Factors Affecting the Folding and Association of Flounder Muscle Glyceraldehyde-3-phosphate Dehydrogenase, *in Vitro*†

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ABSTRACT: A partially purified preparation of flounder muscle glyceraldehyde-3-phosphate dehydrogenase was reversibly dissociated utilizing 8 M urea as the dissociating agent. The regain of enzyme activity was found to be dependent on a number of factors other than just dilution of the urea. The optimal reversal conditions for this enzyme were determined to be incubation in a solution containing 0.24 M KCl, 0.12 M 2-mercaptoethanol, 40 mM NAD^+, and 0.1 M imidazole (pH 7.2). Enzyme activity recoveries of 80% were consistently obtained. The reversal process was concentration dependent displaying a maximum at 0.1 mg/ml of total protein. Recoveries of less than 10% were obtained if 2-mercaptoethanol was omitted from the reversal mixture. NAD^+, the cofactor of the glyceraldehyde-3-phosphate dehydrogenase reaction, was required at the initiation of the reversal process. Salts in general also had a stimulatory effect on the reversal process with recoveries of 22% obtained in a KCl concentration of 0.3 M in the absence of NAD^+. $NADP^+$ was able to replace NAD^+, being nearly as effective in catalyzing the reversal process. Other nucleotides (ATP, ADP, AMP, GTP) were relatively without effect on the regain of activity. The reversed and native enzymes were identical as judged by their sedimentation rates in sucrose gradients and their multiple form patterns in isoelectric focusing experiments. The results indicate that the primary structure of flounder muscle glyceraldehyde-3-phosphate dehydrogenase does not contain sufficient information to direct the formation of the active enzyme *in vitro*. The cofactor of the enzyme (NAD^+) seems to play a major role in catalyzing the proper folding and assembly of the component subunits with salts also exerting a significant effect. The results demonstrate that specific folding and assembly of the subunit can occur in the presence of other proteins since the enzyme sample used throughout was a crude preparation.

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating) (EC 1.2.1.12)) is a key glycolytic enzyme that has been studied from many sources (Allison and Kaplan, 1964; Wolny, 1968; Harris, 1970; D'Alessio and Josse, 1971). The structural and functional properties of the enzyme from these divergent sources are similar. Molecular weights of 120,000–145,000 have been reported from the sources investigated (Taylor and Lowery, 1956; Allison and Kaplan, 1964; Jaenicke *et al.*, 1968). In the studies made the enzyme exists as a tetramer composed of apparently identical subunits (Harrington and Karr, 1965; Harris and Perham, 1965; Wrigley, 1968). The flounder muscle enzyme also appears to be composed of identical subunits since electrophoresis in 0.1% sodium dodecyl sulfate gels and 8 M urea gels yields one band (Marangos and Constantinides, 1973). Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, yeast, and flounder muscle has been shown to exist as a mixture of multiple forms (Lebherz and Rutter, 1967; Susor *et al.*, 1969; Marangos and Constantinides, 1973). Since the enzyme from each source is composed of one subunit type a problem has arisen in explaining the structural heterogeneity observed. The simple explanation used for tetrameric enzymes having two subunit types (lactic dehydrogenase) that combine in different combinations to yield the structurally distinct forms does not apply. The possibility should be considered that the multiple forms of glyceraldehyde-3-phosphate dehydrogenase from each of the above-mentioned sources may arise from several different folding conformations of their component subunit, and that these conformers combine in different ratios producing the observed heterogeneity. This concept leads to the idea that for these enzymes factors other than primary structure probably affect their folding and association. Many enzymes have been shown to fold spontaneously to their active conformation by simple removal of the dissociating agent (Anfinsen and Haber, 1961; Sund and Weber, 1966). Other enzymes such as mitochondrial malate dehydrogenase are able to be reversibly denatured but the renatured enzyme is structurally different from the native enzyme (Kitto *et al.*, 1970), indicating that the enzyme was able to assume more than one active conformation (Kitto *et al.*, 1966). It has also been demonstrated that pancreatic ribonuclease is able to assume a conformation distinct from that of the native enzyme following partial denaturation (Pflumm and Beychok, 1969). Enzymes such as yeast glyceraldehyde-3-phosphate dehydrogenase (Deal, 1969), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Deal and Constantinides, 1967; Teipel and Koshland, 1971), and dogfish lactic dehydrogenase (Levi and Kaplan, 1971) regain little or no activity when renaturation is attempted unless other factors such as salts or metabolites are added.

The question of what factors control protein folding and conformation is of prime importance in biochemistry since a specific tertiary structure is required for an enzyme to be active. The phenomenon of reversible enzyme dissociation provides a powerful tool for studying protein folding if the

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assumption is made that the regain of activity is accompanied by the recovery of the specific native conformation of the enzyme. The effects of metabolites such as NAD^+ (Deal, 1969) and ATP (Constantinides and Deal, 1969) and salts such as KCl and ammonium sulfate (Constantinides and Deal, 1970) on the reversible dissociation process of glyceraldehyde-3-phosphate dehydrogenase *in vitro* strongly suggest that factors other than primary structure significantly affect the subunit folding and association of this enzyme. An analogous process occurring *in vivo* could be a significant factor in the posttranslational regulation of this enzyme.

