Reactions of D-Glyceraldehyde 3-Phosphate Dehydrogenase Facilitated by Oxidized Nicotinamide–Adenine Dinucleotide

By D. R. TRENTHAM

Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

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Transient kinetic methods have been used to study the influence of NAD^+ on the rate of elementary processes of the reversible oxidative phosphorylation of D-glyceraldehyde 3-phosphate catalysed by D-glyceraldehyde 3-phosphate dehydrogenase. In the pH range 5–8 NAD^+ is bound to the enzyme during the following elementary processes of the mechanism: phosphorylation of the acyl-enzyme, its formation from 1,3-diphosphoglycerate and the enzyme and the formation and breakdown of the glyceraldehyde 3-phosphate–enzyme complex. The rates of these four elementary processes only equal or exceed the turnover rate of the enzyme when NAD^+ is bound and are as much as 10^4 times the rates in the absence of NAD^+ . Autocatalysis of the reductive dephosphorylation of 1,3-diphosphoglycerate occurs when glyceraldehyde 3-phosphate release is rate determining because NAD^+ is a reaction product. An important feature of the enzyme mechanism is that the negative-free-energy change of a chemical reaction, acyl-enzyme formation, is linked in a simple way to the positive-free-energy change of a dissociation reaction, NAD^+ release.

D-Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) is a tetrameric glycolytic enzyme that catalyses the oxidative phosphorylation of D-glyceraldehyde 3-phosphate. All species of the enzyme so far examined contain an exceptionally reactive thiol group that is essential for enzyme activity. The amino acid sequence of the tryptic peptide that contains this cysteine is conserved to a remarkable extent (Allison & Harris, 1965). There is good evidence that this thiol is acylated by the substrate during each turnover of the enzyme (Krimsky & Racker, 1955). Most mammalian sources of D-glyceraldehyde 3-phosphate dehydrogenase bind NAD^+ strongly. The reaction rate of the essential thiol group with a variety of reagents is dependent on the presence of NAD^+ (Harting & Velick, 1954; Krimsky & Racker, 1955; Park, Meriwether, Clodfelder & Cunningham, 1961; Malhotra & Bernhard, 1968).

De Vijlder, Hilvers, Van Lis & Slater (1969) and Trentham (1969) have investigated the role of NAD^+ on the acylation rate of D-glyceraldehyde 3-phosphate dehydrogenase by 1,3-diphosphoglycerate. The work presented here describes the influence of enzyme-bound NAD^+ on the rate of the reversible reactions of D-glyceraldehyde 3-phosphate and 1,3-diphosphoglycerate with D-glyceraldehyde 3-phosphate dehydrogenase over a wider range of pH and reaction conditions than reported before. These

reactions are indicated in **bold type** in Scheme 1. The influence of NADH on the rate of acyl-enzyme formation is relevant to understanding the mechanism of D-glyceraldehyde 3-phosphate dehydrogenase and is also carefully considered.

D-Glyceraldehyde 3-phosphate dehydrogenase from two sources, sturgeon and lobster, was selected for the studies. D-Glyceraldehyde 3-phosphate dehydrogenase from sturgeon muscle is a crystalline stable NAD^+ -free form of the enzyme and has two advantages. First, removal of NAD^+ from most forms of the enzyme requires charcoal treatment and the resulting enzyme is labile. Secondly, the NAD^+ -free D-glyceraldehyde 3-phosphate dehydrogenase from the yeast and rabbit muscle enzymes can exist in a catalytically inactive form until it has undergone a substrate-induced transformation that occurs at a slower rate than the catalytic-centre activity (Kirschner, 1967; Sapag-Hagar, 1969). Such forms of the enzyme are unsuitable for the studies of transients described here. In contrast the apoenzyme of sturgeon D-glyceraldehyde 3-phosphate dehydrogenase is shown here to be fully active in less than 3 ms. The enzyme from lobster tail muscle is crystalline and stable, and the crystals contain one molecule of bound NAD^+ /subunit, which is advantageous for stoichiometric studies of single turnovers of the enzyme. Moreover lobster enzyme has a known amino acid sequence

Scheme 1.

(Davidson, Sajgò, Noller & Harris, 1967) and work on the determination of the tertiary structure is in progress (Wassarman & Watson, 1968); so this is the enzyme of choice for correlation of structural and dynamic properties. The two enzymes therefore offer complementary advantages for elucidating the mechanism and study of the reactions facilitated by NAD^+ . It is important to know if all sources of D-glyceraldehyde 3-phosphate dehydrogenase, and particularly the two enzymes under study, have a common mechanism. For this reason a comparison of the functional identity of the two enzymes has been made.

The method of stopped-flow kinetics has been used that enables transients of the overall reactions or partial reactions of a single turnover of the enzyme to be observed directly.

