Huxley, H. E. (1969), *Science 164*, 1356.
Krimm, S. J. (1962), *J. Mol. Biol. 4*, 528.
Lecocq, J., and Inesi, G. (1966), *Anal. Biochem. 15*, 160.
Lenormant, H., Baudras, A., and Blout, E. R. (1958), *J. Amer. Chem. Soc. 80*, 6191.
Lowey, S., and Risby, D. (1971), *Nature (London) 234*, 81.
Miyazawa, T. (1960), *J. Chem. Phys. 32*, 1647.
Miyazawa, T., and Blout, E. R. (1961), *J. Amer. Chem. Soc. 83*, 712.
Miyazawa, T., Fukushima, K., Sugano, S., and Masuda, Y. (1967), *in* Conformation of Biopolymers, Ramachandran, G. N., Vol. 2, New York, N. Y., Academic Press, p 557.
Mommaerts, W. F. H. M. (1966), *J. Mol. Biol. 15*, 377.
Oikawa, K., Kay, C. M., and McCubbin, W. D. (1968), *Biochim. Biophys. Acta 168*, 164.
Prior, M. G. M. (1950), *Progr. Biophys. 1*, 216.
Seidel, J. C. (1967), *J. Biol. Chem. 242*, 5623.
Susi, H. (1969), *in* Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, Chapter 7.
Suzuki, S., Iwashita, Y., Shimanouchi, T., and Tsuboi, M. (1966), *Biopolymers 4*, 337.
Timasheff, S. N., and Gorbunoff, M. J. (1967), *Annu. Rev. Biochem. 37*, 13.
Timasheff, S. N., and Susi, H. J. (1966), *J. Biol. Chem. 241*, 249.
Tonomura, Y., Appel, P., and Morales, M. F. (1966), *Biochemistry 5*, 515.
Tonomura, Y., Sekiya, K., Imamura, K., and Tokiwa, T. (1963), *Biochim. Biophys. Acta 69*, 305.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Properties of Crystalline Kynureninase from *Pseudomonas marginalis*†

Mitsuaki Moriguchi,‡ Tatsuo Yamamoto, and Kenji Soda*

ABSTRACT: The distribution of microbial kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) among various strains was investigated and *Pseudomonas marginalis* was found to have the highest activity of enzyme, which was induced by the addition of L-tryptophan to the medium. The kynureninase was purified and crystallized from *Ps. marginalis*. The purified enzyme is homogeneous by the criteria of ultracentrifugation ($s^0_{20,w}$ = 5.87 S) and disc gel electrophoresis. The mol wt is 100,000, assuming a partial specific volume of 0.74. The enzyme exhibits absorption maxima at 280, 337, and 430 mμ. No appreciable spectral change was observed on varying the pH between 5.4 and 9.0. The holoenzyme can be resolved to the apoenzyme by incubation with hydroxylamine, L-alanine, and L-ornithine, and reconstituted by the addition of pyridoxal 5′-phosphate. One mole of pyridoxal 5′-phosphate is bound per mole of enzyme. The formyl group of pyridoxal 5′-phosphate is bound in an aldimine link to the ε-amino group of a lysine residue of the protein. The enzyme exhibits maximum reactivity at about pH 8.0; it is stable over the pH range 5.8–8.0. Neither D-kynurenine nor *N*-formyl-L-kynurenine is hydrolyzed by the enzyme. The Michaelis constants were determined as follows: L-kynurenine, 3.5×10^{-5} M, and pyridoxal 5′-phosphate, 2.3×10^{-7} M (0.065 M Tris-HCl buffer, pH 8.0). The enzyme activity was inhibited by carbonyl reagents and thiol reagents.

Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) is the enzyme which catalyzes the hydrolysis of L-kynurenine to L-alanine and anthranilate. Since the first observation of kynureninase in a mammalian liver extract (Kotake and Nakayama, 1941), several reports on the enzyme have appeared. The enzyme was partially purified from a pseudomonad (Hayaishi and Stanier, 1951), *Neurospora crassa* (Jakoby and Bonner, 1953a,b), and rat liver (Knox, 1953), and characterized. It has been also demonstrated that pyridoxal-5′-P[1] is required as a coenzyme for the reaction (Braunstein *et al.*, 1949; Wiss, 1949). Turner and Drucker (1971) recently presented evidence that two forms of tryptophan-inducible kynureninase exist in *Neurospora crassa*, which differ in kinetic properties, and in the reaction to pyridoxal-5′-P. Gaertner *et al.* (1971) also reported the presence of two types of kynureninase, *i.e.*, kynureninase and hydroxykynureninase; hydroxykynureninase is not inducible by tryptophan, but the other is, and their kinetic properties differ strikingly in their response to L-kynurenine and L-3-hydroxykynurenine.

