The Glyceraldehyde 3-Phosphate Dehydrogenases of Liver and Muscle

COOPERATIVE INTERACTIONS AND CONDITIONS FOR FUNCTIONAL REVERSIBILITY*

(Received for publication, September 13, 1971)

COLLEEN M. SMITH† AND SIDNEY F. VELICK
From the Department of Biological Chemistry, University of Utah College of Medicine, Salt Lake City, Utah 84112

SUMMARY

A method is described for the isolation of glyceraldehyde 3-phosphate dehydrogenase from rabbit liver. The enzyme has been crystallized as the NAD complex and its chemical and physical properties have been compared with those of the muscle enzyme. The kinetics of the reversible reaction catalyzed by the dehydrogenases is sensitive to temperature and ionic strength and has been examined at 37°, pH 7.4, in a solvent of ionic strength 0.1. Under these conditions the outstanding new feature of the reverse reaction, the reductive dephosphorylation of 3-phosphoglyceroyl phosphate, is the positive cooperativity of the response of both enzymes to concentration increments of the acyl phosphate and the increase in this cooperativity as a function of concentration of the cosubstrate, NADH. At low concentrations of the acyl phosphate, the reduced coenzyme exerts an inhibitory function which is abolished by high concentrations of acyl phosphate or by low concentrations of NAD acting as a heterotropic effector. The same concentrations of NAD which activate at low acyl phosphate concentration inhibit at high concentrations of the 3-carbon substrate by competing with NADH. Activation of the reaction by NAD involves a transition of the acyl phosphate saturation function from a sigmoidal to the hyperbolic form and hence sensitizes the enzyme to lower concentrations of acyl phosphate.

The substrate inhibition by NADH and the activation by NAD may be accounted for by the binding of the nucleotides to catalytic sites of the tetrameric protein that are not occupied by the acyl enzyme intermediate. Although the competitive advantage in binding to the apoprotein strongly favors NAD over NADH, the competition under reaction conditions tends to be equalized at nonacylated sites of the acylated protein and is drastically reversed at the acylated sites. Thus at kinetic saturation levels of the acyl phosphate $K_{\text{NADH}}$ remains in the 10-μM range, but half-maximal inhibition by NAD occurs at concentrations of the latter that are in excess of 1000 μM. These properties involve both ligand-induced conformational transitions of the protein and a local steric effect of the acyl group that interferes with the binding of the oxidized but not the reduced form of the pyridine nucleotide.

In the forward or oxidative phosphorylation reaction the reciprocal kinetic plots are entirely linear except under conditions of product inhibition by NADH. The preferential binding of NADH at an acylated site is not strongly expressed in the inhibition by added NAD because such sites are already occupied by product NADH that is released subsequent to the rate-limiting acyl group transfer from enzyme to orthophosphate ion. The acyl phosphate is a strong inhibitor of the forward reaction, noncompetitive with NAD and strictly competitive with aldehyde.

Intracellularly, the enzyme always operates in the presence of high concentrations of NAD which are required to provide the driving force for the energy-conserving reaction. Functional reversibility, under the adverse substrate concentration distributions in the aerobic hepatocyte, is maintained by regulatory effects of NAD binding which promote the reaction of extremely low concentrations of acyl phosphate, and by the differential effects of acylation of the protein which allow access of substrate NADH and at the same time eliminate a prohibitive inhibition by the high concentrations of NAD. The catalytic properties of the liver and muscle enzyme are qualitatively similar but exhibit some quantitative differences that would favor the more diversified metabolic requirements of liver. However, no definitive structural differences have been identified.

A more detailed understanding of the kinetic properties of glyceraldehyde 3-phosphate dehydrogenase is required to rationalize its behavior in liver where it functions in the direction of gluconeogenesis against the high steady state concentrations of NAD and orthophosphate ion that prevail in the predominantly aerobic hepatocyte. If the kinetic parameters previously reported for the muscle enzyme (1) were applicable to the intracellular performance of the liver enzyme, the reaction in the direction of NADH oxidation would be subject to continuously strong product inhibition. However, substrate analyses of quick frozen liver samples have indicated that the coupled reac-
tions catalyzed by the dehydrogenase and 3-phosphoglycerate kinase remain in equilibrium over a wide range of metabolic conditions (2).

To examine the possibility that the dehydrogenase is synthesized in tissue-specific forms the rabbit liver enzyme has been crystallized and analyzed. Although the occurrence of one or a few conservative amino acid replacements has not been excluded, no definitive structural differences have been identified. The previous kinetic analysis had been done at 26°C and low ionic strength. Since the enzyme has a strong affinity for anions (3) and has several properties which exhibit a marked temperature dependence (4), the reaction kinetics has been re-examined at a physiological temperature and in a solvent of an ionic strength that more closely approximates natural levels. Under these conditions, the reciprocal interactions between NAD and the acyl enzyme intermediate which had been assigned a prominent role in the kinetics of glyceraldehyde 3-phosphate oxidation, but not in its reversal, are now found also to dominate the steady state kinetic behavior of the enzyme in the reduction of 3-phosphoglyceroyl phosphate.

Under the new experimental conditions, which simulate critical features of the natural reaction environment, the dehydrogenase exhibits a combination of homotropic and heterotropic cooperative responses that operate in a way to keep the reaction freely reversible under conditions that would be highly adverse to the reversible action of an enzyme monomer. This is in contrast with the behavior of numerous other allosteric enzymes, the kinetic behaviors of which serve to regulate the activity of a metabolic pathway in a single direction.

