Pyridoxal Catalysis in the α,β-Elimination of S-(p-Substituted phenyl)cysteines[1]

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The pyridoxal-catalyzed α,β-elimination of S-(p-substituted phenyl)cysteines was studied at 50 °C and μ= 0.10 (KNO_3) in 79% (v/v) aqueous acetonitrile in the presence of organic bases of different strength. The rate increased with an increase in basicity. The kinetic solvent isotope effect (k_{H_2O}/k_{D_2O}) of 1.15 is consistent with the reaction mechanism in which the α-proton abstraction occurs by the direct attack of a base on the proton. The elimination rate was insensitive to a change in the electronic nature of a leaving thiophenol, indicating that the α-proton abstraction can be referred to the rate-determining step. The rate showed a considerable dependence on the water content, as reflected on the activation parameters for reactions in 79% (v/v) aqueous acetonitrile as well as in water. The larger destabilization due to water-solvation at the transition state in the aqueous reaction system seems to cause a lower reactivity relative to the organic solvent system. The catalysis of benzene analogs demonstrated that the hydroxyl group adjacent to the aldehyde in pyridoxal plays a major role in the nonenzymatic α,β-elimination reactions.

Pyridoxal-dependent enzymes catalyze various metabolic reactions of amino acids such as transamination, decarboxylation, and elimination reactions. Pyridoxal alone can go through some of these reactions in cooperation with metal ions.[2] The reaction mechanism for the pyridoxal-metal ion catalysis of the α,β-elimination reaction of O-phosphothreonine was elucidated in a previous paper.[3] Nonenzymatic pyridoxal-catalyzed reactions in the absence of metal ions, on the other hand, have been studied only to a limited extent partly because of the poor reactivity of amino acids in such systems.

Recently, De Marco and Rinaldi reported that pyridoxal phosphate catalyzes the α,β-elimination reactions of some cysteine derivatives, cystathionine and lanthionine, at 38 °C in weakly alkaline media.[4] S-Phenylcysteine itself was deaminated only at high temperature (100 °C) in an alkaline solution in the absence of both pyridoxal and metal ions.[5] However, no mechanistic elucidation was attempted in those studies. We have found that S-(p-substituted phenyl)-cysteines decompose by pyridoxal catalysis under comparatively mild conditions. Since S-substituted cysteines can be the substrates for the tryptophanase catalysis,[6] this work may provide a valuable information for elucidation of the enzymatic mechanism and for devising effective apoenzyme models.

Experimental

Materials. S-(p-Substituted phenyl)cysteines were prepared by the reactions of the corresponding thiophenols with α-acetoamidoacrylic acid and the subsequent acid hydrolysis.[7] 4-Nitrosalicylaldehyde was synthesized according to the procedure given in the literature.[8] Pyridoxal hydrochloride was obtained from Mann Research Laboratories, Inc., New York, U.S.A. and deuterium oxide (>99.7%) was from Carl Roth OHG, Karlsruhe, West Germany. The other commercial reagents were purified before use when necessary.

Kinetic Measurements. The reaction rate was followed by determining pyruvate as the 2,4-dinitrophenylhydrazone. The analytical method was essentially based on that of Metzler and Snell.[9] A solution containing 0.44×10^{-3} M substrate, 1.00×10^{-3} M pyridoxal, 0.10 M potassium nitrate, and a specified amount of a base was placed in stoppered test tubes which were then immersed in a constant temperature bath at 50.0±0.1 °C. The test tubes were taken out one by one at appropriate time intervals. A 5-ml sample from each test tube was transferred to a 25-ml volumetric flask containing 2 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M hydrochloric acid, and then allowed to stand at room temperature for 30 min. To this mixture was added 10 ml (or less for high concentrations of piperazine and piperidine) of the potassium phosphate–sodium hydroxide buffer, which was prepared by dissolving potassium dihydrogen phosphate (5.4 g), disodium hydrogen phosphate dodecahydrate (21.4 g), and sodium hydroxide (8.0 g) in 500 ml of water. The whole volume was diluted with water to 25 ml. The precipitated pyridoxal hydrazone was filtered off with fluted filter paper. The filtrate was treated with 10 ml of toluene to remove excess 2,4-dinitrophenylhydrazine. A 15-ml sample solution was taken out from the lower aqueous layer and diluted to 25 ml with 2.5 M sodium hydroxide. The absorbance at 530 nm was measured within 30 min on a Hitachi EPS-2 recording spectrophotometer.