In an attempt to elucidate the properties of kynureninase, a unique pyridoxal-5′-P enzyme catalyzing the hydrolytic β,γ cleavage of aryl-substituted γ-keto-α-amino acids, we have purified the enzyme to homogeneity from *Pseudomonas marginalis*, and crystallized the enzyme by the addition of ammonium sulfate (Moriguchi *et al.*, 1971a,b). In this paper more detailed studies on enzymological and physicochemical properties of crystalline kynureninase are described.

Experimental Section

Materials. L-Kynurenine sulfate was synthesized from L-tryptophan by the method of Warnell and Berg (1954). *N*-Formyl-L-kynurenine was prepared from L-kynurenine with

† From the Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan. *Received April 2, 1973.* A preliminary report of part of this work has been presented (Moriguchi *et al.*, 1971a,b).

‡ Present address: Department of Food Science and Technology, Kyoto University, Kyoto 606, Japan.

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

TABLE I: Distribution of Kynureninase Activity in Various Strains.[a]

Strain	Sp Act. (Units/mg)
Aerobacter aerogenes (IFO 3320)	0.001
Bacillus cereus (IFO 3001)	0.004
Proteus vulgaris (IFO 3167)	0.001
Pseudomonas aeruginosa (IFO 3080)	0.009
aeruginosa (IFO 3924)	0.029
dacunhae (IAM 1048)	0.012
marginalis (IFO 3925)	0.069
polycolor (IFO 3918)	0.012
syncyanea (IFO 3757)	0.046
synxantha (IFO 3906)	0.022

[a] The crude enzyme was prepared essentially according to the purification procedure (step 1) described previously (Moriguchi *et al.*, 1971a,b), and dialyzed at 4° for 20 hr against 0.01 M potassium phosphate buffer (pH 7.2) containing 20 μM pyridoxal-5′-P and 0.01% 2-mercaptoethanol. The conditions of culture and enzyme assay are given in the Experimental Section. No activity was found in the following organisms: *Agrobacterium tumefaciens* (IAM 26-1), *Bacillus subtilis* var. *natto* Sawamura, (ICR B-1350), *B. roseus* Migula (IFO 3041), *B. sphaericus* (IFO 3525), *Bacterium mycoides* (IFO 3040), *B. cadaveris* (IFO 3731), *Brevibacterium ammoniagenes* (IFO 12072), *Corynebacterium sepedonicum* (IFO 3306), *Escherichia coli* 26 T (ICR B-0020), *E. coli* E 9 (ICR B-0040), *Pseudomonas fluorescens* (IFO 3461), *Ps. fragi* (IFO 3458), *Ps. iodinum* (IFO 3558), *Ps. ovalis* (IFO 3738), *Ps. riboflavina* Foster (IFO 3140), *Ps. segnis* (IAM 1311), *Ps. solanacearum* (IFO 3509), *Ps. striata* (ICR B-3200), *Saccharomyces cerevisiae* (IFO 0305), *S. fragilis* (IFO 0288), *S. marxianus* (IFO 0277), *Schizosaccharomyces liquefaciens* (IFO 0358), *Schwanniomyces occidentalis* (IFO 0371), *Candida utilis* (IFO 0396), *Cryptococcus albidus* (IFO 0378), *Debaryomyces globosus* (IFO 0016), *D. hansenii* (IFO 0023), *Endomyces hordei* (IFO 0104), *Hansenula wingei* (IFO 0976), *H. anomala* (IFO 0135), *H. beijerinckii* (IFO 0981), *H. jadinii* (IFO 0987), *H. matritensis* (IFO 0954), *Pichia polymorpha* (IFO 0195).

formic acetic anhydride according to the method of Dalgliesh (1952). Hydroxylapatite was prepared according to the method of Tiselius *et al.* (1956).

DEAE-cellulose was obtained from Midori Juji Co., Osaka, Japan, pyridoxamine-5′-P from Sigma, and Sephadex G-150 from Pharmacia, Uppsalla. Pyridoxal-5′-P and L- and D-amino acids were products of Kyowa Hakko Kogyo Co., Tokyo, Japan, and Ajinomoto Co., Tokyo, Japan, respectively. Pyridoxal-5′-P and pyridoxamine-5′-P were chromatographically purified by the procedure of Peterson and Sober (1954). The other chemicals were analytical grade reagents.

The solutions of reagents used for assay of the enzyme were adjusted to a pH of about 8 just before their use.

Concentrations of the following reagents were estimated by measuring the absorbance with their molar absorptivities: L-kynurenine, 4500 at 360 mμ (pH 7.0); *N*-formyl-L-kynurenine, 3750 at 321 mμ (pH 7.0) (Auerbach and Knox, 1957); pyridoxal-5′-P, 6600 at 388 mμ (0.1 N NaOH); pyridoxamine-5′-P, 8000 at 308 mμ (0.1 N NaOH) (Peterson and Sober, 1954).

