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COLD INACTIVATION OF GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE FROM RAT SKELETAL MUSCLE

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Summary

Inactivation of apo-glyceraldehyde-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase(phosphorylating) (EC 1.2.1.12) from rat skeletal muscle at 4°C in 0.15 M NaCl, 5 mM EDTA, 4 mM 2-mercaptoethanol pH 7.2 is a first-order reaction. The rate constant of inactivation depends on protein concentration. With one molecule of NAD bound per tetrameric enzyme, a 50% loss in activity is observed and the rate constant of inactivation becomes independent of the protein concentration over a 30-fold range. Two moles of NAD bound per mole of enzyme fully protect it against inactivation. NADH affords a cooperative effect on enzyme structure similar to that of NAD.

Inactivation of 7.8 S apoenzyme is reflected in its dissociation into 4.8-S dimers. In the case of enzyme—NAD_1 complex, no direct relationship between the extent of inactivation and dissociation is observed, suggesting that these two processes do not occur simultaneously; we may say that dissociation is slower than inactivation. A mechanism in which the rate-limiting step for inactivation is a conformational change in the tetramer occurring prior to dissociation and affecting only the structure of the non-liganded dimer, is consistent with the experimental observations.

Inorganic phosphate protects apoenzyme against inactivation. Its effect is shown to be due to the anion binding at specific sites on the protein with a dissociation constant of 2.6 ± 0.4 mM. The NaCl-induced cold inactivation of glyceraldehyde-phosphate dehydrogenase is fully reversible at 25°C in the presence of 20 mM dithiothreitol and 50 mM inorganic phosphate. The rate of reactivation is independent of protein concentration. Inactivated enzyme retains the ability to bind specific antibodies produced in rabbits, but diminishes its precipitating capability.

Introduction

Study of the reversible dissociation of an oligomeric protein has proved to be a valuable approach to the problem of intersubunit interactions in the molecule. Tetrameric glyceraldehyde-phosphate dehydrogenase has been shown to dissociate reversibly in the cold under a variety of conditions: low ionic strength buffers [1], the presence of ATP [2] or salts of monovalent anions [3,4]. The extent of inactivation and dissociation under a given set of conditions was found to differ with enzymes obtained from various sources [2,5,6], which reflects some differences in the conformation of polypeptide chains and the nature of their interactions in the oligomer.

We have previously reported that glyceraldehyde-phosphate dehydrogenase isolated from rat skeletal muscle becomes markedly destabilized at 4° C in the presence of salts of monovalent anions, undergoing reversible inactivation and dissociation into dimers [4]. The subject of this paper is a detailed study of NaCl-induced cold inactivation of apo-glyceraldehyde-phosphate dehydrogenase and of the effect of one and two firmly bound coenzyme molecules on the stability of tetrameric enzyme structure.

Materials and Methods

NAD, NADH, EDTA, CM—cellulose were obtained from Reanal, 2-mercaptoethanol was an Austrowaren product, dithiothreitol from Serva. D-Glyceraldehyde 3-phosphate was prepared from D-fructose 1,6-diphosphate (Reanal) by the method of Szewczuk et al. [7]. NAD, NADH and D-glyceraldehyde 3-phosphate concentrations were estimated enzymatically. Glyceraldehyde-phosphate dehydrogenase was isolated from rat skeletal muscle as previously described [8]. All the solutions throughout the work contained 5 mM EDTA and 4 mM 2-mercaptoethanol. For preparation of apoenzyme (all the experiments described below were carried out at 20° C) a crystalline suspension of dehydrogenase (40 mg, $A_{280} : A_{260} = 1.2$) was dissolved in 1.5 ml 10 mM sodium phosphate pH 6,7 and passed through a column (2 cm × 18 cm) of Sephadex G-50 equilibrated with the same buffer. The eluate (35 mg of protein, volume 5 ml, $A_{280} : A_{260} = 1.4$) was applied onto a 2 cm × 4 cm CM—cellulose column, equilibrated with the above buffer. Only apoenzyme proved to adsorb on the CM—cellulose under these conditions. It was eluted either with 0.1 M sodium phosphate pH 7.2 or with 0.15 M NaCl pH 7.2. In the latter case 10 mM NaCl pH 6.7 was passed through the column before elution to remove inorganic phosphate. The apoenzyme solution thus obtained had a $A_{280} : A_{260}$ ratio of 1.9—2.0. Protein concentration was estimated from absorbance at 280 nm using an extinction coefficient of 0.83 $cm^2 \cdot mg^{-1}$ for apoenzyme. Enzyme activity, assayed in 0.1 M glycine—NaOH buffer pH 8.7, 5 mM EDTA with 5 mM sodium arsenate, 0.5 mM NAD, 0.5 mM glyceraldehyde-3-phosphate at 25° C, corresponded to 105 μmoles NADH/min per mg of protein.

