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Phosphorus-31 Nuclear Magnetic Resonance Study of D-Serine Dehydratase: Pyridoxal Phosphate Binding Site†

Klaus D. Schnackerz,* Knut Feldmann, and William E. Hull

ABSTRACT: The pyridoxal phosphate dependent enzyme D-serine dehydratase has been investigated using ^{31}P nuclear magnetic resonance (NMR) at 72.86 MHz. In the native enzyme, the pyridoxal phosphate ^{31}P chemical shift is pH dependent with $pK_a = 6.4$, indicating exposure of the phosphate group to solvent. Binding of the competitive inhibitor isoserine results in the formation of the isoserine–pyridoxal phosphate complex. This transaldimination complex is fixed to the enzyme via the phosphate group of the cofactor as the dianion, independent of pH. At pH 6.6 the dissociation constant K_D for isoserine determined by NMR is 0.43 mM. Reconstitution of the apoenzyme with pyridoxal phosphate monomethyl ester produces an inactive enzyme. NMR and fluorescence measurements show that this enzyme does not form the transaldimination complex, indicating that the fixation of the dianionic phosphate (probably via a salt bridge with an arginine residue) observed in the native enzyme is required for the transaldimination step of the catalytic mechanism.

Crystalline D-serine dehydratase (EC 4.2.1.14) from *Escherichia coli* is a monomeric enzyme containing a single polypeptide chain (M_r = 45 500) and has one catalytically essential pyridoxal-P[1] per molecule (Dowhan & Snell, 1970a). From reconstitution studies of D-serine apodehydratase with various pyridoxal-P analogues, it was concluded that substitutions at positions 2 and 6 of pyridoxal-P are not critical for catalytic activity (Dowhan & Snell, 1970b). On the other hand, a phosphate dianion at the 5′ position of the coenzyme is essential for catalysis but not required for the binding of the cofactor (Groman et al., 1972; Kazarinoff & Snell, 1976).

Reconstitution experiments monitored by UV and visible absorption spectroscopy indicate an extension of the π-electron system of the pyridine chromophore consistent with the formation of a Schiff base between cofactor and an ϵ-amino group of a lysyl residue of the protein. This technique, however, provides no information about the environment of the essential phosphate group of the cofactor. ^{31}P nuclear magnetic resonance spectroscopy has been shown to be a powerful tool to obtain direct evidence on the environment of the phosphate group of pyridoxal-P bound to enzymes (Feldmann & Hull, 1977; Martinez-Carrion, 1975). We have therefore utilized this NMR method to gather specific knowledge about the ionization state of pyridoxal-P bound to D-serine dehydratase and its interactions with the protein during catalysis.

Experimental Procedures

Materials. Isoserine was purchased from ICN Pharmaceuticals Inc. (Cleveland, OH). 2-(*N*-Morpholino)ethanesulfonic acid (Mes), 3-(*N*-morpholino)propanesulfonic acid (Mops), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) were obtained from Serva (Heidelberg, West Germany). Pyridoxal-P monomethyl ester was prepared according to the method of Pfeuffer et al. (1972). All other chemicals were of analytical reagent grade from Merck (Darmstadt, West Germany).

Enzymes. D-Serine dehydratase was isolated from *Escherichia coli* K 12 mutant C 6 as described previously (Schiltz & Schnackerz, 1976). Enzymatic activity and protein concentrations were determined according to published procedures (Schiltz & Schnackerz, 1976). D-Serine apodehydratase was prepared by using the resolution procedure of Dowhan & Snell (1970a). The apoenzyme had a residual activity of 1.9 units/mg of protein. The specific activity of pyridoxal-P reconstituted dehydratase was 100. Reconstitution of apodehydratase with pyridoxal-P monomethyl ester was achieved by incubating apoenzyme (350 μM) with a fivefold excess of cofactor analogue for 1 h at 25 °C in the dark. The excess

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[1] Abbreviations used: NMR, nuclear magnetic resonance; UV, ultraviolet; AMP, 5′-adenosine monophosphate; pyridoxal-P, pyridoxal phosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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of cofactor analogue was removed by passing the incubation mixture through a Sephadex G-25 column (3.4 × 40 cm) equilibrated with the same buffer used for NMR measurements.