Spectral signals from NADH absorption (λ_{max} , 340nm) and from the NAD^+ -D-glyceraldehyde 3-phosphate dehydrogenase absorption band, 'the Racker band' (λ_{max} , 365nm) (Racker & Krimsky, 1952), were used to monitor the transients. The extinction coefficient of NADH as measured in the ternary 3-phosphoglyceryl-NADH-enzyme complex or in the binary NADH-enzyme complex differs by a small amount from that of free NADH (Smith, 1966; Fisher, Adija & Cross, 1969). For these studies the extinction due to NADH is assumed to be constant. The 'Racker-band' extinction is in general decreased when the essential thiol group is acylated or alkylated or when phosphate or other anions are present in solution (Trentham, 1968; Boross, Cseke & Vas, 1969). Only 4% of D-glyceraldehyde 3-phosphate is in the catalytically active aldehyde form. The conversion rate from diol into aldehyde is relatively slow (Trentham, McMurray & Pogson, 1969). Therefore quite large concentrations of D-glyceraldehyde 3-phosphate are required for transient experiments. Knowledge of this aspect of the chemistry of D-glyceraldehyde 3-phosphate and of the phosphate perturbation of the Racker-band extinction is important back-

ground for these studies. The Racker-band-absorption changes can be used to monitor two of the reactions of Scheme 1 (eqns. 4 and 6). In the other cases less direct methods have to be used in which the redox reaction:

is involved. These are now explained to help understanding of the results.

If a transient is observed when substrates are mixed with the enzyme then the rate-determining step of the reaction occurs after the production or disappearance of the chromophore. By careful distribution of substrates and enzyme in the syringes of the stopped-flow apparatus both the rate-determining step and the process controlling the rate of the transient production can be identified. This approach is used to characterize the reaction of D-glyceraldehyde 3-phosphate with the holoenzyme (eqn. 3) and to resolve the rates of the processes associated with aldehyde release, acyl-enzyme formation, the binding of NADH and the redox reaction (eqns. 3, 2 and 4).

For relatively slow reactions a second type of rate measurement can be made. The reagents whose reaction rate is being measured are incubated in one syringe of the stopped-flow apparatus and then mixed at measured time-intervals with another reagent which undergoes a rapid chromophoric change with a product of the first reaction. In this case the rate of change of amplitude of the chromophore gives the rate of the slow reaction. This method is used to measure the rates of reaction of D-glyceraldehyde 3-phosphate and 1,3-diphosphoglycerate with the apoenzyme (eqns. 1 and 2).

MATERIALS AND METHODS

Isolation of D-glyceraldehyde 3-phosphate dehydrogenase from lobster tail muscle. The enzyme from lobster tail muscle was isolated and assayed as described by

Trentham (1968). The triethanolamine-phosphate buffer medium was altered in that triethanolamine hydrochloride replaced triethanolamine and the pH was adjusted to 8.7 with NH_3 . Triethanolamine buffer solutions were used within one week of preparation since otherwise they became increasingly inhibitory. The specific activity of the enzyme was $200 \pm 20 \mu\text{mol}$ of NADH produced/min per mg of enzyme at 25°C .

The molecular weight of the enzyme is 146000 (Davidson *et al.* 1967). $E_{1\text{cm}}^{1\%}$ at 276 nm is 10.1 and the E_{276}/E_{260} ratio, when the enzyme contains its complement of 4 mol of NAD^+ /mol (Trentham, 1968), is 1.10. Crystals of the enzyme were washed in 80% (w/v) $(\text{NH}_4)_2\text{SO}_4$ at pH 7 and then dissolved in EDTA (1 mM)–2-mercaptoethanol (1 mM) at pH 7. E_{276}/E_{260} was 1.10 ± 0.02 so that in all the experiments lobster enzyme contained 4 mol of NAD^+ /mol of enzyme.

Isolation and characterization of D-glyceraldehyde 3-phosphate dehydrogenase from sturgeon muscle. The enzyme from sturgeon muscle was isolated essentially by the method of Allison & Kaplan (1964) except that centrifugation at 15000g (MSE 18 centrifuge, 6×250 ml rotor) for 30 min was used instead of the filtration steps. The enzyme was crystallized twice and formed large colourless needles indicating that the crystals contain no bound NAD^+ . $E_{1\text{cm}}^{1\%}$ at 276 nm was 9.7. E_{276}/E_{260} for the freshly dissolved crystalline enzyme was 1.65. This ratio was not altered by exhaustive dialysis, indicating that there is no weakly bound NAD^+ on the crystal. The specific activity of the enzyme was $210 \pm 20 \mu\text{mol}$ of NADH produced/min per mg of enzyme at 25°C . The crystalline enzyme was stored at 5°C and was stable for at least 1 year. In solution at a concentration of 10 mg/ml the enzyme had a half-life of about one day at pH 7 and 5°C .

Allison & Kaplan (1964) concluded that crystalline sturgeon D-glyceraldehyde 3-phosphate dehydrogenase contained no bound NAD^+ . For the transient experiments it was necessary to establish this rigorously and find out if any other nucleotide was bound. After dialysis against EDTA (1 mM) and 2-mercaptoethanol (1 mM), the enzyme (5.4 mg) was incubated in a solution of urea (8 M)–EDTA (0.2 mM) and 2-mercaptoethanol (0.2 mM) and then filtered through a column of Sephadex G-25 (coarse grade) with the urea solution as eluting solvent. The high-molecular-weight material was the only fraction eluted that absorbed light at 260 nm. Crystalline enzyme from sturgeon therefore contains no bound nucleotide.