Microorganisms and Conditions of Culture. Pseudomonas marginalis IFO 3925 and other organisms were grown in the medium containing 0.1% L-tryptophan as described previously (Moriguchi *et al.*, 1971a,b). The harvested cells were washed twice with 0.85% sodium chloride and subsequently with 0.01 M potassium phosphate buffer (pH 7.2). The usual yield of *Ps. marginalis* cells was approximately 4 g (wet weight)/l. of the medium. The washed cells were stored frozen at −20° until used.

Enzyme Preparation. The enzyme was purified about 130-fold from a cell-free extract of *Ps. marginalis* and obtained in a crystalline form as described previously (Moriguchi *et al.*, 1971a,b), but with higher yield (approximately 10%). The specific activity of the crystalline preparation was 9.52.

Assay of Kynureninase. The standard assay system consisted of 200 μmol of Tris-HCl buffer (pH 8.0), 1 μmol of L-kynurenine sulfate, 0.2 μmol of pyridoxal-5′-P, and enzyme in a final volume of 3.1 ml. L-Kynurenine was replaced by water in a blank. The activity of kynureninase was determined by measuring the rate of decrease in absorbance at 360 mμ due to hydrolysis of kynurenine. Incubation was carried out at 25° in a cuvet of 1-cm light path and started by the addition of L-kynurenine sulfate.

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951) using crystalline egg albumin as a standard; with most column fractions, protein elution patterns were estimated by the 280-mμ absorption. Concentrations of the purified enzyme were derived from the absorbance at 280 mμ. The extinction coefficient ($E^{1\%}_{1\,cm}$ = 14.3) was used throughout which was obtained by absorbance and dry weight determinations.

Definition of Units and Specific Activity. One unit of enzyme is defined as the amount of the enzyme that catalyzes the hydrolysis of 1 μmol of kynurenine/min. Specific activity is expressed as units/milligram of protein.

Spectrophotometry. Spectrophotometric measurements were made with a Shimadzu MPS-50L recording spectrophotometer or with a Carl Zeiss PMQ II spectrophotometer with a 1.0-cm light path. Fluorescence measurements were performed with a Hitachi MPF-2 recording spectrofluorometer.

Ultracentrifugal Analysis. The purity of the crystalline enzyme and its sedimentation coefficient were determined with a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm. The molecular weight of the enzyme was determined by the ultracentrifugal sedimentation equilibrium method according to the procedure of Van Holde and Baldwin (1958). The experiments were carried out in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Multicell operation was employed in order to perform the experiment on five samples of different initial concentrations ranging from 0.084 to 0.158% with the use of An-G rotor and double-sector cells of different side-wedge angles. The rotor was centrifuged at 10,589 rpm for 15 hr at 20°. Interference patterns were photographed with Fuji panchromatic process plates at intervals of 30 min to compare and make sure the equilibrium was established. The relation between the concentration of the enzyme and the fringe shift was determined using the synthetic boundary cell.

Results

Distribution of Kynureninase in Microbial Strains. Screening was carried out in order to determine bacterial and yeast strains that would produce a high activity of kynureninase (Table I). *Ps. marginalis* in which kynureninase occurs most

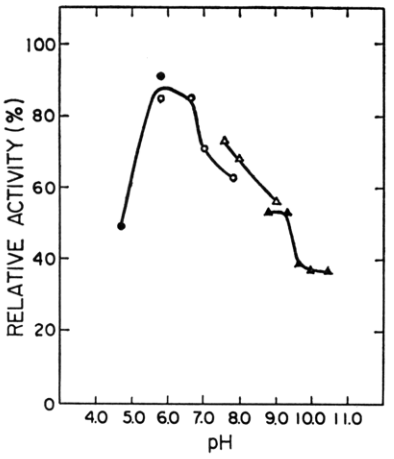

FIGURE 1: Effect of pH on the enzyme stability. The enzyme was heated at 50° for 5 min in 0.02 M buffers of the indicated pH. The buffers used were acetate (●) for pH 4.7–5.8, potassium phosphate (○) for pH 5.8–7.8, Tris-HCl (△) for pH 7.6–9.0, and glycine–NaOH (▲) for pH 8.8–10.5. The remaining activity was determined as described in the Experimental Section.

abundantly was chosen for the purpose of purification of the enzyme, though the considerably high activity was found also in *Ps. aeruginosa*, *Ps. syncyanea*, and *Ps. synxantha*. No activity of kynureninase was observed in all the strains of yeast tested.

Stability of Enzyme. The crystalline enzyme can be stored in 0.01 M potassium phosphate buffer, pH 7.2, containing 2×10^{-5} M pyridoxal-5′-P at 4° for 1 week with about 15% loss of activity. The enzyme is stable in a deep freeze (−20°), although repeated freezing and thawing cause a slight decrease in activity. The enzyme was found considerably stable in the pH range of 5.8–8.0, when heated at 50° for 5 min (Figure 1).