Product Analysis. A reaction mixture (*ca.* 100 ml) containing 1.00×10^{-3} M pyridoxal, 0.44×10^{-3} M S-(p-nitrophenyl)cysteine, and 0.05 M morpholine in 79% (v/v) aqueous acetonitrile was warmed at 50 °C for 3 hr. A portion of the mixture was taken out for the analysis of p-nitrothiophenol and its oxidized product, bis(p-nitrophenyl) disulfide. When assayed by the conventional Folin method,[10] the sample solution developed a blue color characteristic of the reduced phosphotungstate complex. The nitrogen gas was bubbled through the rest of the reaction mixture for 30 min after addition of 10% sodium hydroxide (*ca.* 10 ml). Ammonia collected in 0.05 M hydrochloric acid was assayed by standard Nesslerization. Formation of pyruvic acid was detected by means of visible spectroscopy upon converting it into the corresponding 2,4-dinitrophenylhydrazone. In reference to the above survey, the decomposition of S-(p-nitrophenyl)-cysteine yielded three products, p-nitrothiophenol, pyruvic acid, and ammonia.

Results

The α,β-elimination reactions of S-(p-substituted phenyl)cysteines were carried out with an initial sub-

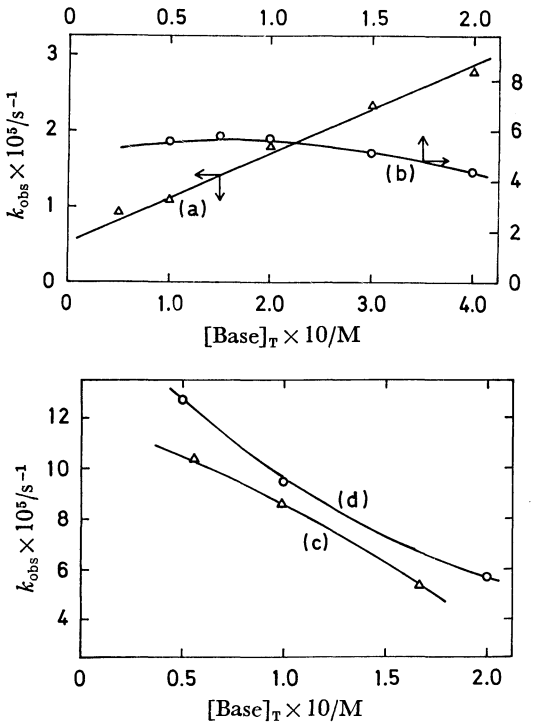


Fig. 1. Correlations between base concentration and apparent first-order rate constant for the pyridoxal-catalyzed α,β-elimination of *S*-(*p*-nitrophenyl)cysteine at 50 °C and $\mu=0.10$ (KNO_3) in 79% (v/v) acetonitrile with the following organic bases: (a), imidazole; (b), morpholine; (c), piperazine; (d), piperidine. Total initial concentrations: substrate, 0.44×10^{-3} M; pyridoxal, 1.00×10^{-3} M.

strate concentration of 0.44×10^{-3} M along with the presence of 1.00×10^{-3} M pyridoxal and a specified amount of organic bases in aqueous acetonitrile. Each reaction rate followed first-order kinetics with respect to the total concentration of unreacted substrate. The correlations between the apparent first-order rate constant and the total base concentration are shown in Fig. 1, *S*-(*p*-nitrophenyl)cysteine being adopted as a substrate. The rate increases as the imidazole concentration increases, but is insensitive to the change of morpholine concentration in the concentration range examined. Increase in either piperazine or piperidine concentration results in the rate decrease. Since the substrates underwent no decomposition in the absence of pyridoxal, the reaction was truly pyridoxal-catalyzed. The product analysis (see Experimental) provides an evidence for the α,β-elimination process.