EXPERIMENTAL PROCEDURE

Materials

Pyridine nucleotides, supplied in previously assayed vials, ATP, and the barium salt of dL-glyceraldehyde 3-phosphate diethyl acetal were products of Sigma Chemical Co. There was less than 0.03% NADH in the NAD. The cyclohexyl amonium salt of d-glyceraldehyde 3-phosphate dimethyl acetal, synthesized by the method of Ballou and Fischer (5) was the gift of Dr. C. D. Gutsche. Phosphoglycerate kinase, crystallized from yeast, and the sodium salt of p-3-phosphoglycerate were obtained from Boehringer and Sons. p-3-Phosphoglyceroyl phosphate, made enzymically from the dl-aldehyde and purified by ion exchange chromatography, was prepared by a previously described method (1). The glyceraldehyde 3-phosphate dehydrogenase of rabbit skeletal muscle was prepared by minor modifications of the method of Cori, Stein, and Cori (6) and maintained throughout the preparation and in storage in the presence of diethiothreitol and EDTA. The dehydrogenase of yeast was prepared essentially by the method of Krebs (7) and handled as described elsewhere (4).

Trypsin, five times recrystallized, was a product of Worthington Chemical Co. 10-Methyl-3(3-dimethylaminopropyl)carbodiimide was obtained from Otke Chemical Co. Reagent grade urea was deionized and recrystallized before use. Imidazole (Aldrich) was treated with charcoal and recrystallized from benzene to remove fluorescent impurities.

Methods

Chemical Analysis—Amino acid analyses of 16- to 72-hour hydrolysates of the S-carboxymethylated protein (8) were made on the long columns of an amino acid analyzer by standard methods. Free carboxyl groups were estimated by the carboximide activation of glycine methyl ester addition and the subsequent measurement of the increase in protein-bound glycine, following the procedure of Hoare and Koshland (9). The reaction was run in 8 M guanidine hydrochloride at pH 4 for 2 hours at 25°C in a pH stat. For trypsin peptide mapping, hydrolysates were carried out at pH 8.5 in 0.1 M ammonium bicarbonate for 12 hours at 24°C with additions of 0.02 mg of trypsin to 4.1 mg of S-carboxymethylated protein at 0, 4, and 7 h hours. Chromatography in 1-butanol-acetic acid-water (4:1:5) and electrophoresis at pH 3.7 in pyridine-acetic acid-water (1:10:289) were performed as described by Katz et al. (10). In addition to ninhydrin staining, replicate maps were also stained with Ehrlich's reagent for tryptophan and with 8-hydroxyquinoline and bromine for arginine (11, 12). Free thiol groups of the native enzyme and of enzyme denatured in 8 M guanidine hydrochloride were measured spectrophotometrically by reaction with sodium dithio-bis-orthoxylitrobenzoate (13). Amino end groups were determined as the 2,4-dinitrophenyl derivatives (14) and by Edman degradation (15).

Electrophoresis—Zone electrophoresis at pH 6.5 and 8.5 was done on cellulose acetate (Microzone) and at the alkaline pH in polyacrylamido gel. Electrofocusing measurements were made in a 110-ml column containing Ampholine (LKB) in a sucrose gradient. The column was operated at 1000 volts, 10°C for 3 to 6 days. No losses of enzyme activity were observed under these conditions. The columns were emptied through an ultraviolet absorbance monitor, and the absorbance and specific enzyme activities of individual fractions were measured on a Cary 15 spectrophotometer.

Sedimentation—Molecular weight measurements by equilibrium sedimentation employing a filled Epon capillary-type double sector centerpiece, column heights of 2.3 to 2.7 mm, and by interference optics were made essentially as described in the Spinco model E manual with the modification that an initial overspeed was used to shorten the equilibration time (16). Fringe displacements were measured in a Gaertner micro-comparator. Sedimentation velocity runs in a 4° single sector aluminum centerpiece were monitored by schlieren optics. Diffusion measurements were made in the free boundary cell of the Amino, model H, electrophoresis apparatus and were monitored by interference optics. We are indebted to Dr. Robert Yue for the diffusion measurements.

Kinetic Measurements—Initial velocity measurements of the forward reaction, that of aldehyde oxidation, were measured fluorometrically. For the purpose, a simple filter fluorimeter was used with a low pressure mercury arc. The signal was taken directly from the photomultiplier to an 11-inch adjustable zero, adjustable range strip chart recorder (Leeds and Northrup) giving a full scale deflection with 0.6 μM NADH. When still higher sensitivity was required, the recorder was replaced by a Tektronix model 568 storage tube oscilloscope sweeping at 10 sec per scale division and allowing the measurement of linear initial rates during the production of as little as 2 × 10⁻⁸ μM NADH. The high sensitivities were required to distinguish between some of the strong primary and secondary actions of substrates and products. The same apparatus could be used for the reverse reaction at initial NADH concentrations of 4 μM or less. However, at higher NADH concentrations, the large zero suppression at high sensitivity resulted in a drifting signal.
susceptible to accelerated decay, activity controls covering the
tions were in the extremely dilute range of 10⁻³ pg ml⁻¹ and hence
required for activity decay of stock solutions over a period of

**Table I**

| Step                          | Volume a) | Total units b) | Specific activity c) |
|------------------------------|-----------|---------------|----------------------|
| Supernatant solution of acidified extract |           | 700           | 150,000              | 0.26 |
| Clarified solution of first ethanol precipitate |           | 125           | 75,000               | 3.4  |
| Clarified solution of second ethanol precipitate |           | 15            | 60,000               | 10.3 |
| Ammonium sulfate fractionation, first crystals |           | 50,000        | 103                  |
| Third crystallization        |           | 40,000        | 154                  |

a) Preparation from 400 g of liver.

b) Practical units, ΔA₄₅₀ min⁻¹ at pH 7.0 and 25° in 0.01 M imidazole, 0.3 mM ATP, 0.3 mM MgCl₂, 0.1 mM NADH, 5 mM p-3-phosphoglycerate, 2 mM dithiothreitol, excess phosphoglycerate kinase, and limiting concentrations of dehydrogenase.

c) Specific activities are in units of micromoles of NADH oxidized per mg of enzyme per min at pH 7.4 and 25°. The activity of the third crystals corresponds to a turnover rate of 22,400 min⁻¹ for a molecular weight of 144,000. The assay method, involving a 3-phosphoglyceroyl phosphate-generating system, was as described previously (4).

due to phototube fatigue. A Cary model 15 spectrophotometer and the 0 to 0.1 absorbance slide wire with cells of 10-cm light path was therefore also used for the reverse reaction. Product inhibition presented no technical problem in this reaction direction, but the filling and mixing time of the 10-cm absorption cells was longer than desirable. In some instances, an open rectangular cell of 5-cm light path was used allowing a dead time after manual enzyme addition of a few seconds.

