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PHOTODYNAMIC PROPERTIES OF PYRIDOXAL PHOSPHATE BOUND TO CYSTATHIONASE-γ-LYASE

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Summary

The cofactor pyridoxal phosphate bound through an aldimine linkage to lysine residues of the enzyme cystathionase (L-Cystathione cysteine-lyase (deaminating), EC 4.4.1.1) is very stable to irradiation with light of 420 nm. The catalytic function of the enzyme remains unaffected indicating that the cofactor is not an efficient photosensitizer of essential amino acid residues. This unusual stability of the cofactor to irradiation can be ascribed to the presence of aldimine linkages as demonstrated by studies conducted on model compounds. The binding of a reversible inhibitor (L-allylglycine) to the catalytic site of the enzyme does not facilitate photooxidation of the cofactor. On the contrary, irradiation of the cofactor in the presence of the inhibitor results in photodestruction of the inhibitor.

Introduction

The potential usefulness of pyridoxal-*P* as an active site reagent was increased when it became known that it can act as an efficient photosensitizer reagent of several enzymes [1—3]. In each instance where pyridoxal-*P* was used as photosensitizer, the modified residue on the enzyme was identified as histidine.

However, it should be noted that different results are obtained when Pyridoxal 5′-*P* is a cofactor of the enzyme. Thus, irradiation of aspartate aminotransferase at absorption wavelengths corresponding to the absorbance maxima of bound pyridoxal-*P* did not affect the catalytic activity and the spectra properties of this enzyme [4]. Furthermore, studies conducted on glutamate decarboxylase revealed that irradiation of the cofactor pyridoxal-*P* brings about photo-oxidation of histidine and cysteine without concomitant changes in the spectroscopic properties of the sensitizer [5].

The present paper deals with the application of pyridoxal-*P* as photosensi-

tizer for the photomodifications of reversible inhibitors bound to the catalytic site of the enzyme, cystathionase (L-cystathione cysteine-lyase (deaminating), EC 4.4.1.1).

Experimental Procedures

Methods

Photoinactivation experiments were carried out in a temperature-controlled cell compartment in the presence of oxygen. The sample of enzyme (3 ml) was contained in a quartz cuvette (1-cm light path). The sample of enzyme was illuminated from the side with a 200 W Xenon-Mercury lamp. The desired wavelengths were selected by a 500 nm Bausch and Lomb monochromator. The energy of the exciting light was measured by the actinometric method of Hatchard and Parker [6].

Fluorescence measurements at 20°C were conducted in a fluorimeter designed in our laboratory [7]. Absorption measurements were carried out in a Cary model 15 spectrophotometer.

Materials

The enzyme cystathionase from rat liver was purified by the procedure of Matsuo and Greenberg [8] with the modification of Churchich et al. [9]. Enzymatic activity was assayed by measuring α-ketobutyric production using L-homoserine as substrate [9]. Resolution of the native enzyme into apoenzyme and free cofactor was performed according to the method of Beeler and Churchich [10]. The synthesis of *P*-pyridoxal-L-homoserine was performed according to the method developed in our laboratory [9].

Materials

Pyridoxal-*P*, L-homoserine, L-lyine, alanine and glycine were obtained from Sigma, carboxymethoxyl amine (amino-oxyacetate) and allylglycine from Aldrich.

Irradiation of the holoenzyme

Irradiation of pyridoxal-*P* in the presence of oxygen at pH 7.4 causes several changes in the spectroscopic properties of this compound. Thus, the absorption at 388 nm is diminished, whereas the absorption at wavelengths shorter than 330 nm is enhanced (Fig. 1). In contrast to free pyridoxal-*P*, the cofactor bound to the enzyme cystathionase through an aldimine linkage is very stable to irradiation. This is clearly illustrated in Fig. 2, where it may be seen that the absorption of the enzyme at 415 nm does not undergo any significant change after exposure to light for 15 min at 10°C. In addition, the absorption properties of irradiated cystathionase are indistinguishable from those of the native enzyme (Fig. 2). This result, taken together with the absence of any noticeable change in the catalytic function of the enzyme, is interpreted to mean that the cofactor is not an efficient photosensitizer of amino acid residues implicated in catalysis.

