 4.4[b]	50.5[b]	6.3	22.8[d]

[a] From Cook et al. [9].
[b] From Schnackerz et al. [10].
[c] L-Cysteine was 10 mM for OASS-A and 30 mM for OASS-B, while L-serine was 36.4 mM for OASS-A and 300 mM for OASS-B.
[d] Numbers are line widths corrected to a 300 MHz spectrometer.

Experiments were carried out at pH 7.0, 25 °C. The excitation and emission slit widths were set to 3 nm and 5 nm, respectively. The excitation wavelength was set to 298 nm, and the emission monochromator was scanned from 310 to 550 nm. The blank consisted of all components except enzyme.

Results

^{31}P NMR spectroscopy

^{31}P NMR spectra obtained for the ISB of native OASS-B from *S. typhimurium* exhibits a ^{31}P chemical shift of 6.2 ppm, Fig. 2. This can be compared to a chemical shift of 5.2 ppm for OASS-A [9]. The line width of the ^{31}P resonance of the ISB of the B-isozyme was 19.4 Hz, similar to the value of 20.5 Hz measured for the A-isozyme. The chemical shift is independent of pH over the range 7.0–8.9 (data not shown), as observed for the A-isozyme [10].

The pH dependence of the dissociation constants for the L-serine and L-cysteine ESB has been obtained previously [6]. The pH independent K_{ESB} values are 3 and 30 mM and are observed above pH 7. Thus, ^{31}P NMR spectra were obtained for the ESB of L-cysteine (30 mM) or L-serine (300 mM) at pH 8 for the B-isozyme; the chemical shift reflects the ESB of both amino acids. Addition of L-cysteine generates the ESB of lanthionine,[2] and its ^{31}P chemical shift moves upfield to 6.0 ppm, with a line width of 21.0 Hz. In the presence of 300 mM L-serine, the chemical shift moves downfield to 6.3 ppm, with a line width of 22.8 Hz. In comparison, the chemical shift of OASS-A for the ESBs with lanthionine and L-serine are 5.3 and 4.4 ppm, with line widths of 32.5 and 50.5 Hz, respectively, indicating significant differences depending on which amino acid forms the external Schiff base [10]. Values of chemical shift (δ) and line width are summarized in Table 1, together with values obtained previously for OASS-A [10].

Resolution of PLP from OASS-B

Several procedures were tested for the preparation of apo OASS-B, including guanidine hydrochloride with OAS, which was effective with the closely related OASS-A [19], L-cysteine with imidazole citrate buffer [26], OAS with a high concentration of inorganic phosphate, and high concentrations of inorganic phosphate at low pH. None of these reagents were able to successfully resolve PLP from OASS-B. Treatment of OASS-B with 1 mM hydroxylamine in 500 mM phosphate, pH 7.6, with 0.2 mM DTT, followed by dialysis against 100 mM phosphate, pH 7.0 with 0.2 mM DTT generated apo OASS-B [27].

The internal Schiff base of OASS-B absorbs maximally at 414 nm with a small shoulder at 330 nm, suggesting the ketoenamine

[2] Cysteine is rapidly converted to the α-aminoacrylate intermediate, which is attacked by the thiol of a second cysteine molecule to generate the external Schiff base of lanthionine [1].

