EVIDENCE FOR HISTIDINE AS ANOTHER FUNCTIONAL GROUP OF δ-AMINOLEVULINIC ACID DEHYDRATASE FROM BEEF LIVER

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SUMMARY: δ-Aminolevulinic acid dehydratase (EC 4.2.1.24) was obtained in highly purified form from beef liver. Upon photooxidation of the enzyme in the presence of methylene blue as a sensitizer led to a loss of the enzymatic activity according to pseudo-first order kinetics. The pronounced pH dependence (pk value of 6.8) of the photooxidation rate and the results of amino acid analysis suggested that the inactivation was largely due to the modification of the histidine residue. The finding of the enzyme with little activity in the presence of diethylpyrocarbonate was consistent with such a speculation. On the basis of these results, it can be postulated that the histidine residue seems to play an important role in the enzymatic activity of δ-aminolevulinic acid dehydratase.

It has been stated that δ-aminolevulinic acid dehydratase (δ-ALAD), which forms the Schiff base intermediate with the substrate, is the sulfhydryl dependent enzyme. That is, δ-ALAD contains two functional groups, one is a specific lysine residue at the active site which reacts with the carbonyl group of the substrate, the other is essential sulfhydryl groups on which activity of the enzyme largely depends. Presently, there is no clear evidence of whether the essential sulfhydryl groups have a primary role in the enzymatic catalysis or whether reaction of sulfhydryl groups results in secondary changes leading to loss of activity.

During the chemical modification of functional groups of δ-ALAD, we have encountered the evidence that the histidine residue may be involved as another functional group, because the enzyme was all inactivated by the treatment with either photooxidation or diethylpyrocarbonate.

MATERIALS AND METHODS:

Enzyme preparation

δ-ALAD was isolated from beef liver and purified by a slightly modified

procedure of Wilson et al.[1]. The enzymatic activity was assayed by a modified method of Coleman[2] using 5 MM dithiothreitol as an activator. The protein concentration was determined by either measuring absorbance at 280 nm, Lowry's method[3], or performing amino acid analysis.

δ-Aminolevulinic acid hydrochloride was purchased from Nakarai Chemicals Ltd.

Photooxidation of the enzyme

Photooxidation was performed in essentially the same way as that of aldolase[4), 5] except the use of methylene blue as a sensitizer. A number of experiments were made to obtain reproducible oxidation rates and the standard conditions are as follows. One ml of enzyme solution (200 μg protein/ml), 0.001% methylene blue freshly prepared, and 0.1 M Tris-acetate buffer, pH 6.8, are placed in each reaction test tube and kept at 25° C. One 500-watt floodlight (Toshiba photoreflector lamp 3200°K) was placed 70 cm above the enzyme solution and photoirradiation was carried out at different time intervals. After that, removal of the methylene blue dye from the oxidized enzyme solution prior to acid hydrolysis was made by passing through a column (1.5 x 15.0 cm) of Sephadex G-25 which had been equilibrated with 0.05 M Tris-acetate buffer, pH 7.0.

Treatment with diethylpyrocarbonate and with hydroxylamine

Treatment of the enzyme (600 μg protein/2 ml) with diethylpyrocarbonate (0.13 mM) was carried out in 0.1 M sodium acetate buffer, pH 6.0, at room temperature[6), 7]. The number of modified histidine residues was determined by measuring absorbance at 240 nm of N-carbethoxy histidine residues using a value of 3,200 M^{-1} cm^{-1} for the molar extinction coefficient[6]. After the treatment, excess of diethylpyrocarbonate was removed by gel filtration or by reaction with excess of histidine, and then the enzymatic activity was determined.

Deacylation at neutral pH was carried out in 10 to 60 min with 0.7 M hydroxylamine[8] and enzymatic activity was determined after removal of excess of hydroxylamine by gel filtration using Sephadex G-25.