Factors affecting the renaturation of unfolded yeast glyceraldehyde-3-phosphate dehydrogenase subunits have been studied in some detail (Deal, 1969). It has been shown that NAD^+ in physiological concentrations and high concentrations of salts greatly stimulate the regain of activity for this enzyme. In this report the factors affecting the reversible dissociation of flounder muscle glyceraldehyde-3-phosphate dehydrogenase were studied with the intention of comparing it to the yeast enzyme and further characterizing the effect of metabolites and salts on the reversal process. The multiple form pattern of the reversed enzyme was also compared to that of the native enzyme. This work represents the first attempt at studying the reversible dissociation process of glyceraldehyde-3-phosphate dehydrogenase in the presence of other proteins, a condition more closely resembling that encountered *in vivo*. The results support the concept of metabolites affecting the folding and association of subunit enzymes.

Experimental Section

Reagents. The barium salt of diethyl acetal glyceraldehyde 3-phosphate was obtained from Sigma and prepared in the manner suggested by them. NAD^+, NADH, $NADP^+$, beef heart lactic dehydrogenase, and 2-mercaptoethanol were also obtained from Sigma. Urea (Sigma) was recrystallized prior to use by dissolving 500 g in 1 l. of boiling 95% ethanol, filtering on a heated Buchner funnel, and cooling the filtrate overnight at 4°. The crystals were collected by filtering at 4° and washed with 400 ml of ice-cold absolute ethanol after which they were oven dried. Ammonium sulfate was special enzyme grade purchased from Schwarz/Mann. All other chemicals were reagent grade and used without further modification.

Enzyme Assays. The method used for the assay of flounder muscle glyceraldehyde-3-phosphate dehydrogenase was that described by Constantinides and Deal (1969), for the rabbit muscle enzyme. Each component of the assay was optimized for the flounder enzyme and found to be similar to that of the rabbit muscle enzyme. In place of cysteine, 2-mercaptoethanol was used (15 mM). The increase in absorbancy at 340 mμ was determined during the first 30 sec of the reaction in a Gilford 240 recording spectrophotometer. The final assay volume was 0.35 ml. The lactic dehydrogenase assay employed has been described (Pesce *et al.*, 1964). Enzyme activity is expressed as micromoles of product per minute per milliliter of enzyme solution or as units which are micromoles of product per minute per milligram of protein. Protein concentrations were determined spectrophotometrically after determining that $E^{0.1\%}_{2.80\ m\mu}$ for the 60–90 fraction was 1.

Enzyme Preparation. Freshly caught flounder muscle (75 g) was diced in 250 ml of ice-cold 1 mM EDTA (pH 7.0) and homogenized in a Waring Blendor (two 5-sec bursts). The homogenate was incubated for 20 min in ice and then centrifuged at 15,000*g* for 20 min. The resulting supernatant solution was then centrifuged at 120,000*g* for 75 min and the soluble supernatant fraction collected. The 60–90% ammonium sulfate fraction was prepared by slowly adding 390 g/l. of solid ammonium sulfate to the soluble fraction with continuous stirring. This 60% salt solution was centrifuged at 15,000*g* for 15 min and the pellet which contained no glyceraldehyde-3-phosphate dehydrogenase activity was discarded. The resulting supernatant solution was filtered with Whatman No. 1 filter paper. To the filtrate 227 g of ammonium sulfate/l. was slowly added with stirring. This solution referred to as the 60–90 fraction was used for all the reversible dissociation experiments. The preparation consistently displayed a specific activity of 20–25 units and was stable to storage at 4° for several months.

Sucrose Density Gradient Centrifugation. The sucrose gradients were prepared and run according to the procedure of Martin and Ames (1961). All density gradients ranged from 5 to 20% with both light and dense solutions (2.30 ml of each) containing 1 mM EDTA (pH 7.0). On the top of each gradient 0.1 ml of a solution containing 1 mM EDTA (pH 7.0), a marker concentration of 0.1 mg/ml, and an enzyme fraction concentration of 0.5 mg/ml were applied. The gradients were centrifuged for 16 hr at 40,000 rpm in an SW-50 rotor at 4°. The marker used was lactic dehydrogenase which has an *s* value of 7.4. The molecular weight of the flounder enzyme was calculated as outlined by Martin and Ames (1961).

Isoelectric Focusing. Electrofocusing was carried out as described in the LKB instruction manual. The 110-ml column was routinely run at 4° utilizing 0.25 M NaOH in 60% sucrose as the cathode solution (bottom of column) and 1% sulfuric acid as the anode solution (top of column). Ampholytes of the desired pH range were used in concentrations of 1% with all columns having 0.01% 2-mercaptoethanol to stabilize the enzyme. The sample usually contained 10 mg of the 60–90 fraction (200 units of glyceraldehyde-3-phosphate dehydrogenase activity) and was mixed with the light solution. The density gradient was prepared using the LKB gradient mixer. The column was emptied at a rate of 1 ml/min and fractions of 0.5 ml were collected. The pH and enzyme activity of each fraction were determined.

