The Reactions of D-Glyceraldehyde 3-Phosphate with Thiols and the Holoenzyme of D-Glyceraldehyde 3-Phosphate Dehydrogenase and of Inorganic Phosphate with the Acyl-Holoenzyme

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D-Glyceraldehyde 3-phosphate forms adducts with thiols. These adducts, which are presumed to be hemithioacetals, equilibrate rapidly with the unhydrated form of the aldehyde, which is the substrate for D-glyceraldehyde 3-phosphate dehydrogenase. The adduct provides a substrate buffer system whereby a constant low free aldehyde concentration can be maintained during the oxidation of aldehyde by the enzyme and NAD^+ . With this system, the kinetics of the association of the aldehyde with the enzyme were examined. The rate profile for this reaction is a single exponential process, showing that all four active sites of the enzyme have equivalent and independent reactivity towards the aldehyde, with an apparent second-order rate constant of $5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 8.0 and 21°C . The second-order rate constant becomes $8 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ when account is taken of the forward and reverse catalytic rate constants of the dehydrogenase. The pH-dependence of the observed rate constant is consistent with a requirement for the unprotonated form of a group of $\text{pK} 6.1$, which is the pK observed for the second ionization of glyceraldehyde 3-phosphate. The rate of phosphorolysis of the acyl-enzyme intermediate during the steady-state oxidative phosphorylation of the aldehyde was studied, and is proportional to the total P_i concentration up to at least 1 mM-P_i at pH 7.5. The pH-dependence of the rate of NADH generation under these conditions can be explained by the rate law $d[\text{NADH}]/dt = k[\text{acyl holoenzyme}][\text{PO}_4^{3-}]$, where $k = 7 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 21°C , indicating that PO_4^{3-} is the species attacking the acyl thioester bond, although kinetically indistinguishable rate equations for the reaction are possible. The rates of the phosphorolysis reaction and of the aldehyde-association reaction decrease with increasing ionic strength, suggesting that the active site of the enzyme has cationic groups which are involved in the reaction of the enzyme with anionic substrates.

The kinetics of the partial reactions catalysed by D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) have been elucidated for various steps in the proposed reaction mechanism (eqns. 1–5) at physiological pH [see Harrigan & Trentham (1974) and references therein].

The rate constants for the forward and reverse reactions of eqn. (1) are k_{+1} and k_{-1} , and $K_1 = k_{+1}/k_{-1}$.

However, it has not been possible to examine the combination of the holoenzyme with D-glyceraldehyde 3-phosphate, except below pH 6, because of the extreme rapidity of the aldehyde-association/redox steps (Trentham, 1971*b*). At higher pH values, stopped-flow techniques have insufficient time resolution to characterize these reactions, so that no transient process is observed, and the reaction is virtually complete within the dead-time of the apparatus (approx. 3 ms). In principle, it should be possible to examine the transient kinetics of the aldehyde and enzyme; however, glyceraldehyde 3-phosphate has characteristics that make this difficult to achieve. Although the reaction is between enzyme and free aldehyde, the latter exists predominantly as its hydrate, a *gem*-diol, so that the effective free aldehyde concentration is only approx. 4% of the total aldehyde present, and the dehydration of the hydrate is a slow process ($k = 0.08 \text{ s}^{-1}$) (Trentham

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et al., 1969). Thus at concentrations of the enzyme suitable for stopped-flow experiments, the rate of the reaction observed tends to be controlled by the dehydration of the aldehyde hydrate, unless high total aldehyde concentrations are used. At such concentrations, aqueous solutions of the aldehyde contain significant amounts of contaminating P_i . As a result, phosphorolysis of the acyl-enzyme takes place, and at pH values above 7 this reaction becomes so rapid that the kinetics of the transient reaction (eqns. 1–4) are obscured.

An observation by Peczon & Spivey (1972), who claimed that the hydration of glyceraldehyde 3-phosphate was pH-dependent, has enabled us to circumvent these difficulties. These workers carried out their experiments at high concentrations of dithiothreitol, presumably to protect sensitive thiol groups on the enzyme. As will be shown in this paper, their results were due not to a change in the hydration equilibrium with pH, but to the presence of the thiol, which gave rise to a marked increase in the amount of aldehyde available to react rapidly with the enzyme. If it is assumed that dithiothreitol reacts with the free aldehyde (RCHO) to form a hemithioacetal, a consideration of the equilibria:

shows that the concentration of free aldehyde must be decreased in the presence of the thiol. (The symbol R'SH is used in this paper to denote any thiol, the specific thiol in question being apparent from the text.) Thus it becomes necessary to postulate that the decomposition of the hemithioacetal is rapid in comparison with the dehydration reaction, so that the hemithioacetal should be capable of acting as a substrate 'buffer' if it decomposes at a rate higher than that of the combination of free aldehyde with the enzyme. This approach has been used to measure the rate of the aldehyde-association reaction for the dehydrogenase with D-glyceraldehyde 3-phosphate as substrate, and to measure the stoichiometry of this reaction.

The catalytic activity of muscle and liver enzymes shows a marked dependence on ionic strength, and decreases as the ionic strength increases. This has been demonstrated for the forward and reverse reactions under steady-state conditions (Smith & Velick, 1972) and for the formation of acyl-enzyme from the holoenzyme and diphosphoglycerate (Harrigan & Trentham, 1973). The effect of increasing ionic strength on the transient kinetics of the oxidation of D-glyceraldehyde 3-phosphate by its dehydrogenase are reported here, and are discussed in terms of the marked difference in reactivity of the enzyme towards D-glyceraldehyde and its 3-phosphate. The phosphorolysis reaction

(eqn. 5) has been re-examined under steady-state conditions at various pH values and ionic strengths, giving further evidence that PO_4^{3-} is the attacking species (Harrigan & Trentham, 1974).

