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A PROPOSED STRUCTURE FOR THE 330-nm CHROMOPHORE OF GLUTAMATE DECARBOXYLASE AND OTHER PYRIDOXAL 5'-PHOSPHATE DEPENDENT ENZYMES

MARION H. O'LEARY
Department of Chemistry, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

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SUMMARY

1. Pyridoxal 5'-phosphate reacts with 1,3-diaminopropane to form a cyclic aldamine structure, rather than a Schiff base. The structure of this compound was proved by direct spectral study and by analogy with the reaction of the coenzyme with *n*-butylamine and the reactions of pyridine-4-aldehyde with *n*-butylamine and with 1,3-diaminopropane.

2. Evidence is presented which suggests that the 330 nm chromophore of glutamate decarboxylase is an aldamine formed by addition of a second amino group to the carbon nitrogen double bond, rather than any other sort of aldamine or a normal Schiff base structure.

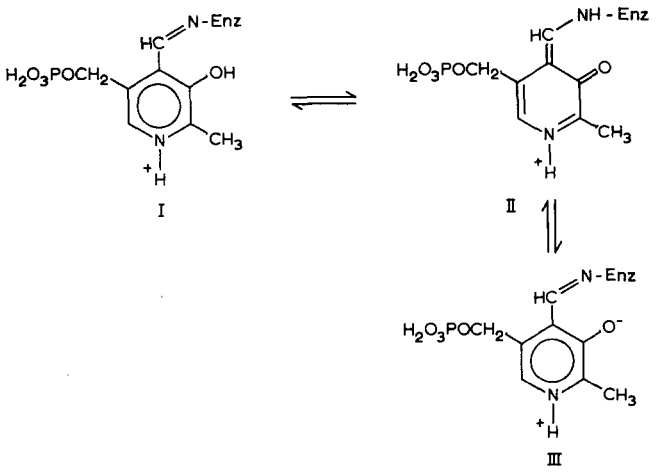
INTRODUCTION

Enzymes which contain pyridoxal 5'-phosphate (pyridoxal-5'-*P*) can be divided into two major groups according to their spectral properties[1,2]. The larger group contains those enzymes which absorb at 360 nm at higher pH and at 420 nm at lower pH. From studies of enzymes and models[2] it is clear that both spectral forms are Schiff bases, and in various cases either form may be catalytically active. The 420 nm absorbing form is tautomer II of Schiff base I*, which is the predominant tautomer in polar solvents. At higher pH, II loses a proton to form III, which absorbs at 360 nm. In all three of these structures the presence of the proton on the pyridine nitrogen cannot be ascertained.

The second group of enzymes has different spectral characteristics**. At lower pH the absorption maximum is again 420 nm, but at higher pH the maximum is 330 nm. Except in the case of glycogen phosphorylase (which we will show later is exceptional in a number of respects) only the form absorbing at 420 nm, which again

* Throughout this discussion we will not consider the ionizations of the phosphate of pyridoxal-5'-*P* and its derivatives. Changes in the ionization state of the phosphate seem to have no appreciable effect on the spectrum in aqueous solution.

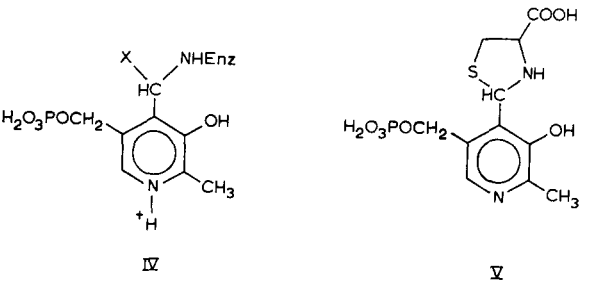
** Among enzymes of this group are glycogen phosphorylase[3], bacterial glutamate decarboxylase[4], bacterial tryptophanase[5], and rat liver alanine aminotransferase[6].



is assigned structure II, is catalytically active. The structure of the 330-nm form remains uncertain. Proposals for its structure are two main types: one based on environment polarity, and one based on aldamine formation.