Dissociation. An aliquot of the 60–90 fraction containing 20 mg of protein was centrifuged at 15,000*g* for 20 min. The supernatant was discarded and the sides of the centrifuge tube were carefully wiped with tissue paper. The pellet was resuspended in 2 ml of the dissociation solution which contained 8 M urea, 1% 2-mercaptoethanol, and 5 mM EDTA (pH 7.0), after which it was incubated at 4° for 1–1.5 hr. The loss of enzyme activity was immediate upon exposure of the enzyme to 8 M urea. Incubation times of up to 3 hr in the dissociation mixture did not affect the per cent of activity recovered.

Reversal of Dissociation. The dissociation mixture (5 μl) was slowly added to 0.50 ml of the ice-cold reversal mixture which contained 0.24 M KCl, 40 mM NAD^+, 15 mM 2-mercaptoethanol, and 0.10 M imidazole (pH 7.2). The solution was gently mixed and incubated in ice for 10 min after which time it was removed and placed in a water bath at 22° for 1 hr. The stock NAD^+ solution (0.2 M) used in preparing the reversal mixture was adjusted to pH 6.0 by the addition of sodium hydroxide and stored at −20°. All recoveries are expressed as per cent of control activity. The controls in each experiment were exposed to the same incubations as the experimental tubes with the only difference being that the urea was omitted from the dissociation mixture and replaced by the appropriate volume of water.

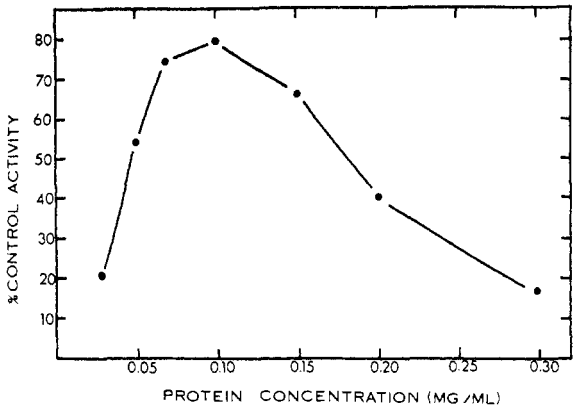

FIGURE 1: Effects of protein concentration on the reversal process. Dissociation mixtures containing 3–30 mg/ml of protein were prepared and reversed by diluting each 100-fold in the reversal mixture. The controls were prepared in an identical manner with the exception that the urea in the dissociation mixture was replaced with water.

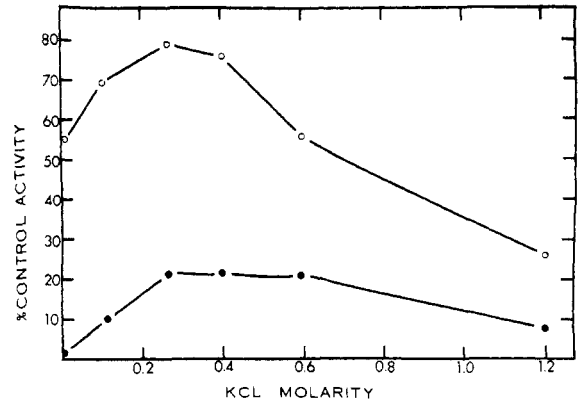

FIGURE 3: Effects of KCl on the reversal process in the presence and absence of NAD^+. Each reversal mixture contained the indicated concentration of KCl in the presence of 40 mM NAD^+ (open circles) and with no NAD^+ (closed circles).

Results

Effects of Temperature on the Reversal Process. A crucial factor in the regain of enzyme activity was that the unfolded urea dissociated subunits be incubated at 0° for several minutes in the reversal mixture before raising the temperature to 22°. If the dissociation mixture was added directly to the reversal mixture at 22° virtually no activity was recovered. Recoveries of >70% were obtained over a temperature range of 16–27°, with an optimum reversal temperature of 22°. A very sharp decline in recovery is observed at 37°. These results are similar to those obtained with the yeast enzyme (Deal, 1969) although the flounder enzyme does not renature as well at 37°.

Protein Concentration in the Reversal Mixture. The effect of protein concentration in the reversal mixture was determined (Figure 1) by incubating several different concentrations of protein in the dissociation mixture and reversing each one of these in the standard manner. The designated protein concentrations represent total protein and not glyceraldehyde-3-phosphate dehydrogenase concentration. Since the specific activity increases five- to sixfold upon isolation of each flounder muscle glyceraldehyde-3-phosphate dehydrogenase form (Marangos and Constantinides, 1973) it can be assumed that glyceraldehyde-3-phosphate dehydrogenase represents about 15% of the total protein in the 60–90 fraction. The results indicate that renaturation is a concentration-dependent process. Recoveries fall off sharply at protein concentrations below 0.07 mg/ml and above 0.10 mg/ml indicating that there is a minimum concentration of subunits necessary for folding and association to occur and that at high concentrations nonspecific aggregation probably occurs. The fact that other proteins are present in the reversal mixture complicates the process since they may be reacting nonspecifically with the glyceraldehyde-3-phoshate dehydrogenase subunits. The fact that 80% of the activity can be recovered in the presence of these other proteins illustrates that the association process is highly specific. The reversal of the yeast enzyme does not display such a strict concentration dependence (Deal, 1969). The kinetics of reversal were also similar to that of the yeast enzyme with a half-time of about 12 min.