Apoenzyme titrations with NAD were carried out by the method of Stockell [9] with a Hitachi-356 spectrophotometer. The measurements were made at two wavelengths, 405 nm and 500 nm, since, as pointed out by

Stockell, it is not practical to measure formation of the compound of enzyme with NAD at its absorption maximum of 365 nm, high concentrations of coenzyme having significant absorption at this wavelength. The readings taken at 500 nm were subtracted from those at 405 nm, where enzyme—NAD compound has half-maximal absorption. Sedimentation velocity experiments were carried out with a Spinco model E analytical ultracentrifuge equipped with an automatic split-beam photoelectric scanning optical system. Sedimentation patterns were monitored at 280, 294 or 340 nm at 60 000 rev./min and 4°C. Sedimentation coefficients were corrected to $S_{20,w}$ according to Schachman [10].

Immunochemical studies were performed with anti-rat glyceraldehyde-phosphate dehydrogenase antibodies produced in rabbits as previously described [11]. γ-Globulin fractions of antisera and normal sera were obtained by ammonium sulfate precipitation at 40% saturation and dialyzed against 0.15 M NaCl, 5 mM EDTA pH 7.2. The quantitative precipitin reaction was performed by the method of Kabat and Mayer [12]. Increasing amounts of antigen dissolved in 0.15 M NaCl, 5 mM EDTA, 4 mM mercaptoethanol were mixed with a constant amount of γ-globulin fraction of antiserum. Each mixture was made up to 0.3 ml with the NaCl solution and kept at 4°C for 24 h. The precipitates were collected by centrifugation, washed twice with cold 0.15 M NaCl pH 7.0 and analyzed for the protein content by the method of Lowry [13]. 95% pure γ-globulins obtained by DEAE—cellulose fractionation were used to prepare a standard protein solution. The supernatants obtained after separation of the antigen—antibody complex, were assayed for the presence of excess antibody and antigen.

Results and Discussion

Apoenzyme solutions prepared by CM—cellulose chromatography can be

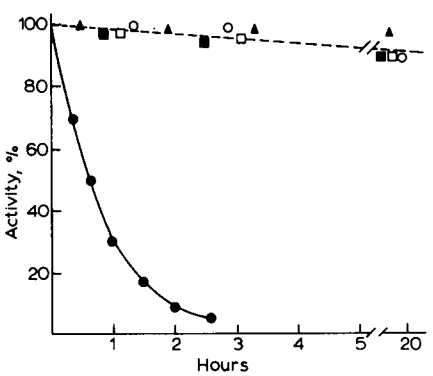


Fig. 1. Cold inactivation of apo-glyceraldehyde-phosphate dehydrogenase. Apoenzyme, eluted from CM-cellulose column with 0.15 M NaCl, 5 mM EDTA, 1 mM dithiothreitol, pH 7.2 was incubated at 25°C (○) or at 4°C with no additions (●) and in presence of 0.1 M Na_2HPO_4, pH 7.2 (▲). A portion of the eluted apoenzyme solution was passed through a Sephadex G-50 column equilibrated with 0.15 M NaCl, 5 mM EDTA, 1 mM dithiothreitol in ^2H_2O, pH 7.2 and incubated at 25°C (□) or at 4°C (■). Protein concentration 1.2 mg/ml. At various time intervals aliquots were removed with chilled pipettes and enzyme activity assayed in 0.1 M glycine—NaOH buffer pH 8.7, 5 mM EDTA, 5 mM sodium arsenate, 0.17 mM glyceraldehyde-3-phosphate, 0.13 mM NAD at 20°C. Activity measured at the beginning of cold inactivation corresponds to 100%.