The tautomerism between structures like I and II is sensitive to solvent polarity[7]. If a pH-dependent conformational change in a pyridoxal-5'-*P*-containing enzyme were to enclose the coenzyme in a hydrophobic pocket at high pH, then tautomer II, which absorbs at 420 nm, would predominate at low pH and tautomer I, which absorbs at 330 nm, would predominate at high pH. A convincing argument has recently been given for the occurrence of this phenomenon in glycogen phosphorylase[7]. However, as we shall show later, it is unlikely that this is a general phenomenon.

Alternatively, the 330-nm absorption might be due to formation of an aldamine, IV, if some group X on the enzyme were sufficiently close to the Schiff base



that addition to the carbon–nitrogen double bond could occur. Aldamine structures are known in a number of cases. For example, carbinolamines (IV, X = OH) are intermediates in the formation of Schiff bases from pyridoxal-5'-*P* and primary amines[8]. Cysteine reacts with pyridoxal-5'-*P* to form thiazolidine V (refs. 9, 10). Other mercapto and hydroxy amines also react to form cyclic aldamine structures, as do histidine[9,11] and tryptophan[9].

The purpose of this paper is to present a new possible mechanism for aldamine formation and to give evidence in favor of this mechanism in the case of bacterial

glutamate decarboxylase. The mechanism which we present is the first such mechanism which is pH dependent without requiring that a pH dependent conformation change of the enzyme occur. Our mechanism also satisfies other criteria not satisfied by the previously suggested mechanisms.

MATERIALS AND METHODS

All chemicals were reagent grade and were used as received. Ultraviolet spectra were obtained with a Cary 15 recording spectrometer. NMR spectra were obtained with a Varian A60-A or T-60 machine. Infrared spectra were obtained with a Beckman IR-8. pH measurements were made on a Radiometer Model 26 pH meter calibrated by the two-buffer method.

The ultraviolet spectrum of Schiff base VI formed by reaction of pyridine-4-aldehyde with *n*-butylamine was obtained in aqueous solution with 0.3 mM aldehyde and 0.13 M amine. The pH was adjusted with HCl. Hexahydropyrimidine VII, formed by reaction of this aldehyde with 1,3-diaminopropane, was made similarly.

A pure sample of Schiff base VI was prepared by reaction of 15 g of pyridine-4-aldehyde with 35 g *n*-butylamine in 200 ml of benzene. Benzene, water, and amine were removed by distillation at atmospheric pressure, and VI was distilled at reduced pressure, b.p. 62° (0.20 mm).

Hexahydropyrimidine VII was prepared by dissolving 1.0 g of pyridine-4-aldehyde in 20 ml of carbon tetrachloride and adding an equivalent quantity of 1,3-diaminopropane. The solution was dried with $CaSO_4$ and spectra were taken without further purification.

Spectra of pyridoxal-5′-*P*–amine compounds were taken at various pH's on solutions containing 0.2 mM pyridoxal-5′-*P*, 0.1–0.5 M amine, and 0.1 M sodium phosphate buffer. pH adjustments were made with concentrated HCl. Since the amount of acid added was always quite small, sharp isosbestic points could be ob-

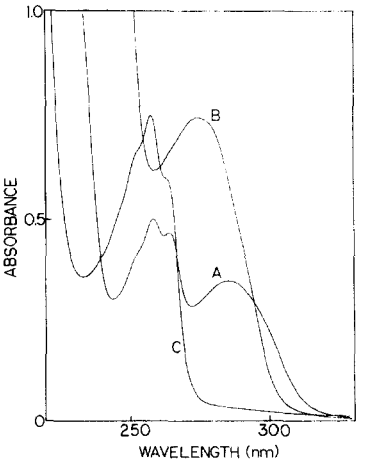


Fig. 1. Ultraviolet spectra of pyridine-4-aldehyde (A), Schiff base VI (B), and hexahydropyrimidine VII (C) at pH 10.

