Biochimica et Biophysica Acta, 293 (1973) 506–513

BBA 66808

THE ENZYMATIC INACTIVATION OF PORPHOBILINOGEN DEAMINASE

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(Received July 27th, 1972)

SUMMARY

1. Porphobilinogen deaminase was inhibited by pyrrolooxygenase, a mixed-function oxidase which inhibits tryptophan-containing enzymes in the presence of sodium dithionite.

2. Both enzymes were isolated from wheat germ but it was found that pyrrolooxygenase acted on deaminases from different origins.

3. Porphobilinogen deaminase was inactivated by chemical reagents which oxidize tryptophan residues in proteins, and the disappearance in tryptophan content in the deaminase was paralleled by the loss in its enzymatic activity.

4. Spectrophotometric studies indicated that pyrrolooxygenase may oxidize only a few specific tryptophan residues of porphobilinogen deaminase, causing deaminase inhibition.

INTRODUCTION

The enzymatic polymerization of porphobilinogen yields the cyclic tetrapyrrole derivatives known as porphyrins and chlorins[1]. This polymerization is carried out by an enzyme called porphobilinogen deaminase, which transforms prophobilinogen into uroporphyrinogen I. The metabolic polymerization is directed toward the biosynthesis of uroporphyrinogen III in the presence of a second enzyme known as uroporphyrinogen III cosynthetase, which by itself does not consume porphobilinogen[2]. Uroporphyrinogen III is the precursor of both heme and the chlorophylls.

It has never been demonstrated before that porphobilinogen deaminase, the porphobilinogen-consuming enzyme, is subjected to any kind of metabolic control. As will be shown below porphobilinogen deaminase was efficiently inactivated by pyrrolooxygenase, a newly-discovered enzyme present in the same extracts as deaminase. Pyrrolooxygenase was found to inactivate a large number of enzymes containing tryptophan residues essential for their activity[3,5]. It is a mixed-function oxidase which oxidizes the tryptophan residues of the enzymes, inhibiting their activity[5]. Since pyrrolooxygenase is an ubiquitous enzyme present in both plants and animals[4], its interaction with porphobilinogen deaminase may be of importance in the regulation of porphyrin metabolism.

MATERIALS AND METHODS

Porphobilinogen was obtained by synthesis[6]. L-Tryptophan, L-histidine, skatole, *N*-bromosuccinimide, sodium dithionite and all other reagents were commercial products of analytical grade. Wheat germ was a gift of Molinos Rio de la Plata. Spectrophotometric determinations were done with a Beckman DU spectrophotometer. Fluorimetric measurements were carried out with an Aminco Bowman spectrofluorimeter.

Porphobilinogen deaminase was isolated and purified from wheat germ. 100 g of wheat germ were extracted with 400 ml of water. The slurry was filtered through a nylon cloth and centrifuged at 20 000 × *g* for 15 min. The supernatant fluid was adjusted to pH 5 with 1 M acetic acid and kept for 1 h at 0–4 °C. After centrifugation at 20 000 × *g* for 15 min, the supernatant solution was fractionated with solid $(NH_4)_2SO_4$. The 30–50% fraction (AS 30–50) was reprecipitated again with a neutralized $(NH_4)_2SO_4$ solution and the fraction precipitating between 30 and 70% saturation (AS II) was dissolved in 0.01 M Tris–HCl (pH 7.6), dialyzed against 4 l of water and applied to a 3 cm × 20 cm DEAE-cellulose column equilibrated with 0.01 M Tris–HCl buffer. The deaminase activity was eluted with 0.2 M NaCl, while pyrrolooxygenase activity was eluted with the 0.01 M Tris–HCl buffer, (Fig. 1). The

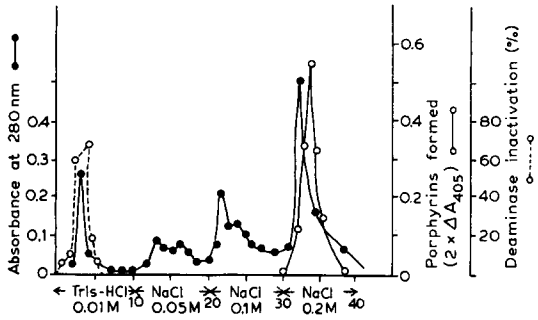

Fig. 1. Separation of pyrrolooxygenase and porphobilinogen deaminase. The conditions of the adsorption and elution are described in the text. Fraction AS II (100 mg of protein) were adsorbed on the column. Activities were measured as described in *Assays*.

deaminase was further purified as described elsewhere[7]. The eluted pyrrolooxygenase was filtered through Sephadex G-100 (2.5 cm × 40 cm) as described[4], concentrated, and used for subsequent work. Porphobilinogen deaminase and uroporphyrinogen III cosynthetase from human erythrocytes were prepared and separated as described[8]. Usually the DEAE-cellulose fractionation completely separated the deaminase from the pyrrolooxygenase in the wheat germ preparations. However, in several preparations some pyrrolooxygenase activity remained in the deaminase fraction.