Purity and Molecular Weight. The crystalline enzyme (Figure 2) was shown to be homogeneous upon ultracentrifugation. The sedimentation coefficient of the enzyme, calculated for water at 20° and zero protein concentration, is 5.87 S. The molecular weight of the enzyme was determined by the sedimentation equilibrium method. Enzyme solutions at five different concentrations, 0.84, 1.05, 1.26, 1.40, and 1.58 mg/ml, were centrifuged at 20°. Assuming a partial specific volume of 0.74, a mol wt of 100,000 was obtained. The molecular weight of kynureninase was also determined by the Sephadex G-150 gel filtration method (Andrews, 1964, 1965). The enzyme was eluted with 0.01 M potassium phosphate buffer (pH 7.2) containing 0.1 M NaCl. The flow rate was 2 ml/hr and 2-ml aliquots of eluate were collected. The Sephadex G-150 column was standardized with catalase (mol wt 244,000), asparaginase of *Escherichia coli* (mol wt 140,000) (Nakamura *et al.*, 1971), serum albumin (mol wt 68,000), egg albumin (mol wt 45,000), and RNase I (mol wt 12,000). On the basis of the plots of the reduced elution volumes (V_e/V_0) *vs.* log mol wt, the mol wt of the enzyme was calculated to be 91,000.

The enzyme was also found homogeneous by disc gel electrophoresis as described previously (Moriguchi *et al.*, 1971a).

Absorption Spectrum of the Enzyme and Reduction with Sodium Borohydride. The absorption spectrum of kynureninase has maxima at 280, 337, and 430 mμ (curve A in Figure 3). No shift in the spectrum occurred by variations in pH between 5.4 and 9.0. The occurrence of the absorption peak at

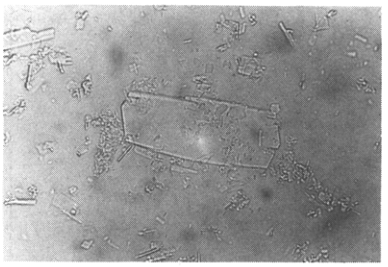

FIGURE 2: Crystals of kynureninase.

430 mμ suggests that the formyl group of the bound pyridoxal-5′-P forms an azomethine linkage with an amino group of the protein, as in other pyridoxal-5′-P enzymes thus far studied. Enzyme (1.6 mg) was treated with 5 mM sodium borohydride at about 5° for 10 min by the dialysis method of Matsuo and Greenberg (1959), and was then dialyzed for 18 hr against two changes of 1 l. of 0.01 M potassium phosphate buffer (pH 7.2). Treatment of sodium borohydride led to immediate bleaching of the yellow color of the enzyme solution coupled with a loss of the enzyme activity. Spectral measurements showed that the 430-mμ peak disappeared with an appearance of a new peak at 330 mμ (curve B in Figure 3). This peak did not change on further dialysis against 0.01 M potassium phosphate buffer (pH 7.2) for 18 hr. The presence or absence of pyridoxal-5′-P did not affect the catalytic inactivity of the reduced enzyme. These findings suggest that the borohydride reduces the aldimine linkage formed between the 4-formyl group of pyridoxal-5′-P and the amino group of the protein to yield the aldamine bond. An attempt was made to identify the amino acid residue with which pyridoxal-5′-P is bound in the enzyme. The enzyme (4.5 mg) reduced with sodium borohydride as described above was hydrolyzed in 6 N HCl at 105° for 24 hr in an evacuated and sealed tube. The hydrolysate was evaporated thoroughly to remove hydrochloric acid and the residue was dissolved in a small volume of water. An aliquot was analyzed with a Yanagimoto automatic amino acid analyzer, Model LC-5S. A column (0.9 × 10 cm) of Aminex A-5 was employed and

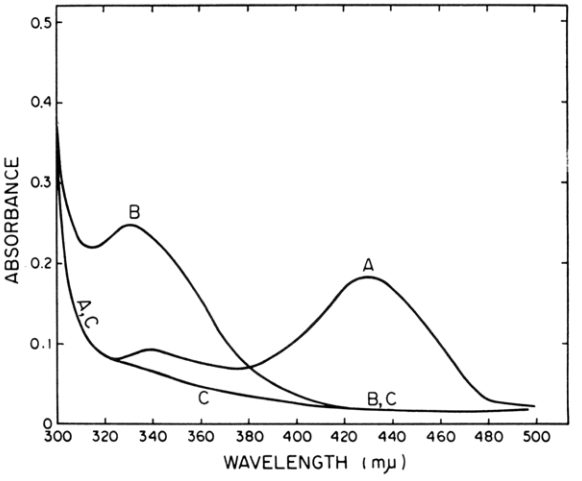

FIGURE 3: Absorption spectra of kynureninase: curve A, a 0.16% holoenzyme solution in 0.01 M potassium phosphate buffer, pH 7.2, was used; curve B, the same concentration of enzyme reduced with sodium borohydride and dialyzed was used; curve C, the same concentration of apoenzyme in 0.01 M potassium phosphate buffer, pH 7.2, was used. Other conditions are given in the text.