Stock dilutions of enzyme were kept at the highest allowable concentration, and additions to the reaction mixtures were made with 5- or 10-µl pipettes. Small normalizing corrections were required for activity decay of stock solutions over a period of several hours. Since the enzyme concentrations in the test solutions were in the extremely dilute range of 10⁻³ µg ml⁻¹ and hence susceptible to accelerated decay, activity controls covering the duration of a single initial velocity measurement were also made by sampling this concentration of enzyme incubated for various periods of time in several incomplete reaction mixtures. Linear initial velocities lasting up to 2 min were acceptable without correction.

**Purification and Analysis of Liver Enzyme**

*Initial Extract—* The livers of six adult rabbits were promptly removed, diced, and homogenized in a blender at low speed for 20 sec in 2 volumes of cold 0.4 M sucrose containing 1% sodium chloride, 5 mM EDTA, and dithiothreitol. The pH of the suspension was adjusted to 5.8 with hydrochloric acid and the suspension was centrifuged at 23,000 × g for 20 min at 0°.

*Precipitations with Ethanol—* After adjusting the pH of the extract to 6.8, 1 volume of 95% ethanol previously chilled to −5° was added at a rate that maintained the solution temperature below +5°. The suspension was then spun at 20,000 × g for 15 min at −10°. Approximately 65% of the original activity was precipitated in this step, together with a large amount of denatured protein. The packed precipitate was resuspended by a brief homogenization in 100 to 150 ml of 0.05 M potassium phosphate, pH 7.0, containing 5 mM EDTA, 5 mM dithiothreitol, and 1 mM NAD. Insoluble material was removed by ultracentrifugation at 75,000 × g for 3 hours at 0°. The enzyme in the supernatant solution was reprecipitated exactly as described above, extracted from the precipitate with 5 to 15 ml of the same extracting solution, and clarified by ultracentrifugation as before.

*Ammonium Sulfate Fractionation—* The clarified enzyme solution was dialyzed in a 1-cm diameter casing against 150 ml of the phosphate potassium chloride buffer to remove residual ethanol. Weighed portions of ammonium sulfate were added, basing the concentrations upon the saturation table of Green and Hughes (17). Precipitates obtained at 0.55 and 0.59 saturation were allowed to form for 1 hour in the cold and were removed by centrifuagration and discarded. The ammonium sulfate was then increased to 0.64 saturation, and the pH was raised to 8.3 with ammonium hydroxide. Crystals appeared overnight at 4°. They were usually contaminated with a smaller molecular weight protein that was retained on a DEAE-Sephadex column equilibrated with 0.01 M potassium phosphate, pH 7.0. On such a column the dehydrogenase emerges with the solvent front. Second crystals obtained under the above conditions contained approximately 3 moles of NAD per 144,000 of protein. The purification is summarized in Table I. If NAD is omitted from the extraction solutions in the above procedure apoenzyme in small yield may be obtained after arduous refractionation with ammonium sulfate. Crystals of the holoenzyme may be obtained either as thin plates resembling those of the muscle enzyme or as stout rods.

**Molecular Weight—** Sedimentation constants of the liver and muscle enzyme as a function of protein concentration are plotted in Fig. 1. The reported protein concentrations are averages over the time of the experiment, corrected for radial dilution. Within small limits of error, the sets of points extrapolate to the same value of 1/80 and exhibit the same slope. $g_{w, w}$ for both enzymes is 7.60. Converted to standard conditions, the diffusion constant is $D_{20, w} = (5.12 \pm 0.041) \times 10^{-7} \text{cm}^2 \text{sec}^{-1}$, compared with the value of $(4.97 \pm 0.03) \times 10^{-7}$ for the muscle enzyme reported by Fox and Dandliker (18). With the calculated anhydrous partial specific volume of 0.740, the molecular weight of the liver enzyme by this method is 139,000. Sedimentation

![Fig. 1. Reciprocal sedimentation coefficients as a function of protein concentration at 4°. The buffer in both sets is 0.05 M potassium phosphate, 0.001 M EDTA, pH 6.98. The upper set, in addition, contains 0.1 M potassium chloride. □, •, gyceraldehyde-3-P dehydrogenase of muscle; □, ○, the liver enzyme.](http://www.jbc.org/content/275/1/275.large)
TABLE II