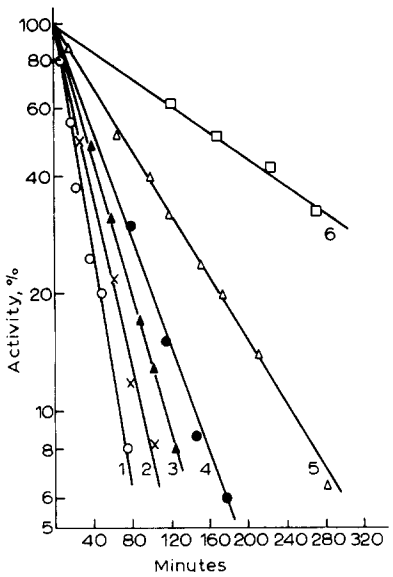


Fig. 2. Effect of inorganic phosphate on cold inactivation of apo-glyceraldehyde-phosphate dehydrogenase. Apoenzyme (0.41 mg/ml) was incubated at pH 7.2 in 0.15 M NaCl, 5 mM EDTA, 4 mM 2-mercaptoethanol at 4°C with no additions (1) and in presence of 1 mM (2), 1.7 mM (3), 2.5 mM (4), 5 mM (5) and 10 mM (6) Na_2HPO_4; pH of Na_2HPO_4 was adjusted to 7.2. Aliquots were removed and assayed as described in Fig. 1.

stored for several days in 0.1 M sodium phosphate, 5 mM EDTA, 1 mM dithiothreitol, pH 7.2 without any loss in activity either at 25°C or at 4°C. However, when chloride is substituted for phosphate, a rapid temperature-dependent inactivation occurs (Fig. 1). This marked temperature effect suggests that a weakening of hydrophobic interactions is involved in destabilization of the protein structure in the presence of a monovalent anion. As shown in Fig. 1, this destabilization is fully prevented either in the presence of ^2H_2O or in 0.1 M sodium phosphate. The non-specific protective effect of ^2H_2O may be explained by strengthening non-covalent interactions (such as hydrophobic and hydrogen bonds) in the enzyme molecule [14,15].

On the other hand, the effect of phosphate seems to be due to the anion binding at specific sites on protein. This conclusion is based on the following experiments. As shown in Fig. 2, the enzyme inactivation is a first-order reaction, and addition of increasing phosphate concentrations diminishes the inactivation rate. Assuming that the protective effect is due to the reversible association of phosphate with the enzyme to form a complex which is inactivated slower than the free enzyme, we may write the following equilibrium:

$$\begin{array}{ccc} E + P & \overset{K_D}{\rightleftharpoons} & E\text{—}P \\ \downarrow k_1 & & \downarrow k_2 \\ E^* & & E^*\text{—}P \end{array}$$

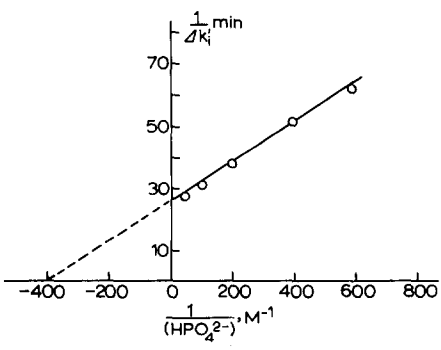
where apoenzyme (E), having a first-order rate constant of inactivation (k_1) combines with inorganic phosphate (P) to form a complex (E—P) with a first-order rate constant of inactivation, k_2. E* and E*—P stand for inactivated

forms of the enzyme produced irreversibly under the conditions of the experiment. The dissociation constant, K_D of the E—P complex, is related to the rate constants according to the following expression:

$$\frac{1}{\Delta k_i} = \frac{1}{k_1 - k_2} + \frac{K_D}{(k_1 - k_2)} \cdot \frac{1}{P}$$

