J. Enzyme Inhibition, 1990, Vol. 3, pp. 295–302
Reprints available directly from the publisher
Photocopying permitted by license only

FURTHER EVIDENCE FOR AN ESSENTIAL HISTIDYL RESIDUE AT THE ACTIVE SITE OF PIG LIVER 5-AMINOLEVULINIC ACID DEHYDRATASE†

HAYDEE FUKUDA, YOLANDA E. SOPENA DE KRACOFF, LIDIA E. IÑIGO, SERGIO R. PAREDES, ANA M. FERRAMOLA DE SANCOVICH, HORACIO A. SANCOVICH and ALCIRA M. DEL C. BATLLE*

Department of Biochemistry, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, Pabellón II, 1428 Nuñez, Buenos Aires, Argentine

(Received 3 April 1989)

Photoxidation with methylene blue and rose bengal and chemical modification by diethylpyrocarbonate of pig liver 5-aminolevulinic acid dehydratase produced strong inactivation of the enzyme which was concentration dependent.

Loss of enzyme activity by both photoxidation and ethoxyformylation was pH and time-dependent and protected by the presence of the substate and competitive inhibitors. The rate of inactivation was directly related to the state of protonation of histidyl groups, the unprotonated from being modified at a much faster rate than the protonated form.

Plots of the pseudo-first order rate constants for 5-aminolevulinic acid dehydratase inactivation against pH resulted in typical titration curves showing inflection points at about pH 6.4 for methylene blue and rose bengal and 6.8 for diethylpyrocarbonate providing further and unequivocal evidence for the existence of critical histidyl groups at the active centre of the enzyme.

KEY WORDS: 5-Aminolevulinic acid dehydratase, methylene blue, rose bengal, diethylpyrocarbonate, histidyl residues.

INTRODUCTION

5-Aminolevulinic acid dehydratase (5-aminolevulinic acid hydrolyase, EC 4.2.1.24) catalyzes the condenstaion of two molecules of 5-aminolevulinic acid (ALA) into the unique monopyrrolic intermediate porphobilinogen (PBG), a key precursor in the synthesis of haems, chlorophylls, corrins and other tetrapyrrole compounds.

Porphobilinogen biosynthesis occurs in a manner reminiscent of the Knorr pyrrole synthesis. An analysis of the characteristics of the 5-aminolevulinic acid dehydratase isolated from different sources reveals that most of its properties and its reaction mechanism appears to be very similar in both eukariotic and prokariotic cells. This complex reaction starts with a stepwise formation of two Schiff bases between 5-aminolevulinic acid molecules and ε-aminolysyl groups of the enzyme. The 5-aminolevulinic acid molecule which gives rise to the propionic acid side chain of porphobilinogen, is the one that initially joins to the enzyme, and its amino group reacts as a nucleophile towards the C_4 of a second 5-aminolevulinic acid molecule. Subsequent steps involving ring closure with the participation of amino acid residues

†Dedicated to Professor Ken Rees on the occasion of his retirement.

*Correspondence to Prof. Dr. Alcira Batlle, Viamonte 1881 10°A, 1056 Buenos Aires, Argentine.

currently implicated in protonation and proton abstraction reactions lead up to the final monopyrrolic ring[1-4].

In the elucidation of enzyme reaction mechanisms it is of importance to identify the amino acids at the enzyme active site. However this is not an easy task because of the lack of very specific amino acid reagents. In the mechanism of porphobilinogen biosynthesis, histidyl residues would be the most likey candidates as the groups acting as proton donors and acceptor within the protein molecule, because they have been identified in the active centre of the enzyme, from other sources[5-7], and because of their particular pKa value[7]. Dye-sensitized photooxidations of enzymes allow the modification of certain amino acid residues with a rather high degree of selectivity therefore providing information as to their role in biological action; in particular photochemical oxidation of histidine can be sufficiently specific to implicate this group in catalysis[8,9].

Because of the known photooxidizing efficiency of a large number of dyes[10], in the present work we have selected methylene blue (MB) and rose bengal (RB) as the photosensitizers to demonstate the presence of an essential imidazole group in the pig liver 5-aminolevulinic acid dehydratase. To obtain more specific evidence for the role assigned to histidine in the enzymic reaction we have also studied the effects of chemically modifying time enzyme by diethylpyrocarbonate (DEP), a reagent which at neutral or slightly acidic pH values has been shown to modify histidyl residues in proteins with considerable specificity without affecting any other amino acid residues[11-13].