Amino acid composition of glyceraldehyde 3-phosphate dehydrogenases of rabbit liver, rabbit muscle, and pig muscle

| Amino acid   | GPD-M (Rabbit) | GPD-L (Rabbit) | GPD-M (Pig) |
|--------------|----------------|----------------|------------|
|              | 24 hrs | 48 hrs | 24 hrs | 72 hrs | From amino acid sequence | 24 hrs | 48 hrs |
| Aspartic acid | 37.97  | 37.81  | 36.8  | 38.4  | 38  | 38.2 | 39.2 |
| Threonine     | 20.11  | 19.94  | 19.88 | 19.87 | 20  | 21.2 | 20.6 |
| Serine        | 17.75  | 16.84  | 17.22 | 17.94 | 19  | 19.2 | 15.4 |
| Glutamic acid | 19.3   | 19.3   | 18.9  | 19.6  | 18  | 19.0 | 19.2 |
| Proline       | 11.0   | 11.3   | 11.9  | 12    | 12  | 13.0 | 12.4 |
| Glycine       | 31.51  | 30.5   | 30.4  | 30.8  | 32  | 33.6 | 33.7 |
| Alanine       | 32.0   | 32.0   | 32.0  | 32.0  | 32  | 32.6 | 32.6 |
| Valine        | 24.8   | 28.0   | 24.6  | 31.9  | 34  | 26.3 | 29.9 |
| Methionine    | 8.6    | 8.8    | 8.3   | 9     | 8.9 | 8.8 |
| Isoleucine    | 15.2   | 17.0   | 15.3  | 18.3  | 21  | 15.7 | 18.0 |
| Leucine       | 18.0   | 17.2   | 18.0  | 17.9  | 18  | 18.0 | 18.2 |
| Tyrosine      | 8.6    | 8.6    | 8.5   | 8.0   | 0   | 8.6 | 8.8 |
| Phenylalanine | 13.4   | 13.3   | 13.5  | 13.7  | 14  | 13.5 | 13.8 |
| Cysteine*     | 4.07   | 3.6    | 3.55  | 4     |     |     |
| Tryptophan    |        |        | 3     |       |     |     |
| Lysine        | 26.2   |        | 24.7  | 26.9  | 26  | 25.8 | 26.9 |
| Histidine     | 19.7   |        | 10.4  | 11.1  | 11  | 10.6 | 11.1 |
| Arginine      | 9.8    | 9.4    | 9.9   | 9.7   | 10  | 10.0 | 10.0 |

* The abbreviations used are: GPD-M, glyceraldehyde 3-phosphate dehydrogenase of muscle; GPD-L, glyceraldehyde 3-phosphate dehydrogenase of liver.

Equilibrium runs on two preparations give linear plots of ln J against r². At protein concentrations of 1.5 and 4.6 mg ml⁻¹, molecular weights of 143,000 and 139,000, respectively, were obtained.

Schlieren patterns of sedimentation velocity runs in 5 M guanidine hydrochloride showed no sign of asymmetry. The sedimentation constant of the dissociated liver enzyme is ε₀M = 1.9 ± 0.09 compared with a value of 1.82 reported by Harrington and Karr (19) for the rabbit enzyme.

Amino Acid Analyses—The results of amino acid analysis are listed in Table II. Although a few single amino acid replacements cannot be excluded, there are no definitive differences in the composition of the rabbit liver and muscle enzymes. Moreover, the results are in close agreement with those tabulated from the total sequence analysis of the pig muscle enzyme by Harris and Perham (20). The NH₂-terminal groups were identified as valine by paper chromatography of the dinitrophenyl derivatives and were quantitated as the phenyl thiodyanolines, 3.9 ± 0.1 moles per mole of tetramer for both proteins. In the free carboxyl group analysis, 30.7 and 32.3 moles of extra glycine per mole of monomer were incorporated, respectively, in the liver and muscle enzymes. If the value of 39 aspartyl plus glutamyl residues per subunit of the pig muscle enzyme applies to the rabbit enzymes, the observed 31 to 32 free carboxyl groups in the latter correspond to an 80% recovery and the difference may not be significant. Yields of 80 to 90% had been reported for proteins of known structure (9). Our own control analysis of lysozyme by this method appeared to be quantitative.

Within the limits of variation of independent runs on hydrolysates of the same protein, the tryptic peptide maps of the liver and muscle enzymes showed no reproducible differences. As required for four identical subunits, 10 of the resolved spots stained for arginine. However, in both cases only two of the expected three stained for tryptophan.

The free thiol group content of the liver enzyme, as routinely isolated and measured by titration with dithio-bis-orthonitrobenzoate, tends to be low, but the expected 8 mole equivalents per mole of native tetramer are obtained after incubation with diithiothreitol and dialysis; 16 mole equivalents (±0.5) are measured after similar treatment in 4 M urea. A low thiol content has been reported for the calf liver enzyme by Heinz and Kühl (22) who isolated the apoprotein. Several marked differences between the calf liver and rabbit muscle enzymes were reported by these workers, but because of some inconsistencies in the analyses the calf protein requires further examination.

Electrophoresis—The liver and muscle enzymes migrate as single slightly diffuse bands in polyacrylamide gel or cellulose

![Fig. 2](image-url)  
**Fig. 2.** Electrophoresis of the apoglyceraldehyde-3-P dehydrogenase of rabbit muscle after 135 hours at 10°C in a pH 8 to 10 amphotolinesusarose gradient. Activity is expressed in units of pA₄₅₀ min⁻¹ under the standard assay conditions of Table I.

![Fig. 3](image-url)  
**Fig. 3.** Electrophoresis of the apoglyceraldehyde-3-P dehydrogenase of rabbit liver for 65 hours under the conditions of Fig. 2.
acetate zone electrophoresis over a pH range in which they are stable and are not resolved by such methods when previously mixed. The observation of multiple active bands by electrofocusing, as reported by Susor et al. (23) for the muscle enzyme is readily confirmed with holoenzyme preparations from both tissues. However, we have obtained similar results with the pig muscle enzyme which has exhibited no structural variation in a total sequence analysis (22). The heterogeneity is greatly decreased when enzyme, stripped of its bound NAD by charcoal treatment in dithiothreitol, is examined. Electrofocusing curves obtained with the muscle and liver apoenzymes are shown in Figs. 2 and 3. The isoelectric point of the major component in both cases is 8.52 ± 0.02 as expected from the amino acid compositions and as predicted from the ionic strength dependence of the isoelectric points and the anion-binding properties (24). The isoelectric point of the minor component, constituting about 15% of the total is 8.32 ± 0.03. The major band exhibits maximal or near-maximal enzyme activity even after 3 to 5 days of electrofocusing. The specific activity of minor band is only slightly lower after an approximate correction for the somewhat variable base-line absorbance. When the bands are isolated and refocused, they concentrate at their original positions. A small pI (isoelectric point) difference in the direction observed for the minor band could arise from a component that still carried bound nucleotide, from differential ampholine binding, from amide group variation, or from a conformational isomerism. A thiol to disulfide conversion would shift the pI in the opposite direction since it is likely that the exposed thiol groups are ionized at pH 8.5 (25).

