Hybridization of Native and Chemically Modified Enzymes. II. Native and Succinylated Glyceraldehyde 3-Phosphate Dehydrogenase*

E. A. Meighen† and H. K. Schachman

ABSTRACT: The succinylation of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase was investigated with the aim of producing a homogeneous derivative having a quaternary structure similar to the native enzyme. Although this proved difficult because of the concomitant dissociation of some of the enzyme molecules, a suitable, inactive, electrophoretic variant was obtained and used for hybridization with the native enzyme. Hybridization was accomplished in both 3 M NaCl and 2 M Tris-chloride solutions; freezing and thawing of the solutions proved unnecessary. Cellulose acetate electrophoresis showed that the hybrid set contained five members, a result consistent with the tetramer structure of the enzyme. Activity measurements indicated that individual native chains functioned enzymically in the hybrid molecules as if each subunit acted independently. High concentrations of electrolyte were required for hybridization. This finding was consistent with sedimentation studies which showed that there was very little dissociation of the enzyme in dilute salt solutions and extensive dissociation into subunits, probably dimers, when solutions of high ionic strength were examined. A combination of the hybridization results and those from sedimentation experiments indicated that the tetramers derived their structure from isologous associations between subunits. Although monomers were not observed in the sedimentation studies their presence in small amounts was inferred from the detection in the electrophoresis patterns of one species composed of one native chain mixed with three succinylated polypeptide chains and another hybrid species containing three native chains and one succinylated chain.

Glyceraldehyde 3-phosphate dehydrogenase (GPDH)[1] from rabbit muscle is a glycolytic enzyme consisting of four polypeptide chains (Perham and Harris, 1963; Harris and Perham, 1965; Harrington and Karr, 1965; Harris and Perham, 1968). Although this enzyme has been studied extensively and a variety of naturally occurring forms (pig, rabbit, lobster, and yeast) are available, detailed reports of successful hybridization experiments have not as yet been presented. The absence of such experiments can be attributed probably to the lack of suitable electrophoretic variants and the difficulty encountered in the reconstitution of the enzyme after disruption of the tertiary and quaternary structures (Chilson *et al.*, 1966). Hence studies were initiated on the chemical modification of GPDH in an effort to produce a homogeneous, electrophoretic derivative suitable for hybridization with the native enzyme. Succinylation of GPDH, though leading to the dissociation and aggregation of some of the protein molecules, did yield some intact, inactive molecules which could be hybridized with the unmodified enzyme molecules. The results of the hybridization experiments are consistent with the behavior expected from the known structure of the enzyme (Harris and Perham, 1968) in that three enzymically active hybrids were found.

The hybridization of most enzymes has been based on the exchange of different subunits by mixing the two forms of a single enzyme under conditions that not only cause the dissociation of the oligomeric protein but also lead to the disruption of the tertiary structure. Initial hybridization experiments with native and succinylated GPDH were attempted in urea solutions under conditions known to dissociate the enzyme (Deal and Holleman, 1964). However, a major part of the protein precipitated upon removal of the denaturant. In contrast, hybridization experiments conducted with the freeze-thaw technique developed for lactate dehydrogenase (Markert, 1963) resulted in a high recovery of the hybrid set. Furthermore, additional experiments showed that GPDH could be hybridized in concentrated NaCl solutions (3 M) without freezing and thawing. Sedimentation velocity and equilibrium experiments confirmed that GPDH dissociates readily at low protein concentrations. Presumably the subunit exchange in the hybridization of the native and succinylated enzyme occurs *via* an association–dissociation equilibrium without the disruption of the tertiary structures of the subunits.

GPDH has been shown to exist in multiple molecular forms in tissue extracts (Lebherz and Rutter, 1967); however, interpretation of *in vivo* experiments is complicated because variants may arise from causes other than mixing of different subunits in oligomeric proteins (Vesell, 1968; Markert, 1968; Epstein and Schecter, 1968; Kaplan, 1968). The results of the present investigations illustrate the value of *in vitro*

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† Submitted in partial fulfillment for the Ph.D. requirements in Biochemistry at the University of California, Berkeley. Present address: The Biological Laboratories, Harvard University, Cambridge, Mass.

[1] Abbreviation used is: GPDH, glyceraldehyde 3-phosphate dehydrogenase.

TABLE I: The Effect of Succinylation on the Sedimentation Behavior of GPDH.[a]

Sample	Moles Added[c]/ Mole of Lysyl Residues	Slow Component		Fast Component	
		$s_{20,w}$	$s^0_{20,w}$[b]	$s_{20,w}$	$s^0_{20,w}$[b]
A	0			6.9	7.4
B	1.0	5.5	5.5	7.1	7.4
C	1.5	4.6	4.7	7.5	7.7
D	2.0	4.5	4.6	7.4	7.6
E	3.0	4.6	4.6	7.3	7.5

[a] Sedimentation velocity experiments were performed on solutions containing 0.5 M NaCl, 0.02 M potassium phosphate, 0.002 M EDTA, and 5×10^{-4} M dithiothreitol, at pH 6.50. Other details are given in the Experimental Section. [b] The sedimentation coefficients of the slow and fast components were assumed to have the same concentration dependence as native GPDH (Jaenicke *et al.*, 1968). Corrections were made according to the method described by Schachman (1959). [c] Of succinic anhydride.

hybridization experiments in which the production of various molecular forms can be related directly to the exchange of different subunits in oligomeric proteins. Furthermore, such hybridization experiments serve as a sensitive and convenient probe for the analysis of association–dissociation equilibria for an oligomeric protein under different conditions. These results support the suggestion that chemical modification of proteins with succinic anhydride should prove a generally applicable technique for the production of electrophoretic variants suitable for hybridization experiments (Meighen and Schachman, 1970; Meighen *et al.*, 1970).

