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

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The kinetics of the acylation of D-glyceraldehyde 3-phosphate dehydrogenase from pig muscle by 1,3-diphosphoglycerate in the presence of NAD^+ has been analysed by using the relaxation temperature-jump method. At pH7.2 and 8°C the rate of acylation of the NAD^+-bound (or holo-) enzyme was $3.3\times10^5 M^{-1}\cdot s^{-1}$ and the rate of phosphorolysis, the reverse reaction, was $7.5\times10^3 M^{-1}\cdot s^{-1}$. After a temperature-jump perturbation the equilibrium of NAD^+ binding to the acyl-enzyme was re-established more rapidly than that of the acylation. The rate of phosphorolysis of the apoacylenzyme from sturgeon muscle and of aldehyde release from the D-glyceraldehyde 3-phosphate–apoenzyme complex were $\leqslant 40 M^{-1}\cdot s^{-1}$ and $\leqslant 12 s^{-1}$ respectively at pH8.0 and 22°C, which means that both processes are too slow to contribute significantly to the reaction pathway of the reversible NAD^+-linked oxidative phosphorylation of D-glyceraldehyde 3-phosphate. Phosphorolysis of both acyl-apoenzyme and acyl-holoenzyme was first-order in P_i up to 100mM-P_i and more. PO_4^{3-} could be the reactive species of the phosphorolysis of the acyl-holoenzyme, in which case phosphorolysis is a diffusion-controlled reaction, although other kinetically indistinguishable rate equations for the reaction are possible.

The role of NAD^+ in facilitating the addition of the substrates 1,3-diphosphoglycerate and D-glyceraldehyde 3-phosphate to D-glyceraldehyde 3-phosphate dehydrogenase has been established (Trentham, 1971*a*, and references cited therein). The effect of NAD^+ on the reverse reactions is an important counterpart. The work now reported describes various kinetic aspects of the reversible phosphorolysis with the lobster, pig and sturgeon muscle D-glyceraldehyde 3-phosphate dehydrogenases. The sturgeon enzyme, which has the particular advantage of crystallizing as the apoenzyme (Allison & Kaplan, 1964; Trentham, 1971*a*), was also used to study the kinetics of D-glyceraldehyde 3-phosphate release from the apoenzyme.

The experiments reported in the present paper are designed to test further the hypothesis that during the enzyme-catalysed reversible oxidative phosphorylation of D-glyceraldehyde 3-phosphate it is necessary for NAD^+ to be bound to the enzyme for any reaction involving cysteine-149 with D-glyceraldehyde 3-phosphate or 1,3-diphosphoglycerate (eqn. 1):

$$E_{NAD^+} + \text{aldehyde} \rightleftharpoons E^{Ald}_{NAD^+} \rightleftharpoons E^{Acyl}_{NADH} \rightleftharpoons E^{Acyl} + NADH$$

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$$E^{Acyl} + NAD^+ \rightleftharpoons E^{Acyl}_{NAD^+} + P_i \rightleftharpoons E_{NAD^+} + \text{acyl phosphate} \quad (1)$$

The kinetics of the reversible phosphorolysis of the acyl-enzyme can be studied by the temperature-jump technique by measuring the rate of the extinction change at 365nm of the 'Racker' band (Racker & Krimsky, 1952; Velick, 1953) as the concentration of the holoenzyme changes. The stability of the acyl-enzyme of D-glyceraldehyde 3-phosphate dehydrogenase is an important factor in such studies, since the acyl-enzyme participates in two side reactions: hydrolysis in which water replaces P_i as the acyl group acceptor, and transfer of the acyl group from the active-centre thiol, cysteine-149, to lysine-183, generating an inactive enzyme (Park *et al.*, 1966; Polgar, 1966; Davidson, 1970). Both these side reactions accelerate at high pH, and the stability of the acyl-enzyme has to be measured as a necessary preliminary to the temperature-jump relaxation experiments.

Since modification of cysteine-149 generally results in an increase in the dissociation constant of NAD^+ to the enzyme, phosphorolysis is likely to be a two-step process (eqn. 2):

$$E_{NAD^+} + \text{acyl phosphate} \underset{}{\overset{K_1}{\rightleftharpoons}} E^{Acyl}_{NAD^+} + P_i \overset{K_2}{\rightleftharpoons} E^{Acyl} + NAD^+ \quad (2)$$

where

$$K_1 = \frac{k_{+1}}{k_{-1}} = \frac{[E^{Acyl}_{NAD^+}][P_i]}{[E_{NAD^+}][\text{acyl phospate}]} \text{ and}$$

$$K_2 = \frac{k_{+2}}{k_{-2}} = \frac{[E^{Acyl}][NAD^+]}{[E^{Acyl}_{NAD^+}]}$$

K_1 can be determined from spectroscopic measurements of E_{NAD^+} concentration. K_2 has not yet been measured directly, but it can be evaluated indirectly from steady-state kinetic measurements.

Phosphorolysis of the acyl-apoenzyme is easier to study since its half-life is relatively long (Harrigan & Trentham, 1971). However, there is no chromophoric change analogous to that of the Racker band. The reaction can be followed spectrophotometrically by linkage to a second process involving a rapid chromophoric change. Cysteine-149 is liberated during phosphorolysis, and so its production can be followed by using chromophoric reagents that react rapidly with thiol groups. A similar method can be used to determine the rate of hydrolysis of the acyl-enzyme in the presence of NAD^+ and the rate of release of D-glyceraldehyde 3-phosphate from the apoenzyme.

Since an underlying theme of this work is to understand the mechanism of D-glyceraldehyde 3-phosphate dehydrogenase under near-physiological conditions, the specific activity, and hence catalytic-centre activity, of the enzyme was determined in conditions close to those used to study the above reactions of the enzyme. In this way the requirement or otherwise of NAD^+ for various steps in the mechanism can be established.

Materials and Methods

Enzyme

D-Glyceraldehyde 3-phosphate dehydrogenases from pig, lobster and sturgeon muscle were isolated, crystallized, characterized and found to have similar properties to those described previously (Trentham, 1971*a*; Harrigan & Trentham, 1973). On the basis of the E_{276}/E_{260} ratio the pig and lobster enzymes were found to contain 4mol of NAD^+/mol of tetramer initially in all experiments. The crystalline sturgeon enzyme contained no bound NAD^+. Before experiments involving the use of chromophoric organomercurials enzyme solutions were treated with 1mM-2-mercaptoethanol in 5mM-EDTA adjusted to pH7.0 with NaOH and then chromatographed through fine-grade Sephadex G-25 with the 5mM-EDTA buffer as eluting solvent at pH7 and 5°C. Otherwise enzyme crystals were washed in a mixture of 80%-saturated $(NH_4)_2SO_4$, 1mM-2-mercaptoethanol and the 5mM-EDTA buffer at pH7. After centrifugation the supernatant was discarded and the enzyme was dissolved in the appropriate solvent.