Rate constants for the pyridoxal-catalyzed elimination of *S*-(*p*-substituted phenyl)cysteines in 79% (v/v) acetonitrile in the presence of 0.10 M morpholine were plotted against Hammett σ-values for the *p*-substituents of the leaving thiophenols (Fig. 2); the slope being 0.17. This small value of the reaction constant implies that the β-elimination is hardly affected by the electronic nature of the leaving group.

The apparent first-order rate constant for the pyridoxal-catalyzed elimination of *S*-(*p*-nitrophenyl)cysteine in the presence of 0.10 M morpholine in 79% (v/v) aqueous acetonitrile was 5.67×10^{-5} s^{-1} and the corresponding value in acetonitrile–deuterium oxide (79: 21 by volume) was 4.93×10^{-5} s^{-1}; the solvent isotope effect (k_{H_2O}/k_{D_2O}) is 1.15.

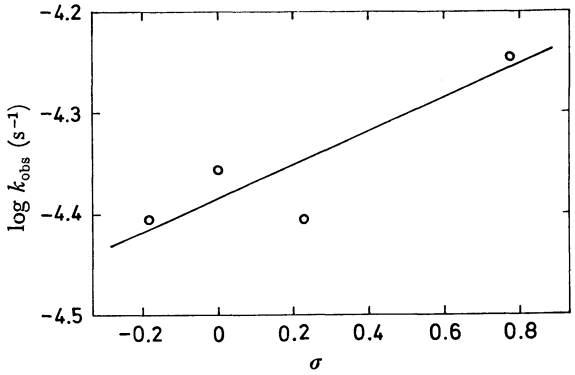


Fig. 2. Hammett plot for the pyridoxal-catalyzed α,β-elimination of *S*-(*p*-substituted phenyl)cysteines at 50 °C and $\mu=0.10$ (KNO_3) in 79% (v/v) acetonitrile with the total initial concentrations: substrate, 0.44×10^{-3} M; pyridoxal, 1.00×10^{-3} M; morpholine, 0.10 M.

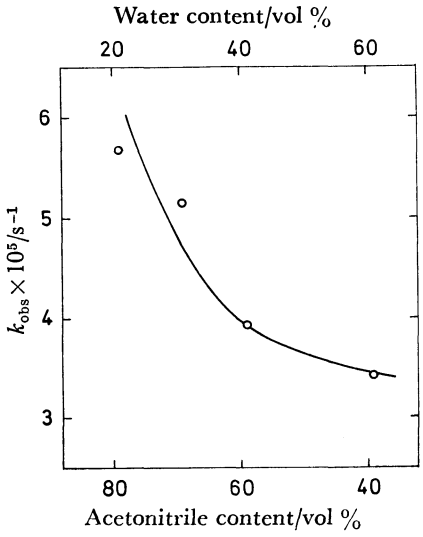


Fig. 3. Correlation between water content and reaction rate for the pyridoxal-catalyzed α,β-elimination of *S*-(*p*-nitrophenyl)cysteine at 50 °C and $\mu=0.10$ (KNO_3) with the total initial concentrations: substrate, 0.44×10^{-3} M; pyridoxal, 1.00×10^{-3} M; morpholine, 0.10 M.

The correlation between elimination rate of *S*-(*p*-nitrophenyl)cysteine and solvent composition was also examined (Fig. 3). The rate decreased considerably with the increase in water content. Activation parameters for the pyridoxal-catalyzed elimination of *S*-(*p*-nitrophenyl)cysteine and *S*-phenylcysteine in 79% (v/v) aqueous acetonitrile as well as in water are listed in Table 1. The activation entropy of large negative value is in line with the bimolecular nature of the reactions.