MATERIALS AND METHODS

Chemicals

5-Aminolevulinic acid, dithiotreitol (DTT) and diethylpyrocarbonate (DEP) were obtained from Sigma Chem. Co. Filtration gels were from Pharmacia Fine Chemicals. Imidazole was from Eastman Kodak. All other chemicals were of the highest purity obtained from different commercial sources.

Enzyme purification and assay

This were performed as already described[14]. A preparation (480-fold purified) with a specific activity of 9,140 nmol PBG/h mg was used throughout the study.

Photooxidation with methylene blue and rose bengal

Photooxidations were performed in essentially the same manner as described by Ray[15] and Hoffe *et al.*[16]. The standard conditions were as follows. One ml of enzyme solution (1.2–1.5 mg, subunit concentration 3.4×10^{-5} to 4.3×10^{-5} M) and the photoactivated oxidant at the concentration, pH and temperature as indicated in each experiment, were placed 30 cm below a 200 watt light and photoirradiation was carried out for the times indicated. For protection studies of pig liver 5-aminolevulinic acid dehydratase against photo-sensitized degradation the enzyme was preincubated

with the substrate or the competitive inhibitors for 10 minutes prior to photoirradiation. Aliquots of the treated enzyme were taken at varying intervals and their activity determined. Prior to enzyme activity determination removal of the sensitizer from the oxidized enzyme solution was accomplished by passing the mixture through a Sephadex G-25 column (2 × 30 cm) which had been equilibrated with potassium phosphate buffer (0.1 m, pH 6.8). Unilluminated samples kept under the same conditions as those irradiated, provided the 100% references in both methylene blue and rose bengal assays.

Chemical modification with diethylpyrocarbonate

The method of Miles[17] was adapted for treatment of pig liver 5-aminolevulinic acid dehydratase with diethylpyrocarbonate. The purified enzyme (115–230 μg, subunit concentrations 6.6×10^{-6} to 8.2×10^{-6} M) in 0.1 M potassium phosphate buffer (or as indicated) was preincubated with 5 mM dithiotreitol at 37°C, pH 6.8 for 10 min. For protection studies of the enzyme against diethylpyrocarbonate inhibition, pig liver 5-aminolevulinic acid dehydratase was preincubated with the substrate or competitive inhibitors for 10 min at 0°C previous to modification. Further treatment of the activated enzyme with diethylpyrocarbonate was carried out at 0°C. An appropriate concentration of diethylpyrocarbonate (range 0.1–0.35 mM) in ethanol (final ethanol concentation 2%) was added to the enzyme. At varying time intervals aliquots (50 μl) were withdrawn and both activity and protein concentration were determined. The ethoxy formylation reaction was stopped by addition of 10 mM imidazole at pH 7.5.

All other materials and methods not specified here were those already reported[14,18].

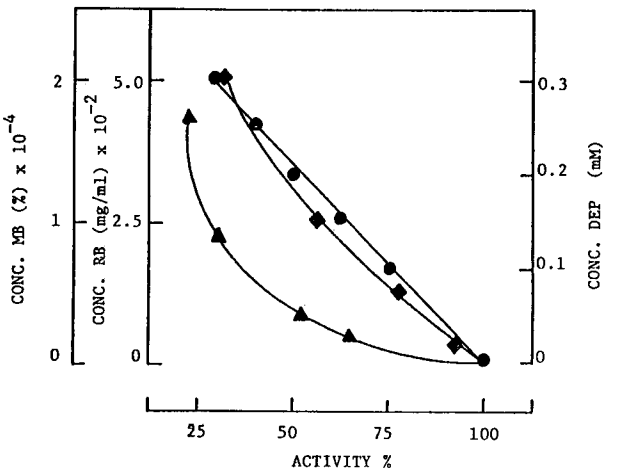

FIGURE 1 Modification of pig liver 5-aminolevulinic acid dehydratase by sensitized photooxidation with (◆) methylene blue at pH 6.8 and (▲) rose bengal at pH 6.6 at different sensitizer concentrations. The reactions were carried out at 20°C during 30 min. (●) Inactivation at different diethylpyrocarbonate concentrations. The reactions were carried out at 0°C during 5 min and pH 6.8. Other experimental conditions were as indicated in the text.

RESULTS AND DISCUSSION

Inactivation of 5-aminolevulinic acid dehydratase

The photoxidation of 5-aminolevulinic acid dehydratase sensitized by both methylene blue and rose bengal was assessed. It was found that pig liver 5-aminolevulinic acid dehydratase sensitized photodegradation increased with increasing dye concentrations (Figure 1). However it is interesting to note that at relatively low values, maximum effects are obtained and the concentration of dye is no longer rate-limiting. It was also observed that rose bengal was a more powerful photosensitizer than methylene blue, as has already been reported for histidine photooxidation[19].

