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Reaction of Pyridoxal 5′-Sulfate with Apoenzyme of Aspartate Aminotransferase. Covalent Labeling of the Protein with Elimination of Sulfate†

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ABSTRACT: The 5′-sulfate esters of pyridoxine, pyridoxal, and pyridoxamine have been prepared and their absorption spectra and dissociation constants have been determined. Spectra have been resolved using log normal distribution functions. The pK_a values are compared with those of related compounds. That for the pyridinium group is strikingly lower in pyridoxal sulfate than in pyridoxal phosphate. When pyridoxal sulfate reacts at pH 8.3 with apoaspartate aminotransferase it slowly forms an absorption band at 24.9 kK (402 nm) which is narrow and displays marked vibronic fine structure. (This is in sharp contrast to the broader band at 27.4 kK [364 nm] formed with pyridoxal phosphate.) At lower pH the peak shifted to 25.9 kK (386 nm) and at higher pH to 27.4 kK. The pyridoxal sulfate–enzyme form absorbing at 24.9 kK has positive circular dichroism (like the native enzyme). Upon denaturation with acid the chromophore precipitated with the protein suggesting covalent attachment. Cysteine, β-mercaptoethylamine, ethylenediamine, ethanolamine, serine, and arginine all react with pyridoxal sulfate with gradual production of narrow absorption bands similar to that formed with the apoenzyme. Investigation of the reaction with cysteine reveals that inorganic sulfate is eliminated during the reaction at a rate that is identical with that of formation of the 24.9-kK chromophore. Investigation with nmr shows that formation of a thiazolidine ring is followed by loss of the 4′ proton and disappearance of the 5′-CH_2 peak. Elimination of sulfate to a quinonoid form that tautomerizes to a cyclic substituted Schiff base is proposed. It is speculated that an unknown group X in the enzyme replaces the SH group of cysteine in a similar reaction.

One approach to understanding the function of the phosphate group in the coenzyme pyridoxal 5′-phosphate (pyridoxal-P)[1] is to study analogs of similar geometry and charge type but containing different anionic side chains. Thus, replacement of the phosphorylated hydroxymethyl side chain of pyridoxal-P with a propionic acid side chain (5-deoxy-5′-carboxymethylpyridoxal) yields an analog of the coenzyme that binds to several apoenzymes. However, the activity with apo aspartate aminotransferase (apoAAT) is at most 3% of that of

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[1] Abbreviations used are: AAT, aspartate aminotransferase; pyridoxal-P, pyridoxal 5′-phosphate.

TABLE I: Nmr Spectral Data.[a]

Assignment	Chemical Shifts, δ		
	Pyridoxine 5′-Sulfate	Pyridoxamine 5′-Sulfate	Pyridoxal 5′-Sulfate
2-CH_3	2.64	2.53	2.42
5-CH_2	5.19	5.20	5.26
4-CH_2	5.02	4.38	
6-H	8.14	7.79	7.60
4-CH			10.44[b]
pD	~7	5.6	7.5

[a] Spectra obtained in D_2O + NaOD with the Varian A60 spectrometer. [b] δ = 6.53 at pD 2, indicating formation of a hydrate.

pyridoxal-P (Furbish *et al.*, 1969) and the compound has no activity with arginine decarboxylase, D-serine dehydratase, and tryptophanase (Groman *et al.*, 1972). Likewise, pyridoxal 5′-sulfate is reported to inhibit brain glutamate decarboxylase (Matsuda and Makino, 1961) and D-serine dehydratase but it partially activates arginine decarboxylase and tryptophanase (Groman *et al.*, 1972).

The lack of adequate descriptions of the synthesis and properties of the 5′-sulfate esters of pyridoxine, pyridoxal, and pyridoxamine has led us to synthesize these compounds, to determine their acid–base properties and absorption spectra, and to test them with an additional enzyme, aspartate aminotransferase of pig heart. Pyridoxal 5′-sulfate was found inactive as a coenzyme and, when bound to the enzyme, it was also observed to undergo an unexpected slow change in absorption spectrum. The reaction resulted in formation of a relatively stable covalent linkage to the protein. A closely similar reaction was found to occur with cysteine and with a variety of β-substituted amines as detailed in the following report. While the exact nature of the reaction with the enzyme remains uncertain, the results suggest that pyridoxal 5′-sulfate may be a useful and specific active site labeling reagent for certain proteins.

Experimental Section

The α subform of cytoplasmic aspartate aminotransferase of pig heart was prepared by the method of Jenkins *et al.* (1959) as modified by Martinez-Carrion *et al.* (1967, method A). ApoAAT was prepared according to the method of Scardi *et al.* (1963) as modified by Furbish *et al.* (1969). However, Tris buffer was used instead of triethanolamine buffer. The residual specific activity of the apoAAT was less than 0.2% of that of the holoenzyme. It could be reactivated, with an excess of pyridoxal-P, to 85–100% of the specific activity of the holoenzyme.

Enzyme activity was determined according to Furbish *et al.* (1969). The specific activity was 220 ± 10 μmol min^{-1} mg^{-1} at 25 °. Protein concentrations were determined from the absorbance at a wave number of 35.8 kK (279.3 nm) using 1-cm cuvets with spacers to reduce the light path to 0.1–0.2 cm. Molar absorptivities of 6.36×10^4 and 6.55×10^4 (Furbish *et al.*, 1969) were assumed for apoenzyme and holoenzyme, respectively. The molecular weight of the subunit of the dimeric enzyme was taken as 4.63×10^4 (Ovchinnikov *et al.*, 1973).

