A ROLE OF SULFHYDRYL GROUPS IN IMIDAZOLEACETATE MONOOXYGENASE[1]

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Imidazoleacetate monooxygenase catalyzes the conversion of imidazoleacetate to imidazoloneacetate with the stoichiometric utilization of DPNH and molecular oxygen (Hayaishi et al., 1957; Rothberg and Hayaishi, 1957). From Pseudomonas sp. (ATCC 11299B), this enzyme was crystallized by Maki et al. (1966). The crystalline enzyme was shown to be a flavo-protein with 1 mole of FAD per mole of enzyme, the molecular weight of which was estimated to be approximately 90,000. Metal components were not detected in the enzyme (Yamamoto et al., 1966).

In this paper, we wish to present some evidence indicating that the monooxygenase contains 2 moles of titratable sulfhydryl groups, one of which is essential, possibly as a substrate-binding site, for the oxygenation of imidazoleacetate. Whereas, these

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sulfhydryl groups seem not to be involved in the oxidase reaction, i.e. the oxidation of DPNH which is catalyzed by the enzyme in the absence of imidazoleacetate.

EXPERIMENTAL

Crystalline imidazoleacetate monooxygenase was prepared as described previously (Maki et al., 1966). Sulfhydryl groups were titrated both with silver nitrate at room temperature (Benesch et al., 1955) and with p-MB[3] at 24° (Boyer, 1954). The enzyme activity was measured at 24° by the decrease in optical density at 340 mμ due to the imidazoleacetate-dependent oxidation of DPNH (Hayaishi et al., 1957) using a Cary 15 spectrophotometer. Oxidase activity of imidazoleacetate monooxygenase was measured spectrophotometrically by the disappearance of DPNH in the absence of imidazoleacetate, and, if necessary, polarographically with a rotating platinum electrode as described elsewhere (Maki et al. in preparation).

RESULTS

I. Titration of Sulfhydryl Groups

Titration of sulfhydryl groups of crystalline imidazoleacetate monooxygenase was carried out. In Table I is shown the number of sulfhydryl groups per mole of enzyme, as determined amperometrically with silver nitrate and spectrophotometrically with p-MB. Two sulfhydryl groups were titratable in the native protein. It should

[3] Abbreviation used: p-MB, p-chloromercuribenzoate.

TABLE I

Amperometric and spectrophotometric titrations of sulfhydryl groups of imidazoleacetate monooxygenase

Amperometric titration; titration mixture contained in 5-ml final volume, 100 mM Tris-acetate buffer, pH 7.5, 10 mM KCl, and 16 mμmoles imidazoleacetate monooxygenase with respect to FAD. In experiment in the presence of imidazoleacetate, 8 mM imidazoleacetate was added and the mixture was preincubated for 5 min at room temperature. Aliquots (5 μl) of 2 mM silver nitrate were added, allowing 3 to 5 min between each addition and reading. After silver ion appeared in excess, more titrant was added to permit extrapolation to the baseline.

Spectrophotometric titration; 1.0 ml of 100 mM potassium phosphate buffer, pH 7.0, was placed in a 1.0-cm spectrophotometer cell and an equal volume of enzyme (8 mμmoles) dissolved in the same buffer was added to a matched cell. Aliquots (5 μl) of 1 mM p-MB solution were added to both cells and the optical density was read at 250 mμ after 10 min. In experiment in the presence of imidazoleacetate, 4 mM imidazoleacetate was added and was preincubated for 2 min at 24°.

Addition	Sulfhydryl group titrated with	
	$AgNO_3$	p-MB
	moles per enzyme-FAD	
None	1.94	1.86
Imidazoleacetate	0.82	1.04

be noted that in the presence of the substrate, imidazoleacetate, only one sulfhydryl group was titrated, indicating that at least one sulfhydryl group was involved in a substrate-binding site.