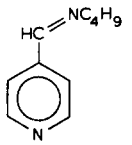
tained by running all spectra on the same solution, with appropriate pH adjustments in between.

RESULTS

Pyridine-4-aldehyde

For our initial studies we used pyridine-4-aldehyde as a model for pyridoxal-5'-*P*. The simplification resulting from the absence of the hydroxyl group is very useful in our initial considerations[8].

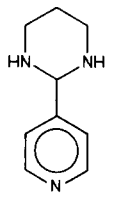
When pyridine-4-aldehyde is mixed with an excess of *n*-butylamine in aqueous solution at pH 10, Schiff base VI is rapidly formed. The ultraviolet spectra of the parent aldehyde and of the Schiff base are similar[12], as shown in Fig. 1. The C=N absorption of the Schiff base in the infrared occurs at 6.08 μm, as is usual for such compounds[13]. The benzylic hydrogen signal in the NMR appears as a singlet at 8.21 ppm downfield from tetramethylsilane.



VI

Reaction of pyridine-4-aldehyde with 1,3-diaminopropane results in a product VII having different properties from those of the Schiff base above, in spite of the fact that the two amines under consideration have very similar pK_a values[16]. The ultraviolet spectrum of VII at pH 10 is shown in Fig. 1. The position of the absorption maximum of VII suggests that it is less conjugated than Schiff base VI. The characteristic double bond absorption of VI at 6.08 μm is absent in the infrared spectrum of VII. The NMR signal of VI at 8.21 δ is shifted upfield to 4.47 δ in VII. Slight changes also occur in the positions, but not in the symmetries, of the aromatic hydrogen absorptions in the NMR.

All of the above data are uniquely consistent with the hexahydropyrimidine structure below for this compound. A few hexahydropyrimidines have been reported



VII

previously[14], although compound VII was not previously known. Such compounds are usually formed from 1,3 diamines and aldehydes and ketones with electron-withdrawing substituents. Although benzaldehyde forms only a Schiff base and not a hexahydropyrimidine on reaction with 1,3-diaminopropane[14], formation of the hexa-

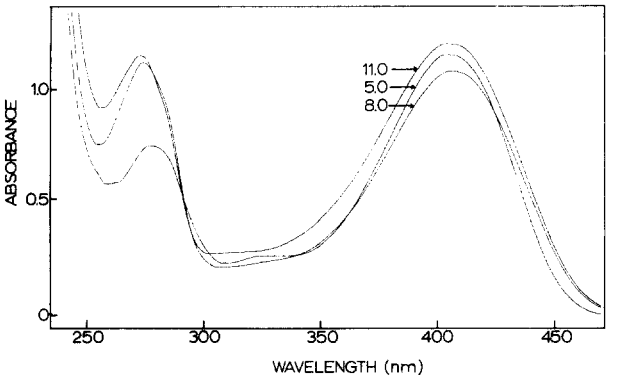
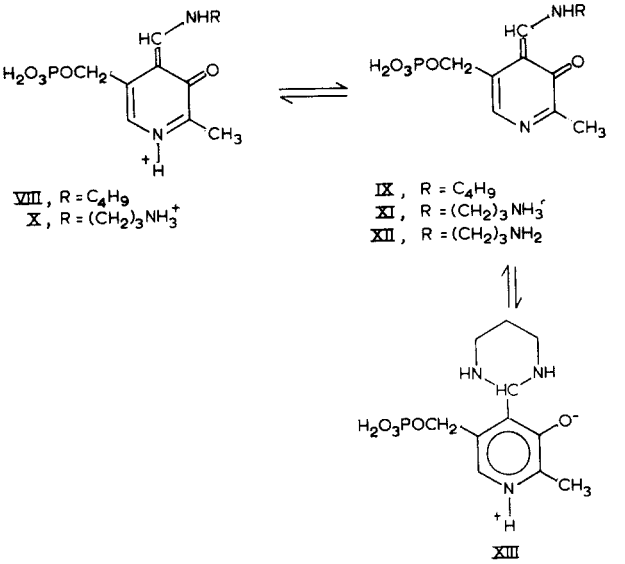