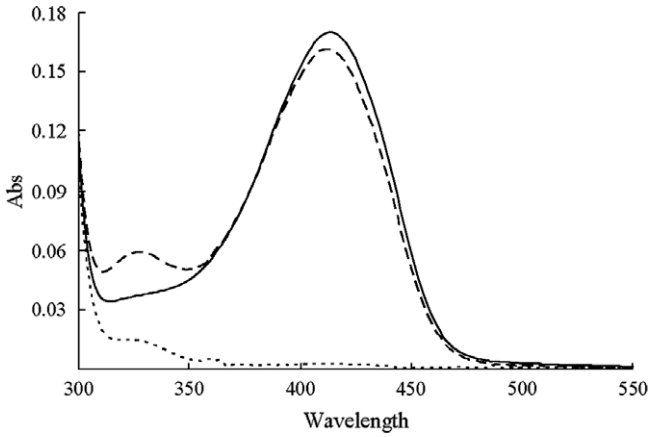

Fig. 3. UV–visible absorption spectra of OASS-B. Spectra are for native enzyme prior to preparation of apoenzyme (—), apoenzyme (· · · ·), and reconstituted holoenzyme (- - - -). The spectra of holoenzyme and reconstituted enzyme were collected in 100 mM Hepes, at pH 6.5 and 7.0, respectively. The spectrum of apoenzyme was collected in 100 mM phosphate, pH 7.0.

tautomer is favored, Fig. 3 [6]. The absorbance spectrum of apo OASS-B, Fig. 3, exhibits no absorbance at 414 nm and only slight absorbance at 330 nm, consistent with removal of most of the PLP cofactor. Fluorescence emission spectra were obtained by excitation at 298 nm. The holoenzyme emits predominantly at 336 nm, Fig. 4, consistent with previously measured spectra [6]. A very low-intensity emission band is also observed for holoenzyme and is centered at 500 nm. The long wavelength band results from resonance energy transfer from tryptophan to PLP. Apoenzyme exhibits an emission band at 336 nm with an intensity that is about 3.3 times greater than holoenzyme, while the 500 nm band is absent.

Reconstitution of PLP with apo OASS-B

The holoenzyme form of OASS-B was reconstituted by adding PLP at a 3:1 M ratio to apoenzyme, as shown by regain of activity as a function of time. Reconstitution of OASS-B was pseudo-first ordered, as shown in Fig. 5, and gave a rate constant of about 0.035 min^{-1}, a $t_{1/2}$ of 20 min. Reconstituted OASS-B had 81.4% activity of the native enzyme. The absorption spectrum of the reconstituted OASS-B (Fig. 3) is nearly identical to that of the wild

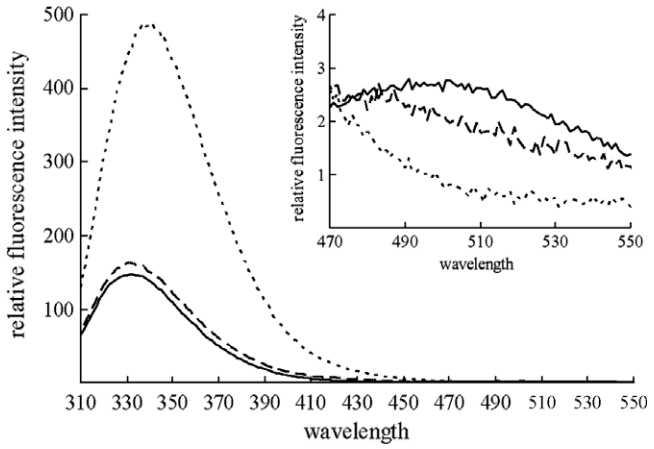

Fig. 4. Steady-state fluorescence spectra of OASS-B. Spectra are for native enzyme prior to preparation of apoenzyme (—), apoenzyme (· · · ·), and reconstituted holoenzyme (- - - -). The spectra of holoenzyme and reconstituted enzyme were collected in 100 mM Hepes, at pH 7.0, while that of apoenzyme was collected at pH 7.0, 100 mM phosphate. The long wavelength fluorescence emissions are shown more detailed in inset.