II. Inactivation of Imidazoleacetate Monooxygenase with Sulfhydryl Reagents

As shown in Fig. 1, when imidazoleacetate monooxygenase and varying amounts of sulfhydryl reagents, p-MB or silver nitrate, were admixed in a buffer solution and incubated, the oxygenase activity was observed to decrease as a linear function of the amounts

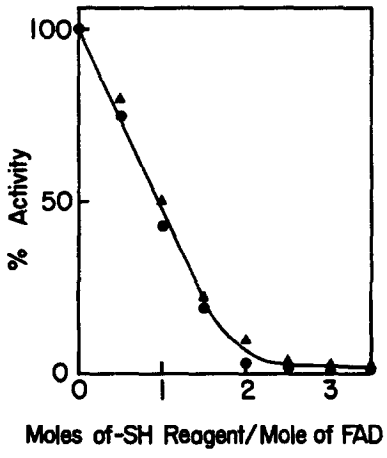

Fig. 1. Effect of Sulfhydryl Reagents on Oxygenase Activity. Enzyme (0.08 - 0.18 mμmole, with respect to enzyme-FAD) and varying amounts of p-MB (●) or silver nitrate (▲) were admixed in 0.12 ml of 50 mM Tris-HCl buffer, pH 7.6, in a quartz cuvette and incubated for 2 min at 24°. The reaction was started at 24° by the addition of 2.88 ml of 50 mM Tris-HCl buffer, pH 7.6, which contained 0.14 mM DPNH and 2 mM imidazoleacetate. All activities were related to those of an equivalent quantity of native, untreated enzyme (520 moles DPNH oxidized/min/mole of enzyme).

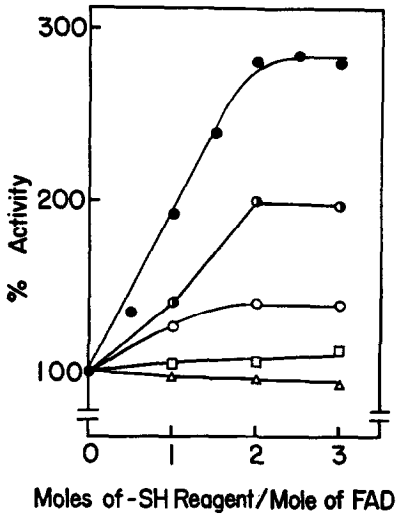

Fig. 2. Effects of Sulfhydryl Reagents on Oxidase Activity. Enzyme (0.8 mμmole, with respect to enzyme-FAD) and varying amounts of sulfhydryl reagents were admixed in 0.10 ml of 50 mM Tris-HCl, pH 7.6, in a quartz cuvette and incubated for 2 min at 24°. The reaction was started at 24° by the addition of 0.90 ml of 50 mM Tris-HCl buffer, pH 7.6, which contained 0.15 mM DPNH. All activities were related to that of an equivalent quantity of native, untreated enzyme (8.9 moles DPNH oxidized/min/mole of enzyme). —●—, Mersalyl; —◐—, ethyl-mercuric chloride; —○—, methyl-mercuric chloride; —□—, phenyl-mercuric acetate; and —△—, silver nitrate.

of reagents added, and the maximum inactivation was obtained at a ratio about 2 moles of p-MB or silver nitrate per mole of enzyme. Quite similar results were also obtained with other sulfhydryl reagents such as sodium mersalyl, phenyl-mercuric acetate, methyl-mercuric chloride or ethyl-mercuric chloride. Neither arsenite nor Cd^{++}, which are known to react with dithiol groups, had any

effect on the oxygenase activity. Protection of oxygenase activity was observed when p-MB was added to the enzyme which had been pretreated with imidazoleacetate; the oxygenase activity remained to the extent of 75% in the presence of 2 moles p-MB per mole of enzyme.

III. Effects of Sulfhydryl Reagents on Oxidase Activity

In the absence of substrate, crystalline imidazoleacetate monooxygenase showed oxidase activity as its other activity. The rate of oxidation of DPNH by the enzyme in the absence of imidazoleacetate was about 1.7% compared to that in the oxygenase reaction. As described above, one of the sulfhydryl groups, possibly as a substrate-binding site, was shown to be essential for the oxygenation of imidazoleacetate. It seemed worthy to study influence of sulfhydryl reagents upon the oxidase activity of the enzyme.