Kinetic Results

Glyceraldehyde 3-phosphate dehydrogenases isolated from widely diverse species in the evolutionary scale constitute a family of homologous tetramers each composed of a single type of subunit (26, 27). The reversible catalytic reaction has been studied by a variety of independent methods (24) and proceeds through a substituted enzyme intermediate. In the direction of aldehyde oxidation, an acyl enzyme thioester is formed by the oxidation by NAD of a presumed thiohemiacetal adduct of aldehyde and enzyme. The acyl group is then transferred to orthophosphate ion. The acyl enzyme intermediate in the reverse reaction is formed by phosphate displacement from the acyl phosphate and is then reduced by NADH. In the absence of NADH there is a powerful activation by NAD of phosphate exchange between acyl phosphates and orthophosphate (28). The interaction between NAD and the acyl enzyme intermediate is a reciprocal one since acylation of the enzyme promotes the release of bound NAD (1) and high NAD concentrations shift the acyl group transfer equilibrium in the direction of acyl phosphate formation (29). An activation by NAD of the reductive dephosphorylation of acyl phosphate, implied by the above results, was not observed in steady state kinetics at pH 7.5 and 25° at low ionic strength (1) but was found qualitatively by de Vijlder et al. (30) to occur under special conditions. This effect has been studied in greater detail by Trentham (31) who employed the lobster and sturgeon muscle enzymes and measured kinetic transients by the stopped flow method. Trentham's work has provided independent evidence that bound NAD promotes the acyl group transfer in both reaction directions and also accelerates the formation and dissociation of the enzyme complex with glyceraldehyde 3-phosphate.

Fig. 4. The dependence of the initial velocities of 3-P-glyceroyl-P reductive dephosphorylation upon potassium chloride concentrations in 0.01 M imidazole (chloride), 2 mM EDTA, and 2 mM dithiothreitol at pH 7.4 and 37°. In Set A the NADH concentration is 11.9 μM and the concentrations of acyl phosphate, reading downward, are 0, 5, and 3 μM. In Set B the NADH and acyl-P concentrations of Curves a and b are, respectively, 220 and 200 μM, and 210 and 60 μM. Set A was measured with a 10-cm light path on a 0 to 0.1 absorbance scale. Set B was measured with a 1.0-cm light path and a 0 to 1.0 absorbance scale. Velocities are in arbitrary units, different in the two sets.

Kinetic transients provide a powerful approach to certain properties of the catalysis but have been restricted to high enzyme and substrate concentrations. However, the dehydrogenase acts on extremely low concentrations of acyl phosphate and NADH. These concentrations may be studied by the steady state kinetic method, and under the appropriate conditions the multiple functions of NAD and new properties of the enzyme interactions with the acyl phosphate and NADH are observable. Our initial objective was to search for functionally significant differences between the liver and muscle enzymes. As it turns out, the two enzymes are qualitatively quite similar in their properties and have been fully recognized and which are pertinent both to mechanism and intracellular function.

Reductive Dephosphorylation of 3-Phosphoglyceroyl Phosphate

Salt Effects—Initial velocities at sets of fixed substrate concentrations were measured in 0.01 M imidazole at pH 7.4 and 37° as a function of the concentrations of a series of neutral univalent electrolytes. The results were similar with the salts examined and involve some minor ion specificities superimposed upon a general ion strength effect. The largest effects were given by sodium and potassium chlorides and are illustrated for potassium chloride in Fig. 4. Initial velocities increase with the concentration of potassium chloride to a maximum at about 0.06 M and then decline with increasing concentrations of the salt. The form of the kinetics as well as the absolute rates change as one passes from the rising to the descending portions of the salt concentration curve. Linear reciprocal plots are obtained only at low ionic strength in the absence of potassium chloride. In the measurements to be described 0.1 M potassium chloride was selected as the supporting electrolyte. At this salt concentration, the enzyme, although not at the activity maximum, is still in the salt-activated state and also exhibits optimal stability. The initial velocities are also more reproducible where the ionic strength functions are less steep.

Cooperative Effects—Reciprocal plots of initial velocities at sets of fixed concentrations of the cosubstrates are shown in Fig. 5. With acyl phosphate as the independent variable, the major portions of the nonlinear curves occur in reverse order and intersect near a common point in the upper right-hand quadrant. The
Cooperative Interactions of Glyceraldehyde 3-Phosphate Dehydrogenase

Reciprocal NADH lines are also curved but diverge with increasing concentrations of NADH. Such behavior is indicative of a strong substrate inhibition by NADH that is opposed by increasing concentrations of the acyl phosphate. Thus, at 20 μM acyl phosphate, the reciprocal NADH plot is linear, corresponding to a hyperbolic saturation function and an apparent $K_{NADH}$ of about 7 μM. Direct plots of $v_i$ against [Acyl-P] are sigmoidal, and the apparent cooperativity increases with NADH concentration.