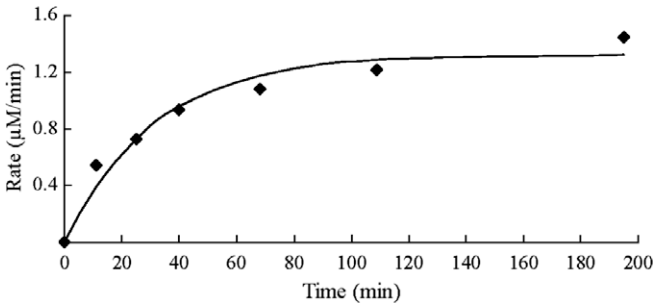

Fig. 5. Kinetics of reconstitution of apo OASS-B upon addition of PLP. The points shown are the experimentally determined values. The curve is theoretical based on a fit of the data using the equation for a first order reaction.

type, with the exception of a small broad-peak centered at about 330 nm. The extinction coefficient at 414 nm is 6400 M^{-1} cm^{-1}, slightly lower than the wild type value of 6800 M^{-1} cm^{-1}. The fluorescence emission spectrum of reconstituted OASS-B was also very similar to that of the native enzyme with excitation at 298 nm, Fig. 4.

Discussion

Pyridoxal 5′-phosphate binding site of OASS

The overall topology and the PLP binding site of OASS-A and -B are very similar, which is not surprising given the 40% sequence identity of the two isozymes [6,8]. The PLP cofactor is buried deeply between the large and small domains in both isozymes. The 5′-phosphate group of PLP in OASS-B interacts with the positive end of the dipole of helix 7 and the phosphate-binding loop (174-GTTGT-178) [6]. The loop donates 6 hydrogen bonds to the 5′-phosphate of PLP. In addition, 2 hydrogen bonds are donated to the 5′-phosphate by active site water molecules. A hydrogen-bonding network is formed by these 8 hydrogen bonds, anchoring the phosphate moiety of PLP to the protein matrix. In OASS-A, the phosphate-binding loop is comprised of 176-GTGGT-180 [7], where T176 in OASS-B replaces G178 in OASS-A. However, the hydroxyl group of T176 points away from the 5′-phosphate and does not participate in the hydrogen-bonding network. Two active site water molecules are also available for hydrogen bond formation in OASS-A. The pattern and position of the hydrogen-bonding network are thus nearly identical for both isozymes, Fig. 6.

^{31}P NMR spectra

The ^{31}P chemical shift of the ISB of native OASS-B is 6.2 ppm. The chemical shift is about 1 ppm higher than that of OASS-A [9] and the highest reported for any PLP-dependent enzyme. The 5′-phosphate is dianionic given data measured for the pH dependence of the chemical shift of free PLP [12]. The line width of 19.4 Hz is similar to data obtained for OASS-A, indicating a tightly bound phosphate group, the motion of which is determined by that of the protein [9,10].

At first glance, the phosphate binding sites of PLP in OASS-A and OASS-B, especially around the 5′-phosphate group, are nearly identical, Fig. 6. The higher chemical shift for B-isozyme may thus result from a tighter binding of the 5′-phosphate. The distance between the Schiff base nitrogen of K41 and O4′ (the bridge oxygen of the phosphate ester) is 4.37 and 4.17 Å, respectively, in OASS-A and OASS-B, Fig. 7. In addition, the torsion angle Φ (O4′–C5′–C5–C4), responsible for the rotation around the C5–C5′ bond, is reduced from 67.8° in OASS-A to 55.6° in OASS-B, Fig. 7. A similar phenomenon was observed in ^{31}P NMR spectra of aspartate aminotransfer-

