CRYSTALLIZATION OF IMIDAZOLEACETATE MONOOXYGENASE AND ITS CHARACTERIZATION AS FLAVOPROTEIN*

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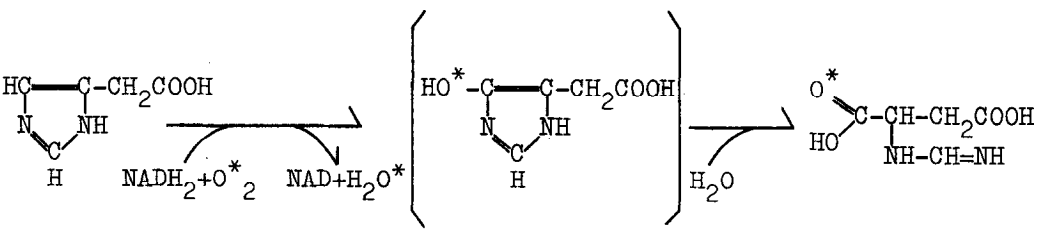
Imidazoleacetate is an intermediate compound in histamine catabolism. In mammalian systems, imidazoleacetate is converted to imidazoleacetate riboside, which is excreted (Tabor et al., 1955). In microbial systems, by contrast, it has been reported to be further metabolized to aspartic acid by way of formiminoaspartic acid (Hayaishi, 1955, Ohmura et al., 1957). A pseudomonad enzyme which catalyzes the conversion of imidazoleacetate to form formiminoaspartic acid, probably via imidazoloneacetate, was first described as imidazoleacetate oxidase and the enzyme was shown to require both



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NADH and molecular oxygen (Hayaishi et al., 1954, 1957). Subsequently the oxygen atom which was incorporated into the reaction product, was shown to be derived from molecular oxygen by ^{18}O experiments (Rothberg et al., 1957). The enzyme is therefore preferably referred to as imidazoleacetate monooxygenase. The present paper describes purification and crystallization of the enzyme and its characterization as a flavoprotein.

Pseudomonas sp. (ATCC 11299B) was grown in a medium containing imidazoleacetate, with shaking, for 18 hours at 26°. The cell paste, weighing 200 g, was suspended in 800 ml of 0.05 M Tris-HCl buffer, pH 8.0, disrupted by sonic oscillation for 15 minutes and centrifuged. One fifth volume of 3% protamine sulfate suspension at pH 8.0 was added to the supernatant. The precipitate was centrifuged off and the supernatant solution was fractionated with solid ammonium sulfate (30 - 50% saturation), followed by dialysis against 0.005 M potassium phosphate buffer, pH 7.2. The dialysate was applied on a TEAE-cellulose column (6.5 x 20 cm) equilibrated with 0.01 M Tris-HCl buffer, pH 8.0. After the column was washed with 2 liters of 0.03 M ammonium sulfate solution (pH 8.0), the enzyme was eluted with 0.10 M ammonium sulfate solution (pH 8.0). The active fractions (260 ml) were combined and concentrated by the addition of saturated ammonium sulfate solution at pH 7.2 (60% saturation). The precipitate was collected and dialyzed against 0.005 M potassium phosphate buffer, pH 7.2. Then the enzyme was adsorbed on to calcium phosphate gel (gel/protein ratio, 7.5 in dry weight) and eluted with 0.05 M potassium phosphate buffer, pH 7.2. The eluate was applied on a DEAE-Sephadex column (2 x 50 cm) which had been equilibrated with 0.05 M potassium phosphate buffer, pH 7.2. After washing the column with 500 ml of 0.05 M ammonium sulfate in 0.10 M potassium phosphate buffer, pH 7.2, elution was carried out using

a linear concentration gradient of ammonium sulfate from 0.06 M to 0.12 M, one liter of each solution in 0.10 M potassium phosphate buffer, pH 7.2. The active fractions were again concentrated by treatment with ammonium sulfate solution (60% saturation) and the precipitate formed was collected and dissolved in 0.30 M potassium phosphate buffer, pH 7.2. Finely powdered ammonium sulfate (about 0.2 g per ml) was then gradually added to 0.7% solution of the enzyme until it became slightly turbid. After 30 minutes the precipitate was removed by centrifugation. When the clear supernatant was kept in an ice bath for a few days, a silky sheen was observed and upon microscopic observation yellow crystalline material could be seen. Recrystallizations were carried out by repeating the process described above. A summary of a typical purification procedure is presented in Table I.