On the basis of a subunit molecular weight of 36000 and the observation that all the active sites are simultaneously active (Trentham, 1971*b*), the catalytic-centre activity (s^{-1}) was calculated from the specific activity (μmol of product formed/min per mg of enzyme) by multiplying the latter by the conversion factor 0.6. Allowance for any inactive enzyme present was not made in the present work. (When this is done, the conversion factor should be divided by the fraction of active enzyme: 0.7–1 for the pig and sturgeon enzymes, 1 for the lobster enzyme.)

Acyl-enzyme preparation

Pig acyl-enzyme was prepared at 5°C by mixing 1,3-diphosphoglycerate (final concentration 1.3mM) and pig D-glyceraldehyde 3-phosphate dehydrogenase (final concentration 6mg/ml) in a total of 10ml of 1mM-2-mercaptoethanol in the 5mM-EDTA buffer, pH7.0. The resulting solution was eluted through coarse-grade Sephadex G-25 with 5mM-EDTA adjusted to pH6.5 with NaOH. This procedure removed excess of 1,3-diphosphoglycerate, 2-mercaptoethanol and most of the NAD^+ originally bound to the enzyme. The resulting acyl-enzyme was stored at 5°C and was stable for up to 3 h. The extent of acylation of the enzyme was checked in the stopped-flow spectrophotometer by rapid mixing of the acyl-enzyme with excess of NADH at 22°C in 2mM-EDTA–0.1M-triethanolamine adjusted to pH7.9 with HCl, when NAD^+ formation was observed in a clean exponential process at 340nm. The extent of the acylation of the enzyme was greater than 90%. The acyl-enzyme concentration was determined from $E^{1\%}_{280} = 10.0$ (Cseke & Boross, 1970) for the holoenzyme, corrected for loss of nucleotide, which was measured from the E_{276}/E_{260} ratio (McMurray & Trentham, 1969). Essentially complete acylation was substantiated by the absence of any available cysteine-149 for reaction with the chromophoric organomercurial (see the Results section).

When there was no need to remove NAD^+ or 1,3-diphosphoglycerate from the acyl-enzyme, it was prepared by direct addition of 1,3-diphosphoglycerate to the holoenzyme.

Substrates

D-Glyceraldehyde 3-phosphate and 1,3-diphosphoglycerate were prepared and assayed as described previously (Trentham, 1971*a*). NAD^+ and NADH were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). For experiments

in which the concentration of NAD^+ was above 2mM it was further purified, essentially as described by Stinson & Holbrook (1973).

Reagents

2 - Chloromercuri - 4 - nitrophenol (E_{410} = $1.7\times10^4 M^{-1}\cdot cm^{-1}$ in 0.1M-NaOH) and 2-chloromercuri-4,6-dinitrophenol were prepared as described by McMurray & Trentham (1969). All other reagents were analytical grade and used without further purification. Water was glass-distilled.

Spectrophotometric and kinetic measurements

Steady-state assays were carried out in the stopped-flow apparatus where indicated or in a 1 cm-light-path thermostatically controlled cuvette of a Uvispek H700 (Hilger and Watts) spectrophotometer fitted with a Hilford recording attachment and a Servoscribe log recorder. Time-independent absorption measurements were made in a Zeiss PMQII Spectrophotometer. Stopped-flow measurements were made at 21±2°C as described previously (Harrigan & Trentham, 1971). The temperature-jump apparatus was designed by Professor L. C. De Maeyer and built by Messanlagen G.m.b.H (Göttingen, Germany). This equipment and the theoretical concept of relaxation techniques have been described by Eigen (1968). The initial temperature was maintained by circulating water at constant temperature through the block housing of the reaction cell, which had 5.5ml capacity and 1cm optical path length. Ice-cold water maintained the cell at 5°C and after the capacitor discharge the temperature rose momentarily so that relaxation processes were observed at 8°C. The signal was recorded on a storage oscilloscope, which was triggered simultaneously with the 20kV discharge of the capacitor.

Results

The stability of the acyl-enzyme in the presence of NAD^+ had to be established to determine appropriate conditions for the temperature-jump experiments. The stability of the acyl-enzyme to hydrolysis was measured by a back-titration with the chromophoric organomercurial 2 - chloromercuri - 4 - nitrophenol (RHgCl), since hydrolysis results in the release of cysteine-149, which reacts rapidly with the chromophore (eqn. 3).

$$E^{Acyl}_{NAD^+} + H_2O \rightarrow E_{NAD^+} + 3\ \text{phosphoglycerate} \xrightarrow{RHgCl} E^{HgR}_{NAD^+} \quad (3)$$

Pig D-glyceraldehyde 3-phosphate dehydrogenase reacts with the organomercurial in a monophasic manner with a similar second-order rate constant to that found for the more rapid process of the reaction of organomercurial and lobster holoenzyme (Harrigan & Trentham, 1971). The reaction rate was unaffected by variation of NAD^+ concentration. However, when the acyl-enzyme was mixed with the organomercurial this relatively fast phase was absent. In its place was a much slower phase that seemed to be directly linked to breakdown of the acyl-enzyme. The back-

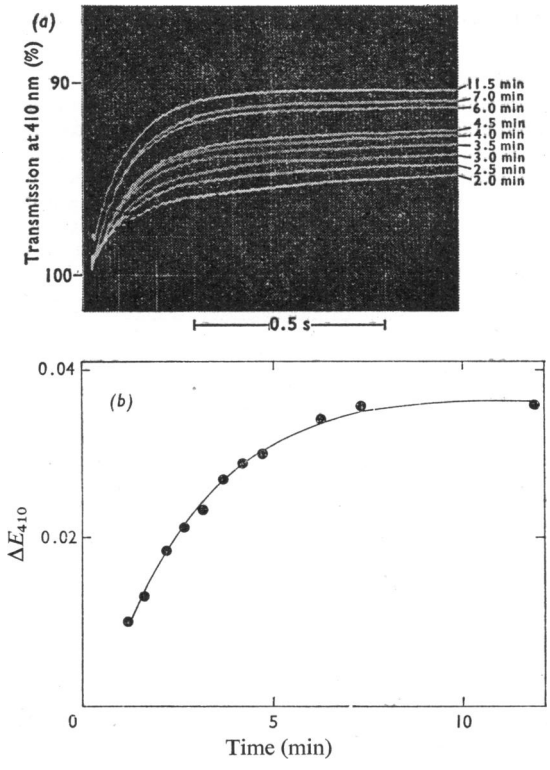

Fig. 1. *Titration of the available thiol groups of the pig enzyme with 2-chloromercuri-4-nitrophenol to monitor the hydrolysis of acyl-enzyme in the presence of NAD^+*

(*a*) Stopped-flow traces recorded at 410nm. The reaction mixture contained (final concentrations) acyl-enzyme (0.20mg/ml; 5.6μM sites), 2-chloromercuri-4-nitrophenol (15μM), NAD^+ (1.15mM), EDTA (2mM) and triethanolamine hydrochloride (0.10M), adjusted to pH7.9 with NaOH. One syringe contained the acyl-enzyme and NAD^+ and the other organomercurial. Triethanolamine hydrochloride and EDTA were in both syringes. The age in minutes of the acyl-enzyme solution when mixed with the organomercurial is indicated. (*b*) Variation of the extinction change in the transient phase in (*a*) with time. The difference between the extinction change at 11.5min and shorter times was taken as proportional to [acyl-enzyme]. From the exponential rate of decay of [acyl-enzyme] its half-life was 1.7min.