Inactivation of pig liver 5-aminolevulinic acid dehydratase by diethylpyrocarbonate occurred very rapidly at room temperature and pH 6.8 and under these conditions and using 0.2 M diethylpyrocarbonate, 70% of activity was lost within 1 minute (data not shown). Instead when the enzyme was treated with diethylpyrocarbonate at 0°C and the same pH, activity decayed at a slower rate and linearly with increasing concentrations of the inhibitor; thus 70% inactivation was only obtained after treatment with 0.3 mM diethylpyrocarbonate for 5 min.

Histidine is one of several amino acids which can be photooxidized in the presence of dyes such as methylene blue and rose bengal[9,15,16,20] and chemical modification of enzymes using the group specific reagent diethylpyrocarbonate has demonstrated that histydyl residues are modified with concomitant loss of catalytic activity[21]. Accordingly, we have also found decreased activity of 5-aminolevulinic acid dehydratase in the presence of such chemicals, indicating that histidyl residues are very likely part of the active centre of pig liver 5-aminolevulinic acid dehydratase.

Effect of pH and exposure time

It is well known that amino acids readily susceptible to photodynamic degradation, can be oxidized at pH values between 4 to 10. Methylene blue (pK_a = 11.6) and rose bengal (pK_a = 4.3) at these pH values exist in solution almost fully ionized as anionic and cationic species respectively. The relative rate at which they undergo photooxidation, however, is dependent on both the pH of the medium and the nature of the sensitizer used. Photodegradation of pig liver 5-aminolevulinic acid dehydratase by both methylene blue and rose bengal, was found to be a function of time and pH (Figures 2a and 2b). Measurable rates of photodegradation were observable within the pH range shown (pH 5.4–8.5). At lower pH values, photooxidation of the enzyme was negligible. The rate of oxidation was proportional to the exposure time, being more rapid as the pH increased, showing that it mainly depends on the state of protonation of the histidyl group and revealing that the unprotonated nucleophile is modified at a much faster rate than its protonated form. Although both dyes were able to sensitize 5-aminolevulinic acid dehydratase photodegradation, the acid dye (rose bengal) again was a markedly more efficient sensitizer than the alkaline dye (methylene blue), within the pH-range studied (Figures 2a and 2b).

It was also found that diethylpyrocarbonate enzyme modification was pH- and time-dependent (Figure 2c), providing further evidence for the presence of histidyl residues at the active centre of pig liver 5-aminolevulinic acid dehydratase.

In contrast with (Figures 2a and 2b) to photodegradation the course of enzyme inactivation by diethylpyrocarbonate is found to be non-linear with the reaction

