CATALYTIC EFFECT OF COPPER ION ON THE REOXIDATION OF REDUCED TAKA-AMYLASE A

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Since the work of White (1960, 1961) on bovine pancreatic ribonuclease, many experimental results were obtained which suggest that proteins, converted to randomly coiled polypeptide chains by reductive cleavage of disulfide bonds in the presence of denaturing agents, can be restored to their native three dimensional forms by reoxidation. Oxidation of sulfhydryl groups in reduced proteins seems to be an important step in renaturation reaction. Little attention, however, has been paid to the mechanism of the oxidation of the sulfhydryl groups in the reduced proteins. On the other hand, it has been well known with thiol compounds of small molecular weight, that their sulfhydryl groups are not easily oxidized by molecular oxygen, but are catalytically air-oxidized in the presence of some of transition metal ions (Barron, 1951 and Boyer, 1959). The present investigation was undertaken to see whether the metal ions catalyze the reoxidation of reduced Taka-amylase A* in the same manner as in thiol compounds of small molecular weight. Among the metal ions investigated, copper ion was

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* Asp. oryzae α-amylase; M.W., ca. 52,000; The enzyme contains four disulfide bonds which cross-link the polypeptide chain intramolecularly, and one sulfhydryl group. For the reversibility of reduction of this enzyme, see previous papers (Isemura et al., 1961 and 1963).

found to be particularly effective as a catalyst for the reoxidation of reduced Taka-amylase A.

EXPERIMENTAL Taka-amylase A (ca. 10 mg/ml) was reduced by treatment with 0.3 M 2-mercaptoethanol in 8 M urea-0.01 M EDTA-0.025 M Tris-Cl, pH 8.0 for 20 hours at 30°C to yield randomly coiled polypeptide chain. After reduction, the reduced enzyme was separated from the reagents by passage through a column of Sephadex G-50 equilibrated with pH 8.0, 0.025 M Tris-Cl buffer. Reoxidation was carried out with a total volume of 5 ml in 50 ml test tubes covered with gauze under shaking in a thermostated incubator at 20°C. Because one gram ion of Ca^{++} ion is necessary per mole of Taka-amylase A for manifestation of enzymatic activity (Oikawa and Maeda, 1957), calcium chloride was added up to the concentration of 2 moles per mole of the reduced enzyme in all experiments. Sulfhydryl content was measured by PCMB-titration. Amylase activity was measured at pH 5.0, 37°C by determining dextrinizing power using amylose as a substrate. At this pH, reactivation during the assay was negligible.

RESULTS Reduced Taka-amylase A (8.0 mg/ml) was oxidized at 20°C in the presence of various transition metal ions (5.0×10^{-5} M). The catalytic effect on the oxidation of sulfhydryl groups in the reduced enzyme can be listed as follows: Cu^{++}(1.1, 0.6)>Co^{++}(6.2, 1.2)>Mn^{++}(6.3, 2.8)>Fe^{++}(5.8, 4.7)>Ni^{++}(6.3, 5.0) >control (7.6, 5.8). The numerals in parentheses show the sulfhydryl content (moles per mole enzyme) after 3 and 20 hours of oxidation respectively. Because Cu^{++} ion excelled others in the catalytic power, special attention was paid to the effect of Cu^{++} ion in the present investigation. Fig.1 shows the time-course of the oxidation of sulfhydryl groups. In a control experiment,

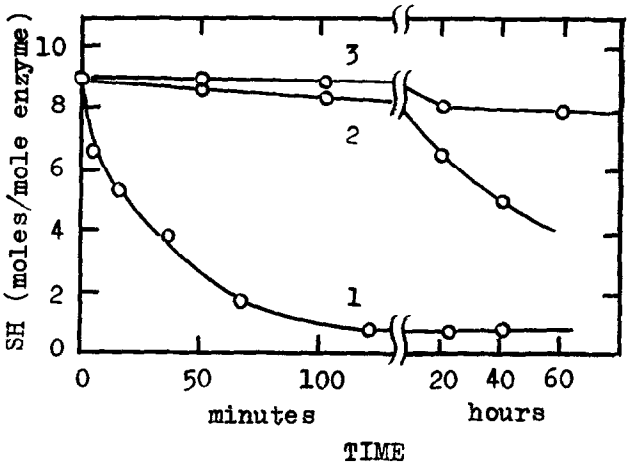

Fig.1 Time-course of the oxidation of sulfhydryl groups in pH 8.0, 0.025 M Tris-Cl buffer at 20° C. 1, 5.2×10^{-5} M $CuSO_4$ added; 2, control; 3, 0.01 M EDTA added. Taka-amylase A, 7-9 mg/ml.