Table 1. *Stability of the pig acyl-enzyme with respect to acyl group transfer to lysine*-183

Each sample contained (final concentrations) pig D-glyceraldehyde 3-phosphate dehydrogenase (1.1mg/ml), 1,3-diphosphoglycerate (as indicated), 2-mercaptoethanol (0.5mM), EDTA (0.2mM) and ethylenediamine hydrochloride (0.10M), adjusted to pH8.0 at room temperature. Specific activity relative to the initial value was measured spectrophotometrically at pH8.7 by the method described by Trentham (1971*a*). After the 20h incubation all the 1,3-diphosphoglycerate had been hydrolysed.

Temp. (°C)	[1,3-Diphosphoglycerate] (μM)	Relative specific activity
20	0	0.77
20	146	0.90
0	0	0.92
0	146	0.90

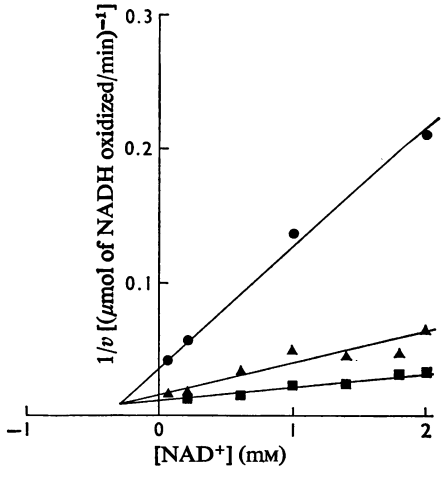

Fig. 2. *Inhibition of NADH oxidation by NAD⁺ at pH*8.0 *and* 25°C

The concentration of 1,3-diphosphoglycerate was fixed at 73μM, and NAD^+ was varied over a series of fixed NADH concentrations; ●, 3.9μM-NADH; ▲, 15.6μM-NADH; ■, 39μM-NADH. In addition the reaction medium contained the pig enzyme, 50μM-2-mercaptoethanol, 0.2mM-EDTA and 0.10M-ethylenediamine hydrochloride, adjusted to pH8.0 with NaOH.

titration was carried out by incubation of the acyl-enzyme at the required temperature and then mixing at various times with the organomercurial in the stopped-flow apparatus (Fig. 1*a*). From these experiments the acyl-enzyme in the presence of 1.15mM-NAD^+ was shown to have a half-life of 1.7min at pH7.9 and 21°C (Fig. 1*b*), 17min at pH7.0 and 21°C and 14min at pH7.9 and 3°C. Satisfactory conditions for the temperature-jump studies were therefore chosen at pH7.2 and 8°C.

No interference arising from acyl group transfer from cysteine-149 to lysine 183 was apparent in the temperature-jump experiments described below. Since Park *et al.* (1966) have shown that the *N*-acylated enzyme is inactive, it was possible to check whether this reaction occurred to any significant extent under conditions more favourable for transfer than in the temperature-jump experiments. Table 1 shows that enzyme incubated with 1,3-diphosphoglycerate had no greater loss of specific activity during a 20h incubation than had holoenzyme.

As a further preliminary to the relaxation studies it was desirable to measure K_1 and K_2 (eqn. 1). K_2, the dissociation constant of NAD^+ from the acyl-enzyme, was evaluated indirectly from K_i, the competitive-inhibition constant of NAD^+ with respect to NADH. K_2 is likely to equal K_i because nicotinamide nucleotide exchange probably occurs on the acyl-enzyme during the overall enzyme-catalysed reaction (Harrigan & Trentham, 1973). K_i was 300μM when measured by the method of Furfine & Velick (1965), both with 200μM-1,3-diphosphoglycerate and the solvent described in Fig. 3 at 5°C and also at pH8 and 25°C (Fig. 2). This value of 300μM was taken for K_2. There is a requirement for low concentrations of NAD^+ to facilitate the reductive dephosphorylation of 1,3-diphosphoglycerate (Trentham, 1971*a*; Smith & Velick, 1972). However, Fig. 2 demonstrates that above a certain concentration of NAD^+ it behaves as a typical competitive inhibitor and, if eqn. (1) is correct, this is because of direct competition with NADH for the acylenzyme. Smith & Velick (1972) have shown that with the rabbit enzyme in the presence of 3μM-NADH the changeover from activation to inhibition occurs at about 50μM-NAD^+ at pH7.4. Since the extinction at 365nm of the holoenzyme is decreased on acylation, the equilibrium constant K_1 could be determined spectrophotometrically. The extinction of a 7.2mg/ml enzyme solution was measured at various 1,3-diphosphoglycerate concentrations for a series of different P_i concentrations at pH7.0 and 5°C, in the presence of 5mM-NAD^+ to prevent dissociation of NAD^+ from the acyl-enzyme. The mean value of K_1 was 44 (S.E.M. was 4).

Temperature-jump studies

The general relation between the relaxation times and rate constants of eqn. (2) is described by eqn. (4), where the two values of λ obtained from solving the quadratic equation equal the reciprocal relaxation times:

$$\lambda^2-\lambda(\alpha+\beta)+\alpha\beta=k_{-1}k_{+2}[P_i] \quad (4)$$

where

$$\alpha = k_{+1}([E_{NAD^+}] + [\text{acyl phosphate}]) + k_{-1}([E^{Acyl}_{NAD^+}] + [P_i])$$

and

$$\beta = k_{+2} + k_{-2}([E^{Acyl}] + [NAD^+])$$

If acylation is characterized by a reciprocal relaxation time $1/\tau_1$, and the NAD^+-binding process to the acyl-enzyme by $1/\tau_2$, two limiting conditions described by eqn. (2) are $1/\tau_1 \ll 1/\tau_2$ and $1/\tau_1 \gg 1/\tau_2$.

