THE MECHANISM OF THE AUTOXIDATION OF OXYGENASES

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In general, a major difficulty in the study of oxygenases is the extreme lability of these enzymes to oxygen (Charalampous, 1959; Iaccarino et al., 1961; Kojima et al., 1961). Attempts to restore the activity once inactivation has occurred have been so far uniformly unsuccessful. However, we have recently reported that the activity of metapyrocatechase could be restored to the original level by the addition of sodium borohydride ($NaBH_4$) after being inactivated by oxygen (Hayaishi et al., 1962). In order to obtain some insight into the role played by $NaBH_4$ in this reactivation reaction, effects of various reducing agents and conditions required for this reactivation were extensively studied.

Metapyrocatechase from Pseudomonas sp. OCI was purified to the step of Cγ gel fractionation, and assayed by the method described previously (Kojima et al., 1961). This preparation had a half life under aerobic conditions of approximately 10 hours at 4° at pH 7.0[1]. The addition of $NaBH_4$ (10^{-2} M) to the partially inactivated enzyme solution in vacuo resulted in almost complete restoration of activity[2]. This process

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[1] The half life value was variable depending on the concentration of the protein and other conditions but it usually ranged between 6 and 10 hours.

[2] The freshly prepared enzyme could be activated to some extent as seen in Fig. 1. This was undoubtedly the result of a certain degree of inactivation by air during preparation of the enzyme.

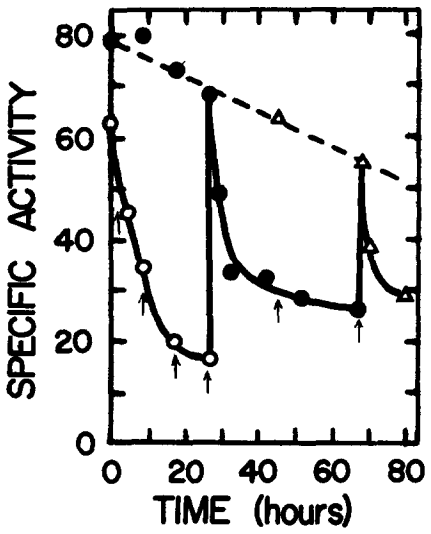

Fig. 1. Reactivation of metapyrocatechase by sodium borohydride. The freshly prepared Cγ gel fraction (40 ml, 0.5 mg protein per ml) of metapyrocatechase was placed in a beaker at 4°. Aliquots were taken out for assays of enzymatic activity or for experiments on the reactivation of the enzyme. The method of reactivation under anaerobic conditions was the following. Each aliquot (2.0 ml) of the enzyme solution was placed in the main portion of a Thunberg tube and 2 mg of $NaBH_4$ (obtained from the Metal Hydride, Inc.) was put in the side arm. After evacuation for 3 minutes at 23°, the components in the closed tube were mixed by inversion and incubated at 23° for 30 minutes, during which period maximum activation of the enzyme by $NaBH_4$ was obtained. After the tube was opened to the atmosphere, 0.02 ml of 1 M KH_2PO_4 was added to reduce the pH of the enzyme solution to below 8 and an aliquot (0.05 ml) was used for assay of enzymatic activity. Another aliquot was reactivated and assayed at each of the times indicated by arrows. At 25.5 hours after starting this experiment, all of the remaining partially inactivated enzyme (20 ml) was reactivated as above and the enzyme inactivation was traced again for an additional 40 hours. After a total of 66 hours, the remaining enzyme preparation (8 ml) was again reactivated and assayed for activity as before during storage for 14 additional hours. (o), (●) and (Δ) indicate activities of the original, once reactivated and twice reactivated enzymes respectively. --- indicates the changes in the maximum activity of the enzyme recoverable by $NaBH_4$ treatment during the experimental periods.

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could be repeated, as seen in Fig. 1[3]. However, the levels of activity of the enzyme during repeated reactivations declined gradually at a rate comparable with the inactivation of the enzyme stored under nitrogen (Kojima et al., 1961)[4]. When $NaBH_4$ was added under aerobic conditions, the extent of reactivation was less than that obtained under anaerobic conditions. Various other reducing substances when tested under aerobic conditions

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[3] Enzyme preparations which had been stored for long periods at 4°, and lost more than 90% of their original activity, reactivated only to values approaching 50% of original activity upon borohydride treatment. Complete restoration has been observed only in rare cases and apparently depended on various conditions.