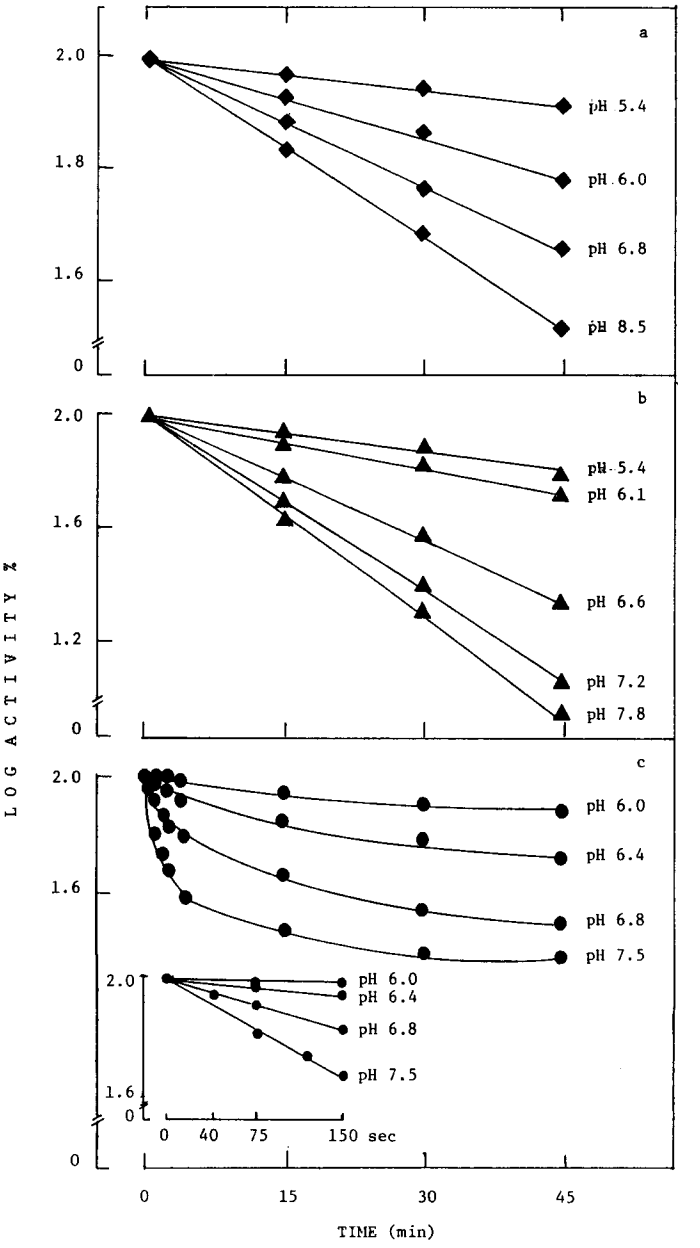

FIGURE 2 Rates of 5-aminolevulinic acid dehydratase photodynamic degradation with (a) methylene blue ($5 \div 10^{-5}$%) and (b) rose bengal (4.4×10^{-3} mg/ml) as sensitizers and (c) diethylpyrocarbonate (0.2 mM) inactivation at various times and pH values. Other experimental conditions were as indicated in the text.

TABLE I
Protection of 5-aminolevulinic acid dehydratase against inactivation by 5-aminolevulinic acid, levulinic acid and pyruvic acid.

Additions	Specific activity (nmol PBG/h/mg)
None	8,655
MB	3,970
MB + ALA	6,925
MB + levulinic acid	6,750
MB + pyruvic acid	6,490
RB	5,625
RB + ALA	7,356
RB + levulinic acid	7,055
RB + pyruvic acid	6,724
DEP	3,460
DEP + ALA	6,665
DEP + levulinic acid	5,885
DEP + pyruvic acid	4,847

Photooxidation with methylene blue 5×10^{-5}% at pH 6.8 and rose bengal 4.4×10^{-3} mg/ml at pH 6.6 were carried out at 20°C during 30 min. Chemical modifications with diethylpyrocarbonate (0.2 mM) were carried out at 9°C during 5 min and pH 6.8. All reactions were performed as described under Materials and Methods in the presence or absence of 5-aminolevulinic acid 5×10^{-3} M, levulinic acid 10^{-3} M or pyruvic acid 10^{-4} M as shown. The remaining specific activity was compared with controls as indicated in Material and Methods.

period studied (Figure 2c) because its rate is higher than that the photooxidation reactions. Due to the high reactivity of diethylpyrocarbonate observed with this enzyme, linear plots could only be recorded at shorter intervals (inset Figure 2c).

The presence of both the substrate and the competitive inhibitors levulinic[22] and pyruvic acid (Sopena de Kracoff, Sancovich, H.A. and Ferramola de Sancovich, A.M, Unpublished data), markedly attenuated the inhibition effect on both photosensitized oxidations and diethylpyrocarbonate inactivation (Table I).

Overall these results indicate that histidyl residues form part of the enzyme active centre or at least participate in the course of the enzymatic catalysis.

pH-Dependence and pseudo-first order rate constants for modification of 5-aminolevulinic acid dehydratase activity

Calculate of the pseudo-first order rate constants for 5-aminolevulinic acid dehydratase inactivation (K_{app}) from the results of Figure 2, and a plot of these values against pH gives three typical titration curves (Figure 3) showing inflection points at pH 6.4 for methylene blue and rose bengal and pH 6.8 for diethylpyrocarbonate were obtained.

The pH-profile for the oxidation of histidine, which is the only photolabile amino acid whose rate of oxidation is not constant between pH 5 and 9[19], and the typical titration curves for histidine inactivation by diethylpyrocarbonate[23] closely resemble the data presentd here for both methylene blue and rose bengal photooxidation and diethylpyrocarbonate chemical inactivation of pig liver 5-aminolevulinic acid dehy-

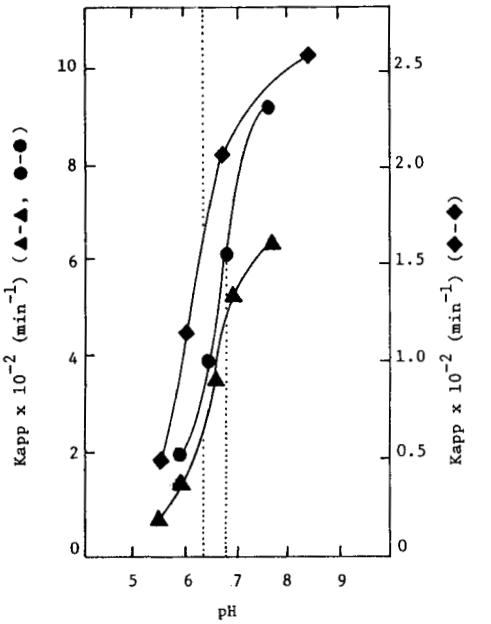

FIGURE 3 pH-dependent of pseudo-first order rate constants (K_{app}) for methylene blue and rose bengal photosensitized degradation and diethylpyrocarbonate inactivation of pig liver 5-aminolevulinic acid dehydratase. Plots are from data in Figure 2. Symbols as in Figure 1.

dratase, providing further proof for the existence of critical histidine residues at the active site of the enzyme.