Catalytic activities of the benzene analogs of pyridoxal were investigated in order to clarify the catalytic roles of the ring-substituents of pyridoxal (Table 2). 4-Nitrosalicylaldehyde showed a catalytic activity to a similar extent. While 4-nitrobenzaldehyde, bearing no *o*-hydroxyl group, was practically inactive, salicylalde-

Table 1. Activation parameters for the pyridoxal-catalyzed α,β-elimination reactions of S-phenyl- and S-(p-nitrophenyl)cysteine in 79% (v/v) acetonitrile and in water[a)]

Substrate	Solvent	E_a kcal mol^{-1}	$\Delta H^{\ddagger}$[b)] kcal mol^{-1}	$\Delta S^{\ddagger}$[b)] e.u.
S-Phenylcysteine	79% (v/v) Acetonitrile	10.2	9.5	−50
S-(p-Nitrophenyl)-cysteine	79% (v/v) Acetonitrile	9.9	9.3	−49
S-(p-Nitrophenyl)-cysteine	Water	13.2	12.5	−41

a) Reactions were carried out at 40, 50, and 60 °C, and μ=0.10 (KNO_3) in the presence of 0.10 M morpholine. b) Values at 50 °C.

Table 2. Catalytic activities of pyridoxal and its benzene analogs in α,β-elimination of S-(p-nitrophenyl)cysteine at 50 °C and μ=0.10 (KNO_3) in 79% (v/v) aqueous acetonitrile[a)]

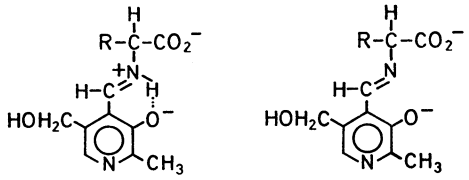
Catalyst	Pyridoxal	4-Nitro-salicyl-aldehyde	4-Nitrobenz-aldehyde	Salicyl-aldehyde
$k_{obs}\times 10^5$/s^{-1}	5.67	5.92	~0	~0.8[b)]

a) Initial total concentrations: substrate, 0.44×10^{-3} M; catalyst, 1.00×10^{-3} M; morpholine, 0.10 M. b) Since the rate showed positive deviation from the first-order plot, the rate constant was estimated from the initial portion of the reaction.

hyde, lacking an electron-withdrawing nitro group, showed some catalytic effect.

Discussion

Reaction Mechanism. The pyridoxal-dependent enzymes may involve various functional groups of nucleophilic and base character in their apoenzyme moieties in a manner as approved for proteinases. The catalytic activity of the latter is provided by the concerted actions of those functional groups at their active sites. In the present study, the catalytic effect of some organic bases of different base-strength was examined in the pyridoxal-catalyzed elimination reaction in connection with the catalytic nature of base moieties which are planted in an apoenzyme skeleton. The rate constants are plotted against the pK_a of the conjugate acids of organic bases obtained in water (Fig. 4), the base concentration for the reaction being maintained at 0.10 M. The rate increases as the basicity of the organic bases rises from imidazole to morpholine, but a leveling-off trend is observed beyond p$K_a\cong$9, *i.e.*, for piperazine and piperidine. The Schiff base formed between pyridoxal and an amino acid can be present in two forms in an alkaline solution, the protonated species (**1**) and the deprotonated one (**2**)



1 2

at the azomethine nitrogen. The former species pyridoxylidenevaline absorbs at 414 nm and the latter at 367 nm in aqueous media.[11)] The corresponding absorption maxima for the same Schiff base in methanol are 418 and 373 nm, respectively.[12)] The pK_a for these protonated aldimines is *ca.* 10 in aqueous phase.[3)] The visible spectra for solutions containing 1.00×10^{-3} M pyridoxal, 1.00×10^{-3} M phenylalanine (pseudo-substrate), and 0.10 M organic base showed λ_{max} (in 79% (v/v) CH_3CN) at 423 nm with imidazole and morpholine, at 382 nm with piperidine, and at the intermediate region with piperazine. This blue shift on going from weak to strong in basicity can be attributed to the deprotonation from the azomethine nitrogen. The deprotonated form can give out a weaker electron-withdrawing effect at the α-carbon, which results in the reduction of reactivity of the amino acids. Thus, the nonlinear Brønsted correlation (Fig. 4) can be interpreted in terms of these protonation-deprotonation equilibria.