where Δk_i is an experimentally determined difference between the rate constant of inactivation of the free enzyme and of the enzyme in the presence of a certain phosphate concentration. The results of determination of K_D for inorganic phosphate by this method [16] are shown in Fig. 3. The intercept at infinite Δk_i is equal to $-\frac{1}{K_D}$, which was found to correspond to 2.6 ± 0.4 mM at pH 7.2 and 4°C. Further experiments using an independent method are needed to determine this value more precisely. The results suggest that the proposed mechanism of the protective effect of inorganic phosphate fits the experimental data. It seems probable that the stabilizing effect of other polyvalent anions (citrate, sulphate [4]) is also due to the interaction with specific anion binding sites. Localization of these sites and possible involvement of the anion-binding sites of the active center of the dehydrogenase [17—19] are to be elucidated.

Fig. 4A shows the effect of inorganic phosphate on the extent of reactivation. The reactivation is influenced by a number of variables and phosphate added before temperature treatment has but a negligible effect. Shown in Fig. 4B are the data on the effect of protein concentration on the reactivation rate.



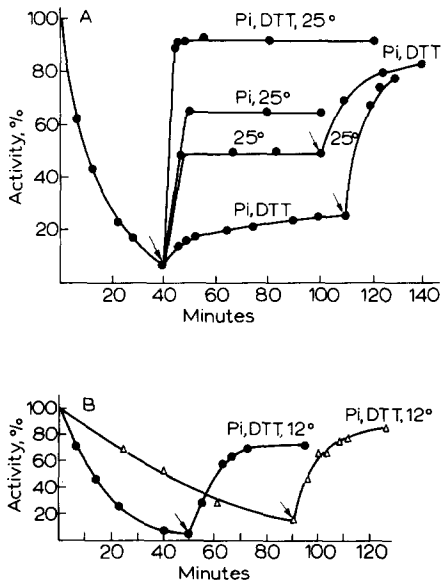


Fig. 3. Determination of the dissociation constant (K_D) for inorganic phosphate. The data from Fig. 2 are plotted as described in the text.

Fig. 4. Reversibility of cold inactivation of apo-glyceraldehyde-phosphate dehydrogenase. Apoenzyme at a concentration of 0.11 mg/ml (•) or 1.12 mg/ml (△) was incubated at pH 7.2 in 0.15 M NaCl, 5 mM EDTA, 1 mM dithiothreitol at 4°C. At time intervals indicated by the arrows, aliquots were withdrawn and treated as indicated in the Fig. DTT, dithiothreitol, 20 mM; P_i—Na_2HPO_4, 50 mM.

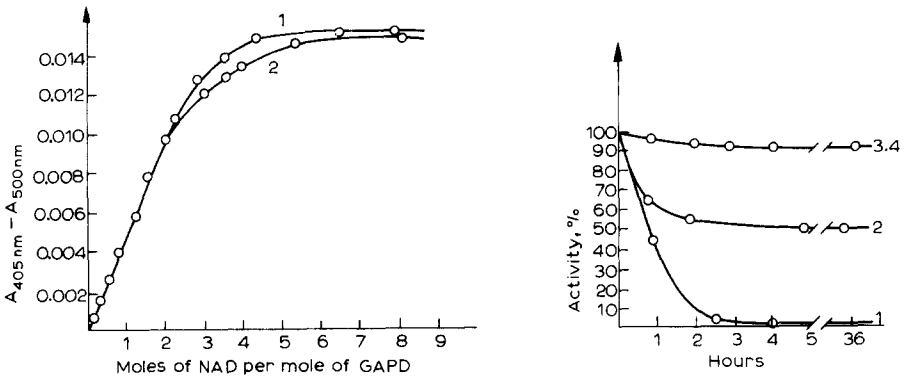


Fig. 5. Titration of apo-glyceraldehyde-phosphate dehydrogenase with NAD. $9 \cdot 10^{-6}$ M apoenzyme (GAPD), in 0.1 M sodium phosphate, 5 mM EDTA, 4 mM 2-mercaptoethanol pH 7.2 at 4° C (1) and 20° C (2).