Fluorescence Measurements. Fluorescence emission and polarization excitation measurements were performed on a Schoeffel fluorimeter Model RRS 1000 equipped with a 450-W xenon lamp. The excitation wavelength for emission measurements was 296 or 415 nm, respectively. The optical density of the samples at the excitation wavelength was always smaller than 0.1 for a pathlength of 1 cm. Dissociation constants for enzyme–inhibitor complexes were obtained from fluorescence emission measurements at 515 nm (excitation at 415 nm) and calculated from double-reciprocal plots of $F - F_0$ vs. inhibitor concentration. F and F_0 are fluorescence emission of dehydratase in the presence and absence of inhibitor, respectively. For fluorescence polarization excitation studies, the analyzing monochromator was set at 515 nm. A depolarizer (Karl Feuer Inc., Upper Montclair, NJ) was introduced into the emission pathway since the fluorimeter used has a grating monochromator at the analyzing unit.

NMR Techniques. Fourier transform ^{31}P NMR spectra were recorded at 72.86 MHz on a Bruker WH-180 wide-bore superconducting spectrometer. Sample volumes of 11–12 mL in 20-mm diameter tubes were used. A concentric 5-mm NMR tube containing ^2H_2O was employed as field/frequency lock. All spectra were recorded with broadband proton decoupling (0.4 W). In general, a 3000-Hz spectral width was acquired in 4096 data points with 60° pulse angle and 1-s repetition time. For enzyme samples the exponential line broadening used prior to Fourier transformation was usually 4 Hz. All line-width data have been corrected for this line-broadening effect. Continuous air flow through the spectrometer probe head kept the temperature at 30 ± 1 °C. Buffers (50 mM) for NMR measurements contained 2 mM EDTA and 50 mM 2-mercaptoethanol and were adjusted to the appropriate pH with KOH. The pH of each enzyme sample was determined before and after the NMR measurements with a Radiometer Model 26 pH meter. D-Serine dehydratase samples lost less than 5% of their initial enzymic activity even after collecting transients for an 8-h period.

Results and Discussion

pH Titration of the Phosphate Group of Pyridoxal-P Bound to D-Serine Dehydratase. A nonclassical pyridoxal-P enzyme, glycogen phosphorylase from skeletal muscle, shows a UV absorption maximum at 333 nm for the cofactor bound as a Schiff base in a hydrophobic pocket (Shaltiel & Cortijo, 1970). In its inactive form as phosphorylase *b*, the phosphate group of the cofactor is protonated, as demonstrated by ^{31}P NMR measurements. Activation of the inactive enzyme by the allosteric modifier 5′-AMP or interconversion to active phosphorylase *a* causes a change of the chemical shift of the phosphate group corresponding to the formation of the dianionic form within a protected binding site, since both ionization states of the phosphate are pH independent in the range from 6.3 to 8.8 (Feldmann & Hull, 1977). On the other hand, a classical pyridoxal-P-dependent enzyme, aspartate aminotransferase, shows a transition in the absorbance maximum of bound pyridoxal-P from 363 to 420 nm in the pH range of 5.6 to 9.2 (Furbish et al., 1969), but no pH-dependent chemical shift of the phosphorus resonance of bound cofactor. This finding was interpreted as a rigid salt bridge of the phosphate with presumably an arginine residue (Martinez-Carrion, 1975). The absorption maximum of the pyridoxal-P bound to D-serine dehydratase at 415 nm, however, is pH independent in the range from 5.5 to 9.5 (Schnackerz et al., 1979). Figure 1 shows the pH dependence of the ^{31}P chemical shift for pyridoxal-P bound to D-serine dehydratase via a Schiff base linkage as well as data for the model Schiff base formed with ε-aminocaproate. The curves calculated according to the Henderson–Hasselbalch equation, assuming a linear relationship between chemical shift and the concentration ratio of protonated and unprotonated phosphate moieties, represent the best fit to the experimental data. pK values of 6.3 and 6.4 were obtained for the ε-aminocaproate–pyridoxal-P model Schiff base and D-serine dehydratase complex, respectively. These pK values are close to that of pyridoxal-P itself (pK = 6.2; Martinez-Carrion, 1975). In both cases, the usual change in the chemical shift from high to low field with increasing pH corresponds to the conversion of the monoanionic to the dianionic form of the phosphoric ester (Horsley & Sternlicht, 1968; Williams & Neilands, 1954); i.e., the pyridoxal-P Schiff base in the dehydratase essentially behaves like the model Schiff base in aqueous medium.