The effect of irradiation on the absorption properties of pyridoxal-*P* bound

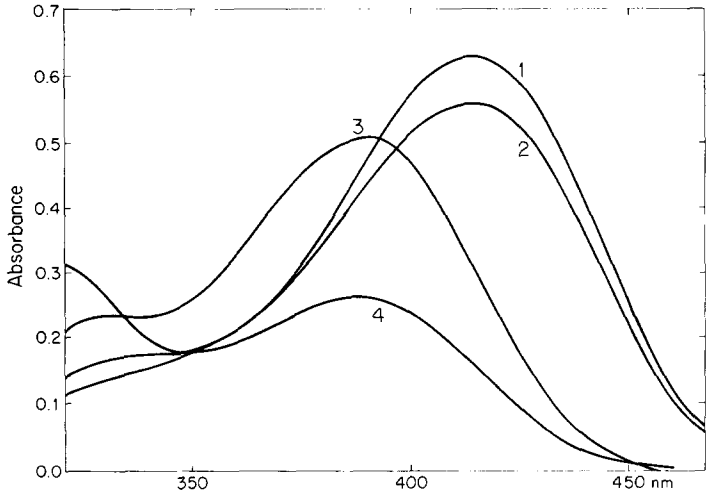


Fig. 1. Absorption spectra of pyridoxal phosphate (3) and pyridoxal phosphate irradiated for 30 min (4). Absorption spectra of pyridoxal phosphate + 0.2 M glycine (M) and pyridoxal phosphate + 0.2 M glycine irradiated for 30 min (2). Experiments conducted at pH 7.4, temperature 10°C. Dose rate: $2.3 \cdot 10^{17}$ photons · min^{-} · cm^{-2}. Samples irradiated at 390 nm in the presence of oxygen.

through an aldimine linkage to several amino acids was also studied in the presence of oxygen. The results of these experiments shown in Figs. 1 and 2 indicate that pyridoxal-*P* is less sensitive to photodestruction when it is irradiated in the presence of the amino acids lysine, alanine and glycine.

Irradiation of the holoenzyme in the presence of a competitive inhibitor

The preceding results indicate that a Schiff's base linkage between pyridoxal-

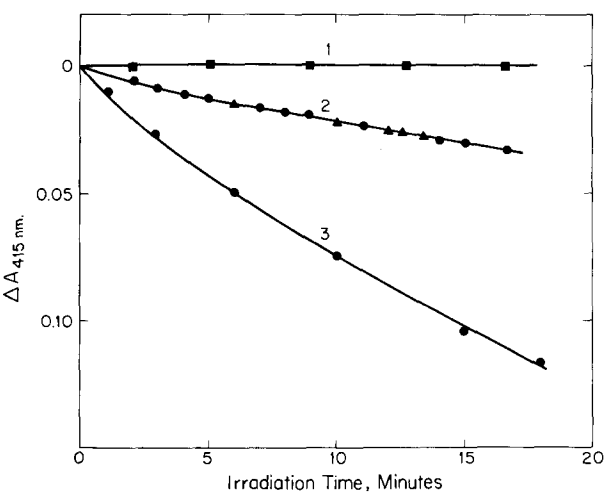


Fig. 2. Change in absorbance at 415 nm upon irradiation of cystathionase (1) at 420 nm. Pyridoxal phosphate + 0.1 M L-alanine (2), pyridoxal phosphate + 0.3 M lysine (2) and pyridoxal phosphate (3) irradiated at 390 min. Experiments conducted at 10°C with a dose rate of $2.3 \cdot 10^{17}$ photons · min^{-1} · cm^{-2} in the presence of oxygen.

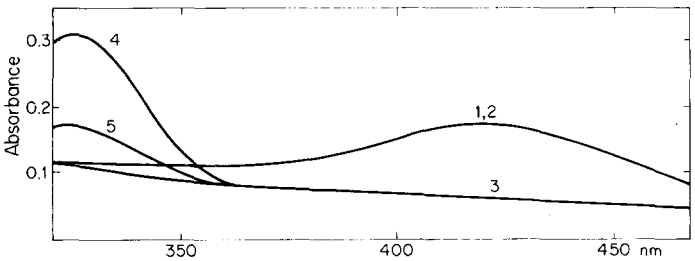


Fig. 3. Absorption spectra of cystathionase (1), cystathionase irradiated for 30 min (2) at 420 nm. Absorption spectra of apocystathionase (3), apocystathionase (5 μM) + *P*-pyridoxal-L-homoserine (20 μM), (4), apocystathionase (5 μM) + *P*-pyridoxal-L-homoserine (20 μM), irradiated for 30 min (5) at 330 nm in the presence of oxygen.

P and the amino acid contributes to the stability of the cofactor during the irradiation process. However, it was thought of interest to known to what extent perturbations of the aldimine linkage in the enzyme might facilitate photoinactivation of cystathionase and/or photodestructions of the cofactor.

In order to perturb the aldimine linkage between pyridoxal-*P* and the enzyme, irradiation experiments were conducted in the presence of a competitive inhibitor which binds to the catalytic site of cystathionase. L-allylglycine was chosen for these experiments for the following reasons: (a) it binds to the catalytic site of the enzyme and brings about a change in the absorption spectrum of pyridoxal-*P* that can be easily monitored during the irradiation process, (b) the binding of L-allylglycine to the enzyme is a reversible process, and (c) removal of the inhibitor is easily achieved by passage of the mixture containing enzyme and allylglycine through a small Sephadex G-25 column.