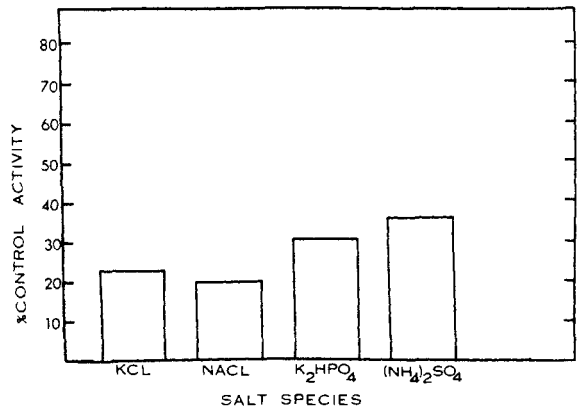

FIGURE 2: Salt requirement in the reversal process. Each reversal mixture contained 0.1 M imidazole buffer (pH 7.2), 0.12 M 2-mercaptoethanol, and 0.3 M of the indicated salt, with no NAD^+ present.

Effect of pH and Salts. Studies with KCl incorporated in the reversal mixture yielded maximal recoveries over a broad pH range of pH 7.0–9.0. The pH chosen for routine use was 7.2 because the renatured enzyme as well as NAD^+ were more stable at this pH. The effect of various salts on the renaturation process in the absence of NAD^+ was tested with the results shown in Figure 2. The divalent salts were in general more effective than the monovalent ions; however, increasing the buffer molarity to 0.4 M had no effect on the recovery observed. KCl was routinely used since it gave the same recoveries as the divalent salts in the complete reversal system. This result suggests that the salt effect was to some degree specific and not just a simple ionic strength effect. The effect of KCl was investigated further in order to determine its efficiency in replacing NAD^+ as a renaturation catalyst. The ability of KCl to catalyze reversal was determined in the presence and absence of NAD^+ with the results shown in Figure 3. It can be seen that the salt in the absence of NAD^+ is able to cause a 22% recovery of enzyme activity in concentrations of 0.24–0.6 M. In the presence of 40 mM NAD^+ the per cent recovery increases from 22 to 80 at 0.24 M KCl. Recoveries of 55% were consistently obtained when KCl was omitted from the reversal mixture. The optimum concentration of KCl for recovery was 0.24 M in contrast to the yeast enzyme which yielded much higher recoveries at higher salt concentrations (Deal, 1969).

Requirement for Sulfhydryl Reducing Agents. The requirement for sulfhydryl reducing agents in the reversal mixture was determined using both 2-mercaptoethanol and glutathione. The results shown in Figure 4 are those obtained in the 2-mercaptoethanol study. Very low recoveries were observed

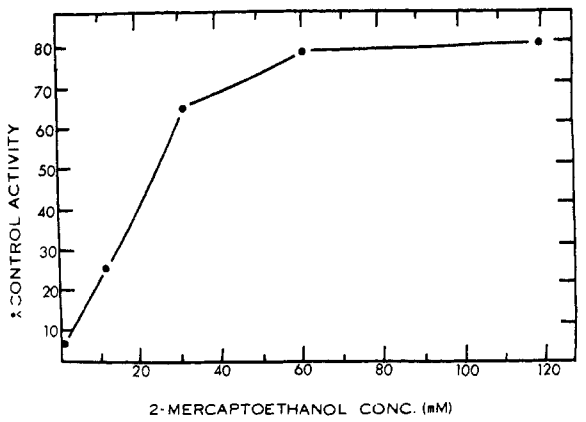

FIGURE 4: Effects of sulfhydryl reducing agents on the reversal process. Each reversal mixture contained the designated concentration of 2-mercaptoethanol with the rest of the components present at optimal levels.

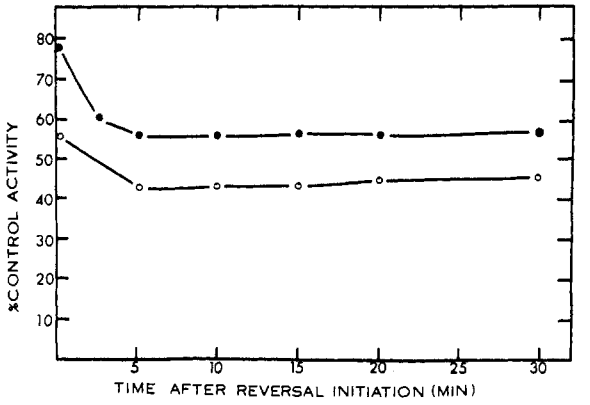

FIGURE 5: Time of 2-mercaptoethanol addition to the reversal mixture. The dissociated enzyme (5 μl) was added to each reversal mixture without mercaptoethanol and incubated at 0° (prereversal) for 10 min. Mercaptoethanol (0.12 M final concentration) was added to each tube at the indicated time intervals after the initiation of the reversal step (22°). The closed circles represent the results obtained when KCl is incorporated in the reversal mixture and the open circles represent the results obtained when KCl is omitted. Each tube was assayed 1 hr after the mercaptoethanol addition.