Fig. 6. Effect of NAD concentration on the cold inactivation of glyceraldehyde-phosphate dehydrogenase. To apoenzyme eluted at 20° C from the CM—cellulose column with 0.15 M NaCl, 5 mM EDTA, 4 mM 2-mercaptoethanol pH 7.2 were added: none (1), one (2), two (3) or six (4) moles of NAD per mole of enzyme. Protein concentration $1.5 \cdot 10^{-5}$ M. After 30 min incubation at 20° C, temperature was lowered to 4° C and enzyme activity determinations were made at various time intervals as in Fig. 1.

Increasing the protein concentrations from 0.11 to 1.12 mg/ml did not affect the rate constant value which corresponded to $11.4 \cdot 10^{-2}$ and $12.4 \cdot 10^{-2}$ min^{-1} respectively suggesting conformational changes to be the rate-limiting step for reactivation. The extent of reactivation was found to depend on the time of incubation under the inactivating conditions. The enzyme which has been inactivated for 4 h regained only 60% of activity and after 22 h 40% of activity could be restored under the conditions of Fig. 4.

We then elucidated the effect of coenzyme on anion-dependent cold inactivation of the dehydrogenase. The protective effect of NAD on cold inactivation and dissociation of glyceraldehyde-phosphate dehydrogenase was reported in a number of publications [1,3,6,20]; in all the cases, the NAD concentrations used were sufficiently high to saturate all coenzyme-binding sites of the tetramer. A more detailed study of the effect of successively bound NAD molecules on the conformational stability of the tetramer under conditions of cold inactivation seemed of interest, taking into account the negative cooperativity of NAD binding to the muscle glyceraldehyde-phosphate dehydrogenases [21—23].

To determine NAD-binding characteristics of the rat muscle enzyme, titration experiments were carried out. As shown in Fig. 5 the titration curves are linear through all experimental points below a NAD—apoenzyme ratio of 2. Below this point the coenzyme is, therefore, stoichiometrically bound to the protein both at 4° C and 20° C. With further addition of NAD the absorbance increase per mole of added NAD declined. On assumption that the binding of the first two molecules of NAD is stoichiometric, the dissociation constant for the third one was determined as being $0.74 \cdot 10^{-6}$ M (at 20° C) and $0.42 \cdot 10^{-6}$ M (at 4° C), which is in good agreement with the data on rabbit muscle glyceraldehyde-phosphate dehydrogenase [24]. We did not use coenzyme con-

centrations sufficiently high for complete saturation of the fourth enzyme site to be ensured.

The dependence of the extent of NaCl-induced cold inactivation on the degree of enzyme saturation with NAD was then studied. As shown in Fig. 6, the rapid activity loss observed with the apoenzyme is completely prevented when the molar ratio of bound NAD to dehydrogenase is 2.0. The effect is the same when more NAD is added to saturate the third binding site of the enzyme molecule. With one molecule of bound NAD per tetrameric enzyme, 50% of activity retains during sufficiently long incubation (up to 36 h).

Fig. 7A shows a kinetic pattern of inactivation of the apoenzyme studied as a function of protein concentration. The loss of enzyme activity is a first-order process. Some acceleration of the inactivation rate with time of inactivation, reflected in non-linearity of the plots, was observed with protein concentrations exceeding 1.5 mg/ml. Apoenzyme instability and aggregation may be responsible for this phenomenon.

First-order rate constants of inactivation calculated from the initial, practically linear parts of semilogarithmic plots, were found to markedly increase with decrease in protein concentration (see legend to Fig. 7A). Concentration dependence of the initial rate constants of inactivation suggests dissociation to be involved in the inactivation process, the dissociated forms being more labile than the tetramer. Conformational changes in dissociated species induced by NaCl and low temperature are supposed to preclude their reassociation, which accounts for the irreversibility of the process.

