A Method Based on the Use of Methanol as a Stabilizing Agent to Prepare Metal-free Glyoxalase I and to Reconstitute Activity by Addition of Bivalent Metal Ions *

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Glyoxalase I is a metalloenzyme which has been found to contain one atom of zinc per subunit in its native state.[1] Various bivalent metal ions have been found to restore activity to glyoxalase I previously inactivated by treatment with chelators such as EDTA.**[1-6] More recent studies on glyoxalase I from human erythrocytes, which has been purified in larger quantities than described for the enzyme from any other source,[7] showed that the enzyme was labile and that previous methods to prepare apoenzyme and to restore activity were inadequate. The availability of glyoxalase I from human erythrocytes in large quantities makes the enzyme from this source a suitable candidate for further physicochemical characterization, *e.g.*, by investigations of the properties of the essential metal ion and its relationship to the catalytic function of the enzyme. It was, therefore, of great importance to develop an improved procedure to prepare apoenzyme and make metal-substitutions. This communication describes such a procedure and the results of reactivation of the apoenzyme of glyoxalase I with Zn^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+}.

Results and discussion. The first attempts to prepare the apoenzyme from human erythrocyte glyoxalase I with minimal irreversible denaturation were made at −30 °C. Methanol in a concentration of 40 % (v/v) was added as an "antifreeze", which under these conditions of concentration and temperature does not change the dielectric constant of the medium from that of water to any great extent.[8] These attempts were successful in giving catalytically inactive apoenzyme that could be reactivated by addition of bivalent metal ions. However, it was later found that subzero temperature was not necessary to prevent denaturation and that methanol rather than the low temperature stabilized the enzyme. Fig. 1 shows a study of the effect of various concentrations of methanol on the stability of native glyoxalase in 10 mM Tris/HCl buffer (pH 7.8) at 4 °C. It was found that methanol concentrations ≥ 10 % (v/v) protected the enzyme from denaturation for at least 11 months, whereas lower concentrations of methanol had smaller or no effect. A concentration of 10 % was chosen for the standard procedure to prepare apoenzyme in order to avoid an unnecessary excess of methanol which might have adverse effects in subsequent use of the enzyme. After developing this procedure it came to our notice that methanol like some additional organic solvents appears to stabilize a variety of proteins against denaturation.[9]

The apoenzyme of glyoxalase I was reactivated in 10 mM Tris/HCl buffer (pH 7.8) containing 10 % (v/v) methanol by addition of salts of Zn^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+}. It was found that although only about 2 Zn per dimeric enzyme molecule are present in the native enzyme (Table 1), about 7 – 10

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** Abbreviation: EDTA, ethylenediaminetetraacetate.

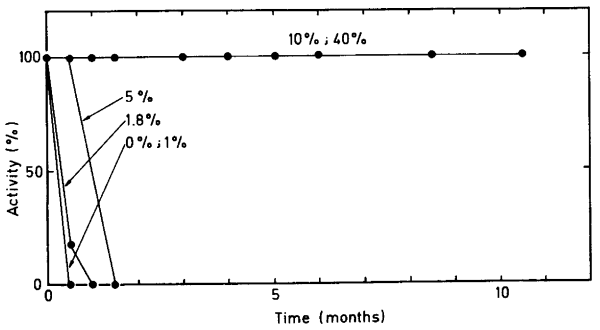

Fig. 1. Stability of native purified glyoxalase I from human erythrocytes at various concentrations of methanol. The enzyme (0.08 μM) was maintained at 4 °C in 10 mM Tris/HCl buffer (pH 7.8) containing the methanol concentrations (%, v/v) indicated.

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Table 1. Metal analyses of various glyoxalase I preparations.

Enzyme	Specific activity (μmol/min per mg of protein)	Metal analyses (mol/mol of enzyme)			
		Zn	Mg	Mn	Co
Native	950	2.5	0.2	–[a]	–
Native, dialyzed	1000	1.6	0.04	–	–
Apoenzyme	12	0.08	0.12	–	–
Zn^{2+}-reactivated	1050	1.6	0.3	–	–
Mg^{2+}-reactivated	1010	0.17	1.4	–	–
Mn^{2+}-reactivated	670	0.05	–	1.9	–
Co^{2+}-reactivated	660	0.07	–	–	1.6

[a] Entries in the table marked with a dash indicate that analyses have not been carried out.