if the reducing agent was omitted from the reversal mixture, indicating that the sulfhydryl groups on this enzyme had to be in a reduced state in order for the active conformation to exist. The same sulfhydryl dependence is observed in the renaturation of the yeast enzyme (Deal, 1969). Similar results were obtained in the glutathione study.

In order to determine if 2-mercaptoethanol was required from the beginning of the reversal process, experiments were performed in which the sulfhydryl reducing agent was added at various time intervals during the reversal process. The results in Figure 5 illustrate that a decrease of only 10% in recovery is observed if 2-mercaptoethanol was added up to 30 min after the initiation of the reversal process. Similar results were obtained when KCl was incorporated in the reversal mixture with the difference being that all recoveries were higher due to the salt. The ability of the sulfhydryl reducing agent to exert its effect after the initiation of the reversal process suggests that it either exerts its effect after subunit folding and association has occurred or that it is able to correct improper folding caused by disulfide bond formation.

NAD Requirements. Experiments were performed attempting to characterize the effect of the cofactor of glyceraldehyde-3-phosphate dehydrogenase on its renaturation. Addition of the dissociated subunits into reversal mixtures containing different concentrations of NAD^+ produced the results shown in Figure 6. The pronounced effect of NAD^+ on the extent of reversal is evident. Further analysis of the data reveals that NAD^+ seems to be affecting two processes since the curve exhibits a plateau between 4 and 10 mM. The first NAD^+ stimulated process accounts for some 55% of the observed recoverable activity and has a K_{NAD^+} (concentration of NAD^+ at which one-half of maximum recovery is obtained) of 1.5×10^{-4} M, a value in the physiological range. The second apparent process increases the recovery by 25% and has a K_{NAD^+} of 1.5×10^{-2} M.

The nature of the process catalyzed by NAD^+ remains speculative but experiments concerning the time of NAD^+ addition during the reversal process suggest that the cofactor is affecting the folding of the glyceraldehyde-3-phosphate dehydrogenase subunits. When NAD^+ is added at time intervals after the initiation of reversal in the absence of KCl, the recoveries obtained drop off sharply (Figure 7). These data clearly indicate that in the absence of KCl, NAD^+ is required from the initiation of the reversal process in order that appreciable activity be recovered. These experiments were done at two different temperatures, 19 and 24°. A greater loss of recoverable activity at the higher temperature is expected if irreversible nonspecific aggregation is occurring prior to the addition of NAD^+. Figure 7 also indicates that if KCl is incorporated into the reversal mixture a much greater amount of activity is recoverable upon addition of NAD^+ at times of up to 30 min after reversal initiation. The rather abrupt initial decrease of 20% in recoverable activity in the salt curve is difficult to rationalize.

$NADP^+$ also had a marked effect on the catalysis of the renaturation process. In the absence of KCl recoveries of 35–40% were consistently obtained with 40 mM $NADP^+$ as opposed to the 55% recoveries obtained with NAD^+ under these conditions. Other nucleotides such as AMP, ADP, ATP, and GTP had very little effect on the recovery of enzyme activity with inactivation of the controls occurring in many cases.

Characteristics of the Reversed Enzyme. It was of interest to determine if the renatured enzyme was structurally similar to the native enzyme in order to determine if the active conformation or conformations were specific. Sucrose density gradient centrifugation was performed on the native and renatured enzymes. The molecular weights calculated for both the native and renatured enzymes were identical (155,000)

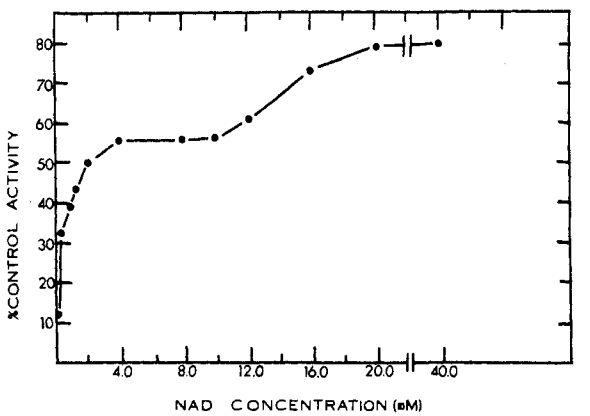

FIGURE 6: The effect of NAD^+ on the reversal process. The dissociation mixture (5 μl) was incubated in the standard reversal mixture with the indicated concentration of NAD^+ present.