For most of the experiments the pig muscle enzyme was used because of its ready availability. Some experiments were carried out with lobster enzyme, which can be prepared readily with full catalytic activity. The kinetic properties of the pig and lobster enzymes appear to be identical above pH 6, but below this pH the pig enzyme is much less stable than the lobster enzyme.

Materials and Methods

Enzyme

Glyceraldehyde 3-phosphate dehydrogenase was prepared from pig muscle, and was assayed and characterized as described by Harrigan & Trentham (1973). The specific activities obtained initially in three preparations were 127 ± 12 units/mg (where 1 unit is the amount of enzyme required to catalyse the formation of $1 \mu\text{mol}$ of NADH/min). Preparations were discarded when the specific activity had fallen below 90 units/mg. Active-site concentrations were calculated from the specific activity of the enzyme assuming that fully active enzyme (four sites) had a specific activity of 200 units/mg (Peczon & Spivey, 1972). The lobster enzyme was prepared and had the same properties as described in Trentham (1971a).

Substrates

D-Glyceraldehyde 3-phosphate was prepared as its calcium salt from D-fructose 1,6-diphosphate by periodate oxidation (Szewczuk *et al.*, 1961). Solutions of the sodium salt were prepared by dissolving the calcium salt in dilute HCl (1–10 mM), adsorbing the Ca^{2+} ions by passage through a small column (8 mm $\times$ 80 mm) of Dowex 50 (H^+ form), and adjusting the eluted material to pH 5–5.5 with 1 M Na_2CO_3 . In a few experiments the aldehyde was prepared from the barium salt of DL-glyceraldehyde 3-phosphate diethyl acetal (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany). NAD $^+$ and NADH were also obtained from C. F. Boehringer und Soehne. NAD $^+$ was further purified as described by Stinson & Holbrook (1973). The concentrations of NAD $^+$ and D-glyceraldehyde 3-phosphate in stock solutions were determined as described by Ferdinand (1964). The pK of D-glyceraldehyde 3-phosphate in 0.2 M NaCl at 20°C was determined by titration and was found to be pK 6.1. β -Mercaptoethanol (Sigma Chemical Co., St. Louis, MO, U.S.A.) and methyl thioglycollate (Ralph N. Emanuel, Wembley, Middx., U.K.) were dissolved in water, and the thiol concentration of these solutions

was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959).

All other reagents were A.R. grade where possible and were used without further purification. Water was glass-distilled.

Spectrophotometric and kinetic measurements

Transient-kinetic experiments were carried out by using both the single-beam (Trentham, 1971*a*) and split-beam instrument (Gutfreund, 1972). Concentrations of reagents refer to the concentration existing after mixing, but before any reaction. The convention describing a stopped-flow reaction $[A+B+\dots]+[X+Y+\dots]$ means that one syringe of the stopped-flow instrument contained A, B etc., and the other syringe contained X, Y etc. All measurements were made at room temperature ($21\pm 2^\circ\text{C}$).

Except where stated otherwise, steady-state assays were carried out at 25°C in a 1 cm light-path thermostatically controlled cuvette in a Uvispek H700 spectrophotometer (Hilger and Watts Ltd.) fitted with a Gilford recording attachment. Time-independent absorbance measurements were made in a Zeiss PMQ II spectrophotometer.

For NADH, $\epsilon_{340} = 6220\text{M}^{-1}\cdot\text{cm}^{-1}$ (Horecker & Kornberg, 1948) and for the 'Racker' band $\epsilon_{365} = 1000\text{M}^{-1}\cdot\text{cm}^{-1}$ at pH 7.8 for each molecule of NAD^+ bound to the enzyme (Cseke & Boross, 1967).

Results

Effect of thiol on apparent aldehyde concentration

When limited concentrations of enzyme catalyse oxidative phosphorylation of glyceraldehyde 3-phosphate in the presence of excess of NAD^+ (that is, when the enzyme turns over a number of times) the formation of NADH follows a biphasic curve with respect to time. The first phase corresponds to the oxidation of free aldehyde, whereas the second slow first-order process corresponds to the rate-limiting dehydration of the *gem*-diol. The rate of this process can be made independent of enzyme concentration and proportional to the total aldehyde concentration (Trentham *et al.*, 1969). The amplitude of the transient NADH formation is an estimate of the concentration of free aldehyde present. Under the conditions used by Peczon & Spivey (1972) the concentration of free aldehyde seemed to increase in going from pH 8.6 through pH 7.4 to 7.0, and they assumed that the hydration equilibrium for aldehyde was pH-sensitive. Their experiments at pH 7.4 and 7.0 were carried out in 5 mM-dithiothreitol. Fig. 1 shows the results obtained by repeating their experiment at pH 7.0 (a) with no added dithiothreitol, (b) with 5 mM-

dithiothreitol added both to the enzyme- NAD^+ solution and to the aldehyde solution before mixing, and (c) (see the legend to Fig. 1) with dithiothreitol added only to the enzyme- NAD^+ solution. The thiol increased the amplitude of the initial burst of NADH formation considerably, but only if it was added to the aldehyde before mixing with the enzyme.

Fig. 2 shows that 'available' aldehyde increased with increasing thiol concentration and a fixed total D-glyceraldehyde 3-phosphate concentration in the system $[\text{enzyme} + \text{P}_i + \text{NAD}^+ + \text{thiol}] + [\text{aldehyde} + \text{P}_i + \text{thiol}]$. In this experiment the transient in all cases corresponded to several turnovers of the enzyme, i.e. the 'available' aldehyde was in excess of the active-site concentration.

The slow phase of NADH generation in Figs. 1 and 2 occurred at the rate expected for the dehydration reaction, this rate being essentially constant over the range 0–10 mM-thiol.