The absorption spectrum of the cofactor is influenced by the addition of the competitive inhibitor allylglycine. As shown in Fig. 4, the addition of L-allylglycine to cystathionase brings about a substantial decrease in the intensity of the absorption band covering the spectral range 360—450 nm. This decrease in the intensity of the absorption band induced by the inhibitor can be used conveniently to determine the affinity constant of L-allylglycine for the enzyme. To this end, samples of enzyme were allowed to react in the dark with increasing concentrations of L-allylglycine at a temperature of 10° C. After incubation

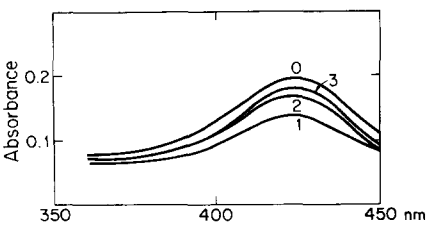


Fig. 4. Irradiation of cystathionase (6 μM) in the presence of L-allylglycine (30 μM). Absorption spectra of cystathionase (0), cystathionase + L-allylglycine (1). Mixture irradiated for 15 (2) and 30 min (3). Dose rate: $8.3 \cdot 10^{16}$ photons $\cdot$ min^{-1} $\cdot$ cm^{-2}. Experimental conditions, pH 7.4, temperature 10° C. Irradiation conducted in the presence of oxygen.

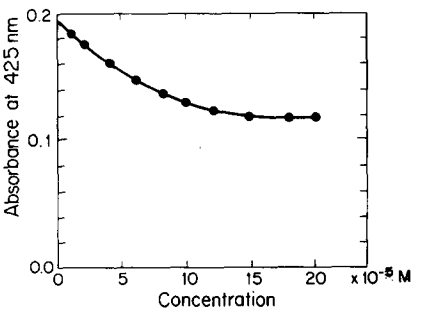
for 30 min, the absorbance at 425 nm was recorded and aliquots were withdrawn from the incubation mixture for activity assay. Fig. 5 includes the results of the spectrophotometric titrations. Assuming each pyridoxal-*P* site (n = 4) on the enzyme binds only one molecule of L-allylglycine, it is possible to determine the equilibrium constant by resorting to Eqns. 1 and 2

$$\frac{[EL]}{[L]} = K([E_0] - [EL]) \quad (1)$$

$$[EL] = \frac{A_0 - A}{A_0 - A_m}[E_0] \quad (2)$$

Where [L] is the concentration of free ligand, [EL] the concentration of complexed ligand, E_o total concentration of binding sites, A_o absorbance of the protein at 425 nm in the absence of ligand, A absorbance of the protein when both free and bound ligand are in equilibrium and A_m absorbance of the enyme saturated with ligand. A plot of [EL]/[L] vs. [EL] yields a straight line, from which the association constant ($1.5 \cdot 10^4$ M^{-1}) is determined. This association constant measured by spectrophotometric titrations of the enzyme is compatible with the dissociation constant ($K_D = 8 \cdot 10^{-5}$ M) obtained from enzymatic activity measurements. Thus, the affinity of L-allylglycine for the enzyme is weaker than the affinity of the cofactor pyridoxal-*P* for the catalytic site of cystathionase ($K_A = 10^6$ M^{-1}) [11].

The irradiation of cystathionase in the presence of L-allylglycine was conducted at 10°C light absorbed by the cofactor. Samples irradiated for different lengths of time were examined in the spectrophotometer and tested for enzymatic activity after dilution with buffer. Fig. 4 shows that upon exposure to light, the intensity of the absorption band due to the inhibitor complexed to the enzyme is increased when compared to a control kept in the dark. As the irradiation progresses, the absorption band of the irradiated samples tend to approach the intensity of the absorption band of native cystathionase. In addition, samples of the enzyme cystathionase, before and after irradiation, were indistinguishable from one another with respect to their catalytic properties.



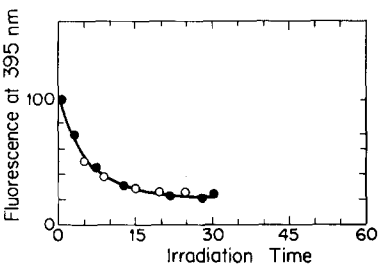


Fig. 5. Titration of cystathionase (6 μM) with L-allylglycine at 10°C, pH 7.4. Change in absorbance at 425 nm as a function of L-allyglycine concentration.