Binding Studies. Usually 0.8–1.0 ml of apoAAT of concentration approximately 10^{-4} M was placed in a semimicro spectrophotometer cuvet. Then a small volume of pyridoxal sulfate or other coenzyme analog (usually of 10^{-2} M concentration) was added. The exact amount added was such that the molar ratio of analog to apoenzyme was less than 1.0. Only the 85–100% of apoenzyme that could be reactivated with excess pyridoxal-P was considered in this calculation. Quantitative delivery of compounds added was performed with a Teflon-tipped syringe (Unimetrics Universal Corp.) and mixing was accomplished by inversion or by gentle mixing with a Teflon stirrer.

An attempt to displace bound pyridoxal 5′-sulfate (10^{-4} M) from the enzyme was carried out by adding a 200-fold excess (0.02 M) of pyridoxal-P to the apoenzyme–analog complex prepared as described in the preceding paragraph. Aliquots, typically 10 μl each, were withdrawn at various time intervals and were diluted approximately 40-fold for assay. Part of the enzyme activity observed initially after addition of the pyridoxal-P and dilution represented AAT formed by combination of small amounts of free apoenzyme (present in excess in the binding experiment) with pyridoxal-P. Additional activity gained over a time period after addition of pyridoxal-P was attributed to displacement of the analog by pyridoxal-P.

Electronic absorption spectra were recorded with a Cary 1501 spectrophotometer equipped with a digital output system that entered data points directly on punched cards. Absorbances from 0 to 2.0 were recorded to the nearest 0.001 unit at regular intervals of wave number, usually 0.05 or 0.20 kK (1 kK = 10^3 wave numbers). The apparent pK_a values in buffers of ionic strength 0.2 and the spectra of individual ionic forms were evaluated by methods described previously (Johnson and Metzler, 1970; Metzler *et al.*, 1973).

Circular dichroism was recorded with a Jasco Model ORD/UV-5 spectropolarimeter equipped with a circular dichroism (CD) recorder and with the Sproul Scientific SS-20-2 modification. Circular dichroism was measured to ±0.1 mdeg ellipticity (3×10^{-6} Δ absorbance units). Scanning was at a low speed of about 1.8 nm min^{-1} and with a time constant of 16 or 64 sec. Recorded ellipticity values transcribed manually at 1-nm intervals were entered on punched cards and the CD spectra were replotted against wave number.

Preparation of Pyridoxine 5′-Sulfate (adapted from Kuroda, 1963). One hundred per cent sulfuric acid was prepared by mixing 100 g of 97% sulfuric acid and 68 g of 20% fuming sulfuric acid. α^4-*O*-Isopropylidenepyridoxine (2.1 g, Korytnyk and Ikawa (1970)) was added, with stirring, over a period of 10 min, to 20 ml of 100% sulfuric acid in a 50-ml round-bottom flask in an ice bath. A drying tube packed with Drierite was fitted to the mouth of the flask. The contents of the flask was stirred in the ice bath for another 30 min with exclusion of moisture. It was then gradually poured, with stirring, into 300 ml of cold (< −30°) anhydrous ether. The mixture was stirred, with exclusion of moisture, until it reached room temperature. The ether was decanted and the coarse precipitate was washed with two 100-ml portions of ether. The precipitate was dried *in vacuo* to remove residual ether and was dissolved in 50 ml of water. Excess sulfuric acid was removed by titration with a saturated solution of barium chloride. Barium sulfate was removed by centrifugation and the supernatant was heated for 30 min in a boiling water bath to precipitate additional barium sulfate. After filtration the clear supernatant was concentrated *in vacuo* to dryness. The crude pyridoxine sulfate was dried *in vacuo* and was recrystallized from water. Yield: 1.85 g (74%).

The nmr spectrum of the compound (Table I) closely resembles that of pyridoxine 5′-phosphate (Korytnyk and Ahrens, 1970) except that no splitting of the 5-CH_2 is observed.

Oxidation of Pyridoxine 5′-Sulfate to Pyridoxal 5′-Sulfate. Active manganese dioxide was prepared according to Mancera *et al.* (1953) with the nitric acid wash recommended by Harfenist *et al.* (1954). Pyridoxine sulfate (250 mg, 1.0 mmol) was

TABLE II: Electronic Absorption Spectra of Sulfate Esters of Vitamin B_6 Compounds.[a]

Compound and Ionic Form	Band No.	Position $\bar{\nu}_0$ (kK)	Height, $\epsilon_0 \times 10^{-3}$ (l. mol^{-1} cm^{-1})	Width, W (kK)	Shewness ρ	Molar area A (km mol^{-1})
Pyridoxine 5′-sulfate						
H_zP	I	34.30	9.14	3.27	1.47	327
	II	44.08	3.06	4.7[c]	1.13	153
HP_z	I	30.69	6.60	3.44	1.33	(246)363
	II	39.18	3.58	4.44	1.63	(177)
	III	45.58	15.42	4.05	1.47	(685)
HP_n	I	35.3	1.85	3.46[b]	1.40[b]	(70)218
P	I	32.21	7.30	3.58	1.33	283
	II	40.69	6.65	4.35	1.33	313
Pyridoxamine 5′-sulfate						
H_3P	I	33.95	9.14	3.24	1.44	323
	II	~44.6	3.00	5.16	1.38	
H_2P_z	I	30.50	8.35	3.33	1.36	(302)342
	II	39.27	4.99	3.84	1.50	(210)
H_2P_n	I	35.3	0.63	3.46[b]	1.40[b]	(24)205
P	I	32.32	7.62	3.35	1.43	278
	II	40.50	6.54	3.95	1.10[b]	276
Pyridoxal 5′-sulfate hydrate						
H_2P	I	33.96	6.87	3.23	1.45[b]	(243)
HP_z	I	30.77	1.23	3.45[b]	1.36[b]	(46)
P	I	32.92	0.51	3.44[b]	1.36[b]	(19)
aldehyde						
H_2P	I	29.81	1.37	4.58	1.25[b]	(67)
HP	I	25.83	4.87	4.98	1.62	(270)
P	I	25.70	6.87	4.43	1.33	(329)