It must be concluded that interaction between thiols and the aldehyde decreases the proportion of *gem*-diol present in the reaction mixture, and the results obtained are consistent with the hypothesis that hemithioacetals of glyceraldehyde 3-phosphate

Fig. 1. Effect of added thiol on 'available' aldehyde

The oxidation of D-glyceraldehyde 3-phosphate to 3-phosphoglycerate was measured essentially under the conditions described by Peczon & Spivey (1972). The reaction mixture after mixing contained 0.05 M-imidazole, 0.1 M-KCl, 1 mM-EDTA, 10 mM-arsenate (pH 7.0), 0.5 mM- NAD^+ , 0.2 mM-aldehyde and 1.57 μM enzyme (6.3 μM in sites). One syringe of the stopped-flow apparatus contained enzyme, NAD^+ and 0.5 mM- β -mercaptoethanol, and the other syringe contained aldehyde. The other components of the reaction mixture were present in both syringes. In (a), the reaction was carried out without further additions. In (b), 5 mM-dithiothreitol was present in both syringes before mixing, and in (c), thiol was added only to the syringe containing enzyme. The rate profile for NADH formation was identical in (a) and (c). The Figure shows the results for (a) in the upper trace, and for (b) in the lower trace.

Fig. 2. Dependence of 'available' aldehyde on thiol concentration

The reaction mixture contained 0.1M-triethanolamine hydrochloride, 0.1M-NaCl, 1mM-EDTA, 0.05M- P_i , pH8.0, 0.5mM-NAD⁺, 0.3mM-aldehyde and 1.5 μ M enzyme. The stock solution of enzyme contained 0.5mM- β -mercaptoethanol and was diluted 50-fold by mixing. The reaction studied was [enzyme+NAD⁺]+[aldehyde+methyl thioglycollate]. The thiol concentration in the substrate syringe is given on the right-hand side of the Figure. The other components of the reaction mixture were present in both syringes. The initial phase of the rate profile of NADH formation corresponds to oxidation of free aldehyde, and the second steady-state phase reflects dehydration of the aldehyde hydrate.

are formed and equilibrate rapidly with free aldehyde, thus increasing the amount of 'available' aldehyde. The formation of the equilibrium concentration of hemithioacetal was relatively rapid, and similar results for the transient phase were obtained whether the thiol/aldehyde mixture was used within about 2min or 20min after preparation. If the dehydration reaction is the only determining factor in the equilibration of aldehyde and thiol the reaction will be within 1% of equilibrium at 60s. However, reaction (c) described in the Fig. 1 legend shows that the slow dehydration of the *gem*-diol prevented the equilibrium from being established within a few milliseconds.

Rate-limiting process

The effect of thiols on 'available' aldehyde suggests a possible method for studying the aldehyde-association reaction, based on the following reactions:

where eqn. (7) condenses the reactions described by eqn. (1) to eqn. (4). The addition of thiol to aqueous solutions of aldehyde has two consequences: first,

it will decrease the concentration of free aldehyde in the solution, and secondly, it will provide a substrate 'buffer' system through the hemithioacetal/aldehyde equilibrium. The ability to achieve low concentrations of free aldehyde means that the rapid second-order process (eqn. 1) may proceed sufficiently slowly to be measurable, whereas the substrate 'buffering' action of the thiol will maintain the free aldehyde concentration at an essentially constant value, thus permitting measurements to be made under pseudo-first-order conditions. If the rate constant of the second-order process is to be measured under these conditions, it is necessary that the rate of hemithioacetal decomposition (eqn. 6) proceeds more rapidly than the aldehyde-association reaction, so that the free aldehyde concentration remains essentially constant throughout the course of the reaction. The rate-determining process in the reactions described by eqns. (6) and (7) was found in the following way.

The decomposition of hemithioacetals occurs largely as a second-order process involving catalysis by OH⁻ ion, and is diffusion-controlled for thiols with pK below 8. For more basic thiols the rate decreases with increasing basicity (Barnett & Jencks, 1969). Thus the OH⁻-catalysed decomposition of the hemithioacetal of methyl thioglycollate (pK = 7.8) proceeds at least 10 times as fast as that for the hemithioacetal of β -mercaptoethanol (pK = 9.5). Table 1 shows that the transient rate of NADH formation is the same for the two thiols, either for the overall oxidative phosphorylation of glyceraldehyde 3-phosphate or for the generation of acyl-enzyme. In either case the transient formation of NADH exhibited first-order kinetics for at least 85% of the process. Therefore hemithioacetal decomposition is rapid in comparison with the aldehyde-association/redox reaction, so that the rate-determining process in acyl-enzyme generation occurs in the complex process (eqn. 7) under these conditions.

Equilibrium constant for hemithioacetal formation

The equilibrium concentration of hemithioacetal in the mixture of aldehyde and thiol can be estimated, in principle, from the amplitude of the transient phase of NADH formation observed in Fig. 2. We have carried out a series of measurements under such conditions, obtaining estimates for the equilibrium constant in the range 600–4500M⁻¹, for both β -mercaptoethanol and methyl thioglycollate. The variability arises mainly from the difficulty in extrapolating the dehydration phase of the reaction to zero time, which is necessary to obtain the amplitude of the transient process. A more reliable procedure for determining the equilibrium constant is described below.

Table 1. Hemithioacetal decomposition as a non-rate-limiting process in the oxidation of D-glyceraldehyde 3-phosphate

These experiments were carried out in a buffer containing 0.1M-triethanolamine/HCl, 0.1M-NaCl and 1mM-EDTA, adjusted to pH8.0 with NaOH. A stock solution of pig enzyme was prepared in the same buffer plus 0.5mM- β -mercaptoethanol. All reaction mixtures contained 0.5mM-NAD⁺. In Expt. (A) the reaction studied was [holoenzyme+thiol+NAD⁺]+[aldehyde+thiol] and in Expt. (B) the reaction was [holoenzyme+thiol+NAD⁺+50mM-P_i]+[aldehyde+thiol+50mM-P_i]. Buffer was present in both syringes. The reaction was monitored at 340nm.