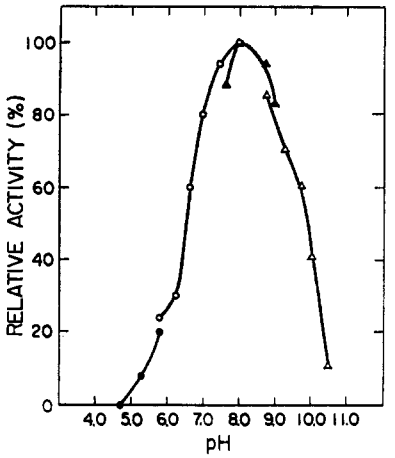

FIGURE 4: Effect of pH on kynureninase activity. The following buffers were used: acetate buffer (●) for pH 4.7–5.8; potassium phosphate buffer (○) for pH 5.8–8.0; Tris-HCl buffer (▲) for pH 7.6–9.0; glycine–NaOH buffer (△) for pH 8.8–10.5.

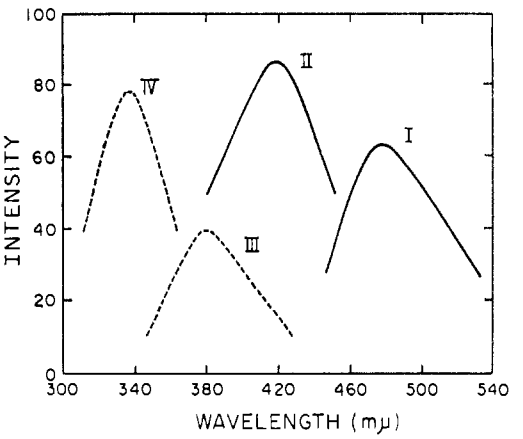

FIGURE 5: Fluorescence spectra of kynureninase. The enzyme was dissolved in 0.01 M potassium phosphate buffer, pH 7.2. Emission spectrum I was measured by exciting at 430 mμ. Excitation spectrum II was taken by analyzing at 480 mμ. Emission spectrum III was measured by exciting at 337 mμ. Excitation spectrum IV was taken by analyzing at 380 mμ.

the elution was carried out with 0.2 M citrate buffer (pH 4.25) for 60 min and then the buffer (pH 5.28) at 56°. The authentic amino acids were eluted in order of lysine, histidine, α-*N*-pyridoxyllysine, ε-*N*-pyridoxyllysine, and arginine. The occurrence of an amino acid identified with authentic ε-*N*-pyridoxyllysine was observed. An aliquot of the hydrolysate was also submitted to paper chromatography with the solvent system, *n*-butyl alcohol–pyridine–acetic acid–water–piperidine (30:20:0.4:24:2). α-*N*-Pyridoxyllysine and ε-*N*-pyridoxyllysine gave fluorescent spots and a blue color when sprayed with 2% 2,6-dichloroquinone chloroimide in toluene. The R_F value (0.34) of the sample was consistent with that of authentic ε-*N*-pyridoxyllysine.

Effect of pH on the Activity of Kynureninase. The enzyme has an optimum reactivity at about pH 8.0, when examined in the presence of Tris-HCl buffer and potassium phosphate buffer as shown in Figure 4. The concentrations of Tris-HCl buffer showed no effect on the enzyme activity under the experimental conditions employed.

Substrate Specificity. L-Kynurenine is the preferred substrate for kynureninase, while D-kynurenine and *N*-formyl-L-kynurenine are inert.

Kinetics. The Michaelis constant for L-kynurenine was determined to be 3.5×10^{-5} M. The constant for the cofactor, pyridoxal-5′-P, was also estimated to be 2.3×10^{-7} M (0.065 M Tris-HCl buffer, pH 8.0) and 2.2×10^{-7} M (0.065 M potassium phosphate buffer, pH 8.0).

Inhibitors. The various compounds were investigated for their inhibitory effects on the activity of kynureninase (Table II). The enzyme is inhibited most strongly by hydroxylamine and phenylhydrazine, both of which are typical inhibitors for pyridoxal-5′-P enzymes. D-Cycloserine, D-penicillamine, potassium cyanide, and semicarbazide are also inhibitory.

Kynureninase displays a high sensitivity to $HgCl_2$ and *p*-chloromercuribenzoate which are known to be inhibitors of thiol enzymes, while iodoacetate and iodoacetamide are not inhibitory. Ethylenediaminetetraacetic acid has no effect on the activity.

Fluorescence Spectrum. The fluorescence spectrum of the enzyme was measured at pH 7.2 (Figure 5). The enzyme showed fluorescence upon excitation at 337 and 430 mμ, and the emission maxima were observed at 380 and 480 mμ, respectively. When the enzyme was analyzed at 380 and 480 mμ, excitation spectra exhibited the maxima of excitation at 337 and 420 mμ, respectively, corresponding to the absorption maxima of the enzyme.