For $1/\tau_1 \ll 1/\tau_2$ and when both $[P_i]$ and $[NAD^+] \gg [E]_0$, where $[E]_0$ = the total enzyme active-site concentration:

$$1/\tau_2 = k_{+2} + k_{-2}[NAD^+] \quad (5)$$

$$1/\tau_1 = k_{+1}([E_{NAD^+}] + [\text{acyl phosphate}]) + k_{-1}[P_i]\left(1 + \frac{K_2}{[NAD^+]}\right)^{-1} \quad (6)$$

Any extinction change at 365nm will be associated with acylation (Harrigan & Trentham, 1973). The condition $1/\tau_1 \ll 1/\tau_2$ means that acylation is the slower process, so that only one relaxation will be observed at 365nm.

For $1/\tau_1 \gg 1/\tau_2$:

$$1/\tau_1 = k_{+1}([E_{NAD^+}] + [\text{acyl phosphate}]) + k_{-1}[P_i] \quad (7)$$

$$1/\tau_2 = k_{+2}\left(1 + \frac{[P_i]}{K_1([E_{NAD^+}] + [\text{acyl phosphate}]) + [E^{Acyl}_{NAD^+}]}\right)^{-1} + k_{-2}[NAD^+] \quad (8)$$

In this case the faster process, which is associated with the spectral change, will re-equilibrate as the slow process relaxes, so that two relaxation processes should be observed. Two relaxations should also be observed for the condition $1/\tau_1 \approx 1/\tau_2$.

When the equilibrium (eqn. 2) was established, one and only one relaxation process was observed at pH7.2 and 8°C (e.g. Fig. 3). No relaxation process was observed if 1,3-diphosphoglycerate or P_i was omitted from the solution. Similarly no relaxation processes were observed in a system containing pig D-glyceraldehyde 3-phosphate dehydrogenase (9.0mg/ml), 1,3-diphosphoglycerate (620μM) and imidazole (50mM) at pH7.2 and 8°C or when the NAD^+ concentration was increased to 0.97mM in this solution.

These results are consistent with eqn. (2) and with the limiting condition $1/\tau_1 \ll 1/\tau_2$, which leads to eqns. (5) and (6). To test this model further, the dependence of the relaxation time on concentrations

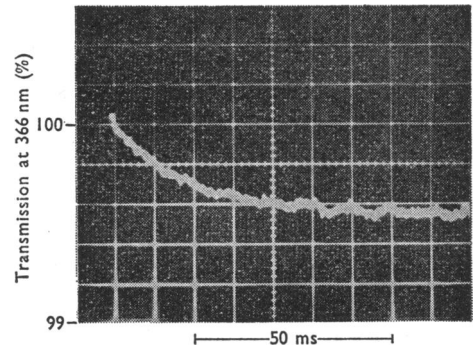

Fig. 3. *Relaxation process observed on temperature-jump perturbation of the acylation/phosphorolysis equilibrium of pig* D-*glyceraldehyde 3-phosphate dehydrogenase measured at 366nm*

The reaction chamber contained (final concentrations) enzyme (4.0mg/ml; 110μM sites), P_i (4.8mM), NAD^+ (2.1mM), 1,3-diphosphoglycerate (210μM), 2-mercaptoethanol (0.2mM), EDTA (0.4mM) and imidazole (67mM) at pH7.2 and 8°C. (The pH was measured at 20°C and then estimated to be pH7.2 at 8°C from a temperature–pH calibration curve of a 67mM-imidazole, 9.5mM-P_i solution whose pH was adjusted to 7.0 at 20°C with 1M-HCl.)

of reaction components was measured. Fig. 4 shows that, within the experimental sensitivity, the reciprocal relaxation time increased linearly with $([E_{NAD^+}] + [\text{acyl phosphate}])$ at effectively constant $[NAD^+]$ and $[P_i]$. From eqn. (6) the slope of Fig. 4 gives $k_{+1} = 3.3 \times 10^5 M^{-1} \cdot s^{-1}$ and the intercept $k_{-1}[P_i](1 + K_2/[NAD^+])^{-1}$, from which $k_{-1} = 7.5 \times 10^3 M^{-1} \cdot s^{-1}$. The line through the experimental points of Fig. 4 was drawn to satisfy the condition $k_{+1}/k_{-1} = K_1 = 44$, the value obtained in the equilibrium studies. Reciprocal relaxation times were also measured in a mixture containing enzyme (4.0mg/ml; 110μM sites), 1,3-diphosphoglycerate (220μM), NAD^+ (2.3mM), imidazole (50mM) and various P_i concentrations at pH7.0. Under these conditions both multiplicands of k_{+1} and k_{-1} in eqn. (6) will vary, leading to a complex relationship between $[P_i]$ and $1/\tau_1$. However, it was possible to measure reciprocal relaxation times over a 17-fold range of $[P_i]$, the lowest being at 1mM-P_i, when it was found that the intercept of the graph of the reciprocal relaxation time and $[P_i]$ intercepted the abscissa (i.e. $[P_i] = 0$) at $40 s^{-1}$. From eqn. (6) this gives

$$1/\tau_1 = k_{+1}([E_{NAD^+}] + [\text{acyl phosphate}]) = k_{+1}(0 + 1.1 \times 10^{-4})s^{-1}$$

and so $k_{+1} = 3.6 \times 10^5 M^{-1} \cdot s^{-1}$, in agreement with the analysis of Fig. 4.

Increasing the added NAD^+ concentration 11-fold from 0.45mM to 5.0mM in a reaction medium similar to that for the experiment described in Fig. 4 with 220μM-1,3-diphosphoglycerate had no measurable effect on the reciprocal relaxation time. According to eqn. (6), after insertion of the appropriate numbers, $1/\tau_1$ should increase by 10% when the NAD^+ concentration is increased, but such a small change is within the experimental sensitivity. Moreover, the concomitant increase in ionic strength would tend to decrease k_{+1} and k_{-1}, counterbalancing any increase in $1/\tau_1$ arising from an increasing multiplicand of k_{-1} (Harrigan & Trentham, 1973).

With 200μM-1,3-diphosphoglycerate and 120μM-NADH as substrates in 50mM-imidazole at pH7.2 and 5°C the specific activity of the enzyme was 34μmol of NAD^+ produced/min per mg of enzyme. With 1.0mM-D-glyceraldehyde 3-phosphate, 0.4mM-NAD^+ and 40mM-P_i as substrates it was 24μmol of NADH produced/min per mg of enzyme. These values correspond to catalytic-centre activities of $20 s^{-1}$ and $14 s^{-1}$ respectively if all four sites per tetramer are simultaneously active.