	Thiol	Aldehyde (μ M)	Enzyme (μ M sites)	$k_{\text{obs.}}$ (s ⁻¹)*
Expt. (A)				
$E_{\text{NAD}^+} \rightarrow E_{\text{NAD}^+}^{\text{Acyl}} + \text{NADH}$	19.4mM- β -Mercaptoethanol	28	10.3	17 $\pm$ 0.8†
	19.4mM-Methyl thioglycollate	28	10.3	16.2 $\pm$ 1.0†
Expt. (B)				
Aldehyde+NAD ⁺ +P _i $\rightarrow$	0.97mM- β -Mercaptoethanol	300	3.0	7.4
Acyl phosphate+NADH	0.97mM-Methyl thioglycollate	300	3.0	8.4
	3.7 mM- β -Mercaptoethanol	300	3.0	5.4
	3.7 mM-Methyl thioglycollate	300	3.0	5.4

* Observed pseudo-first-order rate constant for transient process.

† NADH formation continued subsequent to the initial $E_{\text{NAD}^+}^{\text{Acyl}}$ formation owing to thiolysis of the acyl-holoenzyme. Fig. 6 illustrates this.

When aqueous solutions of glyceraldehyde 3-phosphate and β -mercaptoethanol are mixed there is a change in E_{230} . Similar spectral changes have been observed for aqueous mixtures of other aldehydes and thiols, and evidence has been presented that these changes correspond to the formation of adducts containing one molecule each of aldehyde and thiol. These are believed to be hemithioacetals (Lienhard & Jencks, 1966; Barnett & Jencks, 1969).

The equilibrium constant for hemithioacetal formation from D-glyceraldehyde 3-phosphate and β -mercaptoethanol was determined as follows. A fixed concentration of the thiol (approx. 0.2mM) was mixed with various concentrations of the aldehyde (in 5 to 75 molar excess), the system being buffered in 67mM-sodium phosphate, pH7.0. Tubes containing the reaction mixtures and appropriate blanks were sealed and kept at 20°C for 1h, after which E_{230} was measured. The apparent equilibrium constant (K^*) with excess of aldehyde was calculated from:

$$K^* = \alpha / \{(1-\alpha)[\text{aldehyde}]_{\text{total}}\}$$

where α is the fraction of the total thiol in the adduct. This expression neglects changes in aldehyde concentration as a result of the reaction. However, the changes are small under the conditions used, and would alter the value of K^* by 2% or less. Glyceraldehyde 3-phosphate is labile at high pH values (Weischet & Kirschner, 1976). No decomposition of this type could be detected during the above reaction though in the same solvent over 24h at 20°C a 5mM-aldehyde solution decayed 37% both in the absence and presence of 0.2mM- β -mercaptoethanol.

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The total absorbance E is the sum of the absorbances of aldehyde (E_G), hemithioacetal (E_{HT}) and thiol (E_T), which can be related in turn to the absorbance of thiol alone (E_T^0) and the absorbance if all the thiol had been converted into hemithioacetal (E_{HT}^0). The values of E , E_G and E_T^0 can be measured experimentally, and

$$E_T = (1-\alpha)E_T^0 \quad \text{and} \quad E_{\text{HT}} = \alpha E_{\text{HT}}^0$$

By substitution and rearrangement

$$(E - E_G - E_T^0)^{-1} = (E_{\text{HT}}^0 - E_T^0)^{-1} \times \left[1 + \left(\frac{1}{K^*} \cdot \frac{1}{[\text{Aldehyde}]_{\text{total}}} \right) \right]$$

Thus K^* can be obtained from the ratio intercept/slope for a plot of $(E - E_G - E_T^0)^{-1}$ versus $([\text{aldehyde}]_{\text{total}})^{-1}$. This value must be corrected for the presence of the *gem*-diol as follows:

$$K_{\text{HT}} = K^*(1 + K_H)$$

where K_{HT} and K_H are the equilibrium constants for the formation of the hemithioacetal and *gem*-diol respectively, and $K_H = 29$ (Trentham *et al.*, 1969). On this basis, $K_{\text{HT}} = 3400 \pm 500 \text{ M}^{-1}$ for the reaction of D-glyceraldehyde 3-phosphate and β -mercaptoethanol at room temperature.

Thus with 20mM- β -mercaptoethanol and aldehyde at concentrations of 1mM or less, the equilibrium mixture would contain about 70% hemithioacetal, 29% *gem*-diol and 1% free aldehyde.

Rate of the aldehyde-association reaction

In the absence of P_i, eqn. (7) describes the reaction of aldehyde with the holoenzyme. Provided that

excess of NAD^+ is present, pseudo-first-order conditions are possible in the presence of thiol if the hemithioacetal concentration is several times the active-site concentration. This is because the buffer action of the hemithioacetal keeps the free aldehyde concentration effectively constant. In the Discussion section it is shown that the time-course of NADH formation in the reaction described by eqn. (7) will follow first-order kinetics, and that the observed rate constant will be directly proportional to the total aldehyde concentration within the limits discussed. Fig. 3 shows this to be the case for the reaction $[\text{enzyme} + \text{NAD}^+] + [\text{aldehyde} + \text{thiol}]$ over a concentration range of $0.3\text{--}3\text{ }\mu\text{M}$ free aldehyde ($28\text{--}280\text{ }\mu\text{M}$ total aldehyde). The solid line in Fig. 3 is the least-squares line of best fit passing through the origin, in conformity with eqn. (10). A slightly improved fit to the data is obtained by allowing the line to have a non-zero intercept with the ordinate. However the intercept is not significantly different from zero, and it may be concluded that eqn. (10) is obeyed over the range $0.3\text{--}3\text{ }\mu\text{M}$ free D-glyceraldehyde 3-phosphate.

A second-order rate constant k' may be calculated from the slope of the line. This is related to k'_{+1} (eqn. 10) as follows:

$$k'_{+1} = k'(1 + K_H + K_{HT}[\text{RSH}])$$

Values of k'_{+1} calculated from Fig. 3 and from experiments using β -mercaptoethanol as the added thiol are given in Table 2. In these calculations it has been assumed that K_{HT} is the same for both thiols. This can be justified by the similarity of k_{obs} in Table 1, and of k'_{+1} in Table 2, for the two thiols. In most of the following experiments values of k_{obs} are quoted and no attempt is made to convert these values into second-order rate constants describing k'_{+1} . This is because the linear relationship between k_{obs} and aldehyde concentration was not rigorously established.