[a] Spectra of individual ionic forms were resolved with log normal curves (Johnson and Metzler, 1970; Metzler *et al.*, 1973). H_2P and H_3P represent the most protonated forms and P the completely unprotonated form. HP_n and H_2P_n are forms with neutral, uncharged rings while HP_z and H_2P_z are forms with a zwitterionic β-hydroxypyridine ring. Bands I, II, and III are the three π–π^* transitions in order of increasing energy. Band positions and widths are given in kilokaysers (kK); $\bar{\nu}$(kK) = $10^4/\lambda$(nm). Values of area in parentheses are the *measured* areas for forms existing as tautomeric mixtures or mixtures of aldehyde plus covalent hydrate. [b] Parameters fixed at preselected values; see Metzler *et al.* (1973). [c] Considerable uncertainty exists in parameters of band II of the most protonated forms. For example, this band could also be fitted well with W = 5.4 and ρ = 1.4 with little effect on the parameters of other bands.

dissolved in 25 ml of warm water in a 50-ml clinical centrifuge bottle. When the solution had cooled to room temperature, 500 mg (5.7 mmol) of pulverized, active manganese dioxide was added and the mixture was allowed to stir in the dark. The progress of oxidation was monitored by removing 10-μl aliquots into 3-ml portions of 0.1 N sodium hydroxide. After shaking, the basic mixture was pressed through a syringe equipped with a Millipore filter (HA 0.45 μ). The uv-visible absorption spectrum of the clear filtrate was recorded. The absorbance ratio ($A_{25.8kK}/A_{32.5kK}$) was noted. After about 30 min this ratio attained a maximal value of 5.0. Further reaction would lead to the production of the corresponding acid which absorbed at 32.5 kK and would reduce the ratio previously obtained. At this point, the reaction vessel was placed in a clinical centrifuge and spun for 2 min to remove manganese dioxide. The precipitate was washed with 3 ml of water and was again centrifuged. The combined supernatant was passed through another Millipore filter and the filtrate was concentrated *in vacuo* to 1 ml. The concentrate, intensely yellow and still containing some suspended manganese dioxide, was applied to an 85 × 1.9 cm column of Dowex 50 W-X8 (100–200 mesh) in H^+ form previously equilibrated with water. The column was eluted with water at a flow rate of 25 ml hr^{-1}; 5-ml fractions were collected and were monitored by measuring the absorption spectrum after transfer of 10-μl aliquots to 1-ml portions of 0.1 N sodium hydroxide. The initial fractions with low $A_{25.8kK}/A_{32.5kK}$ ratio were discarded while the fractions with a constant ratio of 10 were pooled, concentrated *in vacuo*, and lyophilized. Yield: 116 mg or 46%.

Pyridoxal 5′-sulfate was recrystallized from warm glacial acetic acid by addition of ethyl acetate and chilling. However, the powdery product had a slightly reduced $A_{25.8kK}/A_{32.5kK}$ ratio, possibly indicating some oxidation of aldehyde to carboxylic acid. Therefore the lyophilized powder was used for all of our experiments.

The nmr spectrum (Table I) of pyridoxal 5′-sulfate showed chemical shifts comparable to those observed for pyridoxal-P (Korytnyk and Ahrens, 1970) except that no splitting of the 5-CH_2 peak was seen. *Anal.* Calcd for $C_8H_9O_6NS \cdot \frac{1}{2}H_2O$: C, 37.10; H, 3.99; S, 11.92. Found, C, 37.50; H, 3.93; S, 12.51.

TABLE III: Apparent pK_a Values and Tautomerization Constants K_z at 25°.[a]

	pK_1	pK_2	pK_3	K_z[c]
Pyridoxine	4.94	8.89		3.9
Pyridoxine 5′-sulfate	4.71	8.72		2.1
Pyridoxamine	3.46	8.13	10.40	8.4
Pyridoxamine 5′-sulfate	3.28	7.82	10.33	7.7
Pyridoxamine 5′-phosphate	3.36	8.46	10.67	9.3
Pyridoxal 5′-sulfate	3.54	7.39		
Pyridoxal 5′-phosphate	3.62	8.33		
5-Deoxypyridoxal	4.14	8.03		

[a] The pK's given are those detected by spectrophotometry. [b] Additional literature values for some of these compounds have been summarized by Metzler *et al.* (1973). [c] The tautomerization constants (K_z = $[HP_z]/[HP]_n$) were evaluated by curve resolution as described by Metzler *et al.* (1973) and assuming that the molar areas are related as follows: A_n = $0.6A_z$.

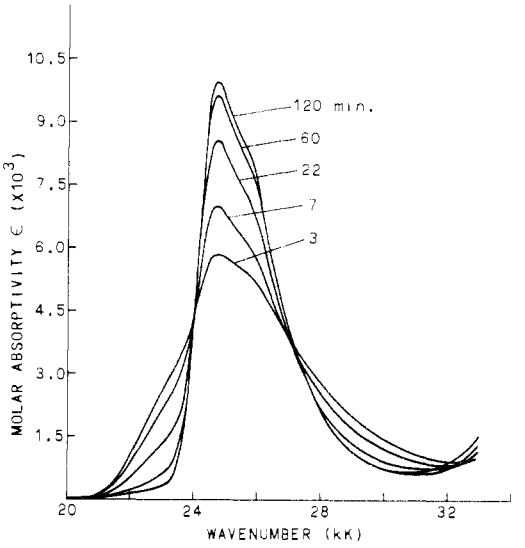

FIGURE 1: Absorption spectrum of the apoenzyme form of aspartate aminotransferase plus pyridoxal 5′-sulfate (1:1) at pH 8.2. The numbers give the time in minutes following the mixing of the two components.