Assays

Deaminase activity was measured by incubating at 37 °C for 60 min in a final volume of 100 μl: 10 μmoles of P_i buffer (pH 7.4), porphobilinogen deaminase, and porphobilinogen (15 nmoles). The uroporphyrinogen formed was determined as uro-

porphyrin after oxidation[7]. Porphobilinogen remaining was measured spectrophotometrically with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in glacial acetic acid–perchloric acid (84:16, v/v)) at 552 nm after previous addition of Hg^{2+}.

Deaminase inactivation by pyrrolooxygenase. A mixture of DEAE-cellulose purified deaminase (forming 0.8–1.6 μmoles of porphyrin), DEAE-cellulose purified pyrrolooxygenase (5–20 μg protein), 10 μmoles of phosphate buffer (pH 7.4) and sodium dithionite (50 μmoles) was incubated at 37 °C for 15 min at a final volume of 100 μl. Residual deaminase activity was then assayed as described above. Controls omitting either dithionite or pyrrolooxygenase were run simultaneously.

RESULTS

Inhibition of porphobilinogen deaminase by pyrrolooxygenase

The addition of wheat germ pyrrolooxygenase and sodium dithionite to the DEAE-cellulose purified porphobilinogen deaminase, resulted in a net decrease in the porphyrin forming capacity of the latter (Table I). In the less-purified prepa-

TABLE I

PORPHOBILINOGEN DEAMINASE INHIBITION BY PYRROLOOXYGENASE

The incubation mixtures contained in a final volume of 100 μl: 10 μmoles of P_i buffer (pH 7.4), porphobilininogen deaminase from the indicated origins at various stages pf purification, porphobilinogen (15 nmoles), sodium dithionite when indicated, and pyrrolooxygenase (DEAE-cellulose purified fraction). Incubations were carried out during 60 min at 37 °C.

Source of porphobilinogen deaminase	*Addition*	*Porphobilinogen consumed (nmoles)*	*Porphyrin formed*	
			nmoles	*Inhibition (%)*
Supernatant pH 5 fraction	—	5.4	0.88	—
(wheat germ)	dithionite	12.4	0.19	78
AS 30–50 fraction	—	5.4	1.23	—
(wheat germ)	dithionite	4.3	0.05	96
AS II fraction	—	4.2	0.88	—
(wheat germ)	dithionite	3.4	0.07	92
DEAE-cellulose fraction	—	4.3	1.0	—
(wheat germ)	dithionite	4.6	1.0	—
	pyrrolooxygenase	4.0	0.96	—
	pyrrolooxygenase + dithionite	5.9	0.05	95
DEAE-cellulose fraction	—	3.8	0.72	—
(human erythrocytes)*	dithionite	3.8	0.75	—
	pyrrolooxygenase	3.4	0.68	—
	pyrrolooxygenase + dithionite	4.0	0.11	85
AS 40–50 fraction**	—	5.4	1.12	—
(*Rhodospirillum rubrum*)	dithionite	5.9	1.18	—
	pyrrolooxygenase + dithionite	5.2	0.16	85
25 000 × *g* supernatant	—	4.4	0.80	—
(Swiss chard)**	dithionite	4.0	0.25	69
DEAE-cellulose fraction	—	4.2	0.78	—
(Swiss Chard)	dithionite	4.2	0.82	—
	pyrrolooxygenase + dithionite	5.5	0.08	90

* Isolated as described in ref. 8.
** Isolated as described in ref. 7.

TABLE II

INACTIVATION OF PORPHOBILINOGEN DEAMINASE BY PYRROLOOXYGENASE

500 μl of the reaction mixture described in Materials and Methods and containing the components indicated above were preincubated for 15 min at 37 °C. An aliquot was withdrawn and assayed for deaminase and porphobilinogen oxygenase[9] activities. The remainder was then dialyzed for 4 h against 4 l of water and then assayed for deaminase and porphobilinogen oxygenase activity after addition of P_i buffer (10 μmoles) to restore the phosphate lost during dialysis.