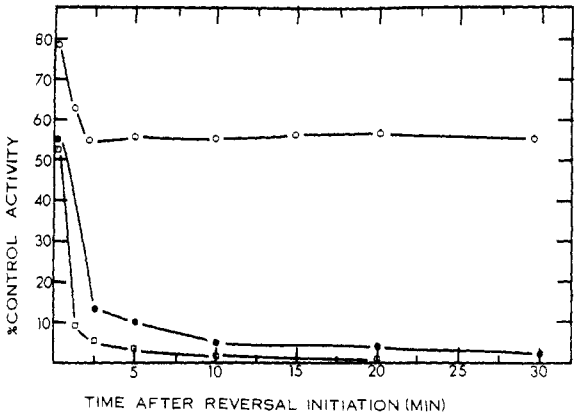

FIGURE 7: Time of NAD^+ addition to the reversal mixture. The dissociation mixture (5 μl) was added to each reversal mixture in the absence of NAD^+ and incubated at 0° for 10 min. NAD^+ was added at the indicated time intervals after the removal of the reversal mixture from ice (initiation of reversal). The final concentration of NAD^+ in each reversal mixture was 40 mM. Each tube was assayed for enzyme activity 1 hr after the NAD^+ addition. The open circles are the results obtained in the presence of KCl. The closed circles and open squares are the results obtained with no KCl in the reversal mixture at 19 and 24°, respectively.

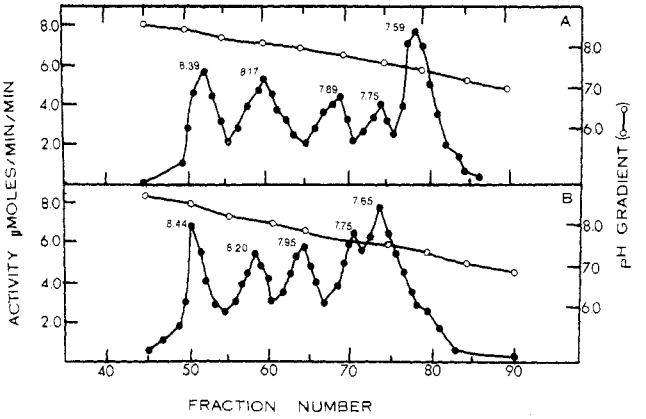

FIGURE 8: Isoelectric focusing of native and renatured glyceraldehyde-3-phosphate dehydrogenase. The dissociation mixture (1 ml) (10 mg of protein) was diluted in 100 ml of the standard reversal mixture and renatured in the usual manner. The solution was concentrated to 10 ml (1 mg/ml of protein) and dialyzed against 7 vol of 1 mM EDTA (pH 7.0) in an Amicon ultrafiltration cell using a PM 10 membrane. This solution was mixed with the light solution of a 110-ml electrofocusing column and electrofocused in the pH 3–10 range for 44 hr with a final voltage of 500 V (A). The control native enzyme fraction was prepared in the same manner with the only difference being the omission of the urea in the dissociation mixture (B). The fraction volume in both cases was 0.5 ml/tube.

indicating that the reversible dissociation process did not create any active enzyme forms of different molecular weight than the native enzyme.

It has been determined that flounder muscle glyceraldehyde-3-phosphate dehydrogenase exists as a mixture of multiple forms separable by isoelectric focusing and polyacrylamide gel electrophoresis (Marangos and Constantinides, 1973). Consequently it became important to compare the multiple form pattern of the reversed enzyme to that of the native enzyme in order to ascertain whether each form was capable of being reversibly dissociated. As a control the native enzyme was incubated in the reversal mixture for 1 hr and dialyzed in an Amicon ultrafiltration cell against 7 vol of 1 mM EDTA (pH 7.0) using a PM 10 membrane. The renatured enzyme was also dialyzed under identical conditions after incubation in the reversal mixture for 1 hr. Each preparation was electrofocused with the results illustrated in Figure 8. Comparison of the two patterns indicates that the same five enzyme forms are present in each case with the pI = 7.6 form being the major one for both the renatured and the native enzyme. Refocusing of each isolated form yields one peak at the expected pH zone indicating that each isolated form is stable. This sensitive means of comparison establishes the fact that the renatured enzyme is structurally similar to or identical with the native enzyme. The fact that the multiple form pattern is regenerated during renaturation indicates that each enzyme form is able to specifically reassociate in the presence of other proteins.

Preliminary experiments have been performed on the reversible dissociation of each isolated flounder muscle glyceraldehyde-3-phosphate dehydrogenase form. The pI = 8.4, 8.2, and 7.9 enzyme forms have all been reversibly dissociated with recoveries ranging from 50 to 60%. Figure 9 illustrates the pattern observed after the renatured 8.4 form is electrofocused. Refocusing of the renatured 8.2 and 7.9 forms produced similar patterns suggesting that for each form the subunit was able to fold in several ways giving rise to tetramers with different conformations. Further reversible dissociation experiments on the isolated forms were not feasible since large enough amounts of each were not readily attainable.

Discussion

In this report the reversible dissociation process of flounder muscle glyceraldehyde-3-phosphate dehydrogenase was studied and characterized. The work represents the first attempt at studying this process under conditions where other proteins are present. The experiments reported were designed to determine the effects of environmental factors on the folding and association of glyceraldehyde-3-phosphate dehydrogenase subunits under these conditions.