The compound migrated electrophoretically at the same rate as a sample of pyridoxal 5′-sulfate supplied by E. E. Snell and prepared by Kuroda.

Preparation of Pyridoxamine 5′-Sulfate. Pyridoxamine dihydrochloride (0.5 g) was added in small portions, with mixing, to 5 ml of 100% H_2SO_4 (cooled with ice), as in the preparation of pyridoxine 5′-sulfate. The mixture was allowed to stand for 30 min at 0° and 100 ml of cold (−30°) ether was added. The ether was decanted from the precipitate and the residue was washed with two 100-ml portions of ether. The solid was then dissolved in 10 ml of water and sulfate was removed by addition of a saturated barium hydroxide solution to give a final pH of about 4.5. The barium sulfate was removed by filtration and the solution was concentrated *in vacuo* to a volume of 1 ml. Boiling methanol (15–20 ml) was added and the product was allowed to crystallize in a refrigerator. Yield: 0.4 g.

Determination of Release of Inorganic Sulfate. The sulfate released by reaction of pyridoxal 5′-sulfate with cysteine was determined quantitatively in two ways. The first was a direct nephelometric procedure based upon those of Meehan and Chiu (1964) and Vogel (1961) as adapted by Johnson (1973). A barium-containing solution was prepared by mixing, in the proportion 1:2:7 (v/v), the following three solutions: (1) glycerol plus absolute alcohol 1:2 (v/v); (2) 60 g of NaCl plus 5 ml of concentrated HCl in 250 ml of solution; (3) barium chloride dihydrate (0.43 g) in 100 ml of solution. The mixture of the three solutions was passed through a Millipore filter prior to use and care was exercised to minimize entrance of atmospheric dust. With stirring, 0.5 ml of the Millipore-filtered reaction mixture of pyridoxal sulfate plus cysteine was added to 10 ml of the barium-containing solution in a capped vial. Stirring (with a tiny magnet) was continued for 90 sec and then the contents were transferred to a light-scattering photocell and left undisturbed for 20 min. Light scattering was then measured in a Sofica light-scattering photometer, Model 701. A standard curve was constructed using sulfate of known concentrations.

Sulfate was also determined by the barium chloroanilate method (Klipp and Barney, 1959).

Results

The 5′-sulfate esters of pyridoxine, pyridoxal, and pyridoxamine have all been obtained in essentially pure form. Their absorption spectra (Table II) are closely similar to those of the corresponding 5′-phosphates (Morozov *et al.*, 1967; Bazhulina *et al.*, 1970; Johnson and Metzler, 1970) and (for pyridoxine and pyridoxamine) to those of the free alcohols (Metzler *et al.*, 1973).

The apparent (ionic strength 0.2) pK_a values for the 5′-sulfates (Table III) are, in most instances similar to those for the 5′-phosphates. However, the second pK of pyridoxal 5′-sulfate is remarkably lower than that of pyridoxal-P or of 5-deoxypyridoxal (7.39 *vs.* 8.33 and 8.03). The amount of covalent hydrate in equilibrium with the aldehyde form is less for pyridoxal sulfate than for pyridoxal-P in both the dipolar ion and anionic forms (Table II, notice the observed area, in parentheses).

When pyridoxal phosphate is mixed with an equivalent amount of freshly prepared apoenzyme of cytoplasmic (α subform) aspartate aminotransferase of pig heart (apoAAT) at pH 8.3 the coenzyme is nearly quantitatively and rapidly rebound at the active site. The spectrum of the regenerated, active aspartate aminotransferase is virtually the same as that of the native enzyme. Thus at pH 8 the rebound coenzyme band has the parameters $\tilde{\nu}_0$ = 27.49 kK, W = 4.0–4.1 kK, ρ = 1.48. For the native enzyme $\tilde{\nu}_0$ = 27.44 kK (364 nm), W = 3.90, and ρ = 1.48. Below pH 5 the peak position, $\tilde{\nu}_0$ shifts to 23.2 kK (Furbish *et al.*, 1969). When pyridoxal sulfate was added to apoenzyme under the same circumstances the spectrum was initially broad and complex in character (Figure 1). However, over a 2-hr period the spectrum became narrower and stable with a peak position of 24.92 kK (402 nm), W = 2.62 kK, ρ = 1.96 (Figure 1). Not only is this band distinctly narrower than that of enzyme-bound Schiff bases observed with pyridoxal phosphate but it shows pronounced vibronic fine structure. This is most clearly seen in the "fine structure plots" obtained by plotting the differences between experimental points and a log normal curve fitted to the points (Johnson and Metzler, 1970; Metzler *et al.*, 1973). Such a plot for the bound pyridoxal sulfate is shown in Figure 2.