Preparation of substrates. D-Glyceraldehyde 3-phosphate was prepared from the calcium salt of D-fructose 1,6-diphosphate (3 mmol) in 27% yield by the method of Szwczuk, Wolny, Wolny & Baranowski (1961). A 350 mg sample of the calcium salt contained 1 mmol of glyceraldehyde 3-phosphate active as a substrate for glyceraldehyde 3-phosphate dehydrogenase. The calcium salt was stable indefinitely at -20°C . The free acid of glyceraldehyde 3-phosphate was prepared by treating the calcium salt with Dowex 50 (H^+ form) cation-exchange resin. The enzyme had the same specific activity with this glyceraldehyde 3-phosphate as for the same concentration of substrate prepared from the diethyl acetal derivative.

1,3-Diphosphoglycerate was prepared from glyceraldehyde 3-phosphate (58 μmol) in 50% yield essentially by the method of Furfine & Velick (1965). 1,3-Diphosphoglycerate was used for transient experiments within 24 h

of its elution from the DEAE-Sephadex column. 1,3-Diphosphoglycerate had a half-life of about two weeks when stored at -20°C as eluate from the column.

Reagents. NAD^+ and NADH were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). HCl and aq. NH_3 were prepared by isopiestic distillation (Irving & Cox, 1958) and were used for pH adjustments where appropriate. Water was glass distilled. All other reagents were A.R. grade where possible and were used without further purification.

Spectroscopic and kinetic measurements. The u.v.-absorption measurements were made in a 1 cm light-path thermostatically controlled cuvette in a Uvispek H700 spectrophotometer (Hilger and Watts Ltd.) with a Gilford recording attachment and a Sunvic recorder (type 10S).

The stopped-flow apparatus used was similar to that described by Gutfreund (1965). The interchangeable mixer and observation cell consisted of eight mixing jets and a 1 cm light-path. Monochromatic light was obtained from a quartz-tungsten-iodine lamp and a Bausch and Lomb grating monochromator by using a Wratten 18A filter to cut down stray light when working below 400 nm. The wavelength drum was calibrated against the 366 nm emission lines of a low-pressure mercury discharge. The dead-time of the instrument was 3 ms.

When reaction rates of incubation mixtures were monitored (Figs. 6 and 7) artifacts that could arise from slow diffusion of the solution in the reaction chamber back into the syringe were avoided by flushing a portion of the solutions through the stopped-flow apparatus immediately before recording the reaction.

The optical yield of the stopped-flow instrument was determined at 340 nm and 412 nm by using standardized solutions of NADH ($E_{340}^{1\text{cm}} 6.2 \text{ mm}^{-1} \cdot \text{cm}^{-1}$; Horecker & Kornberg, 1948) and 3-carboxy-4-nitrothiophenolate ($E_{412}^{1\text{cm}} 13.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$; Ellman, 1959) [the anion obtained by reduction of 5,5'-dithiobis-(2-nitrobenzoic acid)] respectively. The extinction of NADH at 360 nm was determined from a comparison of the extinction of NADH solutions at 340 nm and 360 nm in the stopped-flow instrument. Transmission of light was recorded on a Tektronix storage oscilloscope, which amplified the output of an EMI 9592B photomultiplier. The oscilloscope traces recorded percentage transmission on the ordinate axis with decreasing percentage transmission upwards. In the figures percentage transmission lines are indicated so that extinction changes can be calculated. The 100% transmission line does not necessarily reflect the transmission of the solutions before mixing. Reactions were studied at room temperature ($21 \pm 2^\circ\text{C}$).

RESULTS

Transient kinetic studies of the glyceraldehyde 3-phosphate dehydrogenase-catalysed reaction of 1,3-diphosphoglycerate and NADH should reveal whether the rate-determining step in the reaction occurs concomitantly with or after the redox step. In the latter case a burst of NADH disappearance will be observed. The reaction of [1,3-diphosphoglycerate + NADH] + [lobster enzyme] gave a burst of NADH disappearance at pH 5.3 (Fig. 1) but proceeded immediately at the steady-state rate

at pH 8, showing that there is a change of rate-determining step over this pH range and that the rate-determining step at low pH is associated with the processes on the left-hand side of Scheme 1. (A stopped-flow experiment is described as $[A+B]+[C]$ when reactants A and B in one syringe are mixed with reactant C in the other syringe.)

Transient kinetic studies of NAD^+ facilitation at low pH. Steady-state studies have shown that the reductive dephosphorylation of 1,3-diphosphoglycerate is autocatalytic at low pH and that the production of NAD^+ is responsible for this autocatalysis (Trentham, 1969). These results were

investigated further since, together with the previous experiment, they suggest that NAD^+ is facilitating glyceraldehyde 3-phosphate release at low pH.

Transient kinetic studies of the reaction $[1,3\text{-diphosphoglycerate} + \text{lobster enzyme}] + [NADH]$ were carried out at pH 5.4 (Figs. 2a and 2b). Added NAD^+ accelerated the original steady-state rate threefold when the transient began to disappear and there was a change of rate-determining step. This confirms that there is a process facilitated by NAD^+ that occurs after the redox step (Fig. 1) and is probably glyceraldehyde 3-phosphate release (eqn. 3). In addition, these results show that NAD^+ dissociates rapidly from the aldehyde- NAD^+ -enzyme complex (eqn. 5).