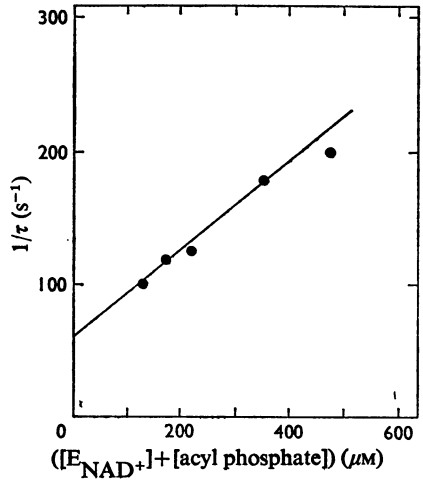

Fig. 4. *Rate of relaxation of the perturbed acylation/phosphorolysis equilibrium as a function of the sum of the equilibrium concentrations of enzyme and* 1,3-*diphosphoglycerate*

Equilibrium concentrations were calculated from the measured equilibrium constant, K_1, of 44. The reaction chamber contained (final concentrations) enzyme (4.0mg/ml; 110μM sites), P_i (9.5mM), NAD^+ (1.7mM), 1,3-diphosphoglycerate, 2-mercaptoethanol (0.2mM), EDTA (0.4mM) and imidazole (60mM) at pH7.2 and 8°C. (pH was measured as described in the legend to Fig. 3.)

Phosphorolysis of the acyl-enzyme

The rate of phosphorolysis of the sturgeon acyl-apoenzyme was determined by measuring the rate of production of active-centre thiol, cysteine-149, by following its reaction with the chromophoric organomercurial 2-chloromercuri-4-nitrophenol. The acyl-apoenzyme does not react with the chromophoric organomercurial. The apoenzyme reacts with a second-order rate constant of $1.4 \times 10^6 M^{-1} \cdot s^{-1}$ at pH7.9 and 20°C (Harrigan & Trentham, 1971), and it was found that this rate constant was unaffected by the presence of 10mM-P_i in the reaction medium.

$$E^{Acyl} + P_i \underset{}{\overset{k}{\rightleftharpoons}} E + \text{acyl phosphate} \xrightarrow{RHgCl} E^{HgR} \quad (9)$$

Eqn. (9) describes the overall reaction scheme. According to this scheme, when the organomercurial, RHgCl, reacts with the acyl-enzyme the observed rate of the spectral change will equal the pseudo-first-order phosphorolysis rate, $k[P_i]$, provided that two conditions are fulfilled. First, the amplitude of the spectral change must be the same as for the reaction between apoenzyme and organomercurial; secondly, the rate of the spectral change must tend to a plateau at constant $[P_i]$ as the organomercurial concentration is increased but increase linearly with $[P_i]$ at constant concentration of organomercurial. The kinetics of such displacement reactions are described in more detail by Harrigan & Trentham (1971).

At pH7.9 and 20°C the half-time for hydrolysis of sturgeon acyl-apoenzyme was 40min (Harrigan & Trentham, 1971), and so the acyl-enzyme was stable over the time of the experiments (in general less than 5min). In addition, the concentration of 1,3-diphosphoglycerate was maintained in excess over enzyme sites to eliminate interference through hydrolysis of the acyl-enzyme.

When sturgeon apoenzyme (0.32mg/ml; 8.9μM sites) was acylated by 20μM-1,3-diphosphoglycerate, no spectral change occurred at 410nm on mixing the product with organomercurial (final concentration 53μM). However, when P_i was added to the reaction medium a spectral change did occur whose amplitude was the same as that observed when no 1,3-diphosphoglycerate was added but whose rate, $k_{obs.}$, was about two orders of magnitude lower, depending on $[P_i]$ (Fig. 5 and Table 2). This suggests that $k_{obs.} = k[P_i]$. $k_{obs.}$ increased sixfold with a tenfold increase in $[P_i]$. It is likely that the increase in ionic strength caused a decrease in the value of k at high $[P_i]$ (Harrigan & Trentham, 1973), though it may indicate that P_i and the acyl-enzyme are forming a Michaelis complex of low affinity. If $[P_i]$ was held constant and the organomercurial concentration was increased, the observed rate tended to a plateau (Table 3), suggesting that $0.4 s^{-1}$ is the phosphorolysis

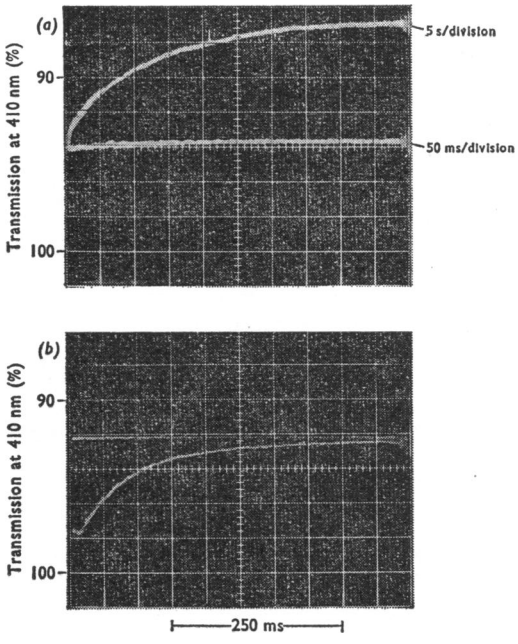

Fig. 5. *Reaction between sturgeon acyl-apoenzyme and P_i followed by measuring the rate of appearance of cysteine-149 with 11.6 μM-2-chloromercuri-4-nitrophenol at 410 nm*

(*a*) Reaction conditions are described in Table 3, with 40 μM-1,3-diphosphoglycerate and 10mM-P_i. The lower trace shows the absence of free cysteine-149 initially. (*b*) Reaction of organomercurial with sturgeon apoenzyme (0.13mg/ml; 3.55 μM sites) in the absence of 1,3-diphosphoglycerate, but otherwise in the conditions described in (*a*). The horizontal trace in (*b*) was recorded a few seconds after the mixing of the reactants and shows end-point stability.

Table 2. *First-order rate constants for the reaction of sturgeon acyl-apoenzyme and 2-chloromercuri-4-nitrophenol in the presence of various concentrations of P_i at pH 7.9 and 20°C*

Rates were measured in a stopped-flow apparatus at 410nm. The reaction mixture contained (final concentrations) sturgeon enzyme (0.32mg/ml; 8.9 μM sites) acylated by 1,3-diphosphoglycerate (20 μM, when present), organomercurial (53.5 μM), P_i (as indicated), EDTA (2mM) and triethanolamine hydrochloride (0.10M) adjusted to pH7.9 with NaOH. One syringe contained the acyl-enzyme and 1,3-diphosphoglycerate, and the other P_i and organomercurial. Triethanolamine hydrochloride and EDTA were in both syringes.