When the binding of pyridoxal sulfate was conducted at pH 4.97 the changing spectrum also became stable after 2.5 hr. However, as shown in Figure 3A, the peak position was at a higher energy (25.93 kK = 386 nm) and the band was wider

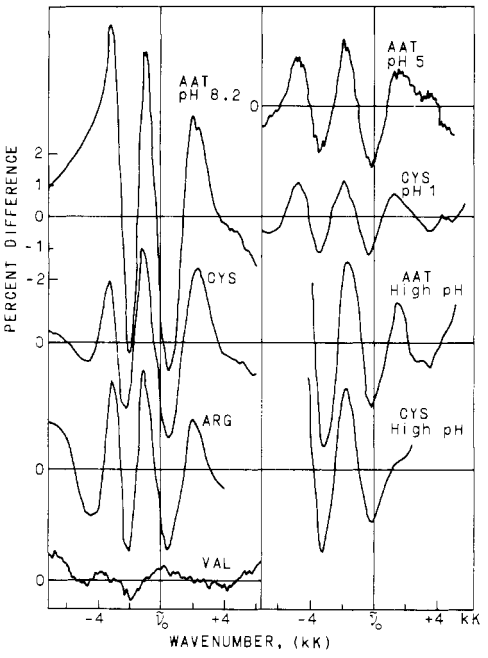

FIGURE 2: Fine structure plots for products of reaction of pyridoxal sulfate with apoAAT, cysteine, arginine, and valine. All plots are scaled the same and the positions of $\tilde{\nu}_0$ have been aligned.

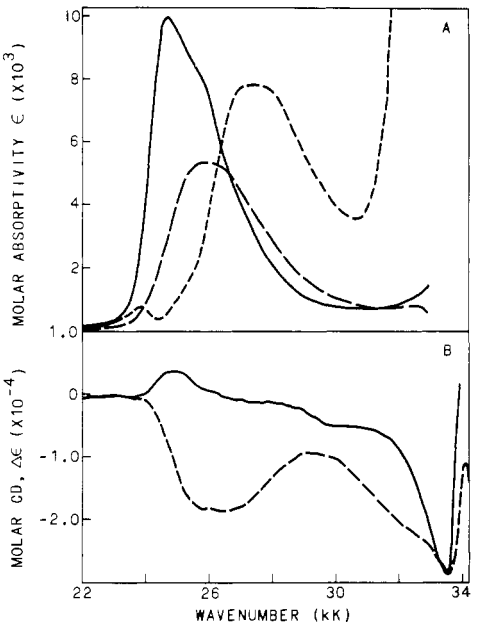

FIGURE 3: Spectra of the compound formed by reaction of apoAAT and pyridoxal 5′-sulfate. (A) Absorption spectra at pH 5.0(- -), pH 8.2(—), and calculated (from data at pH 12.5) (- - -) for the high pH form. (B) Circular dichroism spectra at pH 5.0 (- -) and 8.2(—).

(3.50 kK, ρ = 1.68). The product was highly fluorescent. Again some characteristic fine structure could be seen but much less than that at pH 8. Spectra at intermediate pH values did not form isosbestic points with those at pH 8.2 and 5.0. Thus it is impossible to associate a single pK_a with the spectral change. At high pH the peak also undergoes a hypsochromic shift to 27.4 kK (W = 3.94, ρ = 1.73, Figure 3A). In this instance an apparent pK_a of about 12.2 governs the change in spectrum of the soluble, but presumably denatured, enzyme.

The enzyme-bound pyridoxal sulfate possesses weak optical activity as indicated by the CD spectra shown in Figure 3B. At pH 8.2 the CD spectrum has a weak positive peak at 24.8 kK corresponding to the maximum in the absorption spectrum. In addition there appears to be a small negative band centered at around 30.2 kK and a second negative band centered around 33.6 kK. The latter is also seen in the CD spectrum of the native holoenzyme and is thought to result from one or more tryptophan residues. The CD spectrum at pH 5 is distinctly different and contains a negative band at 26.2 kK, corresponding to the maximum in the electronic absorption spectrum. Additional negative bands are present at higher energies.

Most analogs of pyridoxal-P, when bound to apoAAT, can be displaced by an excess of pyridoxal phosphate. Thus, if the enzyme reconstituted with the analog is enzymically inactive, activity is slowly regained in the presence of an excess of pyridoxal-P. In the case of pyridoxal sulfate, no enzymic activity could be detected for the bound analog. Neither could any displacement of the bound analog by pyridoxal-P be observed. While the peak position at 24.9 kK suggests the presence of a Schiff base, addition of sodium borohydride, either at pH 8.2 or 5.0, had no effect on the spectrum. This lack of reducibility with borohydride is among the pieces of evidence suggesting that pyridoxal sulfate does not bind in the same way as do pyridoxal-P and other pyridoxal-P analogs.

Denaturation of the enzyme–pyridoxal sulfate compound in 1.3 N HCl did not lead to any release of the chromophore from the protein. The protein precipitated and the spectrum of the supernatant showed only a small amount of absorption at 36 kK resulting from dissolved protein. On the other hand, when the precipitate was redissolved in 6 M guanidine hydrochloride, a spectrum with a peak at 26.7 kK was obtained. This indicates that the analog remained on the enzyme during the denaturation. Thus the data suggested that pyridoxal sulfate in some manner interacts with the enzyme and becomes covalently attached.

Addition of 6 M guanidine hydrochloride to the enzyme–pyridoxal sulfate compound at pH 11 led to a rapid loss of the chromophore. Presumably some reaction occurs that leads to a shift of the absorption band into the 36-kK band of the aromatic amino acids of the protein. Even at pH 8.2 this reaction occurred slowly, the chromophore disappearing overnight. The reaction could not be reversed by lowering the pH. However, the low pH form (pH 5 or below) is stable for many days in 6 M guanidine hydrochloride.