[4] The once reactivated enzyme also could be prevented from inactivation by storing under anaerobic conditions as in the case of freshly prepared enzyme.

resulted in either limited reactivation or were inhibitory. However, many of these reducing reagents reactivated the enzyme effectively under anaerobic conditions (Table I).

Ferrous ion, which does not stimulate the activity of intact oxygenase (Kojima et al., 1961), was very effective in restoring the air-inactivated enzyme, but this occurred only under anaerobic conditions (Table I). No additive effects of $NaBH_4$ and ferrous ion were demonstrable. Addition of ascorbate (5×10^{-3} M) during incubation to maintain iron in the ferrous form under aerobic conditions did not result in reactivation either in the presence or absence of catalase (50 μg).

Other oxidizing agents, such as potassium ferricyanide, could also produce reversible inactivation of the enzyme. Thus, the addition of $K_3Fe(CN)_6$ (10^{-3} M), resulted in a complete loss of enzyme activity within 20 minutes at 4°. After dialysis to remove $K_3Fe(CN)_6$ against a large amount of 0.02 M potassium phosphate buffer, pH 7.5, for 3 hours at 4°, the activity was restored to almost the original level by the addition of ferrous ion under anaerobic conditions. This process also could be repeated. $NaBH_4$ and other reducing reagents effective for the reactivation of the air-inactivated enzyme were all effective in reversing the ferricyanide reaction with results similar to those shown in Table I.

Since most reducing substances employed in the above experiments are fairly stable, these results suggest that deoxygenation from the surface of the enzyme molecule may be necessary for the reactivation reaction. Furthermore, the fact that CMB does not inhibit either the enzymatic reaction or the reductive reactivation, indicates that autoxidizable moieties other than sulfhydryl groups of the enzyme may be involved in the reversible inactivation process. Ferrous ion is an essential cofactor in some oxygenases (Suda and Takeda, 1950; Long et al., 1954; Tanaka and Knox, 1959; Taniuchi et al., 1960); therefore, autoxidation of bound Fe^{++} in metapyrocatechase must be considered as a distinct possibility. Although the addition of ferrous ion or metal chelating agents to the reaction mix-

TABLE I

Effect of various reducing reagents on metapyrocatechase inactivated by oxygen

Maximum reactivation occurred after incubation with the reducing agents at 23° for 30 minutes under anaerobic conditions; however, in the case of reactivation by reduced flavins and reduced pyridine nucleotides the activity of the enzyme increased linearly for 2 hours to reach almost the level obtained in the anaerobic reactivation by ferrous ion. Assays were made after 30 minutes incubation in all cases. The concentrations of each flavin, DPNH, ferrous ion and all other reducing reagents were 10^{-4}, 5 X 10^{-4}, 10^{-3} and 10^{-2} M respectively. The concentration of FAD when added together with $SnCl_2$ was 5 X 10^{-4} M. The specific activity of the enzyme was 60 at the time it was freshly prepared.

Additions	Conditions of reactivation	Specific activity	Activity per cent of control
None	Aerobic	16	100
	Anaerobic	16	100
$NaBH_4$*	Aerobic	47	294
	Anaerobic	65	406
Cysteine*	Aerobic	17	106
	Anaerobic	45	281
Thioglycollate*	Aerobic	14	88
	Anaerobic	24	150
Glutathione* (Na salt)	Aerobic	6	37
	Anaerobic	31	194
Mercaptoethanol	Aerobic	15	94
	Anaerobic	36	225
Lipoate	Aerobic	10	63
	Anaerobic	40	250
Ascorbate	Aerobic	20	125
	Anaerobic	37	231
Tetrahydrofolate	Aerobic	4	25
	Anaerobic	26	162
$Na_2S_2O_4$*	Aerobic	14	88
	Anaerobic	39	244
$SnCl_2$*	Aerobic	13	81
	Anaerobic	17	106
$FeSO_4$+	Aerobic	15	94
	Anaerobic	210	1,310
FAD	Aerobic	16	100
	Anaerobic	17	106
FAD + $SnCl_2$*	Aerobic	16	100
	Anaerobic	43	268
DPNH	Aerobic	16	100
	Anaerobic	24	150
FAD + DPNH‡	Aerobic	15	94
	Anaerobic	43	268
FMN + DPNH	Anaerobic	41	256
Riboflavin + DPNH	Anaerobic	53	331