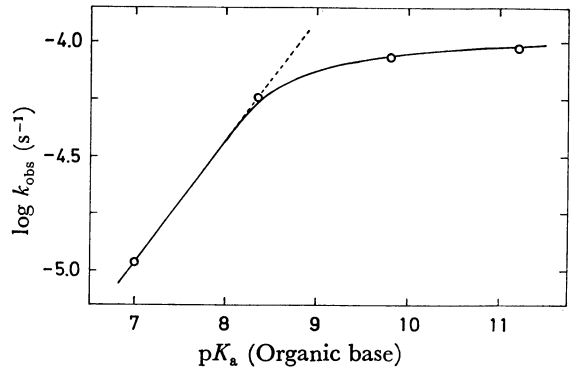
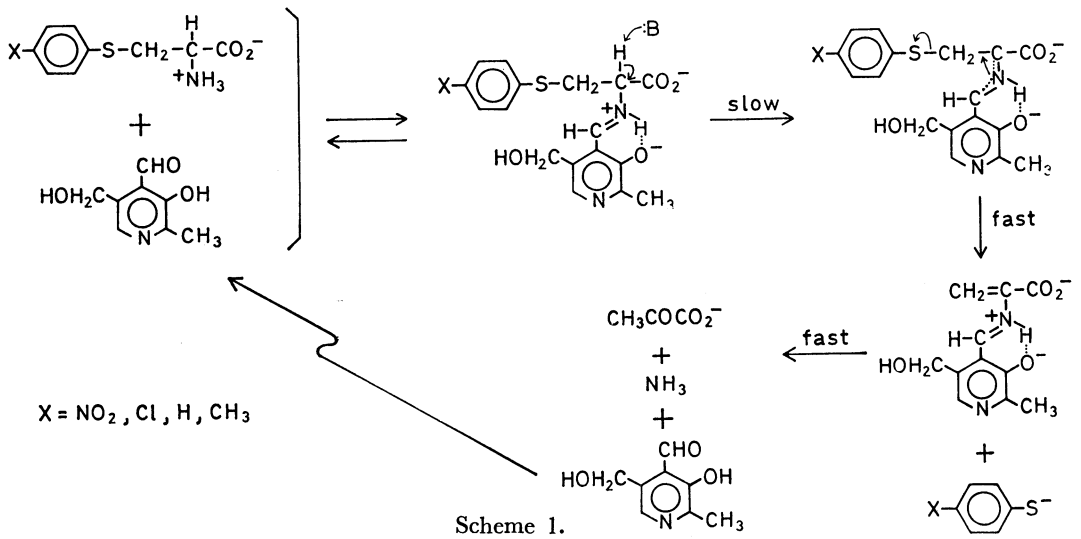


Fig. 4. Brønsted plot for the pyridoxal-catalyzed α,β-elimination of S-(p-nitrophenyl)cysteine at 50 °C and μ=0.10 (KNO_3) in 79% (v/v) acetonitrile with the initial total concentrations: substrate, 0.44×10^{-3} M; pyridoxal, 1.00×10^{-3} M; organic base, 0.10 M. pK_a-Values in aqueous phase have been adopted for organic bases.

The bimolecular attack by an organic base, which can be referred to the α-proton abstraction in the rate-determining step, is reflected on the activation entropy by a large negative value. The problem remains as to whether the α-proton abstraction occurs by the direct attack of a base on the proton or in the presence of a water molecule between them. The small solvent isotope effect (k_{H_2O}/k_{D_2O}) seems to support the former mechanism.