[1,3-Diphosphoglycerate] (μM)	[P_i] (mM)	$k_{obs.}$ (s^{-1})
0	0	81
20	10	0.15
20	50	0.5
20	100	0.9

Table 3. *First-order rate constants for the reaction of sturgeon acyl-apoenzyme and 2-chloromercuri-4-nitrophenol at variable concentrations in the presence of a fixed concentration of P_i at pH 7.9 and 20°C*

Rates were measured as described in Table 2. The reaction mixture contained (final concentrations) sturgeon enzyme (0.18mg/ml; 5.0 μM sites) acylated by 40 μM-1,3-diphosphoglycerate (40 μM, when present), organomercurial (as indicated), P_i (10mM), triethanolamine hydrochloride (0.1M) and EDTA (2mM). The twofold difference in rates between comparable experiments with 10mM-P_i in Tables 2 and 3 may be accounted for by catalytic amounts of NAD^+, as discussed in the text.

[1,3-Diphosphoglycerate] (μM)	[Organomercurial] (μM)	$k_{obs.}$ (s^{-1})
0	11.6	15
40	11.6	0.06
40	58	0.27
40	116	0.33
40	174	0.40

rate in the presence of 10mM-P_i, which leads to a second-order rate constant of 40 $M^{-1} \cdot s^{-1}$ for phosphorolysis. In the presence of 50mM-P_i therefore the phosphorolysis rate will be 2.0 s^{-1}, or somewhat less if P_i and the acyl-enzyme form a Michaelis complex. However, the specific activity of the enzyme at pH7.9 and 20°C in the presence of 50mM-P_i was 83 μmol of NADH produced/min per mg, which corresponds to a catalytic-centre activity of 50 s^{-1} if all four sites are active simultaneously. Phosphorolysis of the acyl-apoenzyme is therefore too slow a process to be on the catalytic pathway of D-glyceraldehyde 3-phosphate dehydrogenase, whereas the relaxation kinetic studies show that phosphorolysis of the acyl-holoenzyme is sufficiently rapid.

It should be noted that 40 $M^{-1} \cdot s^{-1}$ represents an upper limit to the true phosphorolysis rate of the acyl-apoenzyme, since the presence of catalytic amounts of NAD^+ (0.1 μM) would lead to reaction rates similar to those observed if there were a zero phosphorolysis rate for the acyl-apoenzyme. For this calculation the dissociation constant of NAD^+ from the acyl-enzyme and the phosphorolysis rate of the acyl-holoenzyme were taken as 300 μM and 10^5 $M^{-1} \cdot s^{-1}$ respectively.

Phosphorolysis of the acyl-holoenzyme has been identified as the rate-determining step of the oxidative phosphorylation of D-glyceraldehyde 3-phosphate below pH7 (Trentham, 1971*b*), so that the specific activity measured at various P_i concentrations can be used to measure the phosphorolysis rate of the acyl-holoenzyme at low pH. This was carried out at pH5.4 with the lobster enzyme, which appears to be a more stable form of the dehydrogenase at low pH values. Fig. 6 shows that, as with the kinetic studies of

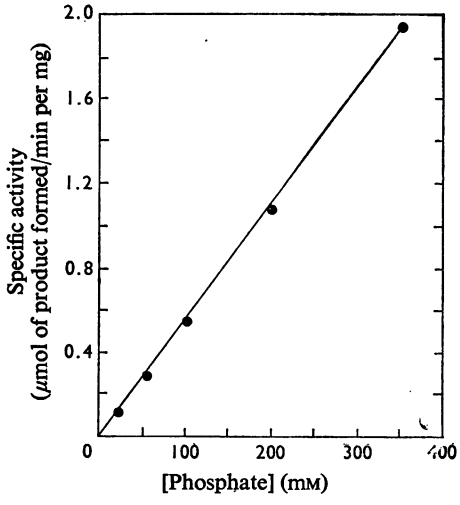

Fig. 6. *Specific activity of lobster* D-*glyceraldehyde* 3-*phosphate dehydrogenase as a function of the concentration of* P_i

Steady-state rates were measured in the stopped-flow apparatus at 340nm. The reaction mixture contained (final concentrations) the enzyme, P_i (as indicated), NAD^+ (4.6mM), D-glyceraldehyde 3-phosphate (2mM), NaCl (to final ionic strength 1.5M), 2-mercaptoethanol (30μM) and EDTA (0.12M) at pH5.4 and 22°C. One syringe contained the enzyme and NAD^+ and the other D-glyceraldehyde 3-phosphate. All other components were present in both syringes.

the phosphorolysis of the sturgeon acyl-apoenzyme there is no evidence for significant Michaelis-complex formation between P_i and the acyl-holoenzyme. The second-order rate constant for the phosphorolysis ($3.3 M^{-1} \cdot s^{-1}$, calculated after the conversion of the specific activities given in Fig. 6 into catalytic-centre activities) is much lower than that calculated at pH7 in the relaxation studies, and is a reflexion of the pH-dependence of the enzyme specific activity (Trentham, 1971*b*) and of the 30-fold increase in ionic strength (Harrigan & Trentham, 1973).

Breakdown of aldehyde–apoenzyme complex

In principle the rate of breakdown of the aldehyde–apoenzyme complex between D-glyceraldehyde 3-phosphate and sturgeon D-glyceraldehyde 3-phosphate dehydrogenase can be determined by measuring the rate of production of active-centre thiol, cysteine-149, from its reaction with the chromophoric organomercurial, just as described above in the previous section. Eqn. (10) describes the overall reaction scheme:

$$E^{Ald} \underset{}{\overset{k'}{\rightleftharpoons}} E + \text{aldehyde} \xrightarrow{RHgCl} E^{HgR} \quad (10)$$

According to this scheme, when the organomercurial reacts with the aldehyde–enzyme complex the observed rate of the spectral change will equal the breakdown rate, k', provided that two conditions are fulfilled. First, as for the phosphorolysis rate determination, the amplitude of the spectral change must be the same as for the reaction between apoenzyme and organomercurial; secondly, the rate of the spectral change must tend to a plateau as the organomercurial concentration is increased. It will not necessarily be possible to fulfil these conditions if the complex between D-glyceraldehyde 3-phosphate and the apoenzyme, E^{Ald}, is a hemithioacetal, because the rate of hemithioacetal formation is typically diffusion-controlled between thiol ion and aldehyde and the equilibrium constant does not favour hemithioacetal formation very markedly (Lienhard & Jencks, 1966; Barnett & Jencks, 1969). Nevertheless the equilibrium between D-glyceraldehyde 3-phosphate and the sturgeon apoenzyme does favour E^{Ald} formation at pH5.4 (Trentham, 1971*a*), and so the chromophoric organomercurial displacement technique could be tested in this pH region by using an organomercurial with a lower phenolic pK value. The results (Table 4) show that (1) 2-chloromercuri-4,6-dinitrophenol reacts with the apoenzyme with a second-order rate constant of $3.6 \times 10^5 M^{-1} \cdot s^{-1}$, (2) the site of organomercurial reaction was blocked by D-glyceraldehyde 3-phosphate and (3) the dissociation rate constant of the aldehyde is $0.12 s^{-1}$.