NAD^+ facilitation of acyl-enzyme formation. This section contains two types of experiment. In one type advantage is taken of the fact that sturgeon enzyme has no bound NAD^+ so that the influence of NAD^+ on the reaction between 1,3-diphosphoglycerate, NADH and enzyme may be studied. In the second type the acyl-enzyme is mixed with NADH and the rate profile of NAD^+ production identifies whether acyl-enzyme formation or some other process is facilitated by NAD^+ .

At pH 5.3 the reaction $[1,3\text{-diphosphoglycerate} + NADH] + [\text{sturgeon enzyme}]$ occurs with virtually no transient phase. The transient is more pronounced and is produced at a greater rate when the enzyme is preincubated with NAD^+ (Figs. 3a and 3b) showing that NAD^+ facilitates a process that occurs before the redox step at pH 5.3 (i.e. on the right-hand side of Scheme 1) and is distinct from the process facilitated by NAD^+ after the redox step at pH 5.4 (Figs. 2a and 2b).

The presence of a transient when the acyl-enzyme is mixed with 1,3-diphosphoglycerate and NADH would show that acyl-enzyme formation is the

Fig. 1. Spectrophotometric record of the reaction of NADH and 1,3-diphosphoglycerate catalysed by lobster enzyme. The stopped-flow trace was recorded at 340 nm. The reaction mixture contained lobster enzyme (0.35 mg/ml), 1,3-diphosphoglycerate (0.1 mM), NADH (0.07 mM), 2-mercaptoethanol (0.1 mM) and EDTA (180 mM) at a final pH of 5.3. One syringe contained enzyme and 2-mercaptoethanol and the other 1,3-diphosphoglycerate and NADH. EDTA was in both syringes.

Fig. 2. Effect of NAD^+ on the steady-state rate of NADH production. Stopped-flow traces were recorded at 360 nm. In (a) the reaction mixture contained lobster enzyme (0.405 mg/ml), 1,3-diphosphoglycerate (0.11 mM), NADH (0.08 mM), EDTA (0.17 M) and 2-mercaptoethanol (0.01 mM) at a final pH of 5.4. In (b) the reaction mixture was identical except that NAD^+ (0.6 mM) was added. In each experiment one syringe contained enzyme, 1,3-diphosphoglycerate, NADH and 2-mercaptoethanol and the other NADH. EDTA was in both syringes. The measured E_{360} of NADH in the stopped-flow instrument was $4.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

Fig. 3. Spectrophotometric record of the reaction of NADH and 1,3-diphosphoglycerate catalysed by sturgeon enzyme showing the effect of NAD^+ . The stopped-flow traces were recorded at 340 nm. In (a) the reaction mixture contained sturgeon enzyme (2.1 mg/ml), 1,3-diphosphoglycerate (0.1 mM), NADH (0.07 mM), 2-mercaptoethanol (0.2 mM) and EDTA (160 mM) at a final pH of 5.3. One syringe contained enzyme and 2-mercaptoethanol and the other 1,3-diphosphoglycerate and NADH. EDTA was in both syringes. In (b) reaction conditions were identical except that NAD^+ (0.03 mM) was present in the enzyme syringe.

Fig. 4. Spectrophotometric record of the reaction of NADH and 1,3-diphosphoglycerate catalysed by sturgeon enzyme showing the effect of added NAD^+ . The stopped-flow traces were recorded at 340 nm. In (a) the reaction mixture contained sturgeon enzyme (0.36 mg/ml), 1,3-diphosphoglycerate (0.09 mM), NADH (0.082 mM), EDTA (2 mM), 2-mercaptoethanol (2 mM) and tris-HCl buffer adjusted to pH 8.2 with NaOH. One syringe contained enzyme and NADH and the other 1,3-diphosphoglycerate. The other reagents were in both syringes. In (b) reaction conditions were identical except that NAD^+ (0.1 mM) was present in the enzyme syringe.

process facilitated by NAD^+ . 1,3-Diphosphoglycerate was incubated with sturgeon enzyme to form the acyl enzyme. The kinetics of this process are described below (Fig. 6). At pH 5.3 the reaction [1,3-diphosphoglycerate + sturgeon acyl-enzyme] + [NADH] did reveal a transient showing that acyl-enzyme formation is facilitated by NAD^+ . Addition of NAD^+ to the enzyme syringe accelerated only the steady-state rate in a similar manner to that already noted with lobster enzyme (Figs. 2a and 2b).

At pH 8.2 the reaction [1,3-diphosphoglycerate] + [NADH + sturgeon enzyme] revealed a lag phase of NAD^+ production that can be eliminated by prior incubation of the enzyme with NAD^+ (Figs. 4a and 4b). In the pH range 7.3–8.3 the reaction