Fig. 2. Spectra of the Schiff base of pyridoxal-5'-*P* with *n*-butylamine at pH 5, 8, and 11.

hydropyrimidine structure in the case of pyridine-4-aldehyde should be favored by the inductive effect of the pyridine ring.

Pyridoxal-5'-P

The reactions of pyridoxal-5'-*P* with *n*-butylamine and with 1,3-diaminopropane were studied. The ultraviolet spectrum of the Schiff base formed by pyridoxal-5'-*P* and *n*-butylamine was measured as a function of pH from pH 5 to pH 11 in the presence of a sufficiently high concentration of amine (0.1 M) to insure that no free aldehyde was present. Three representative spectra are shown in Fig. 2. Only slight changes in the spectrum occur in this pH range.



In an extensive series of spectra *vs.* pH, isosbestic points were observed at 288, 353, and 428 nm in the pH range from 5.5 to 10, indicating that only two species are present in this pH range. The change in absorption at 275 nm can be correlated

with the ionization of a group having a pK_a of 6.2. The two forms of the Schiff base can be assigned the structures VIII and IX by analogy with previous studies[15]. The pK_a of 6.2 observed in this case is similar to the pK_a of 6.51 which was observed by NAGANO AND METZLER[15] for the same transition in the closely analogous case of leucine *plus* 5-deoxypyridoxal.

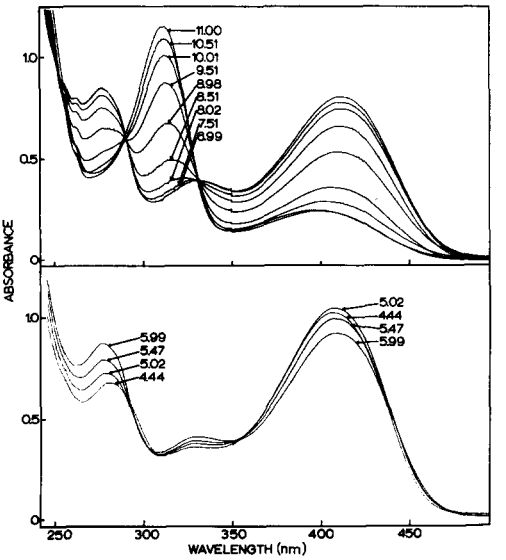


Fig. 3. Spectra of the complex formed by pyridoxal-5'-*P* and 1,3-diaminopropane from pH 4.5 to pH 11.

Spectra of the adduct of pyridoxal-5'-*P* and 1,3-diaminopropane from pH 4.4 to pH 11 are given in Fig. 3. From pH 4.5 to pH 6 the spectra have isosbestic points at 295, 303, and 352 nm and are quite similar to those observed with *n*-butylamine. The transition between the two forms in Fig. 3 is characterized by a pK_a of approx. 5.2, although it is not possible to measure this constant exactly because of the unfavorable formation constant for the Schiff base at this pH.

Above pH 7 the spectra are quite different from those observed with *n*-butylamine. A new species appears which has an absorption maximum at 312 nm and no absorption above 350 nm. The transition between the two forms is characterized by isosbestic points at 291 and 331 nm and a pK_a of 9.2.

The species present between pH 4 and pH 7 can be assigned the structures X and XI by analogy with the spectra of the *n*-butylamine–pyridoxal-5'-*P* Schiff base. As in that case, loss of a proton from the pyridine nitrogen is correlated with the increase in intensity of an absorption band at 275 nm. The spectra above pH 7 have no analog in the *n*-butylamine series, but they are strongly reminiscent of the hexahydropyrimidine spectra obtained with pyridine-4-aldehyde and 1,3-diaminopropane. We suggest that the high pH species is hexahydropyrimidine XIII, possibly in equilibrium with a small amount of the open-chain form XII. The cyclic species can be formed only at sufficiently high pH that the second amino group of XII is unprotonated.