Table 4. *First-order rate constants for the reaction of the complex between* D-*glyceraldehyde* 3-*phosphate and sturgeon apoenzyme and* 2-*chloromercuri*-4,6-*dinitrophenol at pH*5.3 *and* 22°C *measured in the stopped-flow apparatus at* 410*nm*

The reaction mixture contained (final concentrations) sturgeon apoenzyme (0.16mg/ml; 4.5μM sites), organomercurial (as indicated), D-glyceraldehyde 3-phosphate (as indicated) and EDTA (200mM). One syringe contained enzyme and D-glyceraldehyde 3-phosphate when present (at least 10min preincubation was allowed before the mixing with organomercurial) and the other organomercurial. EDTA was present in both syringes.

[Aldehyde] (mM)	[Organomercurial] (μM)	$k'_{obs.}$ (s^{-1})
0	14.5	3.3
0	72.5	25
0	145	54
0.24	14.5	0.08
0.24	72.5	0.12
0.24	145	0.12

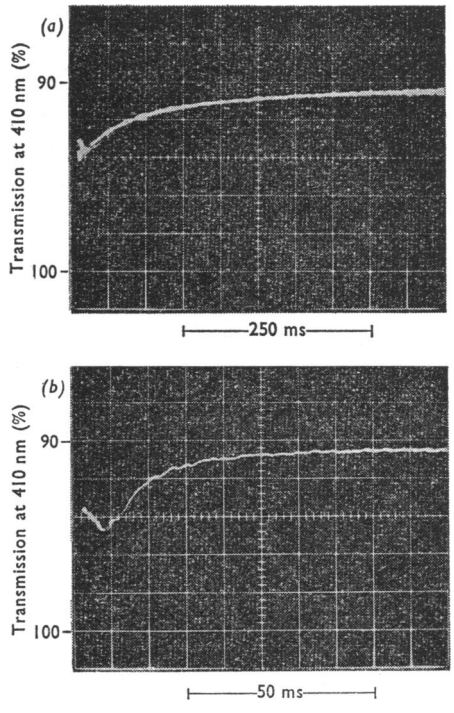

Fig. 7. *Rate of breakdown of the complex between* D-*glyceraldehyde 3-phosphate and sturgeon apoenzyme followed by measuring the rate of appearance of cysteine*-149 *with 2-chloromercuri-4-nitrophenol at* 410 *nm*

(*a*) Reaction conditions are described in Table 5, with 1.1 mM-aldehyde and 134 μM-organomercurial. (*b*) Reaction of organomercurial with sturgeon apoenzyme (0.24 mg/ml; 6.7 μM sites) in the absence of aldehyde, but otherwise in the conditions described in (*a*).

Table 5. *First order rate constants for the reaction of the complex between* D-*glyceraldehyde 3-phosphate and the sturgeon apoenzyme and 2-chloromercuri-4-nitrophenol at pH* 8.0 *and* 22°C *measured in the stopped-flow apparatus at* 410 *nm*

The reaction mixture contained (final concentrations) sturgeon apoenzyme (0.16 mg/ml; 4.5 μM sites), organomercurial (as indicated), D-glyceraldehyde 3-phosphate (1.1 mM), EDTA (2 mM) and triethanolamine hydrochloride (100 mM), adjusted to pH 8.0 with NaOH. One syringe contained the aldehyde and enzyme, and the other organomercurial. All other components were contained in both syringes.

[Organomercurial] (μM)	$k'_{obs.}$ (s^{-1})
13.4	8.5
53.5	12.0
134.0	11.0

At pH 8.0 it was difficult to select an aldehyde concentration in which the kinetic analysis was so clear-cut. It was necessary on the one hand to saturate the enzyme with aldehyde, and on the other hand to keep the kinetic competition between organomercurial and free aldehyde for the enzyme in favour of the organomercurial to prevent the observed displacement rate of aldehyde, $k_{obs.}$, from being a complex function of rate constants [cf. eqn. (3) of Harrigan & Trentham (1971)]. The conditions described in Table 5 satisfied these criteria as well as was practicable, as illustrated by Fig. 7. The results (Table 5) show that the dissociation rate of the aldehyde–apoenzyme complex is $12 s^{-1}$. As for phosphorolysis, $12 s^{-1}$ is more correctly an upper limit because of the possibility of NAD^+ catalysis of the breakdown, although in this case any NAD^+ would be partially reduced.

At pH 8.0 and 22°C the specific activity of the sturgeon enzyme in the presence of saturating concentrations of NADH and 1,3-diphosphoglycerate was 80 μmol of NAD^+ produced/min per mg of enzyme, which corresponds to a catalytic-centre activity of $48 s^{-1}$, and the breakdown of the aldehyde–apoenzyme complex is therefore too slow a process to be on the catalytic pathway of D-glyceraldehyde 3-phosphate dehydrogenase. This is consistent with the observed autocatalysis arising from NAD^+ formation (Trentham, 1971*a*). In fact the rate of aldehyde release from $E^{Ald}_{NAD^+}$ in the catalytic mechanism is considerably greater than $48 s^{-1}$, since transient kinetic studies show that the predominant steady-state complex is E^{Acyl}_{NADH} at pH 8 (Trentham, 1971*b*).

Discussion

The temperature-jump relaxation results are consistent with the model based on eqns. (2), (5) and (6) insofar that only one relaxation process is observed. They also fit into the general pattern of values of rate constants for the enzyme as follows: (1) the acylation rate ($k_{+1} = 3.3 \times 10^5 M^{-1} \cdot s^{-1}$) at pH 7.2 and 8°C compares satisfactorily with the rate ($9.2 \times 10^5 M^{-1} \cdot s^{-1}$) at 22°C and the same ionic strength (Harrigan & Trentham, 1973); (2) the sum of the catalytic-centre activities of the enzyme in the forward and back reactions is $34 s^{-1}$, which is less than the reciprocal relaxation time observed under all conditions, as must be the case when the concentrations of 1,3-diphosphoglycerate, NAD^+ and P_i are all much greater than their K_m values, particularly since under these conditions neither acylation nor phosphorolysis is a rate-determining step of the enzyme-catalysed reaction (Trentham, 1971*b*).

