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The Reversible Inactivation of D-Glyceraldehyde 3-Phosphate Dehydrogenase Following NAD^+ Removal

by

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D-Glyceraldehyde 3-phosphate dehydrogenase (GPDH) isolated from several sources, including rabbit skeletal muscle, binds NAD^+ very tightly (10). NAD^+ may be removed from GPDH by several more or less rigorous procedures including charcoal treatment. It has been reported that removal of the last molecule of NAD^+ from the tetrameric GPDH molecule induces a conformational change (6). Such a conformational change might be expected to induce a simultaneous change in specific activity. The work reported here suggests that this change in specific activity does indeed occur and, furthermore, that the enzymic activity may be restored to a value approaching the original on readdition of NAD^+.

Material and Methods

D-Glyceraldehyde 3-phosphate (G3P) (Ba salt of diethylacetal), NAD^+, NADH, $NADP^+$, ATP, ADP, AMP, 3-phosphoglycerate and 3-phosphoglycerate kinase were obtained from the Boehringer Corp. (London) Ltd. Solutions of G3P were prepared by the method of RACKER, KLYBAS and SCHRAMM (8). Substrates were assayed spectrophotometrically with GPDH. Charcoal (Norit OL; Hopkin & Williams Ltd.) was prepared by boiling with 4 N-HCl for 1 hour. It was washed exhaustively with distilled water, dried at 110° C and finally stored in a dessicator. GPDH activity was measured both in the forward (production of NADH) and backward directions. In the former, the assay medium (modified from FERDINAND (3), consisted of 31 mM-triethanolamine; 39 mM-NaH_2PO_4; 0.8 mM-EDTA; 0.75 mM-2-mercaptoethanol; 0.6 mM-NAD^+; 0.28 mM-G3P; pH 8.8. In the latter, the medium contained 0.1 M-triethanolamine; 1.33 mM-$MgCl_2$; 2.5 mM-2-mercaptoethanol; 3.5 mM-ATP; 0.165 mM-NADH; 3 mM-3-phosphoglycerate; 1.8 U/ml 3-

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phosphoglycerate kinase; pH 7.6. Measurements were carried out using a Hilger-Gilford recording spectrophometer at 25° in a cuvette of 1 cm light-path, final volume 3 ml; reaction was initiated by addition of enzyme. Rates were linear for the first 0.2 OD units and 0.6 OD units in the forward and back reactions respectively.

GPDH was prepared from rabbit skeletal muscle routinely using a slight modification of the method of AMELUNXEN and CARR (1). The final specific activity in the forward assay described was 80 μmoles NADH produced/min/mg enzyme. Enzyme prepared according to FERDINAND (3) and from a commercial source (Boehringer) were also used for comparative purposes. Results with the three preparations were essentially similar.

The $E^{1\%}_{1cm}$ of the native GPDH was taken as 0.92 at 280 mμ. Appropriate corrections were made following removal of NAD^+ to allow for changes in extinction.

NAD^+-free enzyme was made by stirring 1 ml GPDH (containing 6.5 mg and freed of $(NH_4)_2SO_4$ by gel filtration) with 210 mg of freshly activated charcoal for 3 min. at 5°. The mixture was centrifuged at 125.000 g for 45 min. at 5°. Protein recoveries in the supernatant varied from 3 % to 23 % (mean 9 %). After pre-incubation with 5 mM-NAD^+ at pH 7.6 and 4° for 2 hr., the specific activity varied to near a value of 74 % (mean 55 %) of the original value.

Results and Discussion

Progress curves of the rate of NADH production with NAD^+-free GPDH were markedly biphasic. Rates increased over a period of 5-10 min. to maximum steady-state values (Fig. 1). These final steady-state rates were directly proportional to initial enzyme concentration. Experiments with the native enzyme (containing NAD^+), assayed under identical conditions, showed a rate of NADH production linear with time (Fig. 1).

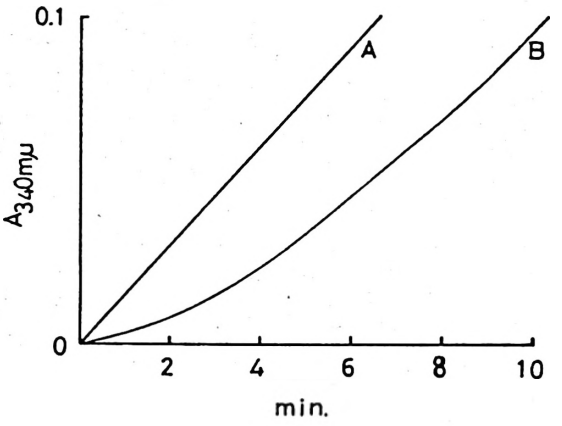

FIG. 1. Progress curves for the forward assay. Conditions as in the text. A) Native GPDH; B) NAD^+-free GPDH.