Each subunit of the dehydrogenase binds 1 molecule of NAD or NADH competitively (32), and the subunits are believed to be equivalent in the apoprotein. Whether or not a given site exhibits a catalytic or an inhibitory function when occupied by NADH should depend upon its occupancy by the cosubstrate. Thus, at low acyl phosphate concentration NADH may bind to an otherwise vacant site and act as an inhibitor. At high acyl phosphate concentrations, the inhibitory sites disappear and only the substrate function of NADH is expressed. In such a model NADH would inhibit by promoting a conformational transition of the protein that weakened either the binding of the acyl phosphate or the intramolecular acyl group transfer from phosphate to the accepting thiol group of the protein. This interpretation is supported by the following effects of NAD upon the rates of acyl phosphate reduction.

**Activation and Inhibition by NAD**—The effects of NAD concentration upon the reductive dephosphorylation of acyl phosphate by NADH are shown for the liver and muscle enzymes in Figs. 6 and 7. At sub saturating concentrations of acyl phosphate, the enzyme activity increases and passes through a maximum as a function of the concentration of NAD. The broad activity maximum occurs in the range of 80 to 150 μM NAD, is not identical for the two enzymes, and is followed by an inhibition that increases slowly with further increments of NAD concentration. The kinetic basis of these effects is indicated by the reciprocal plots of Fig. 8. In the absence of NAD the...
curve is typically steep and concave upward. NAD addition drastically reduces the slope and abolishes the curvature. A change of this type is equivalent in a direct plot to the transformation of a sigmoidal to a hyperbolic saturation function for acyl phosphate. Activation occurs only at low acyl phosphate concentrations and results from a diminution in the effective Michaelis constant of the acyl phosphate. The maximal velocity is diminished to an extent that depends upon the concentration of NAD. In the inhibitory region NAD is competitive with NADH (Fig. 9).

These results follow the pattern exhibited by the NADH inhibition. Activation of the reaction by NAD at low acyl phosphate concentration should result from NAD binding to a site that is unoccupied by the 3-carbon substrate. In so doing, NAD competitively opposes any NADH inhibition at that site and also promotes the intramolecular acylation step on another subunit. A conformational transition of the protein associated with NAD binding is implied. Independent evidence for the occurrence of opposing conformational transitions associated with the binding of the conjugate forms of the pyridine nucleotide is provided by the fact that NAD stabilizes (1) and NADH destabilizes (33) the apoprotein. The activation of the reaction by NAD not only eliminates the cooperative enzyme response to acyl phosphate but also blocks a site which at high acyl phosphate concentration would act catalytically. Hence, at high acyl phosphate concentrations, which require no heterotropic activation, NAD limits the maximal velocity.

There is a great disparity, at low acyl phosphate concentrations, between the NAD concentrations that activate and those that inhibit. Half-maximal activation occurs in the range of 5 to 10 \( \mu M \) NAD depending in part upon the concentration of NADH. Half-maximal inhibition is not clearly defined by the data but occurs at NAD concentrations in excess of 1000 \( \mu M \). The difference may be interpreted as a measure of the relative binding affinities of NAD at nonacylated and acylated sites. In the absence of other ligands, the dissociation constant of the high affinity enzyme-NAD complexes at 37° is about 3 \( \times 10^{-7} \) M (4). The somewhat higher concentration of NAD required for half-maximal activation may be attributed to competition with inhibitory NADH and to indirect conformational effects of acylation. The much weaker binding of NAD at an acylated site, where it would form an inhibitory dead end complex, should arise from direct steric interference by the enzyme-bound acyl group. This behavior is consistent with the reciprocal interactions between NAD and acyl phosphate that have been elicited. An important additional property of the steric interference by the acyl group is that it is specific for the oxidized form of the pyridine nucleotide, since the kinetic \( K_{NADH} \) at high acyl phosphate concentration remains in the low micromolar range. Geometric and possible conformational differences of the bound conjugate forms of the pyridine nucleotides (34) are sufficient in principle to account for this degree of selectivity.

All of the above reactions were initiated by the addition of enzyme-NAD complex at a final concentration of about 10^{-3} M. Holo- rather than apoenzyme was used to improve the stability of the dilute stock solutions. Initial velocities were measured during the production of as little as 2 \( \times 10^{-8} \) M NAD and could be extrapolated back linearly to the origin at zero time. Although it is unlikely, at 37° in the presence of NADH and acyl phosphate, that any significant fraction of the enzyme remained complexed with NAD, the enzyme was added in the active form.
we calculate that at 37° the active aldehyde concentration is 10% of the total α form present. On this basis the aldehyde concentrations in Fig. 10 should be divided by 10, and the aldehyde should be considered to be a relatively strong inhibitor but not anomalously strong as was reported for low ionic strength and 26° (1). The altered behavior of the aldehyde as an inhibitor under the present conditions is primarily a temperature and not an ionic strength effect.

An orthophosphate concentration in the physiological range of 3 mM has no effect on the kinetic parameters of NADII and exerts an approximate 10% inhibition directed against the acyl phosphate at concentrations of the latter from 2 to 8 μM.

Oxidative Phosphorylation of Glucose-6-phosphate Potassium Chloride Effects—As shown in Fig. 11, the effects of potassium chloride concentration upon the initial velocities of the forward reaction are similar to those on the reverse reaction, maximal activation occurring at about 0.06 M followed by a progressive inhibition with increasing salt concentration. It may be noted that at 15 mM phosphate the curve for the muscle but not the liver enzyme is shifted to the left. This is one of several minor differences between the two enzymes that are expressed primarily at low ionic strengths. The kinetic properties of the two enzymes, except for some quantitative differences, are formally quite similar and only the more extensive results obtained with the muscle enzyme will be presented.