This study confirms previous findings[7] postulating that histidyl residues are essential for catalytic activity of pig liver 5-aminolevulinic acid dehydratase. These results are consistent with the participation of the unprotonated and also protonated forms of the imidazole moiety of a putative histidyl residue, in the reversible transfer of protons from an aqueous media to the hydrophobic active centre of the enzyme, according to the mechanism proposed for this reaction.

Acknowledgements

Alcira M. del C. Batlle, Horacio Sancovich and Ana M. Ferramola de Sancovich holds the post of Scientific Researcher at the Argentina National Research Council (CONICET); Haydée Fukuda and Sergio Paredes thank CONICET for fellowships.

This work was supported by grants from CONICET and the Univeristy of Buenos Aires and is part of the Doctoral Thesis of Haydée Fukuda and Yolanda Sopena de Kracoff submitted to the University of Buenos Aires for their PhD degree. We thank Mrs. L. Lescano for expert technical assistance.

References

1. Jordan, P.M. and Seehra, J.S. (1980) *Chem. Comm.*, 240.
2. Jordan, P.M. and Seehra, J.S. (1980) *FEBS Letters*, **114**, 283.
3. Jordan, P.M. and Gibbs, P.N.B. (1985) *Biochem. J.*, **277**, 1015.
4. Gibbs, P.N.B. and Jordan, P.M. (1986) *Biochem. J.*, **236**, 447.

5. Barnard, G.F., Itoh, R., Hohberger, L.H. and Shemin, D. (1977) *J. Biol. Chem.*, **252**, 8965.
6. Batlle, A.M. del C. and Stella, A.M. (1978) *Int. J. Biochem.*, **9**, 861.
7. Tsukamoto, I., Yoshinaga, T. and Sano, S. (1975) *Biochem. Biophys. Res. Commun.*, **67**, 294.
8. Weil, L., James, S. and Buchert, A.R. (1953) *Arch. Biochem. Biophys.*, **46**, 266.
9. Weil, L. and Seibles, T.S. (1955) *Arch. Biochem. Biophys.*, **54**, 368.
10. Oster, G., Bellin, J.S., Kimball, R.W. and Schrader, M.E. (1959) *J. Am. Chem. Soc.*, **81**, 5095.
11. Ovádi, J., Libor, S. and Elödi, P. (1967) *Acta Biochim. Biophys. Acad. Sci. Hung.*, **2**, 455.
12. Müllard, A., Hegyi, G. and Horányi, M. (1969) *Biochim. Biophys. Acta*, **181**, 184.
13. Melchior, W.B. (Jr.) and Fahrney, D. (1970) *Biochemistry*, **9**, 251.
14. Fukuda, H., Paredes, S.R. and Batlle, A.M. del C. (1988) *Comp. Biochem. Physiol.*, **91B**, 285.
15. Ray, W.J. (Jr.) (1967) *Meth. Enzymol.*, **XI**, 490.
16. Hoffee, P., Lai, C.Y., Pugh, E.L. and Horecker, B.L. (1967) *Proc. Natn. Acad. Sci. U.S.A.*, **57**, 107.
17. Miles, E.W. (1977) *Meth. Enzymol.*, **47**, 412.
18. Sopena de Kracoff, Y.E., Kartofel, B.M., Ferramola de Sancovich, A.M. and Sancovich, H.A. (1989) *Anales Asoc. Quim. Arg.*, **77**, 185.
19. Bellin, J.S. and Yankus, C.A. (1967) *Arch. Biochem. Biophys.*, **123**, 18.
20. Westhead, E.W. (1965) *Biochemistry*, **4**, 2139.
21. McCracken, S.R. and Meigen, E.A. (1981) *J. Biol. Chem.*, **256**, 3945.
22. Seehra, J.S. and Jordan, P.M. (1981) *Eur. J. Biochem.*, **113**, 435.
23. Abdulwajid, A.W. and Wu, F.Y.H. (1986) *Biochemistry*, **25**, 8167.