PYRROLOOXYGENASE: A NEW TYPE OF TARGET ENZYME

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Received 16 April 1971

1. Introduction

A new enzyme was detected in plants for which the name pyrrolooxygenase is proposed. The enzyme oxidized the pyrrole ring of tryptophan even when it formed part of proteins. Thus, by the action of the pyrrolooxygenase on chymotrypsin, trypsin, pepsin and lysozyme the tryptophan group of the latter was oxidized and they lost their enzymatic activity. The name of pyrrolooxygenase was proposed since it has an absolute requirement for an exogenous reductor and oxygen and since it differs markedly from tryptophan pyrrolase and from tryptophan hydroxylase.

2. Materials and methods

Trypsin, chymotrypsin, lysozyme and pepsin were commercial products. Potato phosphorylase was prepared as described [1]. L-Tryptophan, D-tryptophan kynurenin, *N*-bromosuccinimide (NBS) and sodium dithionite were commercial samples of analytical grade. Ethyl *N*-acetyl-L-tryptophan, ethyl 2-acetamido-3-(2′-formimidobenzoyl)-propionate and ethyl *N*-acetyl-2-oxo-tryptophan were prepared by synthesis. Wheat germ was a gift of Molinos Rio de la Plata (Buenos Aires).

2.1. *Preparation and partial purification of pyrrolooxygenase*

One hundred grams of wheat germ were extracted with 400 ml of water. The slurry was filtered through a nylon cloth and centrifuged at 15,000 *g* for 15 min. The supernatant fluid was adjusted to pH 5 with 1 N acetic acid and kept for one hour at 0–4°. After centrifugation at 15,000 *g* for 15 min, the supernatant solution was fractionated with solid $(NH_4)_2SO_4$. The 30–50% fraction was dissolved in 0.01 M tris-HCl (pH 7.6), dialyzed against 4 liters of water and clarified by centrifugation. This fraction (100 mg of protein) was applied to a 3 × 20 cm DEAE cellulose column equilibrated with 0.01 M tris-HCl (pH 7.6), and the activity was eluted with the same buffer. The enzyme purified in this manner was filtered through Sephadex G-100 (2.5 × 40 cm), concentrated and used for subsequent work. This preparation was stable at 0–4° for six months.

2.2. *Assay of pyrrolooxygenase*

Unless otherwise indicated, pyrrolooxygenase activity toward tryptophan or ethyl *N*-acetyl tryptophan was routinely assayed as follows: 37 nmoles of substrate, 10 μmoles of phosphate buffer (pH 7.4), 0.05 μmole of sodium dithionite and enzyme (5 to 20 μg of protein) were incubated in a final volume of 100 μl for 30 to 60 min at 37°. Substrate consumption was assayed 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+}. Two blanks were usually run, omitting either dithionite or enzyme. When the pyrrolooxygenase inhibiting activity on a tryptophan containing enzyme was assayed, the following procedure was used: 10 to 20 μg of the crystallized enzyme was preincubated with the pyrrolooxygenase at the above indicated pH, buffer and dithionite concentrations at 37° for 15 to 30 min, and the remaining enzymatic activity was then assayed.