Amino acid analysis

The photooxidation of histidine, methionine, and tyrosine was monitored by amino acid analysis (Hitachi automatic amino acid analyzer (KLA-3B)) on protein samples which had been hydrolyzed with 6 N HCl at 110° C for 24 hrs. Tryptophan was determined according to the methods described by Bencze and Schmid[9], Matsubara[10], or Spande and Witkop[11].

Determination of sulfhydryl contents

The purified δ-ALAD is inactive but activated by reducing agent such as dithiothreitol. The sulfhydryl contents of these inactive or active enzyme preparation were determined as follows. Solutions of purified δ-ALAD was treated with 5,5'-dithiobis (2-nitrobenzoic acid) in 0.1 M Tris-HCl buffer, pH 8.0, for 1 hr at room temperature[12]. The absorbance at 412 nm of each solution and of a reagent blank was recorded with a Shimadzu multipurpose recording spectrophotometer, type MPS-50L. An extinction coefficient of 13,600 M^{-1} cm^{-1} was used to calculate the concentration of sulfhydryl groups. Activated, activator-free δ-ALAD was obtained by passing a solution of the treated enzyme through a column of Sephadex G-25 under anaerobic condition and then the sulfhydryl contents were determined by exactly the same way as described above. The same procedure was applied to the photoinactivated- or diethylpyrocarbonate-treated enzyme preparation.

Sodium dodecylsulfate or urea was used to increase the reactivity of slow-reacting sulfhydryl groups. The combined contents of cystine and cysteine in protein were also determined by the method of performic acid oxidation[13].

RESULTS AND DISCUSSION

Molecular weight of purified enzyme

The purified enzyme preparation gave a single band on polyacrylamide gel electrophoresis carried out at pH 8.3. Molecular weight of each subunit determined by SDS-disc electrophoresis was found to be 35,000, which was in good agreement with the data of Wu et al.[14].

or after integration :

$$\ln \frac{E}{E_o} = \frac{-k_{cat}\, t}{1 + \frac{K_I}{I}}$$

where E_o represents the total amount of enzyme
E the active enzyme at time t
$K_I = k_1 / k_2$

$$\text{or } t_{1/2} = \frac{0.69}{k_{cat}} + \frac{0.69}{k_{cat}} \frac{K_I}{I}$$

As shown in Fig. II, at 25°C and 36°C $t_{1/2}$ is proportional to 1/I. This means that E-I ~ O and that $k_{cat} > k_1$ at these temperatures, in other words the formation of the binary complex is rate limiting. However if the incubation is carried out at 2°C, the catalytic mechanism is slowed down and the kinetic consta were found to be $K_I = 3.4 \times 10^{-4}$M, $t_{1/2}$ for I ~ ∞ = 3.3 min. and $k_{cat} = 3.45 \times 10^{-3}$ sec.$^{-1}$. The K_I of the inhibitor is lower than the K_M of GABA (10^{-3}M).

To ascertain whether the inactivation is catalytic and active site directed, the effect of adding the enzyme substrates was studied (Fig. III). If GABA is added to the incubation mixture, even at a concentration lower than the K_M, the rate of inactivation is greatly decreased. However, if both GABA and α-KG are present in the incubation, the protection against inactivation is lost. At a low inhibitor concentration the rate of inhibition is even higher than in the absence of the substrates. Again, it seems that the formation of the binary complex is rate limiting. GABA-T like many transaminases follows a bi-bi ping pong mechanism (7,15). The protection against enzyme inhibition by GABA is explained by the tran formation of the holoenzyme from the pyridoxal into the pyridoxamine form :

$$E - PLP + GABA \rightleftharpoons E - Pyridoxamine + SSA$$

The pyridoxamine form is then unable to bind and transform the inhibitor. The addition of α-KG displaces the equilibrium to the left, regenerating the PLP holoenzyme which can then bind the inhibitor. The addition of PLP has no marked effect on the inactivation process.