The reaction rate is insensitive to the electronic nature of a leaving thiophenol as can be seen from the Hammett plot (Fig. 2). If the C–S bond fission were to occur in the rate-determining step through either E1 or E1CB mechanism, a larger dependence on the substituent constant would be expected. A comparatively small activation enthalpy also suggests the C–H bond cleavage in the rate-determining step.

On the basis of all the evidences given above, the

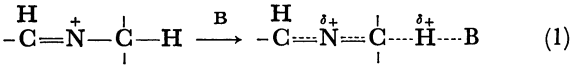


Scheme 1.

most plausible mechanism for the present reaction is as shown in Scheme 1. The Schiff base formation between substrate and pyridoxal occurs in the initial stage as the pre-equilibrium step[3,11] prior to the rate-determining α-proton liberation.

Solvent Effect. In order to observe various reactions of amino acids proceeding to a detectable extent in the absence of any apoenzyme moiety, pyridoxal catalysis requires either an elevated temperature or the presence of metal ions. In organic solvent system, however, these reactions often proceed readily at room temperature even in the absence of metal ions; the transamination from pyridoxamine to α-keto acids and the reverse reaction occurs readily in absolute ethanol without any metal ion.[13] The nonenzymatic reaction between pyridoxamine and α-ketoisovalerate was found to proceed quite smoothly at room temperature in methanol through the cooperation of zinc(II) ion.[14] Our results (Fig. 3) indicate that the α,β-elimination rate depends on the water content of the solvent system in accord with the related reactions of amino acids. The Schiff base formation is generally much favored in organic solvents. However, the rate enhancement due to the solvents cannot be explained in terms of this effect alone, since the Schiff base formation has nothing to do with the rate-determining step.

Alternatively, solvation effects seem to be responsible for the rate acceleration in organic solvents, as evident from the activation parameters listed in Table 1. Since the reaction seems to proceed through the less polar transition state (Scheme 1) relative to the initial one due to the delocalization of a positive charge as represented by Eq. (1), the reaction center may be solvated by organic solvent molecules more effectively and water molecules would be less tightly bound to the center.



(1)

Stabilization of the transition state becomes much greater in 79% (v/v) acetonitrile than in the aqueous system. Contrarily, the polar initial state, due to a positive charge located at the Schiff base nitrogen, can be solvated by water molecules more tightly than by organic solvent molecules. Thus, the initial state would be more stabilized in water than in 79% (v/v) acetonitrile. These effects give rise to a larger activation enthalpy for the aqueous reaction system. This solvation effect is also reflected on the activation entropy values. These features would be more drastic in a higher acetonitrile content, but the lower solubility of the substrates kept us from carrying out the experiments.

Catalysis by Pyridoxal Analogs. In order to clarify the catalytic roles of various functional groups placed in pyridoxal, the catalytic activities of its benzene analogs were examined as listed in Table 2. As a result, the presence of the hydroxyl group *ortho* to the aldehyde is of primary importance for yielding catalytic activity, even though an additional electron-withdrawing group, the nitro group or the pyridyl nitrogen, accelerated the reaction rate to a greater extent. For the noenzymatic transamination reaction between glutamic acid and 3-hydroxypyridine-4-carbaldehyde, Thanassi *et al.* proposed that the general acid catalysis of the hydroxyl group acting on the azomethine bond facilitated the isomerization of this bond.[15] For the present elimination reaction, however, there is no need for such isomerization to be involved and another explanation must be invoked for the role of the hydroxyl group.

The Schiff base formed between pyridoxal and amino acid is known to possess a six-membered ring structure by a strong intramolecular hydrogen bonding (Scheme 1). Thus, the stability of the Schiff base may be greatly reduced by the deprotonation from the azomethine nitrogen or the absence of a hydroxyl group. The deprotonation at the azomethine nitrogen gives out a reduced electron-withdrawing effect on the α-carbon. In conclusion, the hydroxyl group adjacent to the aldehyde in pyridoxal plays an indispensable role in the nonenzymatic α,β-elimination of amino acids.

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