The data clearly indicate that several environmental factors have a marked effect on the renaturation process of flounder glyceraldehyde-3-phosphate dehydrogenase. The process is concentration dependent (Figure 1) with optimal recoveries obtained at 0.10 mg/ml of protein. This high degree of concentration dependence is not observed with a pure preparation of this enzyme from yeast and rabbit muscle (Deal, 1969; Teipel and Koshland, 1971). Renaturation is essentially totally dependent upon the oxidation state of the sulfhydryl groups as is seen by the requirement for 2-mercaptoethanol in the reversal mixture (Figure 4). The sulfhydryl reducing agent is not strictly required from the beginning of the reversal process since it can be added up to 30 min after reversal initiation with significant regain of enzyme activity (Figure 5). This indicates that the sulfhydryl groups can be reduced after folding has occurred to produce the active conformation.

Hydrogen ion concentration does not seem to be a crucial factor in renaturation when KCl is present, since a broad pH optimum is observed under these conditions. KCl in the absence of NAD^+ is able to cause a 22% recovery of enzyme activity (Figure 3). Further investigation relative to the salt effect is provided in the NAD^+ time of addition study (Figure 7) where it was found that in the presence of the KCl, NAD^+ could be added as much as 30 min after reversal initiation with greater than 50% recovery of enzyme activity. In the absence of KCl the recovery falls off sharply if NAD^+ is added at times after reversal initiation. These data indicate that KCl may be preserving the subunits in such a state that when NAD^+ is added they are able to assume the specific active conformation. The salt may be stabilizing the subunits and

decreasing the degree of irreversible nonspecific folding that occurs in the absence of NAD^+. The possibility also exists that KCl decreases the extent of nonspecific binding between the glyceraldehyde-3-phosphate dehydrogenase subunits and other proteins in the preparation. Since salts stabilize or neutralize charge it is possible that KCl exerts its effect by stabilizing the charges on the unfolded subunits and in this way decreases the extent of nonspecific aggregation. It has also been demonstrated that KCl can cause temperature-dependent reversible dissociation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Constantinides and Deal, 1970). Collectively these studies illustrate clearly the profound effect exerted by KCl on the structure of glyceraldehyde-3-phosphate dehydrogenase.

The renaturation process is greatly stimulated by NAD^+, the cofactor of the glyceraldehyde-3-phosphate dehydrogenase reaction. In the absence of KCl, NAD^+ is absolutely required in order for renaturation to occur. In the presence of 0.25 M KCl the cofactor increases the per cent recovery fourfold (Figure 3). The NAD^+ effect appears biphasic with the K_{NAD^+} for the first process being in the physiological range of 1.5×10^{-4} M. Increasing the NAD^+ concentration in the reversal mixture above 20 mM produces an additional 20% increase in recovery. The reason for this further increase at high NAD^+ concentrations remains unclear. The cofactor could be affecting any one of several stages in the reversal process which include subunit folding, subunit assembly to form the tetramer, or allosteric modification of the tetramer to the active conformation. The data relating to the time of NAD^+ addition (Figure 7) support the idea that the cofactor is exerting its effect at the folding level, since it is required at the initiation of renaturation. This in no way excludes the possibility that NAD^+ is affecting the later stages of reversal as well. The structurally similar pyridine nucleotide $NADP^+$ was the only one tested other than NAD^+ that had a significant effect on the regain of enzyme activity; this might have been caused by the existence of a phosphatase in the reversal mixture converting the $NADP^+$ to NAD^+, although it is unlikely that the phosphatase would survive the 8 M urea treatment. The reversal process for the yeast enzyme is not affected by $NADP^+$. It seems that the cofactor specificity is not as strict for the flounder enzyme.

The structural characteristics of the renatured enzyme seem identical with that of the native enzyme. The molecular weight of each enzyme is identical. A more sensitive indication of structural similarity between the renatured and native enzymes is a comparison of their respective multiple form patterns. Such a comparison reveals that the patterns are virtually identical (Figure 8). Each enzyme preparation contains five distinct enzyme forms having isoelectric points of 8.4. 8.2, 7.9, 7.7, and 7.6. These results indicate the high degree of specificity and fidelity inherent in the renaturation process since each enzyme form is apparently regenerated in relative amounts similar to that of the native enzyme.