Experimental Section

Preparation of GPDH Solutions. Rabbit muscle GPDH was a commercial preparation obtained from C. F. Boehringer and Soehne. Aliquots of the ammonium sulfate suspension were spun down in the clinical centrifuge and the precipitate dissolved by dialysis against the desired buffer. The protein concentration (molar) was determined spectrophotometrically on the basis of a specific absorbance coefficient of 1.00 (0.1%, 1 cm) at 280 mμ (Velick *et al.*, 1953; Dandliker and Fox, 1955) and a molecular weight of 1.44×10^5 (Harrington and Karr, 1965; Harris and Perham, 1968).

Succinylation of GPDH. Samples of GPDH were reacted with succinic anhydride (Eastman Organic Chemicals) according to the following procedure. An aliquot of solid anhydride was added to solutions (1.0–1.3%) of GPDH in 0.05 M Tris-chloride at room temperature; the pH was maintained at 8.0 by the automatic addition of 1.0 M NaOH with a Radiometer titrator. A sample of succinylated GPDH was removed after each aliquot of succinic anhydride had reacted as indicated by the termination of the requirement for NaOH. Different extents of succinylation were obtained by the addition of another aliquot of succinic anhydride to the reaction mixture and repeating the procedure. The resulting samples were then dialyzed at 4° against the desired buffer. The percentage of free amino groups in the succinylated protein was estimated by ninhydrin analysis according to the method of Moore and Stein (1948) as modified by Fraenkel-Conrat (1957).

Sedimentation Studies. Sedimentation velocity and equilibrium experiments were performed in a Spinco Model E ultracentrifuge equipped with a cylinder lens schlieren optical system, a rotable light source for Rayleigh optics, and an automatic split-beam photoelectric scanning optical system (Schachman and Edelstein, 1966). Double-sector cells were used routinely with aluminum-filled, epoxy centerpieces and either sapphire windows for interference optics or quartz windows for absorption optics. The temperature was measured and controlled by the RTIC unit supplied by the manufacturer.

Zone Electrophoresis. Zone electrophoresis was conducted routinely on 14.6-cm cellulose polyacetate strips (Gelman Sepraphore III) in a Microzone electrophoresis cell, Model R-101 (Beckman Spinco), as described in the preceding paper (Meighen and Schachman, 1970).

In some experiments, the membranes were stained for enzyme activity according to the method given by Lebherz and Rutter (1967). A 0.5% Noble agar solution in 0.01 M sodium arsenate–0.05 M Tris-chloride (pH 8.3), containing 0.01% crystalline rabbit muscle aldolase (Boehringer), 0.01 M fructose diphosphate (CalBiochem), 0.001 M NAD (Sigma Chemical Co.), 0.025 mg/ml of phenazine methosulfate (Aldrich Chemical Co.), and 0.4 mg/ml of nitroblue tetrazolium chloride (Aldrich Chemical Co.), was poured into shallow Petri dishes (4–10 ml each) at 42° and allowed to solidify at 4°. After electrophoresis, the cellulose acetate strips were placed on this freshly prepared agar and incubated at 37° for 5–30 min to allow color development.

Results

Reaction of Succinic Anhydride with GPDH. Since experiments with aldolase (Meighen and Schachman, 1970) have shown that moderate succinylation leads to a relatively homogeneous derivative with a quaternary structure similar to the native enzyme, similar studies were initiated on GPDH with different amounts of succinic anhydride. Table I summarizes the sedimentation behavior of a variety of succinyl-GPDH preparations. As the extent of succinylation was increased, a slow-sedimenting component (4.6 S) was formed at the expense of the fast-sedimenting component (7.5 S). In addition, aggregates could be observed in the succinylated samples whereas none were present in unmodified GPDH. This latter result made it difficult to estimate the proportion of slow (4.6 S) and fast (7.5 S) components in the different preparations especially since the amount of aggregate changed slowly with time.

Five additional samples of succinyl-GPDH were prepared by reaction of 4.4, 6.0, 8.5, 12.2, and 20.0 moles of succinic anhydride per lysyl residue of native GPDH. The sedimentation velocity patterns were similar to one another and reflected the presence of a large variety of aggregates with the sedimentation coefficient of the slowest species being approximately 8 to 10 S. These results indicated that at higher extents of succinylation the slow component was completely aggregated.