Reactions of Pyridoxal Sulfate with β-Substituted Amines. To obtain some clues about the chemistry of the interaction with apoAAT pyridoxal sulfate was mixed in aqueous solution with a variety of substances. These included amino acids and other compounds containing functional groups of amino acid side chains. Of these, a surprising variety of amino acids and amines with nucleophilic β substituents reacted. Cysteine, β-mercaptoethylamine (cysteamine), ethylenediamine, ethanolamine, serine, and arginine all produced spectral changes strikingly similar to those seen with the enzyme. In each case, a final spectrum with a relatively narrow band, noticeable vibronic fine structure, and an absoprtion maximum at 24.9–25.1 kK was obtained (Figure 2). On the other hand, butylamine and valine gave Schiff bases entirely comparable to those formed with pyridoxal-P and other related aldehydes and completely lacking characteristic fine structure (Figure 2). Imidazole did not react at all and histidine formed a stable cyclic tetrahydropyridine adduct similar to that observed with pyridoxal-P (Abbott and Martell, 1970). β-Mercaptoethanol reacted rapidly to give a compound with an absorption maximum at 31.4 kK (318 nm). This is probably a hemimercaptal (Bergel and Harrap, 1961). No further reaction was evident.

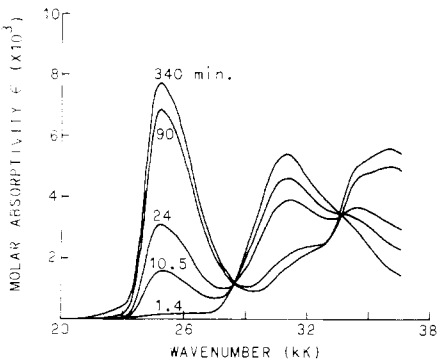

FIGURE 4: Reaction of pyridoxal sulfate (10^{-4} M) with cysteine (0.1 M) at pH 9.0. Spectra are shown for the times in minutes indicated on the figure. At much longer times a slow decay of the 24.8-kK peak took place.

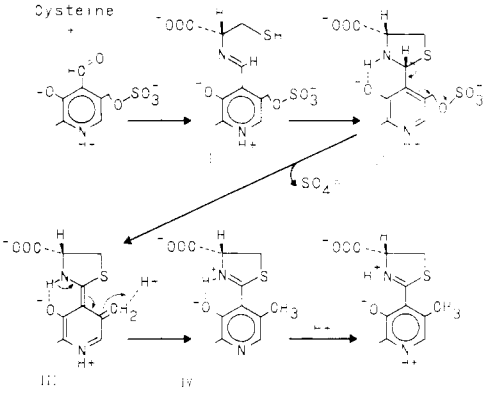

FIGURE 5: Scheme showing proposed reaction mechanism for cysteine and pyridoxal sulfate. It is proposed that at the active site of apoAAT some other nucleophilic group X reacts with a Schiff base of pyridoxal sulfate and the active site lysine initiating a similar reaction sequence.

Spectral changes during the reaction with cysteine at pH 9 are shown in Figure 4. Initially a peak is formed at 31.0 kK (323 nm), apparently representing a thiazolidine (II, Figure 5), similar to that observed as a product of the reaction of pyridoxal phosphate and cysteine (Heyl *et al.*, 1948; Buell and Hansen, 1960). Presumably a Schiff base (I) is an intermediate in formation of II (Figure 5).

At pH 9 the thiazolidine adduct converted rapidly to the 24.9-kK product with a half-time of about 30 min (Figure 4). Like the product obtained with the enzyme the absorption band of the product is narrow (W = 2.71 kK, ρ = 1.80) and displays the same type of distinctive fine structure (Figure 2). Furthermore, upon acidification the absorption maximum shifts to 26.09 kK (W = 3.65 kK, ρ = 1.65) as does the product with the enzyme. However, with cysteine isosbestic points were obtained with spectra at intermediate pH values. An apparent pK_a of 6.1 was estimated. At high pH the peak shifts to ~27.5 kK with $pK_a \approx 12.25$. This behavor in basic solution is strikingly similar to that of the enzyme.

The fact that pyridoxal sulfate reacts in an unexpected way with both apoAAT and cysteine, while pyridoxal-P does not, suggested that inorganic sulfate might be released during the reaction. To test this idea free sulfate was determined nephelometrically after conversion to a barium sulfate suspension. At the same time the absorbance at 24.8 kK was monitored. The results leave no doubt that sulfate is released during the reaction of pyridoxal sulfate and cysteine. The rate of sulfate formation closely paralleled the increase in absorption at 24.8 kK (Figure 6). Moreover, by comparison with the standard curve, the total amount of sulfate released was almost equal to that originally present in the pyridoxal sulfate. This result was verified using the barium chloroanilate method for sulfate determination (Figure 6).

The elimination of sulfate would be expected to produce a quinonoid intermediate (III) which could tautomerize to product IV, a cyclic substituted Schiff base (Figure 5). The formation of an intermediate, as well as the elimination of sulfate from the 5′ carbon, was verified by nmr measurements (Figure 7). The nmr spectrum of the thiazolidine adduct obtained from pyridoxal-P plus cysteine has been reported by Abbott and Martell (1970). We have verified their spectrum and note that the 100-MHz nmr spectrum obtained a few minutes after mixing pyridoxal sulfate with cysteine (Figure 7, top curve) is very nearly the same. From left to right the major peaks represent the 6-H, 4′-H, 5′-CH_2, the α-H and β-CH_2 (multiplets) from the cysteine residue, and the 2-CH_3. Over a period of time, the 4′-CH peak completely disappeared as predicted by the reaction scheme of Figure 5. At the same time, the 5′-CH_2 peak diminished at almost the same rate. Although that peak does not appear to disappear completely, more careful inspection shows that the peak present in the final spectrum is shifted upfield slightly. It doubtless represents an unidentified side product, perhaps one in which displacement of sulfate from the CH_2 has taken place. At an early time, two new peaks appear downfield from the position of the original 6-H. One of these represents the transient intermediate and the one farthest to the left the final product. It is also clear that other transient products arise and that some side products remain at the end of the reaction.