Preincubated system	*Activity (nmoles consumed or formed)*			
	Before dialysis		*After dialysis*	
	Porphobilinogen consumed	*Porphyrin formed*	*Porphobilinogen consumed*	*Porphyrin formed*
Deaminase	4.6	1.05	3.8	0.88
Deaminase + pyrrolooxygenase	4.3	1.02	3.2	0.70
Pyrrolooxygenase	none	—	none	—
Deaminase + dithionite	4.6	0.90	2.7	0.62
Deaminase + pyrrolooxygenase + dithionite	5.9	0.10	0.27	0.02
Pyrrolooxygenase + dithionite	6.7	0	0	0

rations of porphobilinogen deaminase (AS 30–50 and AS II) the sole addition of sodium dithionite produced the same effect, indicating that pyrrolooxygenase was present in those preparations (Table I). It has already been reported that sodium dithionite was necessary for pyrrolooxygenase activity[3,4].

Since porphobilinogen is also a substrate of a dithionite-dependent pyrrolooxygenase (porphobilinogen oxygenase)[9], it remained to be demonstrated that the inhibition of porphyrin formation was not due to a lack of substrate (porphobilinogen). This was achieved by first preincubating deaminase with pyrrolooxygenase and sodium dithionite, and then assaying the porphobilinogen utilization and porphyrin formation before and after dialysis (Table II). The results indicated that deaminase activity was not affected by preincubation with pyrrolooxygenase in the absence of dithionite and was almost totally inhibited in the presence of the latter. Removal of the sodium dithionite by dialysis afforded an equally inactive deaminase even in the presence of added substrate. Both pyrrolooxygenases, the porphobilinogen consuming one and the one which inactivated porphobilinogen deaminase could be partially separated by a DEAE-cellulose exchange (Fig. 2). The pyrrolooxygenase which inactivates porphobilinogen deaminase is probably identical with tryptophan pyrrolooxygenase[4], and differs in many of its properties from porphobilinogen oxygenase[9].

Similar preparations from Swiss chard (Table I) and spinach[4] also contained porphobilinogen deaminase together with pyrrolooxygenase[7], and were separated as described above. They were also found to interact as described. Wheat germ pyrrolooxygenase also inactivated porphobilinogen deaminase from *Rhodospirillum rubrum* and from human erythrocytes (Table I). Pyrrolooxygenase did not affect the activity of uroporphyrinogen III cosynthetase isolated either from wheat germ or from human erythrocytes.

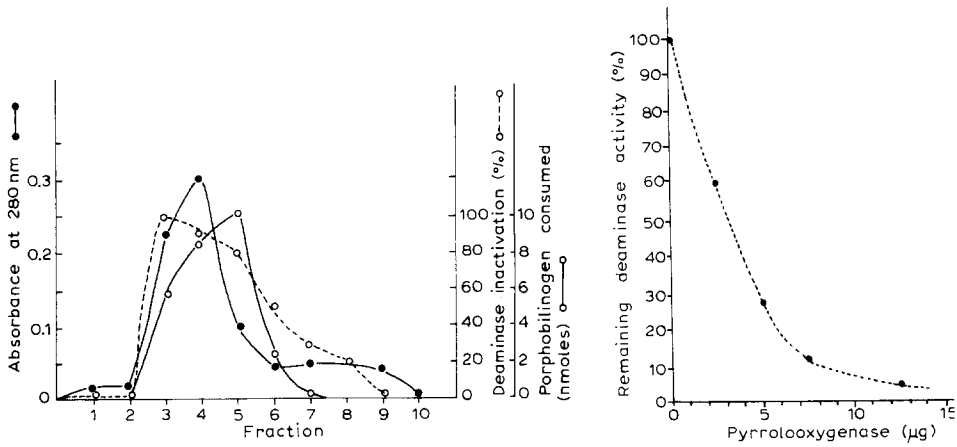

Fig. 2. Partial separation of pyrrolooxygenase and porphobilinogen oxygenase. Fraction AS II (100 mg) were adsorbed on a DEAE-cellulose column (3 cm × 20 cm) equilibrated with 0.01 M Tris–HCl buffer. Activities were eluted with the same buffer. Fractions of 30 ml were collected. Pyrrolooxygenase-inhibiting activity on deaminase was determined as described in *Assays*. Porphobilinogen oxygenase activity was measured as described[9], using 15 nmoles of porphobilinogen.

Fig. 3. Effect of pyrrolooxygenase concentration on deaminase inactivation. The pyrrolooxygenase inhibiting activity was measured as described in Table I, except for the amounts of pyrrolooxygenase used.