In contrast, with the results on the succinylation of aldolase

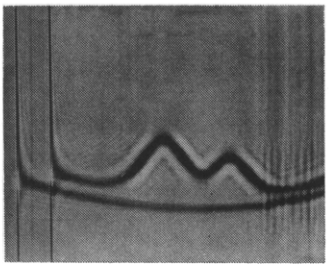


FIGURE 1: Sedimentation velocity pattern of succinyl-GPDH (sample C, Table I) after 51 min at a rotor speed of 60,000 rpm. The photograph was taken with a phase-plate angle of 60°. Movement is from left to right. The protein concentration was 8 mg/ml; additional experimental details are given in Table I.

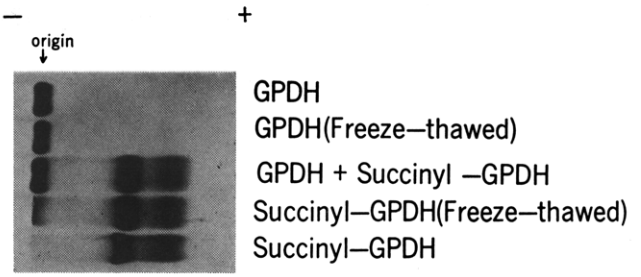


FIGURE 2: Cellulose acetate electrophoresis of the hybridization controls. Electrophoresis was performed as given in the Experimental Section and the membrane stained for protein. In the mixture of native and succinylated GPDH, the components had been frozen and thawed separately.

(Meighen and Schachman, 1970), none of the succinylated-GPDH preparations showed a single sharp boundary in the ultracentrifuge. Accordingly electrophoresis experiments were performed on some of the samples. When sample C (1.5 moles of succinic anhydride/mole of lysyl residues) was examined by electrophoresis, it was found to contain two components. The more rapidly migrating component had a mobility of -1.5×10^{-4} cm²/V sec and the other component migrated at a rate (-1.0×10^{-4} cm²/V sec) intermediate between the fast component of succinyl-GPDH and the native enzyme which had a mobility of -0.05×10^{-4} cm²/V sec. Both components in the succinylated enzyme exhibited sharp bands in the electropherograms. In contrast a preparation of GPDH succinylated to a lesser extent (sample B) exhibited a relatively wide band on electrophoretic analysis with a mobility (-1.0×10^{-4} to -1.3×10^{-4} cm² per V sec) significantly less than the most anodic band in sample C. Since sample B contained a heterogeneous population of succinylated molecules of differing electrophoretic mobilities, it was obviously unsuitable for hybridization experiments. Preparations of GPDH that had been succinylated to a greater extent than sample C had significantly lower amounts of the 7.5S component. Consequently these preparations were not considered suitable for hybridization experiments.

Of all the succinylated preparations of GPDH examined by sedimentation velocity analyses and electrophoresis, sample C appeared most suitable for subsequent hybridization experiments. Figure 1 shows a sedimentation velocity pattern for this preparation with the fast component (7.5 S) corresponding to intact modified enzyme molecules having a mobility of -1.5×10^{-4} cm²/V sec. Ninhydrin analysis on this preparation showed that approximately 27% of the lysyl residues were succinylated.

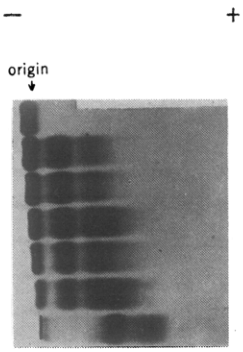
Conditions for Hybridization of Native and Succinylated GPDH. Initial hybridization experiments revealed that species with intermediate electrophoretic mobility were produced after dissociation and reconstitution of mixtures of native GPDH and succinyl-GPDH exposed to 4 M urea solutions. However, in these experiments a large fraction of the protein precipitated after removal of the urea. Although the details of the dissociation and reconstitution of GPDH were not investigated, the experiments indicated that exposure of GPDH to the denaturing activity of 4 M urea solutions for a short period of time led to a product from which it was extremely difficult to recover even a small amount of soluble protein upon removal of the denaturant.

In view of the inadequacy of the technique described above, hybridization of the native and succinyl-GPDH was attempted by the freeze-thaw method first introduced by Markert (1963) in studies on lactic dehydrogenase. These experiments led to the formation of species with electrophoretic mobilities intermediate between native GPDH and succinyl-GPDH. Moreover, very little protein was lost by precipitation during the process of freezing and thawing the solution. Accordingly the following procedure was adopted for the hybridization studies.

GPDH and succinyl-GPDH, at a total protein concentration of about 0.5%, were mixed at varying molar ratios in 3 M NaCl, 0.02 M potassium phosphate, 0.002 M EDTA, and 0.1 M dithiothreitol, pH 6.5 at 4°. The samples were maintained in a frozen state at −10° for 6 hr and then thawed at room temperature. The resulting solutions were dialyzed against 0.02 M potassium phosphate, 0.002 M EDTA, and 5×10^{-4} M dithiothreitol, pH 6.5 at 4°. Small amounts of precipitate were removed in a clinical centrifuge.