[1,3-diphosphoglycerate (1.0 mM) + sturgeon enzyme (0.41 mg/ml)] + [NADH (70 μM)] in triethanolamine hydrochloride (0.1 M) proceeded at the steady-state rate with no lag phase and no rapid transient of NAD^+ production. There was no evidence of a transient whose rate of formation was too fast to measure. This shows that NAD^+ facilitates acyl-enzyme formation at pH 8.2 also (Figs. 4a and 4b). The rate-determining process of the reductive dephosphorylation at pH 8 occurs after acyl-enzyme formation and the subsequent NAD^+ release but concomitantly with the redox reaction. In a control experiment at pH 8 a somewhat less-marked lag phase was observed in the reaction [NADH + 1,3-diphosphoglycerate] + [sturgeon enzyme] showing that, whereas acylation of the NADH-bound

enzyme is slow, a direct measure of the acylation rate of the apoenzyme has not been made. In a further control experiment the reaction [glyceraldehyde 3-phosphate (0.5 mM) + NAD^+ (0.6 mM)] + [sturgeon enzyme (0.17 mg/ml)] in EDTA (1 mM)–2-mercaptoethanol (1 mM)–arsenate (10 mM)–triethanolamine (180 mM) adjusted to pH 8.0 with HCl was monitored in the stopped-flow apparatus. There was a rapid transient followed by the steady-state rate of NADH production, showing that the sturgeon apoenzyme is fully catalytically active on being mixed with NAD^+ within the time resolution of the stopped-flow machine (3 ms).

Reaction of 1,3-diphosphoglycerate with NAD^+ -bound enzyme (eqn. 4). In view of the importance of NAD^+ in acyl-enzyme formation the acylation was studied in isolation both from a kinetic and stoichiometric viewpoint. The reaction can be monitored by following the extinction change of the Racker band which may occur as a result of acyl-enzyme formation. However, since the Racker band disappears in the presence of inorganic phosphate (Trentham, 1968), the mere formation of the Michaelis complex between 1,3-diphosphoglycerate and enzyme may be sufficient for its removal. The rate of disappearance of the Racker band in the reaction [1,3-diphosphoglycerate] + [lobster enzyme] was dependent on 1,3-diphosphoglycerate concentration (Figs. 5a and 5b).

The final extinction coefficient of the enzyme in the reaction mixture is comparable with that of an enzyme solution in the presence of phosphate ions at pH 5.4 (Trentham, 1968), so that the residual extinction of the solution after the reaction is probably due solely to the extinction of the protein. This implies that all four sites on the enzyme have formed the Michaelis complex or been acylated.

Although qualitatively the dependence of the

rate of Racker-band removal on 1,3-diphosphoglycerate concentration is clear-cut, the quantitative description is more complex. This arises because of the rather limited range of acyl phosphate and enzyme concentrations that can be used. Consequently the reaction conditions (Fig. 5a) cannot be accurately described as pseudo-first-order because the acyl phosphate concentration (0.1 mM) is only twice as large as the enzyme-site concentration (0.0396 mM). The observed rate was calculated as a second-order rate constant and is $2.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. The second-order rate constant is $1.7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the higher concentration of 1,3-diphosphoglycerate (Fig. 5b). The reaction rate has a first-order dependence on 1,3-diphosphoglycerate concentration over the fivefold range of concentration.

The effect of NAD^+ on the rate of Racker-band removal was studied. When no NAD^+ was present in addition to that already bound to the enzyme, the amplitude of the observed signal change was the same as before whereas the second-order rate constant decreased to 60% of its original value. The explanation of this decrease in rate constant is not clear although this experiment does indicate that the rate of acyl-enzyme formation is not dependent on NAD^+ dissociation from the acyl enzyme.

The rate of the reaction of 1,3-diphosphoglycerate with enzyme is strongly dependent on the reaction medium. The experiment (Fig. 5a) was repeated except that the sodium chloride concentration was decreased 100-fold and that of EDTA was doubled. The second-order rate constant was $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is an 80-fold enhancement. This medium is similar to that used in the other transient and steady-state experiments. However, it was not possible under these conditions to study the first-order dependence of the rate on 1,3-diphosphoglycerate

Fig. 5. Dependence of the rate of Racker-band disappearance on 1,3-diphosphoglycerate concentration. Stopped-flow traces were recorded at 365 nm. The reaction mixtures contained 1,3-diphosphoglycerate [0.1 mM (a) and 0.5 mM (b)], lobster enzyme (1.44 mg/ml), NAD^+ (0.46 mM), EDTA (70 mM), NaCl (1.1 M) and 2-mercaptoethanol (0.1 mM). The pH of the reaction mixture was 5.7. One syringe contained enzyme, NAD^+ and 2-mercaptoethanol and the other contained 1,3-diphosphoglycerate. EDTA and NaCl were in both syringes. The lower traces show the end point of the reaction and were recorded a few seconds after mixing the reactants.

concentration since the rate was too fast to measure at high concentrations.