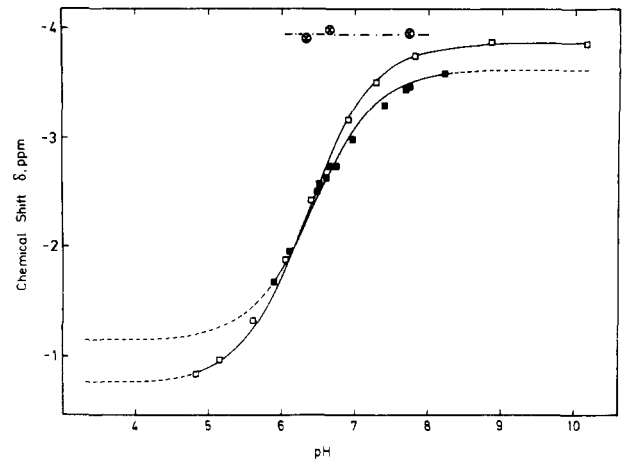

FIGURE 1: pH dependence of ^{31}P chemical shifts of ε-aminocaproate–pyridoxal-P Schiff base (□) and pyridoxal-P bound to D-serine dehydratase (■). The symbol ⊗ denotes experiments with D-serine dehydratase in the presence of 25 mM isoserine. Shifts in ppm are downfield changes relative to triethyl phosphate as external standard. For details, see Materials and Methods.

The line width observed for the phosphate signal of the cofactor in several preparations of D-serine dehydratase lies in the range of 10–14 Hz with no significant pH dependence. The line width observed for phosphorylase *b* or *a* under similar conditions is 55–60 Hz (Feldmann & Hull, 1977). Since in this case the phosphate group is well protected from the solvent, it appears that the phosphate of the pyridoxal-P is rigidly bound to phosphorylase in such a way as to prevent normal pH titration. The predominant relaxation mechanisms governing the ^{31}P line width are expected to be dipole–dipole with the neighboring protons and a contribution from chemical-shift anisotropy. For isotropic tumbling and mol wt ≥ 20 000, the ^{31}P line width is simply proportional to $\tau_c = 1/6D_r$, where D_r is the rotational diffusion constant for a spherical protein molecule (Hull & Sykes, 1975). The Stokes–Einstein relationship predicts that τ_c and, hence, the line width are just proportional to molecular weight. Thus, if dehydratase (mol wt 45 500) has pyridoxal-P binding properties and rotational diffusion properties similar to phosphorylase (mol wt 200 000), we can immediately predict a ^{31}P line width of 12.4–13.5 Hz for the pyridoxal-P phosphate group bound tightly to the dehydratase. If significant internal mobility of the phosphate group about the C–O bond were present for the dehydratase, the ^{31}P line width would be significantly reduced from the predicted value due to additional

FIGURE 2: ^{31}P NMR spectra of pyridoxal-P bound to D-serine dehydratase in the absence of isoserine (A) and in the presence of 0.62 mM (B) and 25.2 mM isoserine (C), respectively. Conditions: enzyme concentration, 120 μM, pH 6.6. The chemical shifts are relative to external triethyl phosphate. The line widths are 10 Hz (A, C) and 28 Hz (B).

modulation of ^1H–^{31}P internuclear vectors and chemical-shift tensor orientation. The observed ^{31}P line width is only slightly less than the predicted value, indicating no significant internal mobility of the phosphate group about the C–O bond. On the other hand, rotation about the O–PO_3 bond has little influence on the quantities responsible for relaxation and is thus invisible for these experiments. O'Leary & Payne (1976) investigated the ^{13}C line width of pyridoxal-P labeled at 4′ and 5′ carbons bound to the dehydratase and also found no substantial internal mobility at these carbon sites. Thus, for both dehydratase and phosphorylase, the phosphate group appears to be located in an enzyme site which prevents large-angle motion about the C–O bond. For the dehydratase, the phosphate group has essentially a normal pK, indicating accessibility to solvent and probably no specific interaction of the phosphate with the protein moiety. On the other hand, for phosphorylase, the phosphate is shielded from solvent and fixed to a binding site whose properties are altered by activation of the enzyme.