With the NAD^+-free enzyme the increase in rate following initiation of the reaction appears to follow first order kinetics (Fig. 2). The first order rate constant for this process at pH 8.8 has been determined as 45×10^{-4} sec.$^{-1}$, corresponding to a relaxation time of 3.7 min

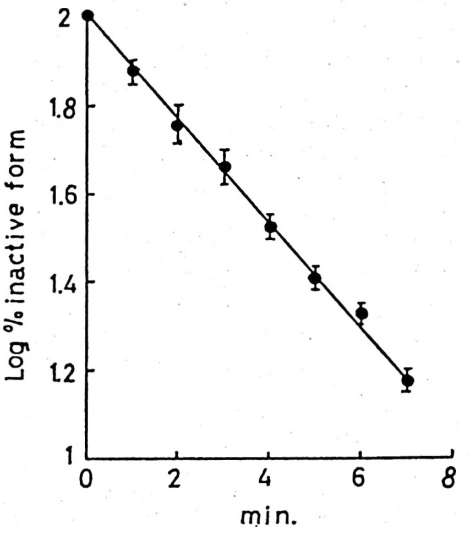

FIG. 2. First order plot of the rate of conversion of inactive to active GPDH. Points are ± S.D. (4 observations).

The possibility of dimer-tetramer interconversion during the initial phase of the reaction has been investigated in three ways: 1) Gel filtration was carried out with Sephadex G-150 at 2° with EDTA (5 mM), NaCl (0.2 M) and 2-mercaptoethanol (1 mM), pH 7.5, as solvent. Native and NAD^+-free GPDH both appeared at a point corresponding to 1.45 times the void volume. The NAD^+-free enzyme was monitored both before and after filtration by 280/260 mμ absorption and by the observation of biphasic kinetics in the forward assay system. Enzyme concentrations varied from 0.02 to 0.2 mg/ml. 2) Electrophoresis on cellulose acetate strips (5) failed to show any difference between the mobilities of the native and NAD^+-free enzymes. 3) Ultracentrifuge studies (Beckman Model E ultracentrifuge) revealed that no significant alteration in sedimentation characteristics is occasioned by NAD^+ removal.

These observations are consistent with the hypothesis that the molecular weight of the NAD^+-free GPDH is similar to that of the native enzyme and that reactivation by NAD^+ involves a slow transition between two tetrameric conformers (corresponding to the R and T forms of MONOD, WYMAN and CHANGEUX (7). In this instance it would appear that the T conformer is catalytically inactive; a similar property has been shown for yeast GPDH (4). Conformational changes due to NAD^+ binding have been reported from ORD studies with the the pig muscle enzyme (2).

NAD^+-free GPDH becomes reactivated during both forward and backward assays. This reactivation was studied further by pre-incubation of the charcoal-treated enzyme (0.31 mg/ml) with a variety of possible effectors (5 mM) at 4° for 2 hr., pH 7.6. GPDH activity was monitored in both forward and back reactions. NAD^+, NADH and G3P reactivated the enzyme and abolished the biphasic response. This result was also obtained with these compounds at 0.5 mM. No effect was observed with P_i, ADP, AMP, 2-mercaptoethanol, Na^+, K^+, Mg^{2+} or Ca^{2+}. 3-Phosphoglycerate and $NADP^+$ produced partial reactivation, the initial rate of NADH production being approx. 50 % of the final steady-state rate. ATP removed the biphasicity but produced a marked degree of inhibition of the steady-state rate (as compared with rates after incubation with NAD^+). These latter observations may be correlated with the report that ATP promotes dissociation of yeast GPDH and also inhibits enzymic activity directly (9).

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Summary

Progress curves of the rate of NADH production with NAD^+-free D-glyceraldehyde 3-phosphate dehydrogenase from rabbit skeletal muscle were markedly biphasic with final steady-state rates directly proportional to initial enzyme concentration. This work suggests that there is a change in specific activity and, furthermore, that the enzymic activity may be restored to a value approaching the original on readdition of NAD^+.

The studies made by gel filtration, electrophoresis and ultracentrifugation are consistent with the hypothesis that the molecular weight of the NAD^+-free enzyme is similar to that of the native enzyme and that reactivation by NAD^+ involves a slow transition between two tetrameric conformers.

A variety of possible effectors were also studied and it was observed that NAD^+, NADH and D-glyceraldehyde 3-phosphate reactivated the enzyme and abolished

the biphasic response. ATP removed the biphasicity but produced a marked degree of inhibition of the steady-state rate. No effect was observed with P_i, ADP, AMP, 2-mercaptoethanol, Na^+, K^+, Mg^{2+} or Ca^{2+}; 3-phosphoglycerate and NADP produced partial reactivation.

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