Effect of pH on the aldehyde-association reaction

Values of k_{obs} for the process in eqns. (6) and (7) were determined over the range pH 6.0–8.25 in buffers of constant ionic strength (Fig. 4). This Figure also shows the results expected if the reaction involved only the basic form of a species with

Fig. 3. Variation of the observed rate constant (k_{obs}) for the aldehyde-association reaction with D-glyceraldehyde 3-phosphate concentration

The formation of acyl-enzyme (eqn. 7) was followed at 340nm. The reaction mixture contained 0.1M-triethanolamine/HCl, 0.1M-NaCl, 1mM-EDTA, pH8.0, 19.4mM- β -mercaptoethanol, 0.5mM-NAD $^+$, 1.5 μM pig enzyme (6 μM sites) and D-glyceraldehyde 3-phosphate at the concentrations indicated. One syringe of the stopped-flow apparatus contained enzyme, NAD $^+$ and thiol, and the other contained aldehyde and thiol. Buffer was present in both syringes. The values of k_{obs} were calculated as pseudo-first-order rate constants by Guggenheim's procedure. The line is the least-squares line $y = bx$.

Table 2. Apparent bimolecular rate constant for the reaction between D-glyceraldehyde 3-phosphate and its dehydrogenase

The values of k'_{+1} were calculated from the observed apparent first-order rate constant as described in the text. Pig enzyme was used in all experiments. The conditions used were as follows. For the regression analysis the data in Fig. 3 were used, and k'_{+1} and its error were calculated from the slope and standard error of the slope of the least squares line $y = bx$. The values for individual determinations were measured in triethanolamine buffers between pH8 and pH9, with 0.5mM-NAD $^+$ and 20mM- β -mercaptoethanol. The values of k'_{+1} at pH5.45 were determined without added thiol in 0.044M-EDTA, 7mM-Tris and 0.75mM-NAD $^+$. Errors are standard errors of the mean.

Method	Thiol	pK	pH	I^*	Number of values	$10^{-7} \times k'_{+1}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Regression	Methyl thioglycollate	7.8	8.0	0.4	11	4.02 ± 0.13
Individual measurement	β -Mercaptoethanol	9.5	8.0–9.0	0.05–0.2	20	5.24 ± 0.14
	None	—	5.45	—	3	0.26 ± 0.02

* Molar ionic strength.

Fig. 4. Effect of pH on the rate of the aldehyde-association reaction

The rate of the association reaction was measured in various buffers at an ionic strength of 0.2 (molar scale). The reaction mixture contained 20 mM- β -mercaptoethanol, 0.5 mM-NAD⁺, 0.1 mM-D-glyceraldehyde 3-phosphate and 5.35 μ M pig enzyme. The reaction was carried out as described in Fig. 3. The buffers used were 0.1 M-imidazole hydrochloride, 0.1 M-NaCl, 1 mM-EDTA for the range pH 6.0–7.0, and 0.1 M-triethanolamine/HCl, 0.1 M-NaCl, 1 mM-EDTA for the range pH 7.0–8.25. The pH values are those measured for the solutions after reaction. —, Dissociation of a proton from a group with pK 6.1; ····, dissociation for a group with pK 5.4, assuming that k_{obs} has a maximum value of 38 s⁻¹ for the unprotonated form of the group.

Fig. 5. Effect of NaCl concentration on the aldehyde-association reaction

The aldehyde-association rate was measured at pH 7.8 (▲) and at pH 5.4 (●) in the presence of increasing concentrations of NaCl. At pH 7.8, the reaction mixture contained 0.1 M-triethanolamine/HCl, 2 mM-EDTA, pH 7.8, 20 mM- β -mercaptoethanol, 0.5 mM-NAD⁺, 0.1 mM-D-glyceraldehyde 3-phosphate and 3.6 μ M pig enzyme (14.4 μ M sites). Measurements were made as described in Fig. 3. At pH 5.4 the reaction mixture contained 0.044 M-EDTA, 6.7 mM-Tris, 5 mM-P_i, pH 5.4, 0.5 mM- β -mercaptoethanol, 0.75 mM-NAD⁺, 1.25 mM-D-glyceraldehyde 3-phosphate and 5.4 μ M pig enzyme (21.5 μ M sites). Buffer, P_i and added NaCl were present in both syringes of the stopped-flow apparatus. One syringe contained enzyme, NAD⁺ and thiol; the other syringe contained aldehyde. In this experiment the aldehyde association and phosphorolysis were well resolved into two phases.

pK 5.4 or pK 6.1. The results are consistent with those expected for a group of pK 6.1, the pK measured for D-glyceraldehyde β -phosphate under these conditions (see the Materials and Methods section). This suggests that the phosphate dianion of the aldehyde is the form of the substrate that combines with the active site. However, the data are not sufficiently firm to consider this an unambiguous assignment. The significance of considering a group with pK 5.4 is that the amino acid residues at the active site of the pig holoenzyme show an ionization with pK 5.4 (Boross & Cseke, 1967). The results in Fig. 4 do not rule out a contribution from such a group to the pH-dependence of aldehyde association; the estimate of k'_{+1} at pH 5.4 in the absence of thiol (Table 2) is considerably lower than would be expected if only a group of pK 6.1 were involved.