Resolution and Reconstitution of Kynureninase. Pyridoxal-5′-P was required for maximum activity of the enzyme. Pyridoxal-5′-P is removed approximately 6% from the holoenzyme by dialysis against 0.01 M potassium phosphate buffer (pH 7.2) for 18 hr. Full resolution of kynureninase was carried out as follows. The enzyme was incubated with either 1 mM hydroxylamine solution (pH 7.2) for 10 min or 10 mM L-alanine or L-ornithine (pH 8.0) for 1 hr, followed by dialysis against three changes of 0.01 M potassium phosphate buffer (pH 7.2) for 18 hr. The enzyme thus treated had no detectable activity in the absence of added pyridoxal-5′-P and no longer exhibited absorption maximum at 430 mμ, but the slight absorption remained at 337 mμ (curve C in Figure 3). The activity can be restored about 90–95% by the addition of pyridoxal-5′-P.

Pyridoxal-5′-P Content. The amount of pyridoxal-5′-P bound with the enzyme was examined by the phenylhydrazine

TABLE II: Effect of Inhibitors on the Activity of Kynureninase.[a]

Compounds	Final Concn (mM)	Rel Act. (%)
None		100
Hydroxylamine	1	0
Semicarbazide	1	63
Potassium cyanide	1	88
Phenylhydrazine-HCl	1	0
D-Cycloserine	1	88
D-Penicillamine	1	85
$HgCl_2$	1	0
Iodoacetate	1	97
Iodoacetamide	1	98
p-Chloromercuribenzoate	0.5	48
Ethylenediaminetetraacetic acid	1	100

[a] The enzyme was incubated with inhibitors at 25° for 10 min. Reaction was started by the addition of L-kynurenine sulfate.

method (Wada and Snell, 1961) and the 3-methyl-2-benzothiazolonehydrazone method (Soda *et al.*, 1969). After the dialyzed enzyme was kept at room temperature for 30 min in the presence of 0.1 N HCl to release the bound coenzyme, the amount of free pyridoxal-5′-P was analyzed. An average pyridoxal 5′-P content of 1 mol/100,000 g of protein was obtained, indicating that 1 mol of pyridoxal-5′-P is bound to 1 mol of the enzyme protein in the holoenzyme.

Discussion

Kynureninase is a key enzyme of the aromatic and NAD^+ pathways in tryptophan metabolism. The physiological importance and induction of the enzyme have been investigated in detail as reviewed by Jakoby (1955), Hayaishi (1955), and Greenberg (1969), Braunstein *et al.* (1949) showed that pyridoxal-5′-P is required as a coenzyme for the kynureninase reaction, and that the enzyme activity is greatly reduced in the livers of pyridoxine-deficient animals. The two probable mechanisms for the reaction involving formation of a Schiff base between kynurenine and pyridoxal-5′-P have been proposed (Longenecker and Snell, 1955; Braunstein, 1960). Previous studies indicated that a single enzyme catalyzes the hydrolytic cleavage of L-kynurenine and L-3-hydroxykynurenine in *Neurospora crassa* (Jakoby and Bonner, 1953a,b) and in other organisms. But the recent reports described separate enzymes which catalyze these reactions (Turner and Drucker, 1971; Gaertner *et al.*, 1971). Thus, purification of the enzyme to homogeneity has been needed to resolve these problems unequivocally.

The studies described here deal with the microbial distribution of kynureninase and the enzymological and physicochemical characterizations of the enzyme. The high activity was found in *Ps. aeruginosa*, *Ps. syncyanea*, *Ps. synxantha*, and *Ps. marginalis*. The last strain in which kynureninase occurs most abundantly was chosen for the purpose of purification of the enzyme. The formation of the enzyme was markedly induced by the addition of L-tryptophan in the growth medium. The crystalline enzyme obtained is homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis. Pyridoxal-5′-P is required as a cofactor for maximum activity of the enzyme. The absorption spectrum of enzyme is characterized by maxima at 337 and 430 mμ. The shape of the spectrum closely resembles those of some other pyridoxal-5′-P enzymes, *e.g.*, L-leucine aminotransferase (λ_{max} 326 and 414 mμ; Taylor and Jenkins, 1966), L-tyrosine aminotransferase (λ_{max} 327 and 425 mμ; Hayashi *et al.*, 1967), L-alanine aminotransferase (λ_{max} 335 and 430 mμ; Matsuzawa and Segal, 1968), and L-ornithine aminotransferase (λ_{max} 330 and 420 mμ; Peraino *et al.*, 1969). In the spectra of these enzymes the absorption at about 430 mμ is generally equal to or higher than that in the region of about 330 mμ. Kynureninase behaves like L-leucine aminotransferase in that the spectra of these enzymes do not appear to change with pH in contrast with L-alanine aminotransferase, the spectrum of which is pH dependent, with an acid peak at 430 mμ and a basic peak at 335 mμ.