Reaction of 1,3-diphosphoglycerate with the apo-

Fig. 6. Dependence of the rate of NADH disappearance on the time of preincubation of 1,3-diphosphoglycerate and sturgeon enzyme before reaction of the mixture with NADH. Stopped-flow traces were recorded at 340 nm. In one syringe of the stopped-flow apparatus 1,3-diphosphoglycerate (0.12 mM) was incubated with sturgeon enzyme (1.46 mg/ml) in 2-mercaptoethanol (1 mM) and EDTA (100 mM) at pH 5.3 for various times: trace A, 0.6 min; trace B, 1.5 min; trace C, 5.0 min; trace D, 16 min, and then mixed with NADH (0.1 mM) in EDTA (200 mM) from the other syringe. The reaction-chamber concentrations were half those in each syringe.

enzyme (eqn. 2). The reaction of 1,3-diphosphoglycerate with sturgeon enzyme can be followed indirectly at pH 5.3. The reaction $[1,3\text{-diphosphoglycerate} + \text{NADH}] + [\text{sturgeon enzyme}]$ occurred with virtually no transient phase (Fig. 3a) in contrast with the reaction of $[1,3\text{-diphosphoglycerate} + \text{acyl-enzyme}] + [\text{NADH}]$ which revealed a transient. It follows that the amplitude of the transient phase reflects the extent of acyl-enzyme formation. 1,3-Diphosphoglycerate (0.12 mM) and sturgeon enzyme were incubated in one syringe of the stopped-flow apparatus and at measured times mixed with NADH. The family of traces (Fig. 6) shows the amplitude of the transient phase increasing with a half-time for the process of about 2 min. This rate is about 10^4 times slower than the acylation rate of the holoenzyme at comparable substrate concentrations and ionic strength (Fig. 3b and previous paragraph).

NAD⁺ facilitation of aldehyde-enzyme complex formation. The reaction of $[\text{glyceraldehyde 3-phosphate}] + [\text{NAD}^+ + \text{sturgeon enzyme}]$ results in NADH and acyl-enzyme formation (Fig. 7a). This reaction was first order with respect to glyceraldehyde 3-phosphate over a threefold increase in glyceraldehyde 3-phosphate concentration. The amount of NADH produced did not vary significantly. At higher substrate concentrations the

Fig. 7. Influence of the order of mixing reagents on the reaction of sturgeon enzyme, glyceraldehyde 3-phosphate and NAD⁺. Stopped-flow traces were recorded at 340 nm. (a) The reaction medium contained glyceraldehyde 3-phosphate (0.17 mM), NAD⁺ (6.0 mM), sturgeon enzyme (0.38 mg/ml) and EDTA (160 mM) at a final pH of 5.4. One syringe contained enzyme and NAD⁺ (0.12 mM) and the other NAD⁺ (12 mM) and glyceraldehyde 3-phosphate. EDTA was in both syringes. (b) NAD⁺ was omitted from the enzyme syringe. (c) As (b) except that glyceraldehyde 3-phosphate was in the enzyme syringe and was preincubated for 6 min with enzyme before the observed reaction trace. (d) As (c) with an incubation time of 20 min. The base-line traces are spectrophotometric records of the reaction medium without the enzyme. (c) and (d) have the same base-line trace since it was possible to erase the upper half of the oscilloscope record leaving the bottom half intact. (e) A control run in the same EDTA buffer, the difference in the upper and lower traces is due to the extinction of sturgeon enzyme (0.38 mg/ml).

reaction was too fast to measure, showing that the rate of the redox step is greater than 400s^{-1} . The observed rate probably corresponds to the rate of aldehyde-NAD⁺-enzyme complex-formation. The experiments described earlier indicate that this complex rapidly loses NAD⁺ as well as undergoing the redox reaction.

The reaction of [glyceraldehyde 3-phosphate + NAD⁺] + [sturgeon enzyme] gave a similar rate of NADH production (Fig. 7b). If glyceraldehyde 3-phosphate and sturgeon enzyme were incubated for various times and then mixed with NAD⁺ the rate of NADH production was biphasic. The more rapid process was too fast to measure whereas the slower process occurred at an identical rate as in the earlier trace (Fig. 7c). After 20 min the whole reaction became too fast to measure except for a small residual process, which is significant and is discussed elsewhere (Fig. 7d) (Trentham, 1971). The apoenzyme retained at least 80% of its activity when allowed to stand for 1 h at room temperature at pH 5.2.

The time-dependent relative amplitude change of the fast and slow processes can be interpreted as showing the aldehyde-enzyme complex/apoenzyme ratio. The residual apoenzyme reacts as before (Fig. 7b) whereas the aldehyde-enzyme complex binds NAD⁺ rapidly and undergoes the redox reaction within 3 ms. It is therefore possible to measure the rate of reaction of glyceraldehyde 3-phosphate with the apoenzyme by following the change of amplitude of the slow process with time (Fig. 8). This reaction was also first order in glyceraldehyde 3-phosphate concentration over a three-fold concentration range, with incubation times up to 30 min. The second-order rate constants based on total glyceraldehyde 3-phosphate concentration for the reactions of this substrate with apoenzyme and holoenzyme (Figs. 8 and 7a) were $4.5\text{M}^{-1}\cdot\text{s}^{-1}$ and $7 \times 10^4\text{M}^{-1}\cdot\text{s}^{-1}$, a 10000-fold rate enhancement.

The rate of aldehyde-enzyme complex formation was measured at pH 7.2. Glyceraldehyde 3-phosphate and sturgeon enzyme were preincubated in 100 mM-triethanolamine adjusted to pH 7.2 with sodium hydroxide and then mixed with a more strongly buffered solution at pH 5.3 containing NAD⁺. From a similar pattern of reaction traces (Fig. 7) a rate constant of $2 \times 10^{-3}\text{s}^{-1}$ was determined at a substrate concentration of $84\mu\text{M}$. The rate of reaction of [glyceraldehyde 3-phosphate] + [NAD⁺ + lobster enzyme] has been shown to have a similar first-order dependence on glyceraldehyde 3-phosphate concentration as in the reaction with sturgeon enzyme (Fig. 7a) (P. J. Harrigan & D. R. Trentham, unpublished work).