Effect of ionic strength on the aldehyde-association reaction

If the combination of glyceraldehyde 3-phosphate with the enzyme involves the substrate dianion and cationic groups at the active site, the aldehyde-association rate should be sensitive to the ionic composition of the reaction medium and should

decrease as the ionic strength increases. The interpretation of ionic-strength effects on the reaction rate is simple, provided that the reaction rate is pH-independent, as is observed for the acylation of holoenzyme by 1,3-diphosphoglycerate (Harrigan & Trentham, 1973), NADH dissociation from the acyl-enzyme (Fig. 3, Trentham, 1971b) and the aldehyde-association reaction at pH 7 or higher (Fig. 4). Where the rate of the reaction varies markedly with pH, e.g. phosphorolysis of acyl-enzyme, any interpretation must take into account the effects of ionic strength on the dissociation of weak acids.

The effect of increasing concentrations of NaCl on the rate of the aldehyde-association reaction at pH 7.8 and at 5.4 is shown in Fig. 5. The reaction rate decreases with increasing ionic strength. This effect is not specific for NaCl, and similar effects are observed with other salts (Table 3). It is noteworthy that glycine (present largely as its zwitterion) has little effect on the reaction rate, as would be expected.

Reactivity of the four enzyme sites with aldehyde

Estimates of the extent of conversion of the holoenzyme into the acyl-enzyme in the hemithioacetal system have been made. Such estimates depend on the extinction coefficient used in converting the absorbance change into NADH concentration, and also require the assumption that the final absorbance change for the reaction corresponds entirely to NADH, and does not include $E_{\text{NADH}}^{\text{Acyl}}$. The latter condition can be fulfilled provided that relatively high NAD^+ concentrations are used, since this will tend to drive the reaction (eqn. 7) to the right, and NADH will be displaced from the enzyme.

The molar extinction change under these conditions can be considered to be that of the free NADH formed minus that associated with the conversion of holoenzyme into acyl-enzyme. The latter can be estimated to be $860\text{M}^{-1}\cdot\text{cm}^{-1}$ from the disappearance of the 'Racker' band on acylation of the holoenzyme (Trentham, 1971*a,b*) and from the ratio of the extinction coefficients of the 'Racker' band at 340nm and 365nm. Therefore the overall molar extinction coefficient for NADH production in the aldehyde-association reaction is $5360\text{M}^{-1}\cdot\text{cm}^{-1}$ in the presence of high NAD^+ concentrations.

Fig. 6(*a*) shows the extinction change at 340nm for the reaction of the aldehyde with lobster holoenzyme. The slow steady-state process corresponds to thiolysis and phosphorolysis (due to P_i contamination of the stock aldehyde solution) of the acyl-enzyme. When this is extrapolated linearly to zero time, making allowance for the 3ms dead-time of the instrument, the absorbance change from zero

time for the reaction of the holoenzyme with aldehyde is 0.0652. The absorbance change expected if all four sites of the holoenzyme are converted quantitatively into acyl-enzyme is 0.0681, equivalent to $12.7\mu\text{M}$ sites. Thus reaction of the aldehyde with the enzyme has occurred at 3.8 sites per tetramer. Further, the reaction profile in Fig. 6(*b*) is a monophasic exponential process, indicating that all four sites of the enzyme have equivalent and independent reactivity towards D-glyceraldehyde 3-phosphate.

The extent of the aldehyde-association reaction with the pig holoenzyme is more difficult to quantify, because the enzyme was not fully active. However, on the basis of the concentrations of active sites

Table 3. *Effect of various salts on the rate of the aldehyde-association reaction*

Observed first-order rate constant of transient NADH formation, k_{obs} (cf. Fig. 6), during the reaction of lobster glyceraldehyde 3-phosphate dehydrogenase ($20\mu\text{M}$ active sites), 1.0mM-NAD^+ and 1mM-EDTA with $76\mu\text{M-D-glyceraldehyde 3-phosphate}$ in a solvent of added salt as tabulated, $10\text{mM-}\beta\text{-mercaptoethanol}$ and 24mM-Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl)ethanesulphonic acid] adjusted to pH 7.45 with NaOH. The reaction was carried out at 21°C in the stopped-flow spectrophotometer. Abbreviation: EGTA, ethanedioxybis(ethylamine)tetraacetic acid.

Additions	$k_{\text{obs.}}$ (s^{-1})
None	39
0.5M-NaCl	11.5
0.5M- NaHCO_3	10.3
0.25M-EGTA (disodium salt)	9.6
0.5M-Glycine	39

Fig. 6. *Single turnover formation of acyl-holoenzyme from lobster holoenzyme*

The formation of acyl-enzyme was followed by the increase in E_{340} in a reaction mixture containing 0.40M-NaCl , 7mM-EDTA , 0.10M-Hepes adjusted to pH 7.4 with NaOH, 6mM-NAD^+ , $0.15\text{mM-D-glyceraldehyde 3-phosphate}$, $20\text{mM-}\beta\text{-mercaptoethanol}$ and lobster enzyme ($12.7\mu\text{M}$ sites). Buffer and thiol were present in both syringes. One syringe contained enzyme and NAD^+ , the other contained aldehyde-hemithioacetal equilibrium mixture. The arrow indicates the origin (no reaction). The slow sweep trace (*a*) shows the amplitude of the rapid process. The fast sweep trace (*b*) shows that the process is monophasic.

Fig. 7. Steady-state oxidative phosphorylation of aldehyde

The reaction mixture contained 0.1M-triethanolamine/HCl, pH8.0, 1mM-EDTA, 1mM- β -mercaptoethanol, 1mM-NAD⁺, 1mM-D-glyceraldehyde 3-phosphate, 10mM-P_i, pH8.0, and 2.2 μ M pig enzyme (8.8 μ M sites). Buffer was present in both syringes. One syringe contained enzyme, NAD⁺ and thiol; the other contained aldehyde and P_i. The upper trace represents the absorbance in the absence of aldehyde, and the lower trace is the absorbance of the reaction mixture with added aldehyde. The arrow indicates the cessation of flow.

deduced from specific-activity measurements as described in the Materials and Methods section, the extent of conversion of holoenzyme into acyl-enzyme was typically 90% complete over the range of conditions described here. This corresponds to the reaction of 3.6 sites per tetramer, and the reaction again behaved as a single exponential process.