mol of bivalent metal ion per mol of enzyme were required to restore maximal catalytic activity to the enzyme. Furthermore, the reactivation of the enzyme appeared to be a cooperative process because a change from an almost inactive state to a fully active state took place over a narrow concentration range following the addition of 6 – 7 mol of metal ion per mol of enzyme (Fig. 2). The shape of the titration curve of Fig. 2 was not time-dependent, and only in the case of Mg^{2+} was any significant increase of activity noted after incubation of the apoenzyme with the metal ion for a longer period of time (*cf.* Ref. 3). The binding of Mn^{2+} to the enzyme could be followed by electron paramagnetic resonance (unpublished work carried out in collaboration with Dr. L. E. G. Eriksson, Department of Biophysics, Arrhenius Laboratory, University of Stockholm) and it was shown that the binding of Mn^{2+} followed a normal linear titration curve with a break-point at the $[Mn^{2+}]$/[enzyme] ratio giving full catalytic activity. Thus, we conclude that the sigmoid shape of the titration curve in Fig. 2 is not due to cooperative binding of metal ions but to a concerted effect of several ions per subunit that induce the changes necessary for restoration of catalytic activity. After regain of the activity the excess of metal ions can be removed by dialysis leaving a maximally active enzyme having the expected stoichiometry of about 2 metal atoms per enzyme molecule (Table 1).

The possibility was considered that the reactivation afforded by addition of Co^{2+}, Mg^{2+} and Mn^{2+} could be due to contaminating Zn^{2+} ions in solutions of these metal ions. Therefore, the analyses of the reactivated enzyme preparations included Zn as well as the metal used for reactivation (Table 1). The results show that the presence of Zn was too low to explain the catalytic activity obtained with Co^{2+}, Mg^{2+} and Mn^{2+}, and, thus, that these metals by themselves can serve as essential metal cofactors of glyoxalase I.

Experimental. Glyoxalase I from human erythrocytes was prepared as previously described; isoenzyme 1 or a mixture of the three isoenzymes was used.[7] All chemicals used were of the highest purity available commercially. Glyoxalase I activity was measured as described, using

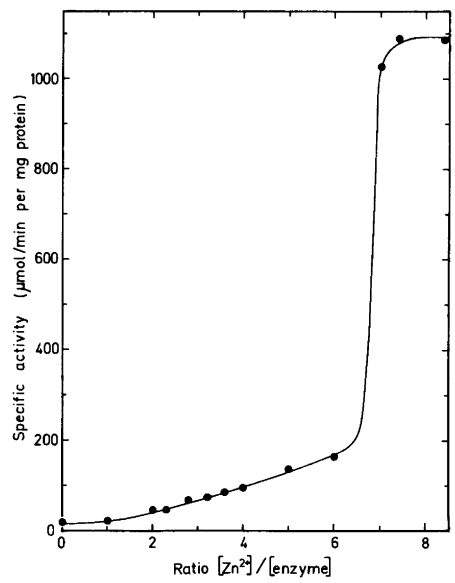

Fig. 2. Reactivation of enzymatic activity by addition of Zn^{2+} to the apoenzyme of glyoxalase I. Apoenzyme (1.88 mg/ml) was kept in 10 mM Tris/HCl buffer (pH 7.8) containing 10% (v/v) methanol and 2 – 5 μl additions of 2 mM $ZnSO_4$ were made. The additions of $ZnSO_4$ are expressed as the molar ratio of total concentrations of Zn^{2+} and enzyme (dimer, $M_r = 46\,000$). The enzymatic activity was determined within 5 min after each addition of $ZnSO_4$.

methylglyoxal as the 2-oxoaldehyde substrate.[10] Apoenzyme was prepared by dialyzing glyoxalase I (about 1.5 mg/ml) for 3 – 4 h at 4 °C against 50 mM imidazole/HCl buffer (pH 6.8) containing 1 mM EDTA and 10 % (v/v) methanol followed by a 12 – 14 h period against a fresh solution of the same buffer. The apoenzyme was then dialyzed three times for 3 h against 10 mM Tris/HCl buffer (pH 7.8) containing 10 % methanol. All buffer solutions were pretreated with Chelex 100 (BioRad).[11] The treatment with Chelex 100 gave lower values of residual Zn content in the buffer solutions than the extraction procedure with dithizone previously used (*cf.* Ref. 5). Whenever possible plastic vessels were used; dialysis tubing and glass-ware were treated as described[5] in order to remove contaminating metal ions.

Restoration of maximal enzymatic activity was made by addition of a 10-fold molar excess of a suitable salt (usually chloride) of the metal to the apoenzyme in 10 mM Tris/HCl buffer (pH 7.8). The excess of metal ions was removed by dialysis at 4 °C against 10 mM Tris/HCl buffer (pH 7.8) containing 10 % (v/v) methanol. After 3 h the buffer was exchanged for a fresh solution. Two 3 h periods were usually sufficient to reach the state at which no further decrease in metal content could be detected.

Metal analyses of enzyme preparations and buffer solutions were made on a Varian Techtron atomic absorption spectrophotometer equipped with a CRA-90 carbon rod atomizer.

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