Mode of Binding of Pyridoxal Phosphate to 5-Aminolevulinate Synthase

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5-Aminolevulinate synthase of *Rhodopseudomonas spheroides* interacts with its cofactor, pyridoxal phosphate, and shows an absorption maximum at 430 nm with a probable shoulder at 320–330 nm. The enzyme-PLP complex absorbing at 430 nm is the predominant species at pH 7.2 and can be reduced by $NaBH_4$ at neutral pH with a spectral shift of the absorption maximum to 325 nm. These data suggests the formation of a Schiff base rather than a substituted aldimine between the enzyme and pyridoxal phosphate. The decrease in absorption at 430 nm and increase in absorption at 325 nm by the addition of 2-mercaptoethanol seem to support Schiff base structures for the absorption bands at 430 nm and 320–330 nm. Both pyridoxal phosphate and glycine can equally protect the enzyme from inactivation by sulfhydryl reagents. The inhibition by *p*-chloromercuribenzoate versus PLP and glycine is noncompetitive and that by N-ethylmaleimide is noncompetitive with glycine and competitive with PLP. These results suggest either a conformational change in the presence of substrates or loss of affinity by the enzyme for PLP, rather than an interaction of PLP with a -SH group of the enzyme. The combined data seems to eliminate the possibility of the formation of a thiohemiacetal or a substituted aldimine and support rather strongly the formation of a Schiff base between the enzyme and pyridoxal phosphate.

It has been claimed by Scholnick *et al.* [1] that 5-aminolevulinate synthase (EC 2.3.1.37) from rat liver, unlike all other PLP-dependent enzymes, forms a thiohemiacetal with pyridoxal phosphate. This was based on their observation that the competitive inhibition of the enzymic activity by N-ethlymaleimide was protected by pyridoxal phosphate. Whiting *et al.* [2], however, suggested a Schiff base structure for the enzyme-PLP complex from the spectral behaviour of chicken embryo liver enzyme in the presence of pyridoxal phosphate. On the basis of resemblance between glycogen phosphorylase and their holoenzyme in regard to their spectral properties and inertness to $NaBH_4$ reduction at pH 7.0, Fanica *et al.* [3] proposed a substituted aldimine structure for the enzyme-PLP complex at neutral pH. I am reporting in this communication experiments with the pure apoenzyme of *Rhodopseudomonas spheroides*, which bear on the most probable nature of binding of pyridoxal phosphate to the enzyme.

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Experimental Procedure

The 5-aminolevulinate synthase was prepared from *Rhodopseudomonas spheroides* and its activity assayed as described previoulsy [4]. Absorption spectra were recorded at room temperature against a buffer in silica cuvettes of 1-cm length with a Cary Model 14 spectrophotometer equipped with 0-0.2 A and 0-2 A slide wires.

Results and Discussion

In the presence of PLP, the apoenzyme shows an absorption spectrum with a maximum at 430 nm and a probable shoulder at 320-33 nm at pH 7.2 (Fig. 1). While the 430 nm band is characteristic of a Schiff base between the enzyme and PLP [5–8] (supported by the spectral shift of the absorption maximum to 325 nm by $NaBH_4$ (Fig. 1) [9]), the 320–330 nm band may represent either a transitional Schiff base [7, 8, 10] or a substituted aldimine [8, 11]. A substituted aldimine represented by the 320–330 nm band is expected to be the predominant species and also inert to $NaBH_4$ reduction at neutral pH. Table I shows that the enzyme can be reduced in the presence of PLP at pH values between 6 and 8, and the 430 nm band rather than 320-33 nm band is the predominant

Table I. Inactivation of 5-aminolevulinate synthase by $NaBH_4$ in the presence of pyridoxal phosphate at different pH.

Reaction mixture	Final pH	Per cent inactivation
Enzyme + $NaBH_4$	6.1	2
Enzyme + $NaBH_4$ + PLP	6.1	70
Enzyme + $NaBH_4$	6.5	3
Enzyme + $NaBH_4$ + PLP	6.5	83
Enzyme + $NaBH_4$	7.1	3
Enzyme + $NaBH_4$ + PLP	7.1	94
Enzyme + $NaBH_4$	7.8	4
Enzyme + $NaBH_4$ + PLP	7.8	95

Borohydride reduction was carried out by adding 0.05 ml of 0.05 M $NaBH_4$ to 0.5 mg of partially purified enzyme in 0.25 M potassium phosphate buffer of appropriate pH containing 0.5 mM PLP in a final volume of 0.5 ml. The reaction mixture was stirred for 1/2 hour at 0–4 °C. The samples were dialyzed against 500 ml of 0.05 M potassium phosphate, pH 7.1, containing 1 mM 2-mercaptoethanol and 5% glycerol. Aliquots were assayed for enzymatic activity and protein contents were determined.

