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WHEAT GERM PHOSPHOGLYCERATE MUTASE: EVIDENCE FOR A METALLOENZYME

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SUMMARY: Wheat germ phosphoglycerate mutase, exposed to 3.4 M guanidinium chloride at 22°C and pH 7.8, slowly undergoes time-dependent inactivation which can be fully reversed by adding excess Co^{2+} or Mn^{2+} to a 50-fold dilution of the denaturing medium. Titration of the denatured enzyme with either Co^{2+} or Mn^{2+} shows that wheat germ mutase preferentially binds Co^{2+}. Assuming 1:1 complexation between metal atom and protein, the apparent dissociation constants (Kd) for E Co^{2+} and E Mn^{2+} at 22°C and pH 8.7 are approximately 1.06 and 1.84, respectively. Other metal atoms (e.g., Cr^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, and Ni^{2+}) have no effect in restoring the apoenzyme's catalytic activity. At low concentrations (0.11-0.23 mM) Zn^{2+} partially restores activity, but promotes protein precipitation at elevated concentrations. Evidence suggests that all bisphosphoglycerate-independent phosphoglycerate mutases require either an intra- or an extramolecular metal atom in order to function. Attempts to characterize wheat germ mutase as a glycoprotein have yielded negative results.

Recently, we reported on the purification and partial characterization of the bisphosphoglycerate-independent phosphoglycerate mutase (PGM, EC 5.4.2.1)[1] from wheat germ (1). Our studies revealed that the enzyme is reversibly inhibited by a variety of polydentate aliphatic and aromatic chelators, suggesting the presence of a tightly bound metal atom(s) within or near the active site, or within a structural domain essential to the enzyme's catalytic integrity.

We now wish to provide confirming evidence for the metalloenzymic nature of wheat germ PGM by showing that the denatured enzyme regains activity only in renaturing media containing specific metal ions.

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[1]Abbreviations: PGM, phosphoglycerate mutase; Gdn-Cl, guanidinium chloride; BPG, bisphosphoglycerate; 3-PGA, D-glycerate-3-P.

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MATERIALS AND METHODS

Materials: Flake wheat germ was purchased from Garden Spot Distributors, New Holland, PA. $CuSO_4$, $NiSO_4$, $FeSO_4$, $CoCl_2$, $FeCl_3$, $MgCl_2$ and $CrCl_2$ were purchased from Fisher. Guanidine hydrochloride was purchased from Sigma. Other materials and chemicals, including purified PGM, were obtained from sources indicated in a previous publication (1). All solutions were prepared with deionized, glass-distilled water.

Activity Measurements: Wheat germ PGM was determined essentially by the enolase-coupled assay of Rodwell et al. (2). The assay mixture was comprised of 20 mM 3-PGA, 8.3 mM $MgCl_2$, 100 mM Tris-Cl (pH 8.7), 1.6 units of enolase, and designated concentrations of metal compounds other than $MgCl_2$. In all cases, 0.02 ml of denaturing medium, containing 0.84 µg PGM, was added to 0.98 ml of assay mixture. Mutase activity was determined by monitoring ultraviolet absorption at 240 nm and 22°C (3).

RESULTS AND DISCUSSION

Previously, we had shown that wheat germ PGM is slowly, but reversibly, inactivated in the presence of polydentate chelators (1). We have now determined that prolonged exposure of the enzyme to concentrated Gdn-Cl, regardless of the presence or absence of chelator, results in an apparently irreversible activity loss which is directly proportional to the length of time spent under denaturing conditions. This phenomenon has been interpreted as a gradual diffusion of metal ion into the medium during and/or after protein unfolding. Corroboration of our interpretation is found by examining the data expressed in Figs. 1 and 2.

Fig. 1A illustrates the regain in phosphoglycerate mutase activity with time in the reconstituting medium after having exposed PGM to 3.4 M Gdn-Cl at pH 7.8 and 22°C for various periods ranging from 5-1080 min (18 h). As shown, each reaction isotherm manifests a lag phase which eventually develops into a region exhibiting steady-state kinetics. The lag phase most likely represents refolding kinetics of the denatured enzyme (i.e., protein plus undiffused metal atom), while each steady-state slope represents the regainable fraction of catalytic activity, expressed as $\Delta A_{240}/\Delta t(\text{min.})$. After 18 h in 3.4 M Gdn-Cl, wheat germ PGM exhibits virtually no regainable activity; however, the addition of Co^{2+} (2.6 mM, final concentration) to the renaturing medium produces instant and complete enzyme reactivation (see Fig. 1A). If the