Implicit in the interpretation of the above experiments on NAD⁺ facilitation of the aldehyde-enzyme complex-formation is the prediction that

Fig. 8. Time-course of complex-formation between glyceraldehyde 3-phosphate and sturgeon enzyme. A is the amplitude of the slow process (see the text) and A_0 is the amplitude at zero time. A/A_0 therefore represents the fraction of unchanged enzyme. The incubation mixture contained glyceraldehyde 3-phosphate (0.42mM) and sturgeon enzyme (1.1mg/ml) in EDTA (180mM) at a final pH of 5.4. This solution was mixed in the stopped-flow apparatus at various times with an equal volume of NAD⁺ (5.6mM), glyceraldehyde 3-phosphate (0.84mM) and EDTA (200mM) also at pH 5.4. A was determined from the spectrophotometric record at 340nm . Extrapolation of the line of best fit to the ordinate axis gave A_0 .

the Racker band will disappear at the same rate as NADH is produced. Above 400nm the Racker band absorbs more strongly than NADH (Chance, 1954), although the extinction coefficient is small. A comparison of the rates of extinction change at 410nm and 340nm during the reaction of [glyceraldehyde 3-phosphate] + [NAD⁺ + lobster enzyme] showed that the Racker band disappeared concomitantly with NADH production (Fig. 9).

The functional identity of the catalytic activity of lobster enzyme and sturgeon enzyme was clear in all experiments where direct comparison was made. In the work described here this identity is apparent in the following experiments: (a) the reaction of glyceraldehyde 3-phosphate with NAD⁺-bound enzyme (Fig. 7a); (b) the reductive dephosphorylation of 1,3-diphosphoglycerate at pH 5.2; (c) the similar specific activities of the enzymes when

Fig. 9. Spectrophotometric record of the reaction between lobster enzyme, glyceraldehyde 3-phosphate and NAD^+ . The reaction medium contained enzyme (3.03 mg/ml), NAD^+ (4.0 mM), glyceraldehyde 3-phosphate (1.59 mM), 2-mercaptoethanol (0.1 mM) and EDTA (160 mM) at a final pH of 5.1. One syringe contained enzyme, NAD^+ and 2-mercaptoethanol and the other glyceraldehyde 3-phosphate. EDTA was in both syringes. The lower trace shows a spectrophotometric record at 340 nm and the horizontal trace just above it a record of the end of the reaction. The upper trace shows a spectrophotometric record at 410 nm.

catalysing the oxidative phosphorylation of glyceraldehyde 3-phosphate. Where differences did arise during the course of experiments it could be traced to the fact that the sturgeon apoenzyme is considerably less stable than the lobster holoenzyme.

DISCUSSION

The validity of using two sources of enzyme to decide the preferred pathway of the mechanism of glyceraldehyde 3-phosphate dehydrogenase must be established. The function identity of sturgeon enzyme and lobster enzyme is apparent from the transient and steady-state studies. In recent years the constant specific activities of new sources of pure enzyme has born out the earlier comparative studies of Allison & Kaplan (1964). Most important, however, for characterization of the nature of NAD^+ facilitation is the fact that sturgeon enzyme is fully active within 3 ms of mixing with NAD^+ . This is in contrast with the slow initial turnover of yeast enzyme at 40°C (Kirschner, 1967) but analogous to the behaviour of NAD^+ -bound lobster enzyme. Therefore NAD^+ facilitation (e.g. Figs. 4a and 4b) is not due to an NAD^+ -induced conformation change analogous to the T to R transformation of the yeast enzyme which must occur before that enzyme is active as a catalyst. The rates of NAD^+ dissociation from the sturgeon and lobster enzymes are more similar than might be anticipated from their difference in crystallization properties (P. J. Harrigan & D. R. Trentham, unpublished work).

Further, the NAD^+ dissociation rate from the yeast enzyme, which has a relatively large NAD^+ dissociation constant, is only 30 s^{-1} at pH 7.5 and 20°C, suggesting that this rate is fairly low for all sources of the enzyme near neutrality (Kirschner & Schuster, 1969).

At both pH 5 and 8 NAD^+ accelerated the rate of acyl-enzyme formation. At pH 8 the rate of acyl-enzyme formation, in the absence of NAD^+ , is slower than the catalytic-centre activity of glyceraldehyde 3-phosphate dehydrogenase. At pH 5 virtually no burst phase is apparent in the reaction $[\text{1,3-diphosphoglycerate} + \text{NADH}] + [\text{sturgeon enzyme}]$ although there is a burst phase in the reaction $[\text{NADH}] + [\text{1,3-diphosphoglycerate} + \text{sturgeon acyl-enzyme}]$. This shows that the rate of acyl-enzyme formation at pH 5 in the absence of NAD^+ only approaches a minimal amount of catalytic-centre activity of enzyme, which is in general much greater as NAD^+ is produced. During the reductive dephosphorylation of 1,3-diphosphoglycerate, NAD^+ -facilitated acyl-enzyme formation is the preferred reaction pathway of the enzyme mechanism.