At the same time that the 5-CH_2 peak disappears the 2-CH_3 peak drops and reappears about 5–6 Hz upfield. We associate this peak with the 2-methyl group of the final product. At first glance, the 2-CH_3 peak of the starting material does not appear to completely disappear but closer examination shows that the peak downfield from the 2-CH_3 of the product is also displaced about 2 Hz from the position of the original CH_3. We believe that this downfield CH_3 peak represents the 5′-CH_2D present in the final product. The height of this peak appears to be somewhat lower than expected, perhaps as a result of exchange reactions. In addition, there is a minor peak on the upfield side of this group of methyl peaks. Another clear change in the spectrum is the loss of the peaks of the α proton and simplification of the β-CH_2 pattern.

While the nmr spectral changes are complicated by formation of side products they strongly substantiate the chemistry suggested. The appearance of a 6-H peak that arises early in the experiment and then decays supports the existence of the proposed quinonoid intermediate (III).

Reactions of other β-substituted amines with pyridoxal sulfate have been investigated spectrophotometrically. Like cysteine, β-mercaptoethylamine reacts to give a product of $\tilde{\nu}_0$ = 25.08 kK and W = 2.76 kK with fine structure. However, a mixture of β-mercaptoethanol plus butylamine did not give a comparable product. Ethylenediamine formed a transient Schiff base at pH 7 and slowly, over a period of 0.5 hr, gave a product with $\tilde{\nu}_0$ = 25.10 kK and W = 3.13 kK. Serine, at pH 7, gave a typical Schiff base with a broad peak at 24.2 kK. This compound was stable at pH 7 but at pH 9 the absorption band narrowed to give a peak at 24.8 kK with characteristic fine structure. A similar reaction occurred with ethanolamine ($\tilde{\nu}_0$ = 25.17 kK). Thus in all these cases, essentially the same kind of reaction appears to take place.

At pH 7, arginine reacted with pyridoxal sulfate instantaneously with formation of a peak at 24.0 kK (Schiff base) which shifted, over a 3-day period, to 31 kK, a position similar to that of thiazolidines and other adducts. Formation of a

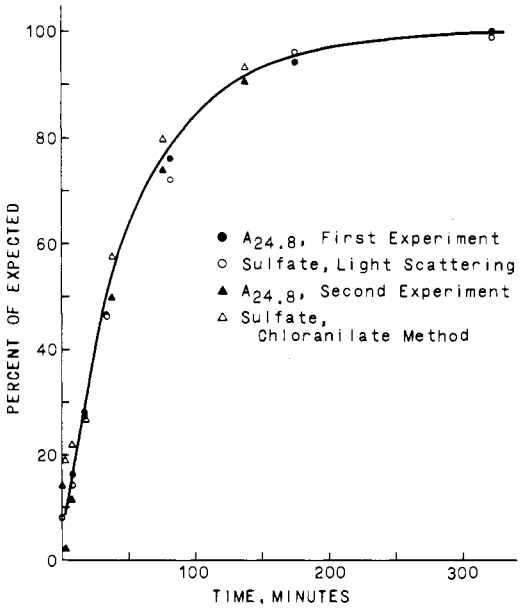

FIGURE 6: Sulfate release during the reaction of pyridoxal sulfate and cysteine. The results are plotted as a percentage of the expected release for 100% reaction of the 1.13 mM pyridoxal sulfate and 0.1 M L-cysteine at pH 9.0. Also plotted is the absorbance at 25.8 kK as a percentage of the final (constant) absorbance at that wave number.

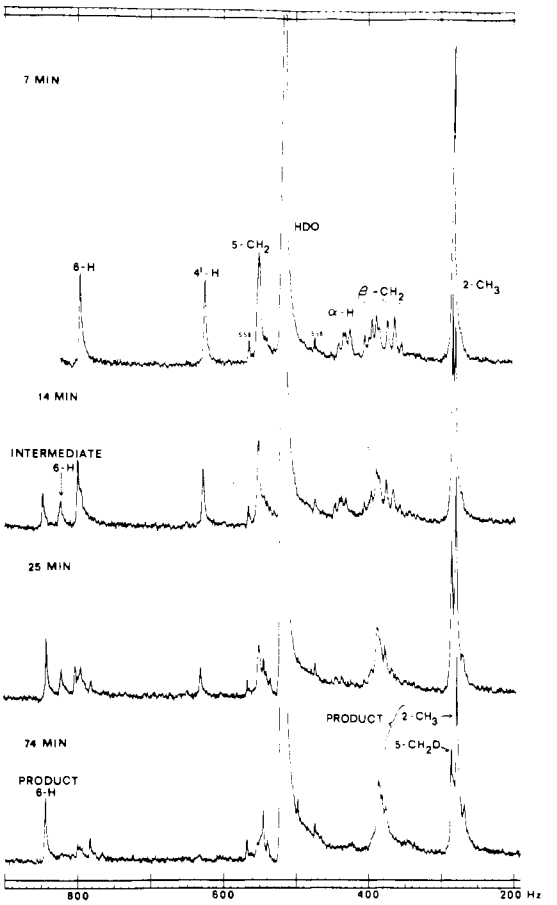

FIGURE 7: The 100-MHz nmr spectrum of an equimolar (0.1 M) solution of pyridoxal sulfate and L-cysteine in D_2O at various times. The observed pH was 8.6. The Varian HA-100 spectrometer was used with Me_4Si as an external standard. The assignments for the initially formed thiazolidine are indicated on the upper curve. The 6-H peak of the intermediate is indicated on the second. Several peaks of the product believed responsible for the 24.8-kK absorption band are indicated at the bottom. Notice that the left end of the first curve was inadvertently omitted. However, replicate experiments with the A-60 spectrometer showed clearly that initially little or none of the 6-H peaks attributed to the intermediate and product were present. Other features of the spectra were identical with those shown here.