The data presented in this report coupled with the work of others on this enzyme from different sources (Deal and Constantinides, 1967; Deal, 1969; Constantinides and Deal, 1969; Teipel and Koshland, 1971) indicate that the primary structure of glyceraldehyde-3-phosphate dehydrogenase does not contain sufficient information to specify its active conformation. It seems obvious that for this enzyme factors other than primary structure, such as metabolites and salts, play a significant role in its folding. This leads to the possibility that the glyceraldehyde-3-phosphate dehydrogenase subunit may have the capacity to fold in more than one conformation

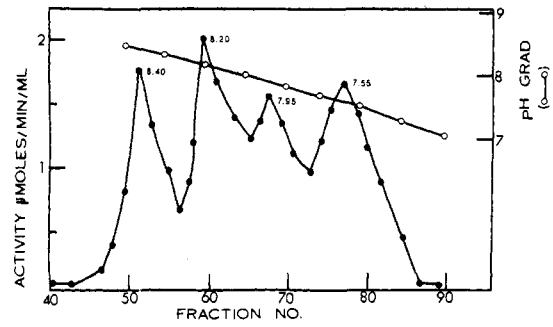

FIGURE 9: Isoelectric focusing of the renatured 8.4 form of glyceraldehyde-3-phosphate dehydrogenase. The 8.4 form (3 mg) was dissociated in 1 ml of the dissociation mixture for 1 hr followed by dilution in 100 ml of the reversal mixture. The sample was prepared for electrofocusing as described in Figure 8A.

(Marangos and Constantinides, 1973). A process such as this would adequately explain the structural basis for the multiple forms of this enzyme from yeast, rabbit muscle, and flounder muscle. The possibility should be considered that fluctuating levels of metabolites in the cell play a role in determining the state of subunit aggregation, thereby regulating the amount of active enzyme present at any one time.

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Inhibition of Acetoacetate Decarboxylase by Ketophosphonates. Structural and Dynamic Probes of the Active Site†

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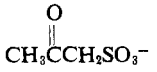
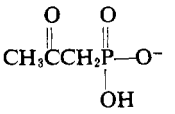
ABSTRACT: Acetonylphosphonate (**2**) (pK = 6.3) is a competitive, rapidly dissociating inhibitor of acetoacetate decarboxylase (K_i = 0.8 mM, pH 5.9). Inhibition decreases sharply with increasing pH due to the dissociation of a proton from the monoacid; compounds without a pK in this region do not show the sharp decrease. Similarly, the monoanion of acetyl phosphate is a much better inhibitor than the dianion. The monomethyl and monoethyl esters of **2** are much poorer inhibitors than the unesterified compound (about 60 times less effective), indicating the presence of steric interactions in the electrophilic region of the active site. Chemical tests for imine formation appear to be highly dependent upon electrostatic effects. Acetoacetate decarboxylase catalyzes the exchange of one of the two protons at the 2 position of the esters of **2** as well as the protons of the 4 position of ethyl acetoacetate but does not catalyze the exchange of the protons of **2**. These results suggest a charge-defined direction of binding of substrate and inhibitors and indicate that **2** binds in a manner unlike that of ketones which are poorer inhibitors. It is suggested that consideration of orbital overlap can account for these observations.

Acetoacetate decarboxylase from *Clostridium acetobutylicum* (Westheimer, 1969) has been the subject of extensive mechanistic studies. These have led to a proposed mechanism (for a discussion, see Fridovich (1972)) which specifies that the enzyme forms a covalent derivative of the substrate, presumably an imine, since complete loss of ^{18}O from that group accompanies decarboxylation (Hamilton and Westheimer, 1959) and sodium borohydride reductively traps a covalent derivative (Fridovich and Westheimer, 1962). The reduced product has been identified as the amine resulting from reduction of an imine derived from acetone and a lysine residue of the enzyme (Warren *et al.*, 1966). Studies of requirements for substrates and inhibitors have appeared and have been reviewed (Fridovich, 1972). The key result of the studies of inhibition which relate to the present work is that acetonylsulfonate (**1**) acts as a competitive



1

inhibitor with respect to substrate and appears to simulate the binding characteristics of the substrate (Fridovich, 1968; Autor and Fridovich, 1970). Although other inhibitors are known, none appear to be accurate mimics of the natural substrate in the manner of acetonylsulfonate since other compounds which are competitive inhibitors by kinetic criteria associate and dissociate with the enzyme very slowly (Tagaki *et al.*, 1968; Autor and Fridovich, 1970). In this work, we have examined the effects of a new substrate analog, sodium acetonylphosphonic acid (**2**) (Kluger and Wasserstein, 1973), and



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derivatives of this and related compounds as a probe of the steric and electronic forces determining the specificity of the enzyme for its substrate. We have also determined the effects of the enzyme upon the dynamic properties of the bound substrate analogs. The phosphonate is particularly useful for these studies since the availability of two anionic groups permits variations of structure and charge not accessible with carboxylate or sulfonate molecules. The carbon–phosphorus bond is not broken in the presence of acetoacetate decarboxylase, enabling us to study steps which are complicated by decarboxylation when acetoacetate is used.

Experimental Section

Materials. Heat activated acetoacetate decarboxylase from *Clostridium acetobutylicum*, prepared by the method described by Westheimer (1969), was obtained in the form of a crystalline suspension from Professor F. H. Westheimer of Harvard University and had been prepared by Mr. Jerome V. Connors. Quantities of enzyme used will be based on units of enzyme activity as defined by Westheimer (1969). Lithium acetoacetate was prepared using a method described by Hall (1963). Lithium acetyl phosphate was prepared by the method of Avison (1955). The sodium salt of acetonylphosphonic acid was syn-

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