Hybridization Controls. The control experiments for the hybridization of succinyl-GPDH and GPDH are illustrated by the electrophoretic patterns shown in Figure 2. As described above, succinylated GPDH contains two components, with the most anodic component corresponding to the 7.5S component and the other component corresponding to the 4.6S species. The freezing and thawing of succinyl-GPDH or native GPDH produced no additional bands. Moreover, no additional bands were detected for GPDH and succinyl-GPDH mixed in the electrophoresis buffer if either the individual components had been frozen and thawed separately in 3 M NaCl or the mixture itself was frozen and thawed in the low ionic strength buffer used for the electrophoresis experiments.

Hybridization of Native and Succinylated GPDH. Figure 3 shows electrophoresis patterns for different hybrid sets of GPDH and succinyl-GPDH. A comparison of the patterns of II to VI with those for GPDH (I) or succinyl-GPDH (VII) shows that intermediate electrophoretic bands were produced upon freezing and thawing mixtures of native GPDH and succinyl-GPDH in 3 M NaCl. It is evident that at least two species were produced with electrophoretic mobilities intermediate between native GPDH and the slow component of succinyl-GPDH. In addition, a third hybrid species with electrophoretic mobility between the slow and fast components of succinyl-GPDH appears to be present in samples IV,



Sample	Composition GPDH	Succ–GPDH
I	100	0
II	59	41
III	50	50
IV	38	62
V	26	74
VI	17	83
VII	0	100

FIGURE 3: Cellulose acetate electrophoresis of hybrid sets of succinyl-GPDH and GPDH. Electrophoresis was conducted as given in the Experimental Section and the membrane was stained for protein. All samples containing the indicated percentages of GPDH and succinyl-GPDH had been frozen and thawed as described previously.

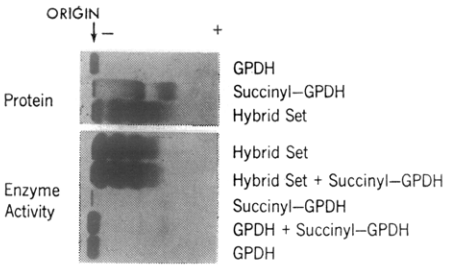


FIGURE 4: Comparison of the distribution of protein and enzyme activity after cellulose acetate electrophoresis of the hybrid sets of GPDH-succinylated GPDH. Electrophoresis was performed as described in the Experimental Section and the membrane was stained for protein or GPDH activity. GPDH, the hybrid set, and succinyl-GPDH, are samples I, VI, and VII, respectively, from Figure 3.

V, and VI. The number and relative positions of the three additional intermediate bands produced upon hybridization of native and succinylated GPDH are in accord with the tetrameric structure of this enzyme (Harris and Perham, 1968).

The variation in intensity of the bands in the hybrid set is only slightly dependent on the relative proportion of GPDH and succinyl-GPDH in the mixture before freezing and thawing. The low amounts of the most anodic bands in the hybrid sets, even at high ratios of succinyl-GPDH to GPDH can be traced to two possible sources; (1) the percentage of each component given in Figure 3 represents the total amount of succinyl-GPDH, whereas the fraction of succinyl-GPDH capable of hybridizing with GPDH is much less. The relative intensities of the slow and fast component in VII illustrates this point since it is likely that only the fast component of succinyl-GPDH is capable of hybridizing with GPDH. (2) The protein which precipitates under the hybridization conditions may be the most anodic members of the hybrid set. Evidence for this hypothesis is seen in the electropherograms of II, III, and IV; the slow component of succinyl-GPDH is missing in these samples presumably as a result of complete precipitation. In addition to these complications there is also the possibility that some of the molecules represented by the slow component of succinyl-GPDH hybridized with native GPDH (especially at low ratios of succinyl-GPDH to GPDH).

Determination of Enzyme Activity of Hybrids Resolved by Electrophoresis. The presence of the slow component of succinyl-GPDH and the small amounts of the anodic members of the hybrid set complicate the interpretation of the results presented in Figure 3. Accordingly, further experiments were conducted in order to demonstrate that the various hybrids detected by electrophoresis were enzymically active. Figure 4 shows the relation between the positions of the bands revealed by enzyme assay and the position of the bands stained for protein content. It is clear that four different active bands are present in the hybrid set of GPDH-succinylated GPDH. In addition, the results show that both the slow and fast component of succinyl-GPDH are inactive. (A very small amount of activity can be detected at the position of these species upon electrophoresis if the membrane is stained for activity for extremely long times). These experiments demonstrate that the hybrid set contains five members: four active species and the one inactive component corresponding to the fast component of succinyl-GPDH (S_4). The most cathodic member of the hybrid set corresponds to native GPDH (G_4), and the intermediate active bands represent the three hybrids of GPDH and succinyl-GPDH designated by G_3S, G_2S_2, and GS_3.