Properties of the inhibition of deaminase by pyrrolooxygenase

The effect of pyrrolooxygenase concentration on deaminase inactivation can be seen in Fig. 3. The inactivation was completely suppressed when the pyrrolooxygenase was heated at 100 °C for 5 min or when it was digested with trypsin (EC 3.4.4.4) (20 μg of protein for 30 min at 37 °C). The pyrrolooxygenase had an absolute requirement for oxygen and a reducing agent. Sodium dithionite was the best reducing agent and could be partially replaced by either sodium bisulfite or sodium sulfite. Other reducing agents were ineffective as was the case with other pyrrolooxygenases[3,4].

Evidence for the oxidation of an essential tryptophanyl residue during the inactivation of porphobilinogen deaminase

Although porphobilinogen deaminase from wheat germ had been considerably purified, the electrophoresis on polyacrylamide gel showed that it was not homogeneous[7]; hence, its amino acid composition could not be determined. However, besides the inactivation by pyrrolooxygenase, a series of chemical modifications carried out with the partially-purified enzyme consistently indicated that tryptophan residues were necessary for its activity. Porphobilinogen deaminases from different origins were efficiently inactivated by photooxidation in the presence of methylene blue and only moderately inactivated in the presence of rose bengal, as would be the case if a tryptophan residue was being oxidized (Table III). The photooxidation in the presence of the methylene blue also resulted in a decrease in the intensity of the tryptophan fluorescence of the deaminase. *N*-Bromosuccinimide, employed under the controlled conditions in which it is known to selectively oxidize tryptophan residues[10],

TABLE III

EFFECT OF PHOTOOXIDATION AND *N*-BROMOSUCCINIMIDE OXIDATION ON DEAMINASE ACTIVITY

Photooxidations were carried out in small test tubes immersed in an ice-bath. A 100-W lamp placed at 20 cm from the tubes was used as a light source. The enzyme (phosphate gel purified deaminase[7]) was illuminated for 10 min in the presence of the sensitizer and in the absence of buffer. Deaminase activity was then assayed as described. Oxidations were also carried out using *N*-bromosuccinimide (0.07 mM) and were performed at either pH 4.5 (acetate buffer), or pH 7.4 (phosphate buffer) for 15 min, as described[10]. The system was assayed for deaminase activity after dialysis.

Treatment	*Porphyrin formed (nmoles)*	*Inhibition (%)*
None	1.41	—
Photooxidation (methylene blue, 0.02%)	0.13	91
Photooxidation (rose bengal, 0.02%)	0.77	46
Preincubated with *N*-bromosuccinimide	0.08	94

inhibited the deaminase activity almost completely (Table III). The inactivation of porphobilinogen deaminase by *N*-bromosuccinimide (Table III) was correlated with a decrease in absorption at 280 nm (Fig. 4a) and in its characteristic tryptophan fluorescence (Fig. 4b). During the inactivation of porphobilinogen deaminase by pyrrolooxygenase a simultaneous decrease in its tryptophan fluorescence and absorbance at 280 nm also took place (Figs 5a and 5b). This decrease was less pronounced than in the case of the *N*-bromosuccinimide oxidation and the inactivated deaminase showed a shift toward higher wavelengths in its tryptophan fluorescence peak (Fig. 5b), indicating that the tryptophan oxidized by pyrrolooxygenase was

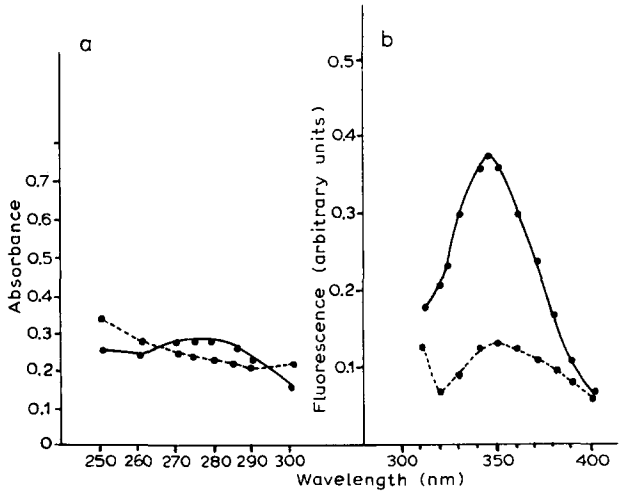

Fig. 4. Oxidation of porphobilinogen deaminase by *N*-bromosuccinimide. (a) Ultraviolet spectra of the *N*-bromosuccinimide-treated deaminase (●---●) and untreated deaminase (●—●). The conditions were as described in Table III. (b) Fluorescence emission spectra of the *N*-bromosuccinimide-treated (●---●) and untreated (●—●) deaminase. Excitation was at 290 nm.