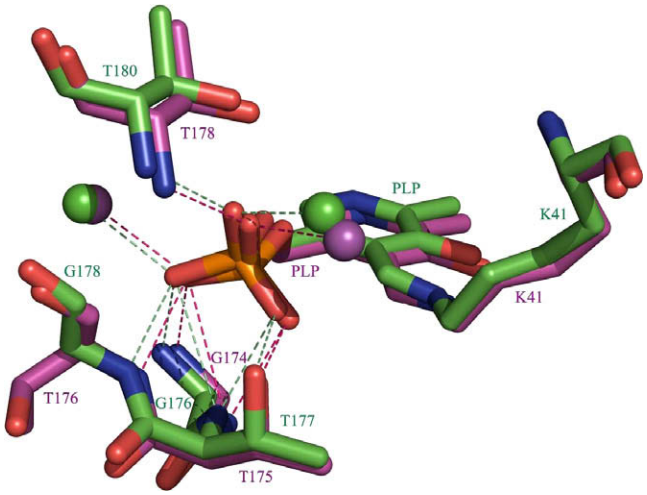

Fig. 6. Superposition of the PLP cofactor in the phosphate binding site of OASS-A (green) and OASS-B (purple) and their respective hydrogen-bonding networks. The dashed lines represent hydrogen bonds and the spheres represent water molecules. This figure was created using Pymol from DeLano Scientific LLC. The structures used in this figure have access number of 1OAS for OASS-A and 2JC3 for OASS-B in the Protein Data Bank. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

ase where the chemical shift of the cofactor is higher at high pH than at low pH [28]. The reduction in torsion angle results *a priori* in a decrease in the distance between C4′ and O4′ [28]. The torsional stain of OASS-B in PLP must be relieved by small changes in bond angle, including those around the phosphorus atom. The ^{31}P chemical shift of phosphate esters correlates with even small changes in O–P–O bond angle [29] and can thus result in higher chemical shift values for the 5′-phosphate.

The 6.0 ppm chemical shift of the lanthionine ESB of OASS-B is upfield from the unliganded B-isozyme. This can be compared to OASS-A, where the chemical shift is found further downfield (5.2 ppm for the ISB and 5.3 ppm for the lanthionine ESB) [10]. The slight upfield shift observed for the lanthionine ESB of OASS-B suggests a slight relaxation of the torsional strain, while the opposite is true for OASS-A. The increased strain in OASS-A was attributed to tight binding of the long lanthionine side chain, while the relaxation observed in OASS-B may reflect the higher degree of flexibility of the active site compared to OASS-A [6].

The further downfield shift of the 5′-phosphate of the serine ESB, 6.3 ppm compared to 6.2 ppm for the ISB, suggests an increase in torsional strain. This would appear to disagree with the explanation provided for the lanthionine ESB, but the serine side chain is much smaller and the orientation of PLP in serine ESB may be somewhat different with that of lanthionine ESB. These factors could contribute to the slight downfield chemical shift of serine ESB. Data for the serine ESB of OASS-A exhibit two conformations in intermediate exchange, one with a chemical shift of 5.3 ppm (slightly downfield to the 5.2 ppm chemical shift of the ISB) and another with an upfield chemical shift of about 3.5 ppm. The ^{31}P chemical shift of the serine ESB of the A-isozyme has a line width of 50.5 Hz, suggesting relatively slow inter-conversion between two tautomers of the serine ESB of OASS-A, the ketoenamine (δ, 5.3 ppm) and the enolimine (δ, 3.5 ppm) [10]. Given values of line width that are very similar for the ISB and ESB of OASS-B, there is no evidence of multiple conformers of the serine ESB in the B-isozyme. In agreement, the UV–visible spectrum of the serine ESB of OASS-B is dominated by the ketoenamine tautomer [6]. This appears to be true for the lanthionine ESB as well. As stated in Introduction, the active site of the B-isozyme is the more hydrophilic than that of the A-isozyme as a result of two additional ionizable residues, C280 and D281, at or near the active site [6].