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the oxidation proceeded slowly (curve 2). The oxidation was accelerated by the addition of a small amount of Cu^{++} ion (curve 1). Addition of EDTA retarded the oxidation (curve 3). Fig.2 shows that the reactivation was also accelerated by the addition of Cu^{++} ion. Fig.3 shows the extent of reactivation after 20 hours of oxidation at 20°C as a function of the concentration of Cu^{++} ion added. The optimum Cu^{++} ion concentration for reactivation depended on the concentration of the reduced enzyme. The extent of the oxidation of sulfhydryl group is also included in Fig.3. Ultracentrifugal analysis showed formation of aggregated material (ca. 40% of the total protein) when the reduced enzyme (9 mg/ml) was oxidized for 20 hours in the presence of 1.4×10^{-3} M Cu^{++} ion. Such an aggregate formation was not observed when the oxidation was carried out in the presence of optimum amount of Cu^{++} ion (5.0×10^{-5} M). Fig.4 shows the effect of the concentration of reduced Taka-amylase A. Reduced Taka-amylase A solution (7.5 mg/ml) was diluted 10- and 100-fold with pH 8.0, 0.025 M Tris-Cl buffer, and the diluted

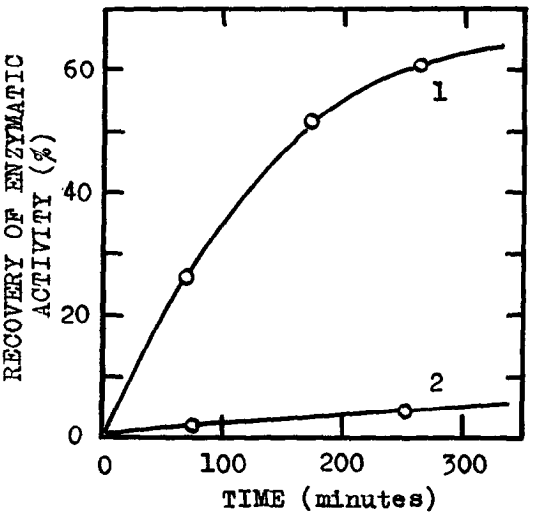


Fig.2 Effect of copper ion on the rate of recovery of enzymatic activity. 1, 5.0×10^{-5} M $CuSO_4$ added; 2, control. Taka-amylase A, 7.5 mg/ml. pH 8.0, 0.025 M Tris-Cl buffer, 20° C.

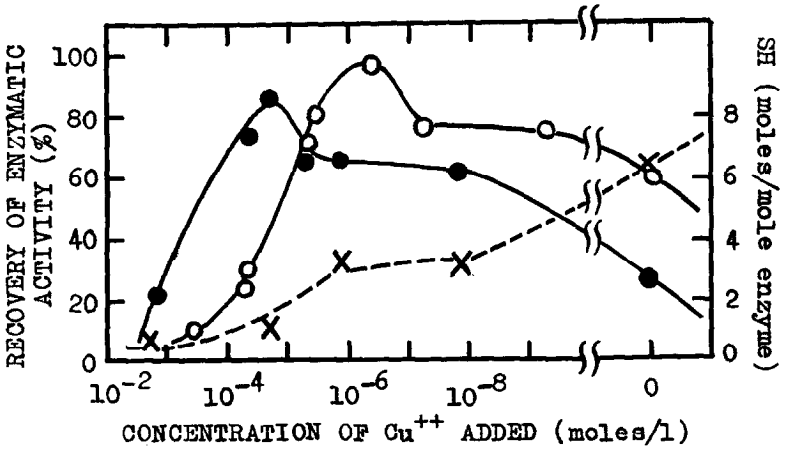
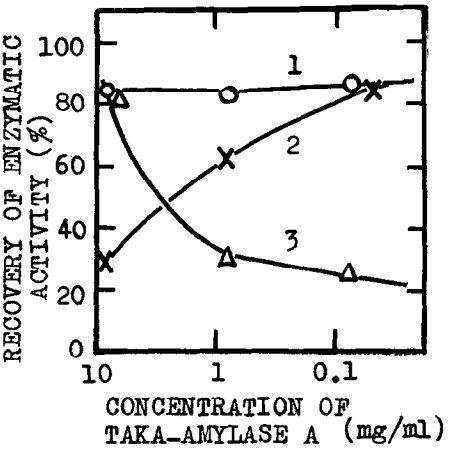