Kinetics of Reaction—The initial velocities in Figs. 12 to 14 are plotted as v/[S] against v. This type of presentation tends to exaggerate the scatter but equalizes the weights attached to the experimental points obtained over a wide concentration range (36). Points obtained at a fixed substrate concentration lie on straight lines radiating from the origin, some of which are marked. Apparent Michaelis constants are given by the slopes, v/[v/[S]].
FIG. 13. The kinetics of the oxidative phosphorylation of glyceraldehyde-3-P (G3P) at 5 mM orthophosphate and a 10-fold concentration range of NAD. Solvent conditions are in Fig. 12.

FIG. 14. The oxidative phosphorylation of glyceraldehyde-3-P (G3P) at 0.5 mM NAD and four concentrations of orthophosphate. The conditions are as in Fig. 12.

The outstanding feature of the results, compared with those of the reverse reaction, is the linearity of the plots. Thus, the NAD concentration function exhibits no significant deviation from a straight line over the range of 20 to 2000 μM. Over the concentration range tested the only substrate cross reaction expressed in the $K_v$ values is between aldehyde and orthophosphate ion. At high concentrations of the latter, the $K_{V,aldehyde}$ is increased. In a direct plot at fixed aldehyde and NAD concentration, this would be expressed as a substrate inhibition by phosphate and is probably the converse of the substrate inhibition by aldehyde previously reported (1). In the latter work, arsenate had been used in place of phosphate and was used at concentrations two to three orders of magnitude lower than the phosphate concentrations employed here. Under the present conditions there is no significant substrate inhibition by aldehyde.

Insofar as can be determined under the present conditions, the enzyme at any fixed concentration of two of its three substrates in the forward reaction functions in a single conformational form.

This may be attributed to the combined effects of NAD as a substrate and activator and to the occurrence of high steady state concentration levels of the acyl enzyme intermediate. As in the earlier work, the acyl group transfer from enzyme to orthophosphate ion is considered to be rate limiting at pH 7.4. The $K_{NAD}$ of about 80 μM satisfies kinetic criteria for a dissociation constant of the complex of NAD with the acylated enzyme. Its magnitude relative to the smaller dissociation constant of the complex in the absence of aldehyde is attributed to the indirect or conformational effect of acylation. The results are consistent with the conclusions of Smith (37) that NADH in the forward reaction is released subsequent to the acyl group transfer. If NAD promoted acyl group transfer to orthophosphate in the forward reaction by displacing product NADH from an acylated site, we would expect nonlinear kinetics and a range of activation by NAD comparable to the extended range of NAD inhibition of the reverse reaction, and this is not observed. However, as shown in the following section, a nonlinear function of NAD concentration is obtained in the presence of NADH added as an inhibitory product.

**Product Inhibitions**—Inhibition of the forward reaction by 1 to 5 μM NADH was measured at a series of fixed concentrations
Cooperative Interactions of Glyceraldehyde S-Phosphate Dehydrogenase

Vol. 247, No. 1

FIG. 17. Competitive product inhibition by 3-P-glyceroyl-P. The fixed NAD and orthophosphate concentrations are 0.1 and 3.0 mM. The acyl-P concentration is zero in the upper curve and 5 μM in the lower. The aldehyde concentration is varied from 0.013 to 0.33 mM under standard conditions at 37°. GSP, glycer-
aldehyde-3-P.

FIG. 18. Noncompetitive product inhibition by 3-P-glycer-
yl-P. The fixed concentrations of glyceraldehyde 3-P and or-
thophosphate are, respectively, 0.043 and 7. mM. NAD concen-
trations range from 0.06 to 0.5 mM under standard conditions at 37°.

TABLE III
Some kinetic parameters of oxidative phosphorylation of glyceraldehyde S-phosphate catalyzed by dehydrogenase from rabbit skeletal muscle*

| Conditions | [NAD] | [G3P] | [P] | V/μM | K'NAD | K'G3P | K'P |
|------------|-------|-------|-----|------|-------|-------|-----|
| 0.02-1.0  | 0.3   | 0.3-1.0 | 0.05 | 5    | 0.00  | 0.00  | 0.05 |
| 0.02-0.5  | 0.03  | 0.3-1.0 | 0.05 | 5    | 0.00  | 0.00  | 0.05 |
| 1.0        | 0.03  | 1      | 1   | Vₜ 0.000 min⁻¹ | Vₜ 0.000 min⁻¹ |

* Conditions: 0.1 M potassium chloride, 0.01 M imidazole, pH 7.4, 37°, 2 mM EDTA, 2 mM dithiothreitol. GSP, glyceraldehyde 3-phosphate.

TABLE IV
Some kinetic parameters of reductive dephosphorylation of 3-phosph-
oglyceroyl phosphate (PGP) catalyzed by glyceraldehyde 3-phosphate dehydrogenase of rabbit skeletal muscle

| Conditions | [PGP] | [NAD] | V/μM | Conditions | [NAD] | V/μM |
|------------|-------|-------|------|------------|-------|------|
| 4          | 0     | 3.5   | 3    | 5          | 0     | 1    |
| 18         | 0     | 6.0   | 3    | 5          | 100   | 3    |
| 3          | 100   | 1.5   | 2    | 5          | 400   | 4    |
| 3          | 400   | 1.0   | 1    | 5          | 400   | 4    |

* Conditions: 0.1 M potassium chloride, 0.01 M imidazole, pH 7.4, 37°, 2 mM EDTA, 2 mM dithiothreitol.

** Concentration required for half-maximal velocity under the specified conditions.

* Relative maximal velocities with respect to the indicated substrate under the specified conditions.

of NAD and glyceraldehyde 3-phosphate. The inhibitions in Dixon type plots, 1/v against [NADH] are linear with respect to NADH and noncompetitive with respect to both substrates. One of these plots is shown in Fig. 15. Similar results at a fixed concentration of NADH with NAD and aldehyde as independent variables are shown in the double reciprocal plots of Fig. 16. At a low concentration of one substrate and high concentrations of the other two, 5 μM NADH is a strong inhibitor. The concentration dependence of the rates of the inhibited reactions are nonlinear with respect to both of the independently varied substrates and resemble the response in the reverse reaction to acyl phos-
phate in the absence of NAD.