On the basis of the characteristic spectrum of the enzyme, and also in view of borohydride reduction of the enzyme, and isolation of ε-*N*-pyridoxyllysine from the hydrolysate of reduced enzyme, it is suggested that pyridoxal-5′-P is bound to an ε-amino group of lysine residue of the protein through an aldimine linkage. The determination of pyridoxal-5′-P shows that 1 mol of the cofactor is bound per mol of enzyme (100,000 g). This value is close to those obtained for L-threonine dehydratase (biosynthetic) (1:97,000; Burns and Zarlengo, 1968a,b), tryptophanase of *Bacillus alvei* (1:125,000; Hoch and Demoss, 1966), and L-lysine decarboxylase (1:100,000; Soda and Moriguchi, 1969), though a value of 1 mol of the cofactor/45,000–60,000 g of protein is characteristic of many pyridoxal-5′-P enzymes (Snell and Di Mari, 1970). The enzyme is resolved to apoenzyme by incubation with hydroxylamine, L-ornithine, or L-alanine, followed by dialysis. Pyridoxal-5′-P of the enzyme must react with hydroxylamine to yield an oxime, which is released from the protein during dialysis. Resolution of the enzyme with L-ornithine or L-alanine is interpreted as the result of formation of pyridoxamine-5′-P by transamination as described in the following paper (Moriguchi and Soda, 1973).

Although it was found that kynureninase is specific for L-kynurenine, and D-kynurenine and *N*-formyl-L-kynurenine are inert, more detailed investigations on the substrate specificity and studies on the reaction mechanism also are currently in progress.

Acknowledgments

We wish to thank Dr. T. Tochikura, Dr. K. Ogata, and Dr. H. Yamada for their helpful discussions. Thanks are also due to Dr. H. Utiyama for ultracentrifugation studies.

References

Andrews, P. (1964), *Biochem. J. 91*, 222.
Andrews, P. (1965), *Biochem. J. 96*, 595.
Auerbach, V. H., and Knox, W. E. (1957), *Methods Enzymol. 3*, 620.
Burns, R. O., and Zarlengo, M. H. (1968a), *J. Biol. Chem. 243*, 178.
Burns, R. O., and Zarlengo, M. H. (1968b), *J. Biol. Chem. 243*, 186.
Braunstein, A. E. (1960), *Enzymes, 2nd Ed., 2*, 170.
Braunstein, A. E., Goryachenkova, E. V., and Pashkina, T. S. (1949), *Biokhimiya 14*, 163.
Dalgliesh, C. E. (1952), *J. Chem. Soc.*, 137.
Gaertner, F. H., Cole, K. W., and Welch, G. R. (1971), *J. Bacteriol. 108*, 902.
Greenberg, D. M. (1969), *in* Metabolic Pathways, Vol. 3, 3rd ed, Greenberg, D. M., Ed., New York, N. Y., Academic Press, p 162.
Hayaishi, O. (1955), *in* A Symposium on Amino Acid Metabolism, McElroy, W. D., and Glass, H. B., Ed., Baltimore, Md., The Johns Hopkins Press, p 914.
Hayaishi, O., and Stanier, R. Y. (1951), *J. Bacteriol. 62*, 691.
Hayashi, S., Granner, D. K., and Tomkins, G. M. (1967), *J. Biol. Chem. 242*, 3998.
Hoch, J. A., and DeMoss, R. D. (1966), *Biochemistry 5*, 3137.
Jakoby, W. B. (1955), *in* A Symposium on Amino Acid Metabolism, McElroy, W. D., and Glass, H. B., Ed., Baltimore, Md., The Johns Hopkins Press, p 909.
Jakoby, W. B., and Bonner, D. M. (1953a), *J. Biol. Chem. 205*, 699.
Jakoby, W. B., and Bonner, D. M. (1953b), *J. Biol. Chem. 205*, 709.
Knox, W. E. (1953), *Biochem. J. 53*, 379.
Kotake, Y., and Nakayama, T. (1941), *Z. Physiol. Chem. 270*, 76.
Longenecker, J. B., and Snell, E. E. (1955), *J. Biol. Chem. 213*, 229.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall,