Table 1 Amino Acid analysis of native and photooxidized δ-ALAD

Amino acid	Native	Photooxidized[a]
	Residues/35,000 g protein[b]	
Aspartic acid	22.4	23.2
Methionine	6.4	6.0
Isoleucine	9.0	9.0
Tyrosine	9.1	8.8
Lysine	12.1	13.3
Histidine	6.4	3.8
Tryptophan[c]	4.0	4.0
" [d]	3.8	3.1
" [e]	3.9	3.5
Cysteine[f]	3.1	1.8
" [g]	5.2	5.2
" [h]	9.1	9.0
" [i]	9.1	9.1

a) enzyme with 5% residual activity obtained by photooxidation at pH 6.8

b) the values are given in terms of molar ratio of amino acids, assuming the number of isoleucine residues to be 9.0

c) determined by the method of Bencze and Schmid[9]

d) determined by the method of Matsubara[10]

e) determined by the method of Spande and Witkop[11]

f) determined with Ellman's reagent on not-activated enzyme

g) determined with Ellman's reagent on activated enzyme

h) determined with Ellman's reagent on activated enzyme in the presence of SDS

i) determined as cysteic acid

prepared under the standard conditions employed in this study were compared with those of the native enzyme. Purified but inactive enzyme solution was found to contain 3 sulfhydryl groups which reacted very rapidly with Ellman's reagent. Upon reduction of the enzyme with 5 mM dithiothreitol, 5 residues were revealed, indicating the presence of a mixed disulfide between the thiol

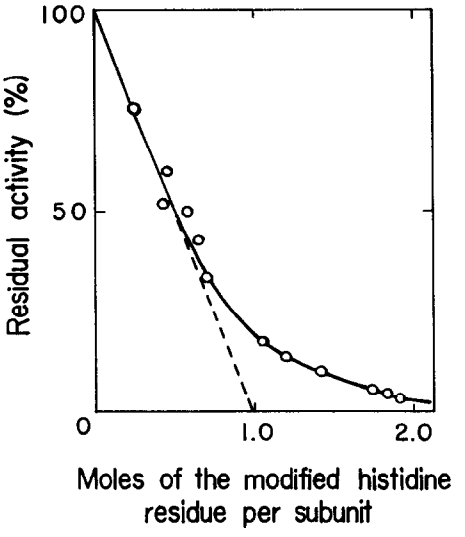

Fig. 2 Relationship between the modified histidine residues per subunit and the enzymatic activities after treatment with 0.13 mM (or 0.3 mM, 0.5 mM) of diethylpyrocarbonate for 5, 15, 20 and 30 min.

group at the active site of the enzyme and cysteine. The other 4 which probably existed in a burried position, appeared only after denaturation (Table 1). In the photoinactivated enzyme, the number of rapidly-reacting thiol groups decreased to 1.8 residues but readily recovered to the original level on treatment with dithiothreitol (Table 1). This indicated that the cysteine residue was not oxidized to cysteic acid. Even after such reduction, the photooxidized enzyme did lead to almost complete inactivation.

Both methionine and tyrosine residues were found to be insensitive and the contents of other amino acids remained practically unchanged. The tryptophan contents of the photooxidized sample determined by three different methods showed loss of one-half residue of tryptophan per subunit.

It was also demonstrated that diethylpyrocarbonate modified one histidine residue per subunit of the enzyme while the enzymatic activity was decreased to less than 5% of the original activity (Fig.2). The inactivation was largely regained by hydroxylamine, indicating that histidine residue but not lysine residue was modified by diethylpyrocarbonate.

In view of these results, it could be postulated that the inactivation was largely due to the modification of the histidine residue and the data appears to dispose decisively of the possibility of histidine group (one or two) involvement as the functional group of δ-ALAD. It is still unknown, however, whether the histidine residue is actively involved in the catalytic site in relation to the concerted action with the sulfhydryl groups, or an unfavorable conformational change in the active site may be induced by the modification of histidine. The change of molecular size of the modified δ-ALAD seemed to be unlikely from the data of gel filtration. Further studies on the inactivation mechanism of δ-ALAD shown in this experiment are needed and the detailed results will be reported at a later date.

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