In terms of the above arguments, the primary action of NAD in overcoming the NADH inhibition should occur in the competi-
tion with NADH for a nonacylated site. Although there is an ex-
tremely strong competitive advantage of NADH over NAD at an acylated site, such sites are already occupied by product NADH which is released subsequent to the acyl group transfer. Hence, competition at these sites is not expressed in the forward reaction. The opposition to NADH inhibition by increasing concentrations of glyceraldehyde 3-phosphate should be a consequence of an increased steady state concentration of the acyl enzyme inter-
mediate and a corresponding decrease in the number of nonacylated sites available for the inhibitory action of the reduced coenzyme.

Product inhibitions by 3-phosphoglyceroyl phosphate are illustrated in the v/[S] against v plots of Figs. 17 and 18. These plots are linear and indicate, in accord with the above interpre-
tations, that the acyl phosphate is competitive with the aldehyde and noncompetitive with respect to NAD.

Kinetic Parameters—The numbers in Tables III and IV are practical quantities, the substrate concentrations which give half-maximal velocities under the defined sets of conditions. In some cases they correspond to apparent Michaelis constants derived from linear reciprocal plots, and in others they are the estimated midpoints of sigmoidal kinetic saturation curves. The important features of the reverse reaction are the small magni-
tudes of the half-saturation values and their dependence upon the positive or negative effector functions of the pyridine nucleo-
tides. Apparent maximal velocities with respect to a given substrate at the designated concentrations of the others are given only in relative units to indicate the magnitudes of the effects.
Observed rates in absolute units of enzyme turnover are in the range of 3000 to 8000 min⁻¹ and are in excess of 20,000 min⁻¹ when extrapolated to the limit for both substrates with maximally active protein. The parameters of the forward reaction require little additional comment other than to mention that \(K_{d\text{ald}}\) may be as low as 2 to 5 μM if the enzyme sees only the unhydrated form at 37°C.

The reductive dephosphorylation of acyl phosphate catalyzed by the dehydrogenase exhibits many of the kinetic properties described by the allosteric transition model of an oligomeric protein proposed by Monod et al. (38). However, complications are introduced by the occurrence and properties of the substituted enzyme intermediate, the multiple functions of the pyridine nucleotides, and the ability of the four active centers of the protein to play either a catalytic or a regulatory role depending upon the partition among them of the five substrates of the reversible reaction. Conformational transitions between enzyme states of different catalytic activity are clearly associated with pyridine nucleotide binding and some of the general relationships that prevail under reaction conditions have been indicated. These properties do not correlate in detail with the observed nucleotide-binding interactions of the mammalian enzyme in the absence of coenzymes (4, 39) chiefly because of the modulating effect of the acyl enzyme intermediate. Simple cooperativity that follows the symmetry restrictions of the allosteric model has so far been exhibited only in NAD binding by the glyceraldehyde 3-phosphate dehydrogenase of yeast at pH 8.5 and 40° (40). At pH 7.4 at 25 or 40°, the yeast enzyme exhibits no measurable cooperativity in NAD binding. However, the steady state kinetics of the reverse reaction catalyzed by the yeast enzyme under the present conditions resemble those of the mammalian enzymes with respect to the enzyme interactions with acyl phosphate and NAD.¹

**REFERENCE**

1. FURFINE, C. S., AND VELICK, S. F. (1965) *J. Biol. Chem.*, 240, 944.

¹ Y. J. Farrar and S. F. Velick, Unpublished observations.
28. Harting, J., and Velick, S. F. (1954) J. Biol. Chem., 207, 867.
29. Malhotra, O. P., and Bernhard, S. A. (1968) J. Biol. Chem., 243, 1243.
30. Devijlder, J. J. M., Hilvers, A. G., van Lio, J. M. J., and Slater, E. C. (1968) Biochim. Biophys. Acta, 191, 221.
31. Trentham, D. R. (1971) Biochem. J., 122, 59.
32. Velick, S. F. (1968) J. Biol. Chem., 243, 1455.
33. Carr, D. O., Amelunxen, R., and Grisolia, S. (1965) Biochim. Biophys. Acta, 110, 507.
34. Velick, S. F. (1961) in W. D. McElroy and H. B. Glass (Editors), Light and Life, p. 108, The Johns Hopkins Press, Baltimore.
35. Trentham, D. R., McMurray, C. H., and Poonson, C. T. (1969) Biochem. J., 114, 19.
36. Dowd, J. E., and Riggs, D. S. (1965) J. Biol. Chem., 240, 863.
37. Smith, T. E. (1966) Biochemistry, 5, 2919.
38. Monod, J., Wyman, J., and Changeux, J.-P. (1965) J. Mol. Biol., 12, 88.
39. Conway, A., and Koshland, D. E., Jr., (1968) Biochemistry, 7, 4011.
40. Kirschner, K., Gallego, E., Schuster, I., and Goodall, D. (1971) J. Mol. Biol., 55, 29.
41. Williamson, J. R. (1965) J. Biol. Chem., 240, 2308.
The Glyceraldehyde 3-Phosphate Dehydrogenases of Liver and Muscle: COOPERATIVE INTERACTIONS AND CONDITIONS FOR FUNCTIONAL REVERSIBILITY

Colleen M. Smith and Sidney F. Velick

*J. Biol. Chem.* 1972, 247:273-284.

Access the most updated version of this article at [http://www.jbc.org/content/247/1/273](http://www.jbc.org/content/247/1/273)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/247/1/273.full.html#ref-list-1](http://www.jbc.org/content/247/1/273.full.html#ref-list-1)