Schiff base was also observed at pH 9, but over a period of time the peak moved from 24.0 to 24.93 kK. The band narrowed to W = 2.96 kK and the characteristic fine structure as seen with the enzyme appeared (Figure 2). Arginine also reacted with pyridoxal-P giving first a Schiff base, then a band at about 31 kK which remained stable. Thus arginine appears to form some kind of cyclic adduct with pyridoxal-P, a fact that seems generally to have been overlooked. Nmr investigations also suggest formation of an adduct, and we are investigating the chemistry of this reaction further.

Discussion

The experiments described show that pyridoxal 5′-sulfate reacts with cysteine with accompanying formation of inorganic sulfate. A reasonable mechanism is indicated in Figure 5. The elimination of the benzylic sulfate is not surprising. While the reaction mechanism proposed seems likely final proof will rest on isolation and characterization of crystalline products containing the new chromophore. The reactions of pyridoxal-P and pyridoxal sulfate with arginine also remain to be elucidated.

The reaction of pyridoxal sulfate with the apoenzyme of aspartate aminotransferase closely resembles the reactions with cysteine, other β-substituted amines, and with arginine. However, the reaction with the enzyme must differ in some ways from the model reaction. The N-terminal alanine could not be the site of reaction. Since reaction with pyridoxal sulfate inactivates the enzyme and prevents binding of pyridoxal phosphate it is probable that the coenzyme analog does react at the active site. From Figure 1 it is clear that a Schiff base is formed initially. It is attractive to think that some group X, held by the enzyme in close proximity to the imine linkage, adds to the C=N bond, just as does the -SH group of cysteine. Further reactions paralleling those shown in Figure 5 could then take place. While we have no direct verification that sulfate is eliminated in the enzymic reaction, the fact that the reaction occurs with pyridoxal sulfate and not with pyridoxal-P strongly suggests that this is the case.

A prime question is, "what is group X in the enzyme?" Attempts are in progress to enzymically digest the low pH form of the product of the reaction of enzyme and pyridoxal sulfate and to isolate a peptide fragment containing the bound chromophore.

The new reaction is at least somewhat specific for aspartate aminotransferase. For example, pyridoxal sulfate reacts with ribonuclease to form a stable Schiff base with essentially the same properties as that formed with pyridoxal-P (Raetz and Auld, 1972) but it does not give the 24.9-kK band. Because of this specificity pyridoxal sulfate may be a useful reagent for the investigation of a variety of enzymes and other proteins.

Acknowledgments

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Substrate Specificity and Mechanism of Action of Acetoacetate Coenzyme A Transferase from Rat Heart†

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ABSTRACT: The specificity of succinyl-CoA:acetoacetate CoA transferase (as a partially purified preparation from rat heart) was examined by employing various analogs of succinate. Diacids with a connecting chain length of ≥3 methylene groups are inactive; oxalate and malonate are competitive inhibitors (K_i = 15 and 21 mM, respectively). Analogs with substitution into the ethylene group of succinate are generally inactive, except for inhibition by 2,2-difluorosuccinate and perfluorosuccinate (K_i = 6.4 and 18 mM, respectively). 3-Sulfopropanoate and compounds with substitution into one of the carboxyl groups (monomethyl succinate, succinamate, maleamate, and *N*-ethylmaleamate) also inhibit competitively (with K_i values from 11 to 33 mM), but do not serve as substrates. Only maleate proved to be a substrate (K_m = 35 mM *vs.* a K_m value for succinate of 28 mM) with a V_{max} one-ninth of that for succinate. Fumarate is ineffective; acetylenedicarboxylate weakly inhibits. The mechanistic implication of these observations is that a cis, coplanar relationship between two proximal carboxyl groups is essential in a substrate molecule in order to have nucleophilic attack by the reactive carboxyl group on the enzyme thiol ester intermediate (with coenzyme A). Finally, usage of acetoacetate is inhibited competitively by succinate and maleate; the other inhibitors to succinate display mixed-type inhibitions for acetoacetate, indicating their potential usefulness for studies on the metabolism of ketone bodies.

For the complete utilization of ketone bodies in mammalian tissues acetoacetate must be converted to the activated coenzyme A derivative. The enzyme succinyl-CoA:acetoacetate CoA transferase performs this function in nonhepatic tissues by means of catalyzing the reaction between acetoacetate and succinyl-CoA (Jencks, 1973). Jencks and his colleagues have unequivocally shown, using kinetic (Hersh and Jencks, 1967) and chemical (Solomon and Jencks, 1969) methods, that the reaction catalyzed by the purfied pig heart enzyme proceeds *via* an enzyme-CoA intermediate. Stern and coworkers have demonstrated the high degree of specificity of the pig heart enzyme with respect to the substrate succinate, finding that only malonate was able to serve as a substrate (Stern *et al.*, 1956; Menon and Stern, 1960). Starting with these observations we have extended the specificity studies using a greater number of succinate analogs with the CoA transferase isolated from rat heart mitochondria. The objectives of these studies have been to gain some added insight into the mechanistic details of the

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