Dissociation of GPDH in 3 M NaCl Solutions. The various hybridization studies on mixtures of GPDH and succinyl-GPDH showed that freezing and thawing of the solutions was not necessary for the formation of hybrids. However, it was essential that the solutions contain a neutral electrolyte like NaCl at high concentration (3 M). No hybrids were detected with analogous mixtures of the two forms of the enzyme in a dilute buffer (0.02 M phosphate). This striking rapidity and efficiency in the subunit exchange between the two oligomers in the 3 M NaCl solutions as contrasted to the absence of hybridization in the solutions of low ionic strength indicated that the dissociation of GPDH into subunits depended on ionic strength. Accordingly sedimentation velocity experiments were conducted on GPDH in the absence and presence of 3 M NaCl in order to obtain direct evidence for the dissociation of the enzyme which would be relevant to the results of the hybridization experiments.

Figure 5 summarizes the sedimentation velocity studies on the native enzyme in the two solvent systems. As seen there, the sedimentation coefficient, $s_{20,w}$, is independent of the ionic strength for relatively concentrated protein solutions (above 2 mg/ml). Indeed, extrapolation of the linear sections of the plots of $s_{20,w}$ *vs.* concentration yielded a value of 7.54 S at infinite dilution. This value corresponds to the sedimentation coefficient of the tetrameric enzyme (Fox and Dandliker, 1956; Taylor *et al.*, 1956). For the solutions of GPDH in the dilute buffer the sedimentation coefficient varied linearly with concentration (there is perhaps a very slight departure from linearity at low protein concentrations). This essentially linear dependence of $s_{20,w}$ on concentration is characteristic of macromolecules which do not exhibit association–dissociation behavior (Schachman, 1959). The enzyme in concentrated NaCl solutions, however, showed markedly different sedimentation behavior in dilute solutions as seen by the substantial decrease in the sedimentation coefficient at protein concentrations below 2 mg/ml. These

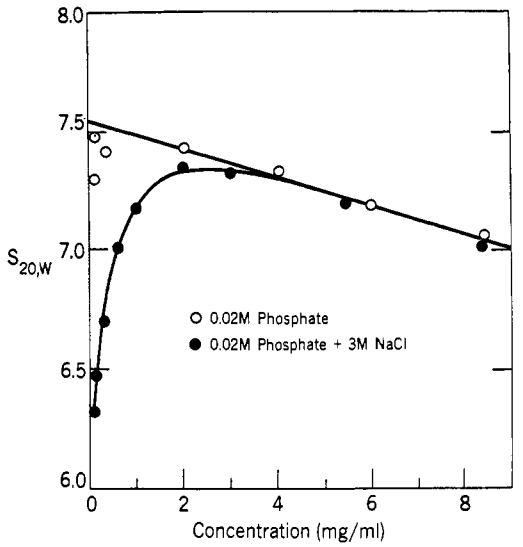


FIGURE 5: The dependence of the sedimentation coefficient of GPDH upon protein concentration in 0.02 M potassium phosphate, 0.002 M EDTA, and 0.001 M dithiothreitol in the presence (●) and absence (○) of 3 M NaCl. The final pH of the solution was 6.50. The protein concentration corresponds to the initial values before sedimentation began. A partial specific volume of 0.791 ml/g for GPDH in 3 M NaCl was used in the calculations to account for preferential interactions in the multicomponent system. Additional experimental details are given in the Experimental section.

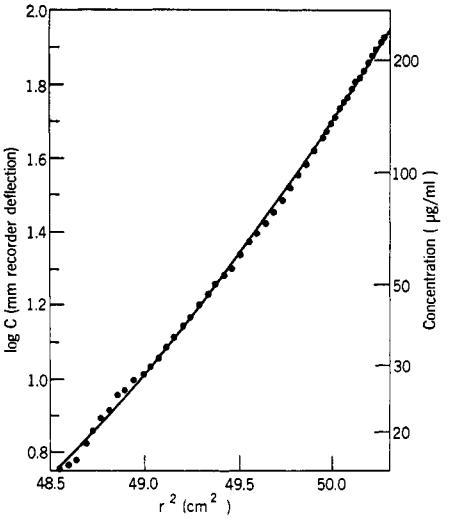


FIGURE 6: High-speed sedimentation equilibrium of GPDH in 3 M NaCl, 0.02 M potassium phosphate, 0.002 M EDTA, and 0.001 M dithiothreitol (pH 6.50) at 23°. The initial protein concentration was 0.5 mg/ml and the speed was 22,000 rpm. A 30-mm cell was used and the concentration distribution was determined with the photoelectric scanning absorption optical system. The left ordinate represents the log of the protein concentration expressed as millimeters of recorder deflection, the right ordinate gives the protein concentration in μg/ml, and the abscissa, r^2, represents the square of the distance (in cm^2) from the axis of rotation.

results indicate clearly that the GPDH molecules in 3 M NaCl are in a rapid dynamic equilibrium between tetramers and subunits. In all experiments a single, symmetrical boundary was observed indicating that the equilibrium probably involved tetramers and dimers (Gilbert, 1959). This conclusion must be viewed with caution since a reliable value of $s_{20,w}$ at infinite dilution was not obtained. If, in fact, subsequent experiments reveal that the sedimentation coefficient approaches a value corresponding to monomers, the observation of a single boundary would indicate that tetramers, dimers, and monomers are implicated in the association–dissociation equilibria.