On the other hand the model described by eqns. (2), (7) and (8) is unsatisfactory because only one relaxation process is observed whose rates from the nature of its dependence on reactant concentration would have to correspond to $1/\tau_1$. A low value for $1/\tau_2$ ($<50 s^{-1}$) in the presence of 1.7mM-NAD^+ would mean an extremely low value for k_{-2} ($<3\times10^4 M^{-1}\cdot s^{-1}$), which is much less than association rate constants of ligands to proteins are in general (typically 10^7–$10^8 M^{-1}\cdot s^{-1}$; Gutfreund, 1971). Further, neither association of NAD^+ to nor dissociation of NAD^+ from the acyl-enzyme (the processes whose rates essentially determine $1/\tau_2$) is rate-determining in the catalytic mechanism, so that the right-hand term of eqn. (8) is $\gg 34 s^{-1}$ (the sum of the catalytic-centre activities), which is incompatible with a value of $<50 s^{-1}$ for $1/\tau_2$.

If our interpretation of the temperature-jump studies is correct, there are three important consequences. First, in any study of enzyme transients there is always the possibility that the kinetics of the first turnover are in some way atypical. The significance of this in the case of D-glyceraldehyde 3-phosphate dehydrogenase has been discussed by Trentham (1971*a*). It is therefore reassuring to find the relaxation-kinetic study ties in satisfactorily with the transient-kinetic studies of acylation (Harrigan & Trentham, 1973). Secondly, the kinetics of phosphorolysis of the acyl-holoenzyme have now been studied in the region of physiological pH and, as shown by the ordinate intercept of Fig. 4 ($65 s^{-1}$), the phosphorolysis rate ($75 s^{-1}$) is greater than the catalytic-centre activity ($14 s^{-1}$), which must be the case since NADH release has been shown to be rate-determining in the enzyme-catalysed oxidative phosphorylation of D-glyceraldehyde 3-phosphate (Trentham, 1971*b*). Thirdly, there is no evidence, from the temperature-jump studies or the steady-state assays at pH5.4 (Fig. 6), that a Michaelis complex of P_i and acyl-holoenzyme is formed to any significant extent during the phosphorolysis of the acyl-holoenzyme. The kinetics of phosphorolysis of the acyl-apoenzyme also suggest that no Michaelis complex is formed between P_i and acyl-apoenzyme (Table 2). P_i-binding sites have been characterized on D-glyceraldehyde 3-phosphate dehydrogenase (Velick & Hayes, 1953; Trentham, 1968), but there is no published evidence that these binding sites lead to phosphorolysis of the acyl-enzyme becoming zero-order in $[P_i]$.

The results reported in the present paper support the mechanism of eqn. (1). Further, Duggleby & Dennis (1974) have shown that the steady-state kinetic properties of the pea seed enzyme are in agreement with this hypothesis. They also suggest why previous steady-state studies have generally not supported this mechanism.

It is useful to relate the observed catalytic-centre activity, $k^*_{obs.}$, of D-glyceraldehyde 3-phosphate oxidative phosphorylation to the kinetics of phosphorolysis of the acyl-holoenzyme when [acyl phosphate] = [NADH] = 0. At pH8 and saturating concentrations of NAD^+, aldehyde and P_i, $k^*_{obs.}$ describes the rate of NADH release, k_x (Trentham, 1971*b*). At lower pH values phosphorolysis of the acyl-enzyme has been shown to be rate-limiting (Trentham, 1971*b*), and this arises because it is not possible to saturate with P_i at reasonable values of ionic strength. Under these conditions at saturating $[NAD^+]$ and [aldehyde] but limiting $[P_i]$, $k^*_{obs.} = k_{-1}[P_i]$. Conditions in which k^*_{obs} is proportional to $[P_i]$ are described in Fig. 6. As already noted, there is a lack of significant Michaelis-complex formation between P_i and $E^{Acyl}_{NAD^+}$ even at those pH values when NADH release is the rate-limiting process of the oxidative phosphorylation of D-glyceraldehyde 3-phosphate. Consequently the phosphorolysis rate can be characterized over a range of pH values: directly from the steady-state rate at low pH, and at high pH from the K_m value for P_i and the catalytic-centre activity, k_x. This is because, at the K_m concentration of P_i, $k_{-1}[P_i] = k_x$, since then:

$$k^*_{obs.} = \frac{k_x}{2} = \frac{k_x k_{-1}[P_i]}{k_x + k_{-1}[P_i]}$$

When this is done, $k_{-1} = 20 M^{-1}\cdot s^{-1}$ at pH5.4, $4\times10^3 M^{-1}\cdot s^{-1}$ at pH6.4 (Fig. 3 of Trentham, 1971*b*) and $4.5\times10^5 M^{-1}\cdot s^{-1}$ at pH8.6 from the K_m (290μM) and the catalytic-centre activity ($120 s^{-1}$) of the rabbit enzyme (Furfine & Velick, 1965; Bloch *et al.*, 1971). The relative phosphorolysis rates at these pH values are approximately proportional to the relative fractions of P_i ions existing as PO_4^{3-}. Although a systematic study with careful control of ionic strength and reaction media is required before the rate equation for phosphorolysis can properly be assigned over a wide pH range, it appears likely to be of the form:

$$\frac{-d[E^{Acyl}_{NAD^+}]}{dt} = k'_{-1}[PO_4^{3-}][E^{Acyl}_{NAD^+}] \qquad (11)$$

or a kinetically indistinguishable alternative (Jencks, 1969), where k'_{-1} is a pH-invariant rate constant and $k_{-1}[P_i] = k'_{-1}[PO_4^{3-}]$. J. McD. Armstrong & D. R. Trentham (unpublished work) have found that phosphorolysis kinetics in the pH range 7.5–8.6 support this conclusion. If eqn. (11) is the correct form of the rate equation, $k_{-1} = 10^9 M^{-1}\cdot s^{-1}$ on the basis of k_{-1} at pH8.6 and pK11.8 for P_i at I 0.1M (Schwarzenbach & Geier, 1963), and so phosphorolysis of the acyl-holoenzyme is a diffusion-controlled process. The low phosphorolysis rate at low pH values results from unfavourable partitioning between PO_4^{3-} and other P_i species. PO_4^{3-} will be the best

nucleophile of the various ionic states of P_i, and it will likewise be the poorest leaving group in the reverse reaction (Di Sabato & Jencks, 1961), although the special role of the positively charged nicotinamide ring might be to overcome this poor leaving-group capacity.

Harrigan & Trentham (1973) have shown that when the holoenzyme is acylated by 1,3-diphosphoglycerate at pH8 a proton is taken up from the reaction medium and also that the acylation rate is pH-independent. This necessarily shows that eqn. (11) or a kinetically indistinguishable alternative is correct at pH8. It is also consistent with PO_4^{3-} being the leaving group, since proton uptake would be associated with PO_4^{3-} protonation and occur concomitantly with acylation but not affect the acylation rate. It should be possible to discriminate between PO_4^{3-} and protonated forms of P_i as the substrate for $E^{Acyl}_{NAD^+}$ from the composition and orientation of active-site residues revealed by the tertiary structure and from whether phosphorolysis has the characteristics of a diffusion-controlled reaction.

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