The reaction of glyceraldehyde 3-phosphate with the apoenzyme is remarkably slow at pH 5.4. Since the unhydrated aldehyde only represents 4% of the total glyceraldehyde 3-phosphate in solution, the second-order rate constant of the reaction between free aldehyde and the apoenzyme is $110\text{ M}^{-1}\cdot\text{s}^{-1}$. The second-order rate constant for the reaction of a typical thiol of pK_a 9 and an aldehyde to form a hemithioacetal would be $120\text{ M}^{-1}\cdot\text{s}^{-1}$ at pH 5.4 (Barnett & Jencks, 1969). Although this rate agreement is not strong evidence for hemithioacetal formation, it does provide a rationale for the observed slow reaction. Smith (1966) noted that the fluorescence change, when glyceraldehyde 3-phosphate was added to NADH -bound enzyme, took up to 5 min to develop at pH 6. This suggests that he was following the time-course of the same aldehyde-enzyme complex-formation, which is not therefore catalysed by NADH .

When glyceraldehyde 3-phosphate reacts with the holoenzyme the Racker band disappears concomitantly with NADH production. This indicates that the rate of the observed process does correspond to the rate of aldehyde- NAD^+ -enzyme complex-formation (Fig. 9). Lack of correlation between the rate of Racker-band disappearance and NADH production has been observed by following the reaction of glyceraldehyde with yeast enzyme in the presence of limiting NAD^+ (Chance, 1954). This probably reflects that the percentage of NAD^+ -bound enzyme sites is constant over the early stage of the reaction, as might be expected from the results of Koshland, Cook & Cornish-Bowden (1969).

The catalytic-centre activity of lobster enzyme at pH 5.4 under similar conditions to those described here is 1 s^{-1} and the K_m of glyceraldehyde 3-phosphate is 10^{-4} M or less (Trentham, 1971, Fig. 1). Therefore the NAD^+ -facilitated reaction whose rate is 65 s^{-1} in the presence of 1.59 mM -glyceraldehyde 3-phosphate (Fig. 9) is on the preferred pathway of the enzyme mechanism at pH 5.1. The same conclusion holds at pH 7.2.

Since acyl-enzyme formation is facilitated by NAD^+ and the dissociation constant of NAD^+ is larger to the acyl enzyme than to the apoenzyme (Trentham, 1968), it follows that phosphorolysis of the acyl-enzyme is facilitated by NAD^+ because the product of the equilibrium constants of eqn. (2) and eqn. (7) equals the product of the equilibrium constants of eqn. (4) and eqn. (6) (Scheme 1). This is borne out by observations of NAD^+ requirement for $[^{32}\text{P}]$ phosphate exchange with acetyl phosphate catalysed by glyceraldehyde 3-phosphate dehydrogenase (Harting & Velick, 1954).

By a similar argument it follows that release of glyceraldehyde 3-phosphate from the aldehyde-enzyme complex is facilitated by NAD^+ . The experiments that show that NAD^+ must be bound to the enzyme when glyceraldehyde 3-phosphate release is rate determining in the reductive dephosphorylation of 1,3-diphosphoglycerate support this conclusion.

In the oxidative phosphorylation of glyceraldehyde 3-phosphate in the pH range 5–7.2 and the reverse reaction in the pH range 5–8, the preferred reaction pathway of the enzyme mechanism is:

In addition E^{Ald} and possibly $\text{E}^{\text{Ald}}_{\text{NADH}}$ are important dead-end complexes. It is noteworthy that, in the above pH ranges, enzyme sites that are not modified by substrates have little significance as intermediates.

Various experiments with lobster enzyme whose stoichiometry is well defined show that all four sites of the tetrameric enzyme are active simultaneously apparently with equal reactivity (Figs. 1, 2a and 5).

The change of NAD^+ dissociation constant after reaction of the essential thiol requires an evaluation as to its effect on the catalytic properties of glyceraldehyde 3-phosphate dehydrogenase. The free energy of hydrolysis of 1,3-diphosphoglycerate at pH 7 is $-14\text{ kcal}\cdot\text{mol}^{-1}$ (Bücher, 1947) and of a

typical thiol ester $-8\text{ kcal}\cdot\text{mol}^{-1}$ (Jencks, Cordes & Carriuolo, 1960). This means that in the absence of other factors the free-energy change associated with phosphorolysis of the acyl-enzyme eqn. 2 (in Scheme 1) would be about $6\text{ kcal}\cdot\text{mol}^{-1}$. However, a concomitant NAD^+ dissociation-constant change by a factor of 10^4 is equivalent to a free-energy change of $6\text{ kcal}\cdot\text{mol}^{-1}$. Thus the overall positive free-energy change associated with phosphorolysis in eqn. (4) (Scheme 1) is decreased by the change of NAD^+ dissociation constant. Since a large positive free-energy change necessarily implies a large activation energy and thus a slow rate for the process, there is an advantage in the large differences in NAD^+ dissociation constants that have evolved. In addition this illustrates an important biological method of transferring the free-energy gain of a chemical reaction to another energy-requiring process. The implications are wide especially in areas where the dynamics of macromolecules are linked to reactions such as ATP hydrolysis.

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