Fig. 6. Change in fluorescence intensity at 395 nm upon irradiation of free (○) and bound *P*-pyridoxal-L-homoserine (●). Experiments conducted at pH 7.4, 10°C. Dose rate: $8.3 \cdot 10^{16}$ photons · min^{-1} · cm^{-2}.

These unexpected results can best be interpreted if one assumes that during irradiation of the enzyme in the presence of inhibitor, the cofactor pyridoxal-*P* acts as an efficient photosensitizer of L-allylglycine. Then, the inhibitor modified by the photo-oxidation process loses the ability to interact with the catalytic site of the enzyme. Thus, the cofactor in the excited state acts as a protector of the enzyme by facilitating the destruction of a competitive inhibitor.

Irradiation of the apoenzyme

The photodynamic properties of *P*-pyridoxyl-L-homoserine bound to the apoprotein of the enzyme cystathionase were investigated by using fluorescence spectroscopy. Previous studies from our laboratory have shown that there is an heterogeneous population of binding sites on the apoprotein characterized by two different association constants [10]. Samples containing apoprotein and *P*-pyridoxyl-L-homoserine were irradiated with light absorbed by the *P*-pyridoxyl analogue, and the fluorescence intensity of the irradiated samples was monitored at 395 nm. As shown in Fig. 6, the fluorescence yield of *P*-pyridoxyl-L-homoserine is drastically changed during the irradiation process. Since the decrease in fluorescence intensity at 395 nm reflects the photodestruction of the *P*-pyridoxyl chromophore, it is evident that the presence of the apoprotein does not afford any protection against the photodestruction of the *P*-pyridoxyl analogue. Thus, it appears that electrostatic forces between the *P*-pyridoxyl analogue and the binding sites of the apoprotein does not prevent the photodestruction of *P*-pyridoxyl-L-homoserine. It should be noted that the photooxidation of *P*-pyridoxyl-L-homoserine is an irreversible process, since illumination of the photoproduct with light of 260 and 280 nm does not restore the original absorption and fluorescence properties of *P*-pyridoxyl-L-homoserine. A comparison of the catalytic properties of native and irradiated cystathionase reveals that samples of apoprotein subjected to irradiation for 30 min lost 10% of the original enzymatic activity. This small decrease in enzymatic activity cannot be correlated with the photo-oxidation of the *P*-pyridoxyl analogue since exposure to light for 30 min results in complete photodestruction of *P*-pyridoxyl-L-homoserine as demonstrated by the results included in Fig. 6.

Discussion

The aim of the experiments described in this paper was to study the effect of irradiation on pyridoxal-*P* and *P*-pyridoxyl analogues bound to the catalytic site of the enzyme cystathionase. The results of our experiments conducted with the holoenzymes indicate that the cofactor pyridoxal-*P* does not act as a photosensitizer of amino acid residues implicated in catalysis. This unusual stability of the cofactor covalently bound to the enzyme can, in part, be ascribed to the presence of an aldimine linkage as demonstrated by the following lines of experimental evidence, (a) the Schiff's base of the amino acids lysine, alanine, and glycine are less sensitive to irradiation than free pyridoxal-*P* in solution, (b) *P*-pyridoxyl-L-homoserine which binds to the catalytic site through electrostatic forces is sensitive to irradiation, and (c) pyridoxal-*P* residues covalently attached to the reduced form of cystathionase are very sensitive to irradiation [9].

An interesting feature of our studies is the finding that perturbation of the aldimine linkage pyridoxal-*P* and the enzyme by additon of a competitive inhibitor induces a change in the intensity of the absorption band due to the cofactor, but it does not facilitate photo-oxidation of the enzyme, On the contrary, the irradiation of the enzyme cystathionase in the presence of L-allylglycine brings about an increase in the intensity of the absorption band covering the spectral range from 360 to 450 nm.

The increase in the intensity of the absorption band upon irradiation can be interpreted as arising from the photo-oxidation of L-allylglycine sensitized by pyridoxal-*P* covalently bound to the catalytic site. Thus, it appears that the cofactor in the excited state sensitize the photo-oxidation of L-allylglycine, and thereafter the photoproduct loses the ability to bind to the enzyme.

The following model is proposed to explain the results:

$$\text{E-PLP} + \text{I} \rightleftharpoons \text{E-PLP-I}$$
$$\text{E-PLP} + \text{I} + h\nu \rightleftharpoons \text{E-PLP} + \text{I}^*$$

where PLP = pyridoxal phosphate.

The competitive inhibitor L-allylglycine (I) forms a ternary complex with pyridoxal-*P* and amino acid residues at the catalytic site. This reaction, which occurs in the dark, is reversible. Upon irradiation of the 'ternary complex', the inhibitor is released from the binding site and converted into a photoproduct (I^*). Thus, the cofactor in the excited state protects the enzyme by facilitating the photo-oxidation of a competitive inhibitor.

Acknowledgement

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