The binding of one equivalent of NAD per enzyme molecule appears to change the inactivation pattern, as evidenced by the results listed in Fig. 7B. A first-order kinetics is observed, as in the case with the apoenzyme, but the rate of inactivation becomes independent of protein concentration over a 30-fold range. A conclusion may be drawn from these results that some intramolecular rearrangement is the rate-limiting step in inactivation of the enzyme—NAD_1

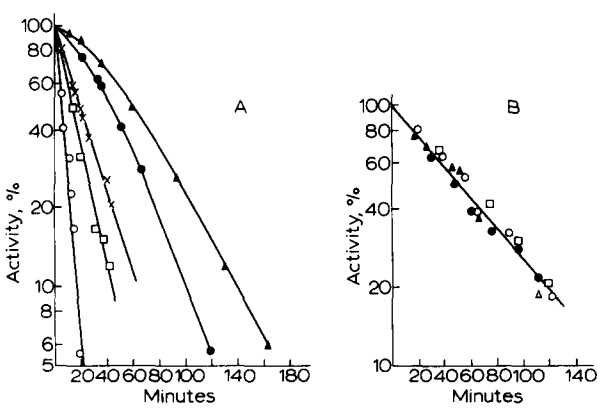


Fig. 7. Semilogarithmic plots of the rate of inactivation of apo-glyceraldehyde-phosphate dehydrogenase (A) and its complex with one mole equivalent of NAD (B). Inactivation was carried out under the conditions of Fig. 2. Aliquots were removed and assayed as usual. A, protein concentrations used were 0.05 (○), 0.1 (□), 0.6 (X) 1.6 (●) and 3.8 (△) mg/ml. The rate constants of inactivation are: $12 \cdot 10^{-2}$, $5.3 \cdot 10^{-2}$, $3.6 \cdot 10^{-2}$, $1.1 \cdot 10^{-2}$ and $0.5 \cdot 10^{-2}$ min^{-1}, respectively. B, protein concentrations: 0.12 (●), 0.34 (▲), 1.0 (○) and 3.7 (□) mg/ml.

complex and its dissociation, if involved, is a secondary process. A similar effect has been observed by Keleti et al. [25] in the study of heat inactivation of holo-glyceraldehyde-phosphate dehydrogenase from swine muscle.

Further studies were carried out to determine the relationship between the extent of enzyme inactivation and dissociation under a variety of conditions. Apoenzyme solutions (0.9—2.2 mg/ml) analyzed in an ultracentrifuge at the end of the inactivation process sedimented as a single component with an $s_{20,w}$ value of 4.8 S, which suggests complete dissociation of tetrameric 7.8-S molecule into dimers. The sedimentation pattern of the enzyme complex with one equivalent of NAD demonstrated two components, 4.8 S and 7.8 S. The amount of 4.8-S species increased with time of incubation under inactivation conditions, until equal distribution of protein between 7.8 S and 4.8 S components was attained. The equal content of dissociated and undissociated forms remained unchanged for several hours after inactivation was completed, which is consistent with 50% of activity being retained (Fig. 6). Fig. 8 shows a time-course of inactivation and dissociation of the enzyme—NAD_1 complex. Disappearance of the 7.8 S undissociated component was slower than the activity loss, whereas quantitative agreement between the rates of these two processes would be expected if inactivation was solely due to the dissociation.

Fig. 9 shows an inactivation pattern of the enzyme complex with one equivalent of NADH per tetramer. A dissociation constant $< 5 \cdot 10^{-7}$ M was assumed to correspond to the binding of the first coenzyme molecule [26]. Independence of the inactivation rate of protein concentration suggests that in this case, as well as in the case of enzyme—NAD_1 complex, inactivation is induced by some intramolecular changes in the tetramer. Sedimentation patterns of the inactivated preparation are shown in Fig. 10. The absorbtion scan carried out at 294 nm demonstrates the presence of two protein components,