The crystalline enzyme preparation was found to be homogeneous upon ultracentrifugation and disc electrophoresis. The sedimentation constant ($S_{20,w}$) of the enzyme was calculated to be 5.26 x 10^{-13} (cm/sec) assuming a partial specific volume of 0.74. The molecular weight was estimated to be 90,000 by the method of Archibald (1947) and an essentially similar value was obtained by the gel filtration method (Siegel et al., 1966, Andrews, 1964). The concentrated solution of purified enzyme was yellow in color. The absorption spectrum of the three times crystallized enzyme in 0.05 M potassium phosphate buffer, pH 7.2, displayed maxima at 270 mμ (ε = 9.61 x 10^4 M^{-1} cm^{-1}), 383 mμ (ε = 1.08 x 10^4 M^{-1} cm^{-1}) and 442 mμ (ε = 1.07 x 10^4 M^{-1} cm^{-1}) with a marked shoulder at 465 mμ. The enzyme treated with acid ammonium sulfate (Warburg et al., 1938) was inactive in the absence of FAD and it required 0.2 μM FAD to restore its half maximal velocity. FAD could not be replaced by FMN or riboflavin. The flavin moiety dissociated from the enzyme by heat

Table I. Purification of Imidazoleacetate Monooxygenase

The standard assay system contained 200 μmoles of Tris-HCl buffer, pH 9.0, 0.6 μmole of NADH, 7.5 μmoles of imidazoleacetate and enzyme in a total volume of 3.0 ml. The enzyme assay was performed spectrophotometrically by measuring the decrease in optical density at 340 mμ. One unit of enzyme activity was defined as the amount of enzyme which catalyzes the substrate-dependent oxidation of 1 μmole of NADH per minute at 24°. Specific activity was defined as units per mg of protein. The protein concentration was determined by the spectrophotometric method of Kalckar (1947).

Fractions	Volume	Total activity	Specific activity	Yield
	ml	units	units/mg	%
Crude extracts	1,000	2,230	0.07	100
Protamine sulfate	1,100	2,250	0.08	101
Ammonium sulfate	83	1,830	0.40	82
TEAE-cellulose	260	1,000	2.75	45
Calcium phosphate gel	41	740	9.06	33
DEAE-Sephadex	40	380	24.30	17
1st crystallization	1.0	178	25.00	8
2nd crystallization	0.8	134	25.10	6
3rd crystallization	0.7	110	25.00	5

treatment displayed the same R_F values as those of FAD by paper chromatography (Sutton, 1955). The enzyme was calculated to contain one mole of FAD per mole of protein, assuming the extinction coefficient of the enzyme-bound FAD at 442 mμ as equal to that of free FAD at 450 mμ ($\varepsilon = 1.13 \times 10^4\ M^{-1}\ cm^{-1}$).

When a catalytic amount of enzyme was anaerobically incubated with an excess amount of FAD and NADH, reduction of FAD could not be observed at an appreciable rate. However, upon the addition of imidazoleacetate, reduction of FAD occurred at a significant rate depending on the concentration of substrate. Similar observations have been reported with salicylate hydroxylase (Katagiri et al., 1965) and p-hydroxybenzoate hydroxylase (Hosokawa et al., 1966). Colorimetric analyses of the enzyme failed to detect iron and copper

in significant quantities; the details concerning the metal analyses will be published elsewhere.

These results suggest that FAD is a sole cofactor of the enzyme and is involved in its catalysis. The crystallizations of lactic oxidative decarboxylase (Sutton, 1957), p-hydroxybenzoate hydroxylase (Hosokawa et al., 1966) and L-lysine oxygenase (Takeda et al., 1966) have been reported and these crystalline monooxygenases have also been established to be flavoproteins. Moreover, several other monooxygenases have been reported to require flavin coenzymes for their catalyses (Conrad et al., 1961, Fulco et al., 1964, Kusunose et al., 1964, Yamamoto et al., 1965, Yano et al., 1966, Mori et al., in press). Although FAD has been suggested as interacting with substrate in some monooxygenases (Suzuki et al., 1966, Hosokawa et al., 1966), the precise role of flavin in monooxygenation reactions remains to be elucidated. Further studies on the reaction mechanism of imidazoleacetate monooxygenase involving FAD are now under investigation.

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