Phosphorolysis of the acyl-enzyme

In the oxidative phosphorylation of D-glyceraldehyde 3-phosphate, the dissociation of NADH from the acyl-enzyme is in general the rate-determining process above pH7 with saturating concentrations of aldehyde, NAD⁺ and P_i, and that phosphorolysis becomes rate-determining only below pH7, the K_m for P_i being given by $k_{+3}/k_{+5}(1+1/K_2)$. Methods for measuring the phosphorolysis rate have been described by Harrigan & Trentham (1974); however, these methods are indirect. The most direct method for following phosphorolysis above pH7 is to measure the steady-state rate of NADH formation at P_i concentrations well below the K_m value for that substrate. A criterion that this condition is satisfied is that the steady-state rate is directly proportional to the P_i concentration for a given set of conditions. One problem with this approach is that, unless a very sensitive spectrophotometer is used, significant product inhibition occurs during steady-state measurements, in particular because of NADH accumulation. To some extent this is not a major problem at low P_i concentrations, since the steady-state rate can be established before

Table 4. Variation of the rate of phosphorolysis of acyl-holoenzyme with pH

The second-order rate constants were calculated either for total P_i (k_{+5}) or for the concentration of PO₄³⁻ present at that pH (k'_{+5}). The experimental method was as described in Fig. 8.

pH	k_{+5} (M ⁻¹ ·s ⁻¹)	$10^{-8} \times k'_{+5}$ (M ⁻¹ ·s ⁻¹)
7.5	3.4×10^4	6.8
8.25	2.3×10^5	8.2
8.6	3.3×10^5	5.2

products have accumulated to a significant extent. The problem is alleviated by the use of a stopped-flow apparatus, since the mixing time is decreased about 5000-fold and this decreases the amount of product formed before measurements can be made. Using this procedure, we have obtained linear steady-state rates of NADH formation that show no evidence of product inhibition from the onset of the steady state (Fig. 7).

Table 4 summarizes the pH-dependence of the steady-state phosphorolysis rate at constant ionic strength. The rate (k_{+5}) showed marked variation with pH, and increased 9.7-fold between pH7.5 and 8.6. NAD⁺ was effectively at saturating concentration, since a threefold increase in its concentration in this pH range only increased the steady-state rate 1.1-fold. That P_i was rate-determining is shown by the proportionality of the steady-state rate to P_i concentration (Fig. 8). In similar experiments D-glyceraldehyde 3-phosphate was also shown to be at saturating concentration. A rate law which describes these results is:

$$\frac{d[E_{\text{NAD}^+}^{\text{Acyl}}]}{dt} = -k'_{+5}[\text{PO}_4^{3-}][E_{\text{NAD}^+}^{\text{Acyl}}] \quad (8)$$

where $k'_{+5}[\text{PO}_4^{3-}] = k_{+5}[\text{P}_i]$ (eqn. 5). The mean value of k'_{+5} is $7 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, based on pK11.8 at $I = 0.1 \text{ mol/l}$ for the third ionization of phosphoric acid (Schwarzenbach & Geier, 1963; Elliot *et al.*, 1958). This is comparable with the value determined by Harrigan & Trentham (1974), and provides further evidence that PO₄³⁻ is the species active in attacking the acyl thioester bond in the acyl-holoenzyme. However, rate equations kinetically indistinguishable from eqn. (8) cannot be ruled out (Jencks, 1969).

The phosphorolysis rate decreased by a factor of 7.5 when the NaCl concentration was increased from 0.1 to 1.0M at pH7.0 (Table 5). In interpreting these results it is necessary to bear in mind that the pK values for all three ionizations of phosphoric acid are markedly dependent on ionic strength (Elliot *et al.*, 1958), and that the phosphorolysis rate is pH-dependent. Table 5 shows that for a tenfold

Fig. 8. Dependence of the steady-state rate of oxidative phosphorylation on phosphate concentration

The steady-state rate of NADH formation was measured in experiments similar to those in Fig. 7. The reaction mixture contained 0.1M-triethanolamine hydrochloride (adjusted to pH7.5 with NaOH), 0.2M-NaCl, 1mM- β -mercaptoethanol, 0.5mM-NAD⁺, 1mM-D-glyceraldehyde 3-phosphate, 0.045 μ M pig enzyme (0.18 μ M sites) and P_i at the concentrations indicated. The finite rate of NADH formation at zero P_i was due to the presence of P_i in the stock aldehyde solution. Results are means of three determinations.

increase in NaCl, pK₂ of P_i is shown to decrease by 0.5 unit. The uncertainty in the actual changes in pK values is in the validity of the formulae derived by Elliot *et al.* (1958) for 38°C and 0.1–0.5M-NaCl, when extrapolated to 21°C and 0.1–1.0M-NaCl. Nevertheless, the actual decrease in phosphorolysis rate must be corrected for these changes in the concentration of the attacking ion, whether it be HPO₄²⁻ or PO₄³⁻. So there is a 40-fold decrease in k'_{+5} when the NaCl is increased 10-fold (Table 5). Whether HPO₄²⁻ or PO₄³⁻ is the correct substrate, it is clear that the reaction between acyl-holoenzyme and P_i is markedly decreased by increasing ionic strength, which strengthens the previous suggestion that the approach of the attacking P_i species to the acyl thioester bond involves cationic groups in the active site of the enzyme.

Discussion

The effect of added thiol in increasing the transient formation of NADH during the oxidative phosphorylation of D-glyceraldehyde 3-phosphate by its dehydrogenase can be explained only if the thiol decreases the proportion of aldehyde hydrate present and makes available more free aldehyde, the substrate for the enzyme (Trentham *et al.*, 1969).