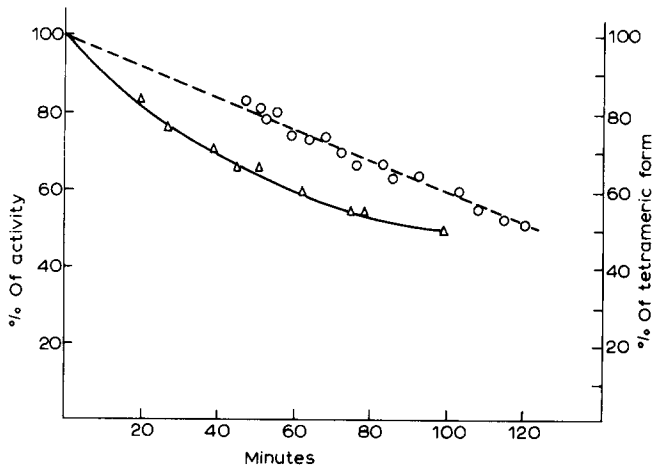


Fig. 8. Time-course of inactivation and dissociation of the enzyme complex with one mole equivalent of NAD. (△), the percentage of enzyme activity. (○), the percentage of tetramer remaining. Enzyme solution (2.2 mg/ml in 0.15 M NaCl, 5 mM EDTA, 4 mM 2-mercaptoethanol pH 7.2 at 4°C) was placed into centrifuge cells at the beginning of inactivation and the centrifuge was immediately started. Enzyme activity was assayed in a parallel sample. The percentage of tetrameric form was determined from the absorbance values of the fast-moving component observed on the scanner traces at the indicated times.

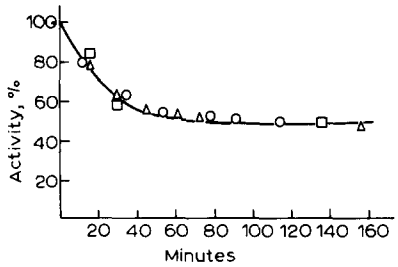


Fig. 9. Effect of NADH on the cold inactivation of apo-glyceraldehyde-phosphate dehydrogenase. Inactivation was carried out under the conditions of Fig. 2. (○), apoenzyme, $2 \cdot 10^{-6}$ M, NAD, $2 \cdot 10^{-6}$ M; (□), apoenzyme, $2 \cdot 10^{-6}$ M, NADH, $2 \cdot 10^{-6}$ M; (△), apoenzyme, $4.26 \cdot 10^{-5}$ M, NADH, $4.26 \cdot 10^{-5}$ M.

corresponding to 7.8-S and 4.8-S species. However, if the absorption scan was carried out at 340 nm, a single component was seen having a sedimentation coefficient of 7.8 S.

This result seems to indicate, that NADH is exclusively bound with tetrameric enzyme species. No protein-bound nucleotide has been detected in the dimeric fraction after 5 h of inactivation, although NADH should be equally distributed between tetrameric enzyme molecules at the beginning of inactivation and no free apoenzyme remained in solution as judged by independence of the inactivation rate constant of protein concentration. It appears therefore that a recombination of NADH-containing dimers takes place as the result of dissociation of the enzyme—$NADH_1$ complex. Conformational changes which become the rate-limiting step of inactivation seem to affect only the structure of non-liganded dimers, the coenzyme-containing ones being fully protected and capable of reassociating in the cold and in the presence of 0.15 M NaCl.

The similarity of the cooperative effects of NAD and NADH suggests that enzyme—coenzyme interactions involving the pyridinium ring of NAD play no role in protecting the dimer from conformational changes. The full protection of the dehydrogenase by two molecules of NAD bound per tetramer was previously observed when proteolytic digestion of the enzyme was studied [27]. However, no protection by two NAD equivalents was found against inactiva-