Effect of Substrate Analogue on the Phosphorus Resonance of Bound Cofactor. The addition of isoserine, a potent competitive inhibitor, to D-serine dehydratase at pH 6.6 results in a downfield shift of the phosphorus signal of bound pyridoxal-P. At less than saturating conditions, a single ^{31}P NMR signal is observed with line width and chemical shift which depend on the inhibitor concentration (Figure 2B). Thus, a fast-exchange kinetic situation arises and the weighted-average chemical shift can be used for binding studies of the inhibitor to the dehydratase. Since the enzyme concentrations for NMR measurements and the K_D value for isoserine are in the same order of magnitude, the conditions for a Lineweaver–Burk representation no longer hold. The dissociation constant K_D of the enzyme–inhibitor complex was therefore determined from eq 1

$$\frac{[I]_0}{\delta_{obsd} - \delta_0} = \frac{[E]_0}{\delta_\infty - \delta_0} + \frac{K_D}{\delta_\infty - \delta_{obsd}} \quad (1)$$

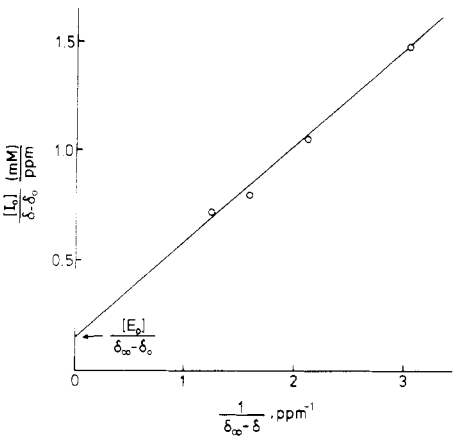

FIGURE 3: Determination of the dissociation constant of the dehydratase–isoserine complex. Conditions: dehydratase concentration, 120 μM, pH 6.6. Initial isoserine concentrations (I_0) were 0.43, 0.62, 0.98, and 1.59 mM, respectively. The slope gives a K_D value of 0.43 mM.

by plotting $1/(\delta_\infty - \delta_{obsd})$ vs. $[I]_0/(\delta_{obsd} - \delta_0)$, where δ_0 is the chemical shift of the phosphate signal of the cofactor when [I] = 0; δ_∞ is the chemical shift at saturation of the dehydratase with I; δ_{obsd} is the chemical shift at less than saturating concentrations of I; $[I]_0$ is the inhibitor concentration applied; and $[E]_0$ is the initial concentration of dehydratase. Equation 1 was derived from eq 2–6 according to Benesi & Hildebrand

$$\frac{[EI]}{[E] + [EI]} = \frac{\delta_{obsd} - \delta_0}{\delta_\infty - \delta_0} \quad (2)$$

$$\frac{[E]}{[E] + [EI]} = \frac{\delta_\infty - \delta_{obsd}}{\delta_\infty - \delta_0} \quad (3)$$

$$[E]_0 = [E] + [EI] \quad (4)$$

$$K_D = [E][I]/[EI] \quad (5)$$

$$[I] = [I]_0 - [E]_0\frac{\delta_{obsd} - \delta_0}{\delta_\infty - \delta_0} \quad (6)$$

(1949), who determined association constants using optical density measurements. Using this approach, we found the dissociation constant of the enzyme–inhibitor complex at pH 6.6 to be 0.43 ± 0.035 mM, as shown in Figure 3. The K_D value determined by measuring the quantity of fluorescence emission of the transaldimination product, isoserine–pyridoxal-P azomethine, was found to be 0.51 ± 0.05 mM. The kinetically determined K_I value is 0.46 ± 0.04 mM. All three measurements are in good agreement. Under saturating inhibitor concentrations at pH 6.3, 6.6, and 7.7, the ^{31}P chemical shift of pyridoxal-P is downfield by about 0.35 ppm of the limiting value from the titration curve in Figure 1 (crossed circles). Similar additional downfield shifts were found for phosphoric esters in their dianionic form when fixed to protein molecules, such as the AMP binding to phosphorylase *b* (Feldmann & Hull, 1977) and the formation of the phosphoserine residue in phosphorylase *a* (Sperling et al., 1979). The same downfield shift was also observed for the tight binding of inorganic phosphate to alkaline phosphatase of *E. coli* at pH 8.0 (Hull et al., 1976). At the chosen pH (6.6), a considerable amount of the cofactor of D-serine dehydratase is in the monoanionic form. Upon addition of isoserine, the transaldimination product is formed and the observed downfield shift indicates that the phosphate group of the cofactor in this cofactor–inhibitor complex is now excluded from interaction with water and fixed in its dianionic