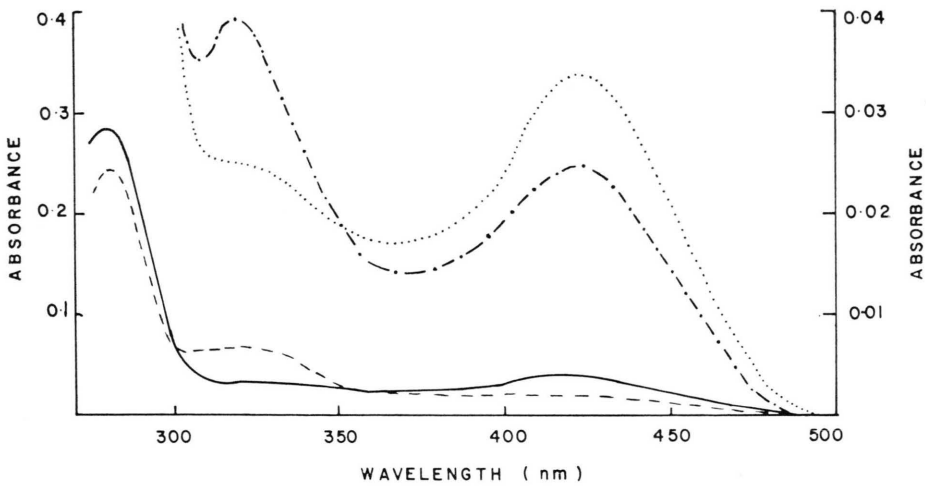

Fig. 1. Absorption spectra of pure 5-aminolevulinate synthase in the presence of PLP and after treatment with $NaBH_4$ or 2-mercaptoethanol. Apoenzyme (0.4 mg/ml) in 0.05 M potassium phosphate buffer, pH 7.2, having equivalent or excess of PLP (solid line). The buffer blank contained equal amount of PLP. The enzyme-PLP complex after reduction with 0.005 M $NaBH_4$ at neutral pH and dialysis against 0.05 M potassium phosphate buffer, pH 7.2 (broken line). The dotted line represents the enzyme-PLP complex on a 10-fold expanded scale; the effect of adding 0.05 M 2-mercaptoethanol to the enzyme-PLP complex is represented by the dashed-dotted line which is also on a 10-fold expanded scale.

species at neutral p H(Fig. 1). The addition of 50 mM 2-mercaptoethanol to the enzyme-PLP complex decreases the absorption at 430 nm and increases the absorption around 325 nm (Fig. 1), probably due to the formation of adducts with the Schiff bases representing the bands at 430 nm and 320 – 330 nm. These results seem to eliminate a substituted aldimine for the enzyme-PLP complex and indicate Schiff base structures for the 430 nm and 320 – 330 nm bands. The ratio of these absorbances probably depends on the polarity of the medium [12]. A small amount of absorbance at 320 – 330 nm at neutral pH may be due to some hydrophobic microenvironment of the coenzyme-binding site [13, 14], and this seems to be supported by the competitive inhibition by low concentrations of urea and guanidine hydrochloride versus pyridoxal phosphate (data not shown), as they can act by reducing hydrophobic interactions.

Scholnick *et al.* [1] suggested the formation of a thiohemiacetal between the enzyme and PLP based on their observation that the competitive inhibition by N-ethylmaleimide was protected by PLP. Our experiments with pure enzyme from *Rhodopseudomonas spheroides* show that both PLP and glycine can equally protect the enzyme from inactivation by sulfhydryl reagents (Table II). Again, *p*-chloromercuribenzoate is noncompetitive to both PLP and glycine, while N-ethylmaleimide is competitive to PLP and noncompetitive to glycine (data not shown). These results seem to suggest that substrates may bring about a conformational change of the protein burying thiol groups, and sulfhydryl

Table II. Protective action of substrates against N-ethylmaleimide or *p*-chloromercuribenzoate inhibition.

Preliminary incubation addition	N-ethylmaleimide (2×10^{-4} M)	*p*-chloromercuribenzoate (1×10^{-5} M)	Percentage activity
None	+		43
PLP+NEM	+		83
Glycine+NEM	+		68
Glycine+PLP+NEM	+		96
None		+	21
PLP+PCMB		+	61
Glycine+PCMB		+	61
Glycine+PLP+PCMB		+	76

The amount of 5-aminolevulinic acid formed at 37 °C was determined in a reaction mixture of 0.05 M Tris-HCl, pH 7.5, 0.1 M glycine, 0.2 mM PLP, 0.2 mM succinyl CoA and 5 μg of enzyme in a final volume of 0.5 ml. The reaction was stopped after 15 min by adding 0.25 ml of 15% of trichloroacetic acid. It was then treated for pyrrole formation and the pyrrole formed was determined with modified Ehrlich's reagent [4]. A 3-minute preliminary incubation was used in these studies. During the first minute of preliminary incubation the entire reaction mixture minus glycine, PLP and succinyl CoA were present except as indicated. After 1 min either *p*-chloromercuribenzoate (PCMB) or N-ethylmaleimide (NEM) was added to the indicated concentrations; at 2 min glycine and/cofactor was added; at 3 min succinyl CoA was added to initiate the reaction.

reagents, on the other hand, may cause a loss of affinity by the enzyme for PLP through the steric hindrance. It may aso be argued that the competitive inhibition by N-ethylmaleimide is probably due to its interaction with the unprotonated amino group of the enzyme which forms a Schiff base with PLP. Furthermore, a thiochemiacetal would not be reduced to a stable covalent linkage by $NaBH_4$ and thus the inactivation caused by reduction (see Table I) would not occur.

Spectral analyses and other related studies thus presented in this paper suggest strongly the formation of a Schiff base between the enzyme, 5-aminoevulinate synthase, and its cofactor, pyridoxal phosphate, rather than a substituted aldimine [3] or a thiohemiacetal [1]. The fact that the enzyme requires thiol compounds such as 2-mercaptoethanol for its stability may implicate a free thiol group(s) for the maintenance of 3-dimensional structure conducive to the efficient functioning of the enzyme.

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