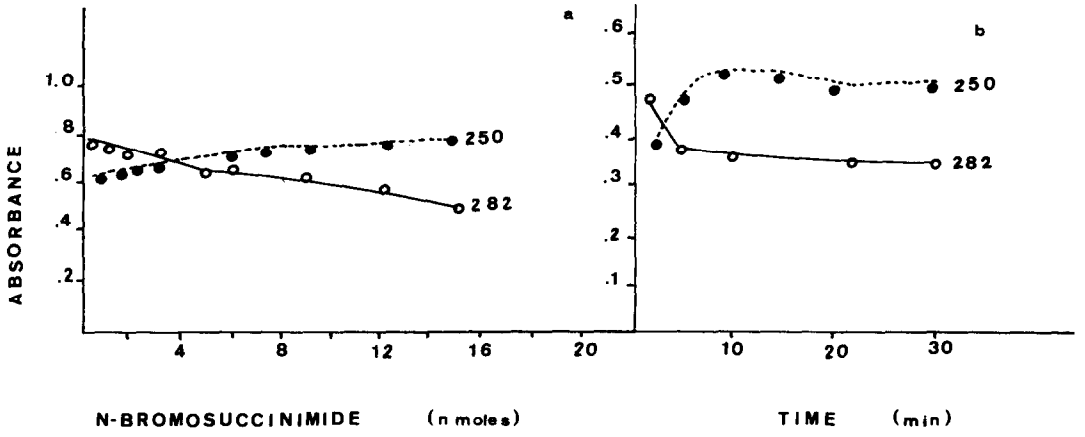


Fig. 1. Correlation of the loss of absorbance at 282 nm (——) with the increase of absorbance at 250 nm (- - - -) in: (a) the chemical (NBS) oxidation of lysozyme and (b) the enzymatic. In the case of the NBS oxidation, increasing amounts of this reagent were added with stirring, to a solution of lysozyme (0.1 mg) in phosphate buffer (10 μmoles), pH 7.4. Final volume, 0.4 ml. Recordings at 282 nm and 250 nm were made immediately after addition. The enzymatic oxidation was carried out in a reaction mixture containing the lysozyme (0.01 mg), oxygenase (5 μg), dithionite (0.5 mM) and the above indicated buffer. Recordings at 282 nm were made at the indicated times. Blanks omitting either sodium dithionite or oxygenase showed no changes with time either at 282 or 250 nm.

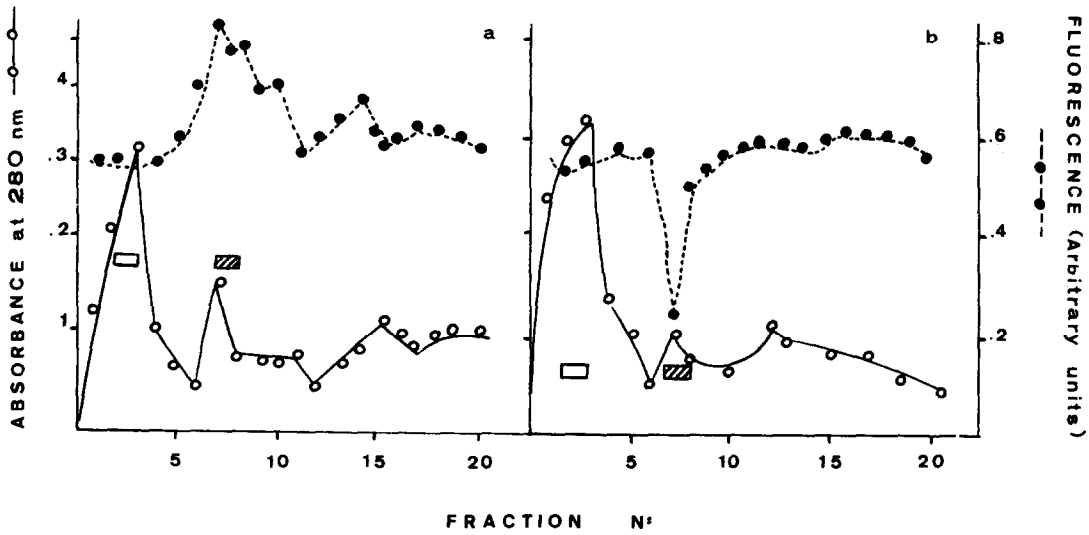


Fig. 2. Separation of chymotrypsin and oxygenase activities. (a) A reaction mixture containing 0.1 mg of chymotrypsin, oxygenase and buffer (pH 7.4) (final volume 300 μl) was incubated for 30 min at 37° and then filtered through a Sephadex G-75 column (1 × 25 cm). The activities of chymotrypsin and oxygenase were assayed as described. Fluorescence of tryptophan at 350 nm was recorded with an Aminco Bowman spectrofluorometer at room temperature. (b) The incubation mixture and conditions were the same as in (a), except for the addition of sodium dithionite (0.5 mM). If the proteolytic activity of chymotrypsin was considered in (a) as 100%, the reamining activity after attack by oxygenase was 15%. ▨ Chymotrypsin; ▭ oxygenase.

3. Results

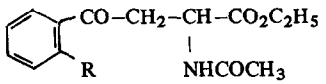
3.1. *Inhibition of enzymes*

The Sephadex G-100 enzyme inhibited a series of enzymes containing tryptophan, as can be seen in table 1. Potato phosphorylase, an enzyme which was not inhibited by tryptophan reagents, was also not inhibited by the pyrrolooxygenase. Table 1 shows that the activity of the pyrrolooxygenase paralleled the inhibition by NBS or by a natural inhibitor (STI). The mode of action of the oxygenase was evident when the correlation between the decrease of the 282 nm absorption and the increase of the 250 nm absorption was measured and compared with the effect of NBS on the same enzymes (fig. 1). This well established effect of NBS is due to oxidation of tryptophan to an oxoindole [2].