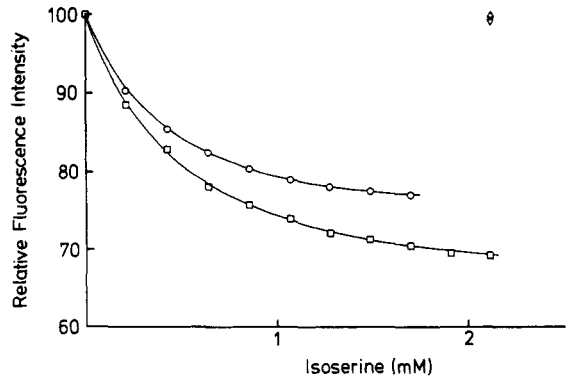

FIGURE 4: Changes in fluorescence emission of D-serine dehydratase reconstituted with pyridoxal-P (○, □) and pyridoxal-P monomethyl ester (△, ▽) upon addition of isoserine. Conditions: excitation wavelengths, 296 nm (○, △) and 415 (□, ▽); emission wavelength, 515 nm; enzyme concentration, 2 μM; pH 7.8 and 25 °C.

form to the protein moiety analogous to the situation in the active form of phosphorylase. This fixation could be achieved via a rigid salt bridge, e.g., to an arginine residue. The requirement of a specific arginine residue for the binding of pyridoxal-P is suggested by the inactivation of D-serine apodehydratase with butanedione or phenylglyoxal which abolishes cofactor binding (Kazarinoff & Snell, 1976).

Pyridoxal-P Monomethyl Ester Reconstituted D-Serine Apodehydratase. Pyridoxal-P monomethyl ester, a coenzymatically inactive cofactor analogue, binds to D-serine apodehydratase via a Schiff base similar to the natural cofactor. This was revealed by the formation of an absorption maximum at 420 nm which shows a positive circular dichroism band similar to that caused by the natural cofactor (Pötsch & Schnackerz, unpublished observations). Moreover, changes in fluorescence emission and an increase in fluorescence polarization from $p = 0.08$ for free cofactor analogue to $p = 0.37$ for the enzyme-bound cofactor analogue at pH 6.6 and 7.8 were found. The fluorescence polarization of pyridoxal-P when bound to apodehydratase was determined to be $p = 0.44$ as compared with a value of 0.05 for pyridoxyl-ε-lysine (Oh & Churchich, 1973), indicating a considerable restriction of the mobility of pyridoxal-P in the active site of the dehydratase. The K_{co} value of pyridoxal-P monomethyl ester bound to apodehydratase, representing the ability of this cofactor analogue to inhibit the reconstitution with pyridoxal-P (Dowhan & Snell, 1970a), was estimated to be 0.43 μM as compared with $K_D = 0.038$ μM for pyridoxal-P (Groman et al., 1972). The weaker binding of the methyl ester may be due to unfavorable steric effects of the methyl group.

^{31}P NMR experiments on pyridoxal-P monomethyl ester reconstituted apodehydratase performed at a protein concentration of 4.8 mg/mL showed a single phosphate signal at 0.44 and 0.38 ppm downfield from external triethyl phosphate at pH 7.8 and 6.6, respectively. In the presence of 24.8 mM isoserine, no change in the location of the phosphate signal (0.39 ppm) occurred, indicating no specific effect on the phosphate group of the cofactor analogue upon addition of inhibitor. This finding was to be expected since, in the case of the monomethyl ester, a dianion cannot be formed. Furthermore, the different chemical shifts of the phosphate group of the natural cofactor are due to changes in the ionization state and are not secondarily caused by structural changes in the surrounding protein moiety.

Specific information on the formation of the transaldimination product was obtained from fluorescence emission data using isoserine. No change was observed with isoserine concentrations up to 10^3-fold excess. Applying 10^4 M excess of isoserine resulted in a small increase in fluorescence emission, when excited either at 296 or 415 nm. In contrast, for natural D-serine dehydratase, addition of substrate analogue in the same concentration range produced a continuous decrease as shown in Figure 4. Thus, the pyridoxal-P monomethyl ester reconstituted apodehydratase is unable to form the transaldimination complex with isoserine. These results indicate that in the native enzyme the fixation of the pyridoxal-P phosphate group as the dianion is a requisite step for the formation of an enzyme–substrate transaldimination complex.

Acknowledgments

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