DISCUSSION

Many aldehydes and ketones, including both pyridine-4-aldehyde and pyridoxal-5'-*P*, react with 1,3-diamines to form hexahydropyrimidines. In the latter case the balance between the open-chain Schiff base structure and the cyclic hexahydropyrimidine structure is very delicate and is pH dependent.

These results suggest that aldamines in pyridoxal-5'-*P*-containing enzymes might be formed by addition of a second amino group to the carbon–nitrogen double bond. According to this mechanism no conformation change of the protein is needed in order to cause aldamine formation—the Schiff base-aldamine equilibrium may be controlled by ionization of an amino group. Although aldamine formation involving a six-membered ring is not possible for enzymes, the proximity of the second amino group in the enzyme to the Schiff base linkage might be such as to allow easy aldamine formation.

The details of the pH dependence of aldamine formation according to this mechanism are interesting. Although the transition from the Schiff base form to the aldamine form is connected with the ionization of an amino group, the observed pK_a is not simply that of the amino group, but instead is given by Eqns. 1–3 for the specific case of pyridoxal-5'-*P* and 1,3-diaminopropane. The observed pK_a, pK_{obs}, of the transition

$$K_a = [H^+][XII]/[XI] \quad (1)$$

$$K_c = [XIII]/[XII] \quad (2)$$

$$K_{obs} = K_a K_c \quad (3)$$

from XI to XIII is 9.2. The pK_a of the second amino group of 1,3-diaminopropane[16] is 10.6, and we can assume that the pK_a of the amino group of Schiff base XI would also be about 10.6. In that case K_c, the pH independent equilibrium constant for the cyclization, is 25. In enzymes, both K_c and K_a might be expected to vary over a wide range, thus giving rise to a range of pK values for the 420–330 nm transition.

Aldamine formation in enzymes

SHALTIEL AND CORTIJO[7] have recently given a convincing explanation for the occurrence of the 330-nm band in glycogen phosphorylase. They showed that the coenzyme binding site in that case is sufficiently nonpolar that the Schiff base exists as tautomer I rather than tautomer II. However, it is likely that phosphorylase is unique in this respect, as we show in the following discussion.

The other well-known enzyme which shows an absorption maximum at 330 nm is bacterial glutamate decarboxylase[4]. However, its spectral properties differ in several important respects from those of phosphorylase. The 420-nm absorption of glutamate decarboxylase is optically active[17,18], whereas that of phosphorylase is not optically active[19]. The 330-nm absorption band of glutamate decarboxylase is optically inactive, whereas that of phosphorylase is active. The fluorescence properties of the 330-nm forms of the two enzymes also differ significantly. When excited at 330 nm, the 330-nm form of phosphorylase shows a fluorescence emission maximum at 530 nm (ref. 7), whereas the maximum for glutamate decarboxylase is at 380 nm (ref. 4).

Unlike the situation with phosphorylase, both the optical activity data and the

fluorescence data indicate that the chromophore of glutamate decarboxylase which absorbs at 330 nm is not a Schiff base buried in a hydrophobic pocket. Such a chromophore would be in an asymmetric environment and would therefore be optically active. The fluorescence emission at 380 nm is not that expected for a Schiff base in a nonpolar environment, but is that expected for an aldamine structure.