R. J. (1951), *J. Biol. Chem. 193*, 265.
Matsuo, Y., and Greenberg, D. M. (1959), *J. Biol. Chem. 234*, 507.
Matsuzawa, T., and Segal, H. L. (1968), *J. Biol. Chem. 243*, 5929.
Moriguchi, M., and Soda, K. (1973), *Biochemistry 12*, 2974.
Moriguchi, M., Yamamoto, T., and Soda, K. (1971a), *Biochem. Biophys. Res. Commun. 44*, 752.
Moriguchi, M., Yamamoto, T., and Soda, K. (1971b), *Biochem. Biophys. Res. Commun. 45*, 828.
Nakamura, N., Morikawa, Y., Fujio, T., and Tanaka, M. (1971), *Agr. Biol. Chem. 35*, 219.
Peraino, C., Bunville, L. G., and Tahmisian, T. N. (1969), *J. Biol. Chem. 244*, 2241.
Peterson, E. A., and Sober, H. A. (1954), *J. Amer. Chem. Soc. 76*, 169.
Snell, E. E., and Di Mari, S. J. (1970), *Enzymes, 3rd Ed., 2*, 363.
Soda, K., and Moriguchi, M. (1969), *Biochem. Biophys. Res. Commun. 34*, 34.
Soda, K., Yorifuji, T., Misono, H., and Moriguchi, M. (1969), *Biochem. J. 114*, 629.
Taylor, R. T., and Jenkins, W. T. (1966), *J. Biol. Chem. 241*, 4396.
Tiselius, A., Hjerten, S., and Levin, O. (1956), *Arch. Biochem. Biophys. 65*, 132.
Turner, J. R., and Drucker, H. (1971), *Biochem. Biophys. Res. Commun. 42*, 698.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Wada, H., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2089.
Warnell, J. L., and Berg, C. P. (1954), *J. Amer. Chem. Soc. 76*, 1708.
Wiss, O. (1949), *Helv. Chem. Acta 32*, 1694.

Transamination Reaction Catalyzed by Kynureninase and Control of the Enzyme Activity†

Mitsuaki Moriguchi‡ and Kenji Soda*

ABSTRACT: The inactivation of kynureninase occurred when the reaction was carried out in the absence of added pyridoxal 5′-phosphate. The degree of inactivation increased with the reaction time. The addition of either pyridoxal 5′-phosphate or pyruvate protected the enzyme from inactivation. Kynureninase was also inactivated by L-alanine, a reaction product, or by L-ornithine. The activity is restored by the addition of pyridoxal 5′-phosphate. Spectrophotometric studies on the inactivation indicate that the addition of L-alanine or L-ornithine to the holoenzyme leads to loss in the peaks at 337 and 430 mμ, and appearance of a new peak at 325 mμ. Apoenzyme was obtained by dialysis of L-ornithine-(or L-alanine)-treated enzyme. The apoenzyme is reactivated by pyridoxamine 5′-phosphate plus pyruvate, or by pyridoxal 5′-phosphate. Thus, the inactivation is due to formation of the bound pyridoxamine 5′-phosphate from the bound pyridoxal 5′-phosphate by transamination with L-alanine or L-ornithine. The product from L-ornithine was identified as Δ^1-pyrroline-2-carboxylic acid, the intramolecularly dehydrated form of α-keto-δ-aminovaleric acid. Kynureninase catalyzes an overall transamination between L-ornithine and pyruvate. There is close correlation between the amino acids that cause inactivation and those that transaminate, and between the α-keto acids that reactivate the inactivated enzyme and those that transaminate. The enzyme can act as an α-aminotransferase of high substrate specificity to regulate the enzyme activity by interconversion of the coenzyme moiety.

Previous articles have described the purification and crystallization of kynureninase from *Pseudomonas marginalis* and some of its properties (Moriguchi *et al.*, 1971a,b, 1973). The enzyme (mol wt 100,000) exhibits absorption maxima at 280, 337, and 430 mμ. One mole of pyridoxal-5′-P[1] is bound per mole of enzyme. The holoenzyme can be resolved to the apoenzyme by incubation with hydroxylamine, L-alanine, and L-ornithine, and reconstituted by the addition of pyridoxal-5′-P.

Recently, inactivation of kynureninase by L-alanine has been reported (Moriguchi *et al.*, 1971c). Kynureninase is inactivated by preincubation with a reaction product, L-alanine, while the addition of L-alanine to the assay system without preincubation causes only a slight decrease in the reaction rate. This activity is restored by the addition of pyridoxal-5′-P. Addition of L-alanine to the enzyme causes a decrease in absorbance at 430 mμ and appearance of a peak at 325 mμ. The inactivated enzyme is resistant to a borohydride reduction. The present investigation was undertaken to elucidate the mechanism of this inactivation and reactivation of the enzyme. Some studies on the regulation of kynureninase activity by the transamination between the enzyme-bound pyridoxal-5′-P (or pyridoxamine-5′-P) and an amino acid, *i.e.* L-alanine or L-ornithine (or pyruvic acid), are described here.

Experimental Section

Materials. D-Ornithine-HCl was prepared by hydrolysis of D-arginine-HCl with concentrated sodium hydroxide and

† From the Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan. *Received April 2, 1973.* A preliminary report of part of this work has been presented (Moriguchi *et al.*, 1971c).

‡ Present address: Department of Food Science and Technology, Kyoto University, Kyoto 606, Japan.

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