It should be noted that the calculations of $s_{20,w}$ for the 3 M NaCl solutions of GPDH involved the use of multicomponent theory and corrections for preferential water binding (Williams *et al.*, 1958; Schachman, 1959). The amount of preferentially bound water was evaluated from a series of sedimentation velocity experiments of the enzyme (at a fixed concentration) in solutions of varying NaCl concentration. The sedimentation coefficient, corrected for the viscosity of the solution, was plotted as a function of the density of the solution (Katz and Schachman, 1955; Cox and Schumaker, 1961). Extrapolation of the data to a value of the density corresponding to zero sedimentation rate yielded a value of 0.791 ml/g for the partial specific volume of the sedimenting unit (protein and preferentially bound water). With this value and the partial specific volume of GPDH in dilute salt solutions, 0.737 ml/g, the preferential hydration was estimated to be 0.26 g of H_2O/g of protein. This value of the preferential interaction and the appropriate multicomponent theory were used for the calculation of the sedimentation coefficients plotted in Figure 5. It is of interest that the preferential interaction estimated in this way is in good agreement with the results for other proteins (Cox and Schumaker, 1961).[2]

Sedimentation equilibrium studies also were performed on GPDH so as to obtain further evidence regarding the state of the enzyme in both the dilute buffer and the high ionic strength solutions. For enzyme in the low ionic strength buffer the sedimentation equilibrium plots of log c *vs.* r^2, where c is the protein concentration and r is the distance from the axis of rotation, were linear as expected for a homogeneous substance. The molecular weight calculated from these plots was 1.45×10^5, in excellent agreement with the earlier result of Harrington and Karr (1965). In contrast the equilibrium plots for GPDH in 3 M NaCl solution were invariably nonlinear as shown in Figure 6. The upward curvature in these plots shows clearly that the molecular weight varied throughout the cell with higher values near the bottom of the solution column where the concentration is relatively high. Such curvature would arise from either heterogeneity in the population of molecules or the existence of an association–dissociation equilibrium. Although detailed investigations at differen-

[2] Sedimentation equilibrium data would have been less ambiguous than the sedimentation velocity data for the evaluation of the preferential interaction since variations in the shape or volume of the sedimenting units as a function of the concentration of NaCl would not affect the equilibrium results as they would the sedimentation velocity data (Schachman and Edelstein, 1966). Thus, it should be recognized that the apparent linearity of the plot of the sedimentation velocity data may be the result of a fortuitous cancellation of different effects (such as the change in shape and preferential interaction as a function of salt concentration) each of which alone would introduce curvature. Variation in the molecular weight of the protein with salt concentration would also introduce curvature in the plot of η_s *vs.* ρ. However, the protein concentration for the sedimentation velocity studies was sufficiently high (8.4 mg/ml) that the bulk of the molecules in all the NaCl solutions must have been in the tetrameric state.

speeds and at various initial concentrations were not conducted, the evidence from the limited studies indicates that the curvature in the plots stems from a concentration-dependent, chemical equilibrium.

The limiting slope at high concentrations (0.5–2.3 mg/ml) corresponded to a molecular weight of 1.42×10^5, in excellent agreement with the value obtained in the dilute buffer. It should be noted that the determination of the molecular weight for GPDH in 3 M NaCl solutions requires correction for the preferentially bound water as described above. Thus the agreement for the two solutions (3 M NaCl and dilute buffer) constitutes evidence for the validity of the correction and the value of the preferential hydration evaluated from the sedimentation velocity experiments in solvents of varying densities.

As seen in Figure 6 the slope of the plot of log c *vs.* r^2 is substantially less at low concentrations (20–30 μg/ml) than at the higher concentrations (200 μg/ml) with the molecular weight varying from 0.95×10^5 to 1.23×10^5 over that concentration range. These results provide strong support for the view that the GPDH molecules dissociate in a reversible manner in 3 M NaCl. Additional studies are clearly needed, especially at lower concentrations, before the sedimentation data can be analyzed in terms of equilibrium constants characterizing the association–dissociation behavior of GPDH in solutions of high ionic strength.

Discussion

Unlike the studies on the mechanism of dissociation of aldolase upon succinylation, the analogous experiments on GPDH are not nearly as extensive; nor are they as clear. Moderate succinylation of GPDH (27% of the lysyl residues were modified in sample C) led to a preparation containing species with sedimentation coefficients of 4.6 and 7.5 S in addition to polydisperse aggregated material. Decreasing the extent of succinylation, as in sample B, did not yield a single molecular species as was observed with aldolase. Similarly, increasing the amount of succinic anhydride relative to GPDH also did not lead to a single component. Perhaps these observations can be attributed, on the one hand, to too much succinylation (even in B, the least modified sample) and, on the other hand, to insufficient succinic anhydride (in E) to stabilize by electrostatic repulsion the subunits produced by dissociation of GPDH. Clearly additional experiments are needed to determine whether intact, succinylated GPDH molecules can be produced without the concomitant formation of smaller components and aggregated material. Also there is a need for data at very high ratios ($\geq$100) of succinic anhydride to lysyl residues to determine whether stable subunits can be isolated and characterized.