We conclude that the 330-nm absorption of glutamate decarboxylase is due to formation of an aldamine rather than to a polarity effect. However, the lack of optical activity in the 330-nm absorption band imposes still further restrictions on the structure of the aldamine. JOHNSON AND GRAVES[19] have shown that thiazolidine V formed by reaction of pyridoxal-5'-*P* with cysteine shows strong optical activity in the 330-nm absorption. Formation of a similar structure in enzymes (IV, X = O or S) should also result in an optically active 330-nm absorption band. In fact, the optical activity of the long wavelength absorption in such a structure should be even greater than that of thiazolidine V because the chiral center in IV is immediately adjacent to the aromatic ring, rather than being two atoms removed. Lack of optical activity in aldamine IV can only occur if the benzylic carbon atom is not chiral—that is, if X = NH.

Thus the available evidence is inconsistent with the 330-nm absorption of glutamate decarboxylase being due to the presence of a Schiff base buried in a hydrophobic environment. Because of the optical activity results the most likely possibility is that the 330-nm absorption is due to formation of an aldamine by reaction of the Schiff base with a second lysine residue of the enzyme. We have provided a simple chemical model which duplicates many spectral properties of glutamate decarboxylase and helps to substantiate our proposed mechanism for aldamine formation. Our results do not in any way preclude the possibility that a conformational change may also occur in glutamate decarboxylase when aldamine formation takes place.

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REFERENCES

1 E. E. SNELL AND S. J. DIMARI, *Enzymes*, 2 (1970) 335.
2 R. J. JOHNSON AND D. E. METZLER, *Methods Enzymol.*, 18A (1970) 433.
3 E. H. FISCHER AND E. G. KREBS, *Fed. Proc.*, 25 (1966) 1511.
4 R. SHUKUYA AND G. W. SCHWERT, *J. Biol. Chem.*, 235 (1960) 1649.
5 Y. MORINO AND E. E. SNELL, *J. Biol. Chem.*, 242 (1967) 2800.
6 H. L. SEGAL, P. W. GATEHOUS AND S. HOPPER, in E. E. SNELL, A. E. BRAUNSTEIN, E. S. SEVERIN, AND Y. M. TORCHINSKY, *Pyridoxal Catalysis: Enzymes and Model Systems*, New York, N.Y., 1966, p. 215.
7 S. SHALTIEL AND M. CORTIJO, *Biochem. Biophys. Res. Commun.*, 41 (1970) 594.
8 T. C. BRUICE AND S. J. BENKOVIC, *Bioorganic Mechanisms*, Benjamin, New York, N.Y., 1966, Vol. 2, Chapter 8.
9 D. HEYL, S. A. HARRIS AND K. FOLKERS, *J. Am. Chem. Soc.*, 70 (1948) 3429.
10 M. V. BUELL AND R. E. HANSEN, *J. Am. Chem. Soc.*, 82 (1960) 6042.
11 D. MACKAY, *Arch. Biochem. Biophys.*, 99 (1962) 93.
12 T. C. BRUICE AND A. LOMBARDO, *J. Am. Chem. Soc.*, 91 (1969) 3009.

13 R. BONNETT, in S. PATAI, *The Chemistry of the Carbon Nitrogen Double Bond*, Interscience New York, N.Y., 1970, Chapter 4.
14 D. J. CURRAN AND S. SIGGIA, in S. PATAI, *The Chemistry of The Carbon Nitrogen Double Bond*, Interscience, New York, N.Y., 1970, p. 163.
15 R. F. EVANS, *Austr. J. Chem.*, 20 (1967) 1643.
16 K. NAGANO AND D. E. METZLER, *J. Am. Chem. Soc.*, 89 (1967) 2891.
17 D. PERRIN, *Dissociation Constants of Organic Bases in Aqueous Solution*, Butterworths, London, 1961.
18 T. E. HUNTLEY AND D. E. METZLER, *Biochem. Biophys. Res. Commun.*, 26 (1967) 109.
19 B. S. SUKHAREVA AND Y. M. TORCHINSKY, *Biochem. Biophys. Res. Commun.*, 25 (1966) 585.
20 G. F. JOHNSON AND D. J. GRAVES, *Biochemistry*, 5 (1966) 2906.