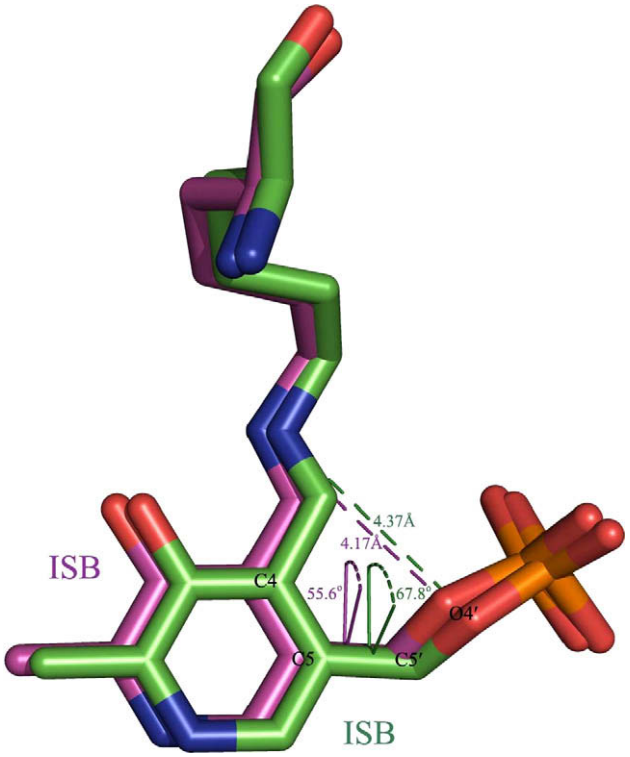

Fig. 7. Superposition of the internal Schiff base of OASS-A (green) and OASS-B (purple). The torsion angle Φ (O4′–C5′–C5–C4) and the distance between K41Nz and O4′ of the phosphate group of PLP are labeled for each isozyme. The dashed lines represent the distance between K41Nz and O4′. Dihedral angles are also labeled. The figure was created using Pymol from DeLano Scientific LLC. The ISB of OASS-A comes from structure 1OAS in the Protein Data Bank, while 2JC3 is for the ISB of OASS-B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

In addition, the active site of the B-isozyme is larger in volume. However, it is not obvious why the serine ESB of the two isozymes exhibits such profound differences. This aspect will have to await future structural studies.

Characterization of apo and reconstituted OASS-B

Similarities in the PLP binding site and the overall structure of the A- and B-isozyme suggested that the method used to resolve PLP from OASS-A might also be applicable to OASS–B. However, OASS-B is not as stable as OASS-A in denaturing reagents and was rapidly and irreversibly denatured in the presence of guanidine hydrochloride. Hydroxylamine was an effective resolving reagent, although it does not work with the A-isozyme [19]. The difference may reside in the higher degree of conformational flexibility of the active site of OASS-B compared to OASS-A, consistent with the broader substrate specificity of the B-isozyme [6]. The reconstitution of the PLP cofactor with apo OASS-B was pseudo-first order as was observed for reconstitution of OASS-A [19].

The UV–visible spectra of the apo and reconstituted OASS-B clearly indicate that both the resolution and the reconstitution of OASS-B were successful. Absorbance at the 414 nm λ_{max} for the ketoenamine tautomer is only observed for the reconstituted enzyme, while it is absent in the apoenzyme. The extinction coefficient at 414 nm for reconstituted OASS-B is very similar to native enzyme as well. In addition, the activity of apo OASS-B is very low and 81.4% of the activity is recovered upon reconstitution. The low-intensity 330 nm band is attributed to the absorbance of a small amount of unreconstituted PLP, which is about 18.6% of the total PLP used in reconstitution.

The quantum yield at 336 nm (excitation at 298 nm) of apo OASS-B is 3.3 times higher than that of native enzyme. The difference in quantum yield indicates that the PLP cofactor is a potent quencher of the tryptophan emission in OASS-B, consistent with the presence of the 500 nm band of native enzyme that results from energy transfer [6]. The same phenomenon was observed for the A-isozyme [22]. A ratio of about 2.5 was observed for fluorescence of native enzyme to apoenzyme, similar to the ratio of 3.3 obtained for the B-isozyme [22]. Fluorescence emission spectra of reconstituted holoenzyme and native OASS-B are nearly identical. The 500 nm emission band is observed in the spectrum of native and reconstituted holoenzyme, but not in that of apoenzyme, indicating that there is no energy transfer from tryptophan to PLP in the apoenzyme, consistent with resolution of the PLP cofactor. A reproducible method for producing stable apoenzyme is important to future lifetime fluorescence studies of OASS-B that will provide information in protein dynamics along the reaction pathway.

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