Although it is evident that increasing the extent of succinylation led to a progressive decrease in the amount of protein with the sedimentation coefficient of the native enzyme (7.5 S), a quantitative description of the relationship has not been established. This could not be achieved because of difficulties in analyzing the ultracentrifuge patterns due to the aggregation which increased at the higher extents of succinylation and as a function of time. The amount of material represented by the sedimentation velocity patterns (*e.g.*, Figure 1) decreased from 100% for sample A to 70% for B, 50% for C, and 36% for D. Also the distribution of components in each succinylated sample varied with time. Whereas the ratio of the 4.6S component to the 7.5S component was 1.6 for sample C shortly after its preparation, after 24 hr the ratio had decreased to 1.0. Despite this change in the relative amounts of the two components, the amount of the 7.5S component appeared constant. We can conclude, therefore, that the aggregates (turbidity was observed visually in many experiments) arose from the 4.6S component and not from the 7.5S component.

The "fast" component (7.5 S) of succinyl-GPDH was not only stable with respect to time but its sedimentation coefficient did not vary with the extent of succinylation (Table I). This value of the sedimentation coefficient corresponds to the tetrameric form of the molecule, and the constant value indicates that the quaternary structure of the protein was not altered substantially despite the large increase in the net charge. It should be noted that the electrophoretic mobility increased progressively from sample A to D and then remained relatively constant as if all of the reactive lysyl residues on the intact molecules had been converted into succinyl-lysyl groups.

GPDH apparently differs substantially from aldolase in the content of discrete classes of lysyl residues which vary in their reactivity toward succinic anhydride. Both enzymes contain very reactive residues which probably are located on the surfaces of the oligomeric structures. Modification of those residues apparently does not destabilize the enzymes. In GPDH, however, there may be an additional group of residues which are only slightly less reactive. When these are converted into the succinylated form, the intersubunit interactions seem to be affected and dissociation (followed by aggregation) occurs. Some of the residues in the less reactive class apparently are modified before all of the residues in the more reactive class become succinylated. As a consequence, with GPDH in contrast to aldolase, it has not as yet been possible to produce moderately succinylated, intact molecules without the concomitant formation of succinylated subunits.

Although the 4.6S component was not purified and characterized by sedimentation equilibrium experiments, it seems likely, from the value of the sedimentation coefficient, that this component corresponds to dimers and not monomers. No evidence was obtained for smaller subunits in any of the samples. This behavior of GPDH is in marked contrast with that of hemerythrin (Klotz and Keresztes-Nagy, 1962) and aldolase (Hass, 1964; Meighen and Schachman, 1970) where succinylation led to stable monomers. Monomers of succinylated GPDH may have been produced but not observed as such because they may have aggregated; alternatively there may have been very little dissociation of the dimers despite the succinylation. Despite our inability to detect the monomers in the sedimentation patterns, we can infer that they were present in the solution from an analysis of the hybridization experiments; otherwise it would be difficult to account for the presence of the hybrids, G_3S and GS_3, where G represents an unmodified GPDH subunit and S corresponds to the succinylated form of the subunit.[3]

[3] It is assumed that the hybrids form by aggregation of independent subunits rather than by a process of subunit exchange at the level of oligomers. Hence hybridization is construed as providing an indication of the dissociation of the oligomers into subunits which can then reassociate at random.

The lack of evidence indicating the presence of monomers of GPDH is particularly interesting in view of the chemical studies (Harris and Perham, 1968) which showed that the enzyme was composed of four identical polypeptide chains. In the dissociation of such a tetramer it would be expected that the various association–dissociation equilibria and the relative amounts of the different species would depend on the types of intersubunit bonding domains (Monod *et al.*, 1965). A closed tetramer involving heterologous associations probably would not exhibit appreciable amounts of intermediate forms in the process of dissociation to form monomers. Only the tetramers and monomers would be observed. In contrast, the dissociation of a tetramer which is stabilized by isologous associations is likely to be accompanied by the formation of appreciable amounts of dimers. The relative amounts of dimers and monomers appearing upon the dissociation of tetramers built through pairs of isologous associations depend upon the tetramer–dimer and dimer–monomer dissociation constants. If the latter were much greater than the former, dimers would be present in only very small amounts. For this special case tetramers constructed through isologous associations would exhibit the same distribution of subunits upon disruption of the quaternary structure as would be found for the tetramers involving heterologous associations. In the light of these considerations the presence of the 4.6S component, which corresponds apparently to dimers, lends support to the view that the structure of GPDH is maintained through isologous associations (Monod *et al.*, 1965). This type of model was proposed earlier by Watson and Banaszak (1964) on the basis of their X-ray diffraction studies of the lobster GPDH–NAD complex. Such a structure is consistent also with the studies of the binding of NAD to GPDH (Chance and Park, 1967; Conway and Koshland, 1968).