In Fig. 2, results obtained on assaying the enzyme after incubation with varying amounts of several sulfhydryl reagents are shown. To both silver nitrate and phenyl-mercuric acetate, oxidase activity was almost insensitive. By mersalyl, ethyl-mercuric chloride, and methyl-mercuric chloride, oxidase activity was activated. This was in sharp contrast to the inhibition observed in the experiment on oxygenase activity of the enzyme. The oxidase activity was observed to increase as a linear function of the amounts of these mercurials added to the enzyme, each reaching a plateau at a ratio of about 2 moles of mercurial per mole of enzyme. The extents of activation produced were 2.8-fold by mersalyl, 2-fold by ethyl-mercuric chloride, and 1.4-fold by methyl-mercuric chloride. The reaction product of both native and acti-

vated oxidase activity was identified as hydrogen peroxide, measured polarographically by the rate of oxygen uptake in the presence and absence of catalase.

DISCUSSION

Amperometric and spectrophotometric measurements of sulfhydryl groups of imidazoleacetate monooxygenase offered evidence for the presence of 2 titratable sulfhydryl groups in the protein. Information concerning the role of the sulfhydryl groups was further obtained from the experiments of titration in the presence of substrate, imidazoleacetate. In the presence of imidazoleacetate only one sulfhydryl group was titrated, indicating that one of the sulfhydryl groups seemed to be essential for the imidazoleacetate oxygenation as a substrate-binding site. When imidazoleacetate monooxygenase was pretreated with varying amounts of mercurials or silver nitrate, the enzyme was almost completely inactivated with respect to oxygenase activity at a ratio about 2 moles of these reagents per mole of enzyme. Evidence for the presence in imidazoleacetate monooxygenase of sulfhydryl group possibly as substrate-binding site was also provided from the protection experiment with substrate on oxygenase activity. On the other hand, the oxidase activity of the enzyme was unaffected by silver nitrate or phenylmercuric acetate. It is of particular interest that some mercurials even caused enhanced oxidase activity. The reaction product of oxidase activity was identified as hydrogen peroxide, while that of oxygenase reaction was water. It has been found that a reduced form of enzyme-FAD can be utilized as a direct hydrogen donor in the imidazoleacetate monooxygenase reaction (Maki *et al.* in preparation). Considering all these observations together, it may be

said that the cleavage of the oxygen-oxygen bond and the reduction of oxygen to H_2O, as well as the monooxygenation reaction, are tightly coupled and catalyzed in a concerted manner probably in a ternary complex of substrate, oxygen, and a reduced form of enzyme-FAD. In either of two cases, absence of substrate or blocking by sulfhydryl reagents of the binding of substrate to enzyme protein, there occur the following phenomena: uncoupling of the oxidation reaction, non-cleavage of oxygen-oxygen bond, and production of hydrogen peroxide.

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REFERENCES

Benesch, R. E., Lardy, H. A., and Benesch, R., J. Biol. Chem., 216, 663 (1955).

Boyer, P. D., J. Amer. Chem. Soc., 76, 4331 (1954).

Hayaishi, O., Tabor, H., and Hayaishi, T., J. Biol. Chem., 227, 161 (1957).

Maki, Y., Yamamoto, S., Nozaki, M., and Hayaishi, O., Biochem. Biophys. Res. Commun., 25, 609 (1966).

Maki, Y., Yamamoto, S., Nozaki, M., and Hayaishi, O., in preparation.

Rothberg, S., and Hayaishi, O., J. Biol. Chem., 229, 897 (1957).

Yamamoto, S., Takeda, H., Maki, Y., and Hayaishi, O., in K. Bloch and O. Hayaishi (Editors), Biological and chemical aspects of oxygenases, Maruzen Company, Ltd., Tokyo, 1966, p. 303.