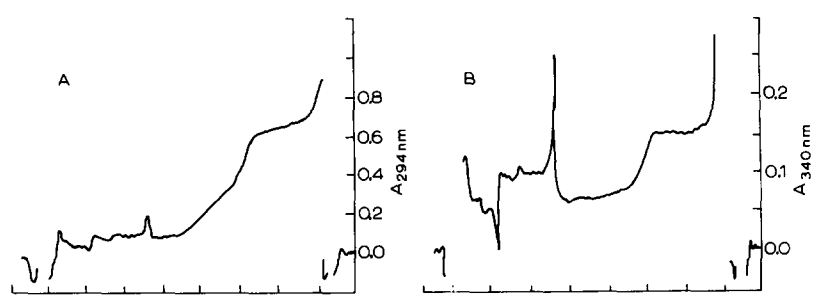


Fig. 10. Scanner traces taken during centrifugation of the enzyme complex with one mole equivalent of NADH. 2.78 mg/ml of the protein analyzed 5.5 h after beginning of inactivation under conditions of Fig. 2 and 60 min after reaching speed. The distribution of material in the cell was measured by scanning for transmitted light of wavelength 294 nm (A) and then immediately afterwards with light of 340 nm wavelength (B). Since the protein does not absorb significantly at 340 nm, the scan at this wavelength provides essentially a measure of the distribution of NADH-containing species, whereas that at 294 nm is a measure of the protein concentration.

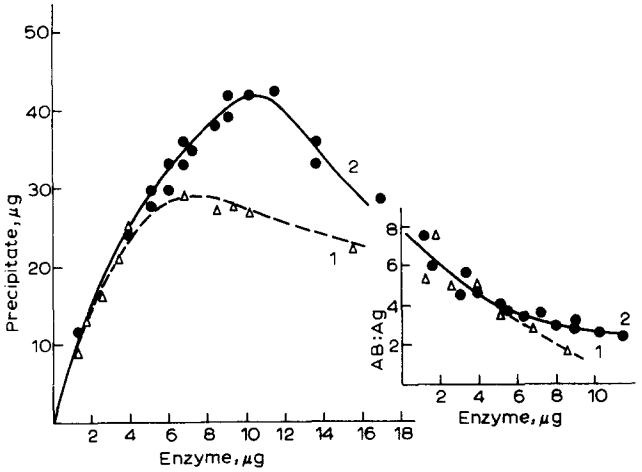


Fig. 11. Quantitative precipitin reaction of glyceraldehyde-phosphate dehydrogenase. Inset: Binding ratio of antibodies to enzyme (Ab : Ag) in the precipitate. Molecular weight of 140 000 was taken for the antigens. 1, apoenzyme inactivated under conditions of Fig. 1. 2, native apoenzyme, in the presence of 4-fold molar excess of NAD.

tion caused by chemical modification of the enzyme under conditions when 4 coenzyme molecules bound per tetramer afforded complete protection [28]. Hence, the effect appears to depend on the nature and extent of conformational changes.

An attempt was made to detect conformational changes induced by cold inactivation in the presence of NaCl by studying immunochemical properties of the enzyme. This was done by using quantitative precipitin techniques with anti-rat glyceraldehyde-phosphate dehydrogenase antibodies produced in rabbits. As seen in Fig. 11, the maximal amount of the precipitate formed by the cold-inactivated enzyme is markedly less as compared with the native enzyme. A possible explanation of this fact is partial loss by the cold-inactivated enzyme of the ability to interact with specific antibodies. To verify this possibility, an analysis of supernatant solutions at equivalence point was carried out for the presence of unbound antibodies. No residual antibody was found. This experiment was repeated, the concentrations of the components being 5 times as high as those in Fig. 11.

In this case, the difference between the amounts of precipitates formed by the native and inactivated enzymes reached 70 μg, but no unreacted antibody could be detected in this case either. It seems therefore, that it is the existence of soluble antigen—antibody complexes that should account for the observed difference in the amounts of precipitates. The maximum number of antibodies attached to one antigen molecule was found to be 8 by extrapolation to the extreme excess of antibody. As is seen in Fig. 11, inset, the inactivated protein fully retains the capability of binding antibodies.

It appears that no significant changes in the antigenic properties of the enzyme are induced by cold inactivation. The lower precipitating ability may result from some alterations of the antibody attachment sites, but the protein retains the capacity of binding the same amount of antibodies as the native enzyme. This indicates that no gross conformational changes resulted from cold

inactivation, which is consistent with reversibility of this process. The changes prove to be local and affect the active site region of the enzyme. The binding sites of rabbit anti-rat glyceraldehyde-phosphate dehydrogenase antibodies do not interfere with the active site region [11] and this may account for the effects observed.

Acknowledgment

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