Fig.3 Effect of copper ion concentration on the extent of recovery of enzymatic activity and the oxidation of sulfhydryl groups. Reduced Taka-amylase A, at concentration of 7.5-9.5 mg/ml (—●— ,--X----) and 0.75-0.80 mg/ml (—○—) was reoxidized in pH 8.0, 0.025 M Tris-Cl buffer, at 20°C in the presence of various amount of copper ion, for 20 hours.

Fig.4 Effect of Taka-amylase A concentration on the extent of recovery of enzymatic activity. The reduced enzyme (8.0 mg/ml) was oxidized after x1, x10, and x100 dilutions

curve	Cu^{++} added (M) original solution	dilutant
1	5.0×10^{-5}	0
2	0	0
3	5.0×10^{-5}	5.0×10^{-5}



solutions were oxidized as well as the original solution for 20 hours at 20°C. In the absence of Cu^{++} ion added, the extent of reactivation increased with decrease in concentration of the reduced enzyme (curve 2). In the presence of optimum amount of Cu^{++} ion, the extent of reactivation was almost independent of concentration of the reduced enzyme (curve 1). The extent of reactivation decreased with decrease in concentration of the reduced enzyme, when a dilutant contained the same amount of Cu^{++} ion as in the original solution (curve 3).

DISCUSSION Cu^{++} ion was found to be an excellent catalyst for the oxidation of sulfhydryl groups in reduced Taka-amylase A. Without the addition of Cu^{++} ion, the oxidation proceeded very slowly. Other transition metal ions were far less effective than Cu^{++} ion. Though the mechanism of the oxidation of thiol compounds catalyzed by the metal ions is not yet clearly understood, following steps are presumed to exist in the oxidation of thioglycolate and cysteine: (1) formation of chelate complex between a metal ion and two or three molecules of these thiol compounds, (2) transfer of electron from

sulfhydryl group to oxygen molecule mediated by the reversible change of valence state of the metal ion, (3) formation of disulfide bond (Boyer, 1959). If a similar mechanism exists in the case of the oxidation of the reduced enzyme, the formation of chelate complex is presumed to be an important step in the refolding process of the polypeptide chain of the reduced enzyme.

Though the oxidation of sulfhydryl groups of the reduced enzyme was catalyzed by Cu^{++} ion, the addition of excess Cu^{++} ion was unfavorable for the recovery of the enzymatic activity, probably due to formation of inactive molecules having incorrect disulfide bonds. Intermolecular disulfide bonds were formed in the presence of excess Cu^{++} ion, when concentration of the reduced enzyme was high. The results of Figs. 3 and 4 show that the catalytic effect of Cu^{++} ion does not depend on the absolute concentration of Cu^{++} ion but on the molar ratio of Cu^{++} ion to the reduced enzyme. Even in the absence of Cu^{++} ion added, appreciable reactivation was observed, and the extent of reactivation inversely depended on the concentration of the reduced enzyme. This result, considered together with the inhibitory effect of EDTA on the oxidation (Fig.1, curve 3), suggests that the contaminating metal ions catalyze the oxidation. Reactivation of reduced enzymes in good yield has been observed for ribonuclease (Epstein et al., 1962), lysozyme (Epstein and Goldberger, 1963), and E. coli alkaline phosphatase (Levinthal et al., 1962) without addition of any catalyst. These experiments were carried out with reduced enzymes of lower concentration than that of reduced Taka-amylase A due to low solubility of these enzymes in reduced state. The results of the present study suggest that the oxidation of sulfhydryl groups in these cases was catalyzed by contaminating metal ions. The

catalytic effect of metal ions is recently suggested from the inhibitory effect of EDTA on the recombination of reduced rabbit antibody fragments (Mandy and Nisonoff, 1963). Microsomal systems from rat liver (Goldberger et al., 1963) and chicken and pigeon pancreas (Venetianer and Straub, 1963) were found to accelerate the reactivation of reduced ribonuclease. As suggested by Venetianer and Straub, the oxidation of sulfhydryl groups may be catalyzed enzymatically in these cases.

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