Although the studies on the dissociation of GPDH lend support to a model implicating isologous association between subunits it should be recognized that the interpretation must be considered as tentative. Not only must the 4.6S component be characterized but also allowances must be made for the possibility that monomers and even trimers were produced but were not detected as a result of their preferential aggregation. The hybridization experiments provide some evidence in this regard since the amounts of the different members of the hybrid set were not in accord with the binomial distribution. In some experiments the results showed relatively large amounts of G_4, G_2S_2, and S_4 and correspondingly small amounts of G_3S and GS_3. This observation indicates that the tetramers undergo dissociation to form dimers which in turn dissociate to monomers with significantly larger interaction energies for the monomer–dimer equilibrium than for the dimer–tetramer equilibrium.

As shown in Figures 3 and 4 the hybridization of native and succinylated GPDH was accomplished by the technique developed for lactic dehydrogenase (Markert, 1963). This procedure requires the freezing and thawing of solutions of the two forms of the protein in the presence of promoter ions (Markert, 1963; Chilson *et al.*, 1965; Markert and Massaro, 1966). Although lactic dehydrogenase can be hybridized in saturated NaCl solutions (about 6 M) without freezing and thawing the solutions, at lower salt concentrations (*e.g.*, 4 M NaCl) freezing and thawing of the solutions is necessary (Chilson *et al.*, 1964). With GPDH, however, hybridization occurred in either 3 M NaCl or 2 M Tris-chloride solutions without freezing and thawing.[4] The latter solvent seems to be very effective in promoting subunit exchange, presumably by favoring the dissociation of the oligomeric proteins into subunits. As yet no detailed investigations have been completed of the relative rates of subunit exchange under different conditions of pH, ionic strength, temperature, and specific ions.

The lack of substantial hybridization in solutions of low ionic strength (0.02 M phosphate) is of considerable interest in view of the recent demonstration by Hoagland and Teller (1969) that GPDH dissociates reversibly into subunits in 0.1 M buffers. These findings should not be considered as conflicting since the sources of the GPDH were different and the buffers themselves were not identical. On the contrary, it should be noted (Figure 5) that the sedimentation coefficient of the protein at very low concentrations (in 0.02 M phosphate) did depart from the linear dependence on concentration observed at the higher concentrations. Further studies of the association–dissociation behavior of GPDH under different conditions would be of considerable value not only in clarifying the nature of the interactions between subunits but also in providing a basis for speculations regarding the mechanism of the hybridization process. In the absence of such sedimentation data, the hybridization experiments provide valuable information regarding the subunit structure and interactions in GPDH and can be used to distinguish between alternative pathways in the dissociation process.

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[4] Recent experiments showed that 2 M Tris-chloride was very effective in promoting efficient and rapid hybridization.

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Studies on the Bioluminescence of *Renilla reniformis*. VII. Conversion of Luciferin into Luciferyl Sulfate by Luciferin Sulfokinase*

Milton J. Cormier, Kazuo Hori, and Yashwant D. Karkhanis

ABSTRACT: An enzyme preparation has been obtained from *Renilla reniformis*, essentially free of luciferase, which catalyzes the 3′,5′-diphosphoadenosine-linked conversion of luciferyl sulfate into luciferin. In the presence of luciferin and [^{35}S]-adenylyl sulfate 3′-phosphate this enzyme catalyzes the formation of [^{35}S]luciferyl sulfate. The enzymatically produced [^{35}S]luciferyl sulfate has the same chromatographic and chemical properties as does the naturally isolated luciferyl sulfate. We propose the name luciferin sulfokinase (3′-phosphoadenylyl sulfate:luciferin sulfotransferase) for this enzyme.

The enzyme is apparently specific for luciferin since it does not exhibit any phenol sulfokinase activity. Luciferin is stored in *Renilla* as luciferyl sulfate. Thus luciferin sulfokinase may play an important role in regulating the levels of luciferin available for bioluminescence in this animal.

It was previously reported (Hori and Cormier, 1965; Cormier, 1962; Hori and Cormier, 1966) that the overall reaction leading to light emission in the sea pansy, *Renilla reniformis*, proceeds as follows:

$$\text{luciferyl-X} \xrightarrow[\text{3′5′-diphosphoadenosine}]{\text{enzyme; } Ca^{2+}} \text{luciferin} + \text{X} \qquad (1)$$

$$\text{luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{light} + \text{oxidized products} \qquad (2)$$

In reference to eq 1 it was not known whether X was transferred to 3′,5′-diphosphoadenosine (DPA).[1] It is known that X is an acid-labile component (Cormier and Hori, 1964) and previous data has shown that X is sulfate. This was based on the fact that acid treatment of luciferyl-X liberates a compound that reacts as and cochromatographs with inorganic sulfate (Cormier *et al.*, 1966).

In the past it was impossible to study the mechanism of reaction 1 owing to the facts that the enzymes catalyzing reactions 1 and 2 were not separated from one another and that sufficient amounts of pure luciferyl-X and luciferin were unavailable. These obstacles have now been overcome and it is the purpose of this report to present data on the mecha-

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[1] The following abbreviations are used: DPA, 3′,5′-diphosphoadenosine; PAPS, 3′-phosphoadenylyl sulfate; APS, adenylyl sulfate.