The tryptophan disappearance by the action of the oxygenase on a tryptophan containing enzyme can be seen in fig. 2. Chymotrypsin (or trypsin) isolated after being attacked by the pyrrolooxygenase lost between 80% and 90% of its enzymatic activity, and this loss was paralleled by the loss of tryptophan fluorescence. Addition of tryptophan prevented the oxidation and inactivation of the hydrolytic enzymes by the pyrrolooxygenase.

3.2. *Properties of the enzymes*

The interaction between tryptophan and the pyrrolooxygenase was examined by using L- or D-tryptophan and ethyl *N*-acetyl-L-tryptophan as substrates of the enzyme. Both were excellent substrates, L- or D-tryptophan being transformed into formylkynurenin and kynurenin (80% yield) and into 2-oxotryptophan (20% yield); while ethyl *N*-acetyl-L-tryptophan was transformed into ethyl *N*-acetylkynurenin (I) and ethyl 2-acetamido-3-(2′-formimidobenzoyl) propionate (II) (80% yield) and ethyl *N*-acetyl-2-oxotryptophan (20% yield).



R: NH_2 (I)
R: NHCHO (II)

The reaction products were separated by TLC and identified by comparison with synthetic samples. A synthetic pentapeptide, 5-carboxy-2-pyrrolidone-tryptophyl-prolyl-arginyl-proline† was also tested as a substrate of the pyrrolooxygenase under the usual incubation conditions. The pentapeptide was then isolated from the incubation mixture, hydrolyzed with

Table 1
Effect of pyrrolooxygenase on enzyme activity.

Enzyme	Preincubated with	Activity (Δ(A)670)	Inhibition (%)
Trypsin	–	0.30	–
	oxygenase + dithionite	0.02	95
	STI*	0	100
Chymotrypsin	–	0.22	–
	oxygenase + dithionite	0.07	69
	NBS	0.06	73
Pepsin	–	0.23	–
	oxygenase + dithionite	0.07	70
	NBS	0.05	75
		Lysozyme activity units	
Lysozyme	–	18	–
	oxygenase + dithionite	0	100
	NBS	0	100
		P_i liberated (μmole)	
Potato phosphorylase	–	0.30	–
	oxygenase + dithionite	0.30	–
	NBS	0.29	–

The indicated enzymes were preincubated for 30 min at 37° with the indicated enzymes or NBS in phosphate buffer, pH 7.4. The activity of the preincubated systems was then assayed for proteolytic activity on bovine serum albumin in the case of trypsin, chymotrypsin and pepsin by the method of Folin Ciocalteau [3]; in the case of lysozyme the lytic activity on *M. lysodeikticus* was measured by turbidimetry [4]. The enzymes preincubated either with oxygenase or dithionite conserved total activity. Potato phosphorylase was assayed as described [1].

* STI: Soybean Trypsin Inhibitor.

† We are indebted to Dr. M.A. Ondetti (New Brunswick, N.J., U.S.A.) for this substance.

6 N HCl, and the aminoacid mixture separated by paper chromatography. All the original tryptophan had been transformed into kynurenin. The oxygenase had a broad optimum pH, between 7.5 and 9. EDTA (1 mM) and α,α'-dipyridil (0.5 mM) inhibited 50% of the tryptophan consuming activity indicating that a metal, probably Fe^{2+}, was bound to the enzyme. *p*-Chloromercuribenzoate (2 mM) and *N*-ethylmaleimide (5 mM) inhibited the oxygenase by 70%. Cyanide (5 mM or higher) had no effect on the enzymatic activity.

4. Discussion

The properties of the new enzyme places it among the oxygenases. It behaves as a true target enzyme since it oxidizes tryptophan when it forms part of an enzyme, and inhibits the activity of the latter. This represents a new biological type of enzyme modification which closely parallels the well known chemical method of modifying an aminoacid in an enzyme to suppress its activity [5]. The described pyrrolooxygenase was isolated from plants but it also exists in animal tissues, as will be reported shortly.

Acknowledgements

This work was supported by the National Institute of Health (GM-11973), the Consejo Nacional de Investigaciones (Argentina) and the Secretaria de Salud Pública (Argentina). One of us (M.L.T.) was a recipient of a Fellowship from Laboratorios Bagó.

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