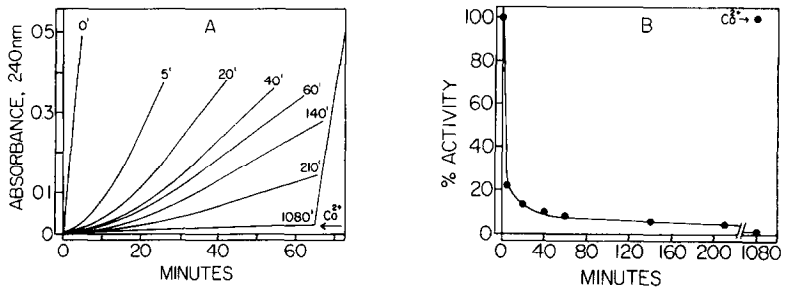

Fig. 1A: Regain of enzyme activity after exposing wheat germ PGM to concentrated Gdn-Cl for varying periods of time. Mutase (42 µg/ml) was incubated in 3.4 M Gdn-Cl, 20 mM Tris-Cl at pH 7.8 and 22°C for periods ranging from 5-1080 min. At the times indicated for each isotherm, 0.02 ml of incubation mixture was added to 0.98 ml of stock assay solution (see Materials and Methods). Enzyme activity at pH 8.7 and 22°C was recorded as uv absorbance (240 nm) vs time (min). After determining activity regain of PGM exposed to Gdn-Cl for 1080 min (18 h), a second activity measurement was made with 2.6 mM Co^{2+} in the assay medium. Under all circumstances, the assay system was found to be unaffected by low concentrations (~0.07 M) of Gdn-Cl.

Fig. 1B: The effect of concentrated Gdn-Cl on wheat germ PGM activity with time. The steady-state slopes (S_t) of the various isotherms illustrated in Fig. 1A were compared with the slope obtained at zero time (S_0) and the ratios $(S_t/S_0)100$ plotted as % activity vs time of enzyme exposure to 3.4 M Gdn-Cl.

steady-state slopes of the various isotherms are compared with that of the isotherm obtained at zero time, and the results are plotted as percent activity vs exposure time to concentrated Gdn-Cl, the plot illustrated in Fig. 1B is obtained. If percent activity represents that fraction of intact metalloenzyme surviving after exposure to Gdn-Cl, then Fig. 1B must represent the kinetics of metal atom loss to the denaturing medium.

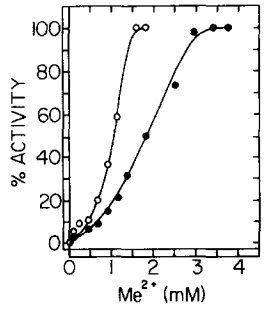

Fig. 2: Titration of guanidine-inactivated wheat germ PGM with Co^{2+} and Mn^{2+}. Mutase (42 µg/ml) was incubated in 3.4 M Gdn-Cl, 20 mM Tris-Cl (ph 7.8 and 22°C) for 20 h. Aliquots (0.02 ml) of incubation mixture were subsequently added to 0.98 ml of stock assay solution (see Materials and Methods) containing increasing concentrations of metal ion and activity was recorded as A_{240} at pH 8.7 and 22°C. The resultant data were analyzed as described in the legend to Fig. 1B.

The addition of either Co^{2+} or Mn^{2+} in increasing concentrations to the reconstituting medium after having exposed PGM to 3.4 M Gdn-Cl for 20 h, induces enzyme activation and allows for construction of the titration curves illustrated in Fig. 2. Although both metal ions are capable of fully restoring catalytic activity, the enzyme manifests a preference for Co^{2+} rather than Mn^{2+}. Assuming that PGM forms a 1:1 complex with metal ion, the apparent dissociation constants (Kd, pH 8.7 and 22°C) obtained from Fig. 2 for $E{\cdot}Co^{2+}$ and $E{\cdot}Mn^{2+}$ are approximately 1.06 and 1.84, respectively. At low concentrations (0.11-0.23 mM), Zn^{2+} restores 1-2% of total mutase activity, but promotes protein precipitation when present at higher concentrations. Other metal ions (e.g., Cr^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} Mg^{2+} and Ni^{2+}) at concentrations as high as 1-2 mM have no effect in restoring activity to the denatured enzyme[2].

Of all of the bisphosphoglycerate-independent phosphoglycerate mutases studied (4), apparently wheat germ PGM is the only one, so far, which has been shown to manifest characteristics attributable to a *bona fide* metalloenzyme; i.e., the enzyme contains a tightly bound metal atom(s) as an integral component of its structural fabric. The exact identification and stoichiometric quantification of that atom must await further investigation. In contrast, the highly purified PGMs from *B. subtilis* (5) and *B. megaterium* (6) require added Mn^{2+} for expression of catalytic activity. These findings suggest that all BPG-independent mutases require the presence of specific metal atoms, in one fashion or another, in order to function.

Employing the method of Lindberg (7) to characterize wheat germ PGM as a glycoprotein, gives negative results. The method requires the formation of methylated sugar alditol acetates prior to residue identification by gas-liquid chromatography.

ACKNOWLEDGEMENT

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[2] Mg^{2+} is a required component of the assay mixture and is always present at 8.3 mM during activity measurements.

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