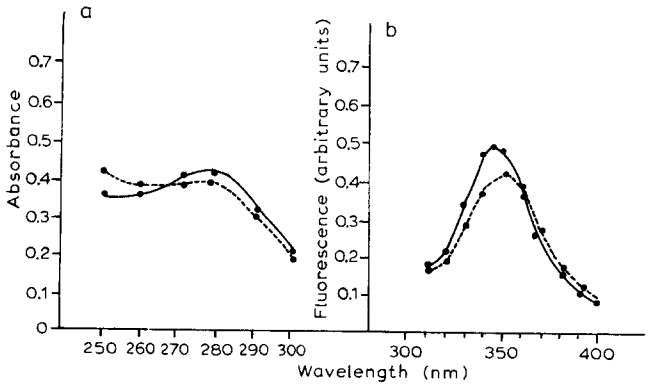

Fig. 5. Oxidation of porphobilinogen deaminase by pyrrolooxygenase. (a) Ultraviolet spectra o the deaminase incubated with pyrrolooxygenase (●—●) and with pyrrolooxygenase *plus* dithio nite (●---●). Incubation conditions were as described in *Assays*. Deaminase used was the calciun phosphate gel enzyme[7]. (b) Fluorescence emission spectra of deaminase *plus* pyrrolooxygenas (●—●) and of deaminase *plus* pyrrolooxygenase and dithionite (●---●).

originally placed in a more hydrophobic region. The addition of tryptophan (5(μmoles) protected porphobilinogen deaminase from its inactivation by pyrrolo oxygenase (50% protection), while histidine (50 μmoles), or skatole (50 μmoles), dic not afford any protection.

By heating the deaminase for 15 min at 70 °C a small increase in its activity could be achieved. When the fluorescence spectra of both the native and the heated deaminase were compared, a 20% decrease in the intensity of the tryptophan fluorescence peak was observed in the heated deaminase, as well as a shift of the maximum toward higher wavelengths (from 350 to 356 nm). This effect could be attributed to a change in the conformation of the heated deaminase: when a trypto phanyl residue becomes buried in a more hydrophobic environment it will not be detected by its fluorescence spectra any longer. The heated enzyme was much less sensitive to pyrrolooxygenase inactivation than the native enzyme (17% inactivation as compared to 91% of inactivation), as could be expected if a tryptophan residue essential for the deaminase activity became protected by an hydrophobic environment Tryptophan residues buried in hydrophobic environments are known to be refractory to *N*-bromosuccinimide oxidation[11]. It should be noted that the tryptophany residues which cannot be detected any more in the heated enzyme seem to be of the same type as those which disappeared during the enzymatic oxidation of the deamin ase (Fig. 5b).

DISCUSSION

From extracts of wheat germ a pyrrolooxygenase has already been isolated which oxidized the tryptophanyl residues of many peptides and enzymes producing inactivation of the latter[3,5]. Since the pyrrolooxygenase which inactivated por phobilinogen deaminase appears to be identical with the above mentioned pyrrolo oxygenase, it is possible that the inactivation of porphobilinogen deaminase is also due to oxidation of a tryptophanyl residue. In support of this assertion it was found that deaminase was efficiently inactivated by oxidizing agents (light in the presence

of methylene blue, *N*-bromosuccinimide), which are known to oxidize tryptophanyl or histidyl residues. Photooxidation in the presence of rose bengal, which is more specific for histidyl residues[12], was only moderately efficient in inhibiting deaminase activity. It was also known that pyrrolooxygenase did not oxidize histidine or tyrosine residues in peptides[4,5]. Tryptophan also protected the deaminase from its oxidation by pyrrolooxygenase, while histidine was without effect.

During the inactivation of deaminase with *N*-bromosuccinimide a net decrease in the intensity of its tryptophan ultraviolet and fluorescence spectra was observed (Figs. 4a and 4b). The enzymatic oxidation by pyrrolooxygenase produced the same effect but was more specific, since only certain tryptophan residues of a more hydrophobic character were oxidized (Figs 5a and 5b).

It remains to be demonstrated that the enzymatic inactivation of porphobilinogen deaminase by pyrrolooxygenase, together with the enzymatic oxidation of porphobilinogen[9], are of relevance for the regulation of porphyrin metabolism.

ACKNOWLEDGMENTS

This work was supported by the United States Institutes of Health (GM-11973), and the Consejo Nacional de Investigaciones (Argentina).

We are grateful to Miss Juana H. Lis for skilled technical assistance.

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