* Solid reagents were added. + $FeCl_3$ (10^{-3} M) had no effect.
‡ TPNH showed similar effects but neither DPN nor TPN did so.

ture failed to affect the activity of metapyrocatechase (Kojima et al., 1961)[5], it is still possible that a tightly bound metal atom which is unable to exchange with external metal ions and to be removed by metal chelating agents under ordinary conditions, may be an essential part of the enzyme. This inference was partly confirmed by the following experiment.

The enzyme was dialyzed against a large volume of 0.05 M potassium phosphate buffer, pH 7.5, containing o-phenanthroline (10^{-3} M)[6] at 4° overnight and then against the same buffer without o-phenanthroline at 4° for 4 hours. This treatment resulted in a marked loss of enzyme activity. In contrast to the untreated enzyme, this preparation could be reactivated effectively by ferrous ion under aerobic conditions. Fe^{+++}, Co^{++} and Ni^{++} could not replace ferrous ion. Borohydride could not reactivate this enzyme under either aerobic or anaerobic conditions (Table II). On the other hand, if the enzyme was dialyzed against the same buffer without o-phenanthroline, it was reactivated by ferrous ion only under anaerobic conditions and it was readily reactivated by $NaBH_4$.

FAD, FMN and riboflavin in the presence of DPNH[7] or $SnCl_2$ were quite effective in reactivating the enzyme under anaerobic conditions (Table I). This indicated that reduced flavins are able to reduce enzyme-bound iron. The effective concentration of the flavins (10^{-6} M) was much less than that of other reducing reagents (10^{-3} - 10^{-2} M), and suggests a possible physiological role for flavin coenzymes in reductive enzyme reactivations.

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[5] Similar phenomena were already reported with pyrocatechase (Hayaishi et al., 1957), but this enzyme also lost 40% of its activity upon treatment with $K_3Fe(CN)_6$ (10^{-3} M).

[6] o-Phenanthroline (10^{-3} M) did not inhibit the enzyme reaction when added to the assay system (Kojima et al., 1961) and had only slight effect on the reactivation of the enzyme by $NaBH_4$; however, it completely prevented the reactivation by ferrous ion when it was added in a molar ratio of 3 to 1 of iron.

[7] DPNH could reduce FAD, FMN and riboflavin in the presence of the Cγ gel fraction, but not with the fraction boiled at 80° for 3 minutes.

TABLE II

Effects of ferrous ion and $NaBH_4$ on metapyrocatechase treated with o-phenanthroline

The Cγ gel fraction used had a specific activity of 60 at the time it was prepared. The concentrations of $NaBH_4$ and $FeSO_4$ were 10^{-2} and 10^{-3} M respectively.

Addition	Condition of reactivation	Specific activity of the dialyzed enzyme	
		with o-phenanthroline	without o-phenanthroline
None		7	1
$NaBH_4$	Anaerobe	15	75
$FeSO_4$	Aerobe	61	16

In a preliminary experiment, another oxygenase, kynurenic acid hydroxylase (Taniuchi et al., 1960)[8], was reactivated by the additions of $NaBH_4$ and other reducing reagents with results similar to those observed with metapyrocatechase[9]. It may be premature to generalize this phenomenon; however, such reactivation procedures may be useful in the purification of various oxygen-sensitive oxygenases. Furthermore, such methods may provide important clues leading to an understanding of the reaction mechanisms of such enzymes[10].

[8] Kynurenic acid hydroxylase is also labile toward oxygen.

[9] Anaerobic conditions were not essential for the reductive reactivation of kynurenic acid hydroxylase, but more effective reactivation was obtained under such conditions.

[10] Recently it has been reported that ferrous ions reactivate p-hydroxyphenylpyruvate oxidase which had been inactivated by treatment with $K_3Fe(CN)_6$ (Goswami et al., 1962); also that ascorbate reactivates 3-hydroxyanthranilate oxidase which had lost activity during catalysis (Mitchell et al., 1962).

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