Table 5. Effect of NaCl concentration on the rate of phosphorolysis of acyl-holoenzyme at pH7.0

The steady-state rate of NADH formation was measured at 340nm in a stopped-flow spectrophotometer using an assay medium of pig glyceraldehyde 3-phosphate dehydrogenase (0.2 μ M sites), 100mM-D-glyceraldehyde 3-phosphate, 100mM-NAD⁺, 100mM-P_i, NaCl as tabulated, 5mM-EDTA, 1mM- β -mercaptoethanol and 50mM-imidazole adjusted with HCl to pH7.00 at 21°C. $k_{rel.}$ gives the ratio of the steady-state rate to that in 0.1M-NaCl. $k'_{+5,rel.}$ gives the ratio of the second-order rate constant as described by eqn. (8) to that in 0.1M-NaCl and allows for the changing pK values of P_i with ionic strength. The changing pK values were calculated by using the data of Elliot *et al.* (1958), and the variations of pK₂ of P_i were checked as follows: two 50mM buffer solutions of imidazole and P_i, each containing 0.1M-NaCl, were adjusted to pH7 at 21°C. The NaCl concentrations were changed as listed, and the differences between the pH values of the pairs of solutions were recorded in the Table as Δ pH. For example, in 1.0M-NaCl the pH of the imidazole solution was 7.07 and that of the P_i solution 6.50. This means that the 50mM-imidazole assay solution at pH7 containing 1mM-P_i was 1.41 times as concentrated in HPO₄²⁻ in 1M-NaCl as the corresponding solution in 0.1M-NaCl. When the P_i concentration was increased from 1 to 5mM, the steady-state rates of NADH formation increased 3.6- and 3.7-fold in 0.1M- and 1M-NaCl respectively. This indicates that the K_m for P_i is about 10mM and that the observed rates at 1mM-P_i do reflect phosphorolysis rates directly.

NaCl (M)	$k_{rel.}$	Δ pH	$k'_{+5,rel.}$
0.1	1	0	1
0.25	0.68	0.15	0.43
0.4	0.32	0.26	0.15
0.6	0.21	0.37	0.068
0.8	0.16	0.50	0.036
1.0	0.13	0.57	0.025

However, the actual concentration of free aldehyde must be decreased, since the rate of the transient NADH formation decreases with increasing thiol concentration. Therefore aldehyde and thiol must react to form some rapidly equilibrating adduct, with a concomitant decrease in the proportion of free aldehyde and its *gem*-diol. Although the chemical identity of this adduct has not been established, the arguments of Lienhard & Jencks (1966) suggest that it is a hemithioacetal, and this is borne out by the ratio K_{HT}/K_H , which is similar to that for the hemithioacetals and hydrates of other aldehydes (Barnett & Jencks, 1969). In the following discussion it will be assumed that the adduct of the aldehyde and thiol is a hemithioacetal. No correction has been made for the ionization constant of the thiol in estimating K_{HT} . For β -mercaptoethanol, such correction should be negligible below pH9.

The use of the hemithioacetal buffering system to maintain a constant low free aldehyde concentration for studies on the reaction between the aldehyde and the enzyme requires that equilibration between the hemithioacetal and aldehyde be rapid in comparison with the reaction between aldehyde and enzyme. The data in Table 2 show that decomposition of the hemithioacetal is rapid in comparison with the enzymic reaction, since there is no change in the rate of NADH generation for two thiols whose hemithioacetals decompose at rates that differ by a factor of 10 at neutral pH (Barnett & Jencks, 1969). Therefore the substrate buffering system is satisfactory for use in studies on the association between aldehyde and enzyme.

Kinetics of the reaction between D-glyceraldehyde 3-phosphate and holoenzyme

For the purposes of this analysis, the reaction described by eqn. (7) is written as:

The following simplifying assumptions are made. The aldehyde concentration is invariant over the course of the reaction; this condition is fulfilled by using the hemithioacetal buffering system, provided that the initial hemithioacetal concentration is several times (≥ 4) the active-site concentration.

The equilibration between $\text{E}_{\text{NAD}^+}^{\text{Ald}}$ and $\text{E}_{\text{NADH}}^{\text{Acyl}}$ is instantaneous; the lack of a kinetic isotope effect in the redox process (Trentham, 1971b) supports this assumption. The formation of $\text{E}_{\text{NADH}}^{\text{Acyl}}$ is essentially irreversible; this should occur if the NAD^+ concentration is high enough to trap all $\text{E}_{\text{NADH}}^{\text{Acyl}}$ as $\text{E}_{\text{NAD}^+}^{\text{Acyl}}$.

Given these conditions, in general, NADH formation will be expressed as a function of two exponential terms. However, provided that $[\text{E}_{\text{NAD}^+}^{\text{Ald}}]$

and $[\text{E}_{\text{NADH}}^{\text{Acyl}}]$ do not build up appreciably, the steady-state approximation will hold (Frost & Pearson, 1961), and:

$$\begin{aligned} \frac{d([\text{E}_{\text{NAD}^+}^{\text{Ald}}] + [\text{E}_{\text{NADH}}^{\text{Acyl}}])}{dt} \\ = k_{+1}[\text{aldehyde}][\text{E}_{\text{NAD}^+}] - k_{-1}[\text{E}_{\text{NAD}^+}^{\text{Ald}}] \\ - k_{+3}[\text{E}_{\text{NADH}}^{\text{Acyl}}] = 0 \end{aligned}$$

Further,

$$K_2[\text{E}_{\text{NAD}^+}^{\text{Ald}}] = [\text{E}_{\text{NADH}}^{\text{Acyl}}]$$

and

$$\begin{aligned} -\frac{d[\text{E}_{\text{NAD}^+}]}{dt} \\ = k_{+1}[\text{aldehyde}][\text{E}_{\text{NAD}^+}] - k_{-1}[\text{E}_{\text{NAD}^+}^{\text{Ald}}] \end{aligned}$$

from which it follows that

$$\begin{aligned} -\frac{d[\text{E}_{\text{NAD}^+}]}{dt} &= \frac{\frac{K_2 k_{+3}}{1+K_2} \cdot k_{+1}[\text{aldehyde}][\text{E}_{\text{NAD}^+}]}{\frac{K_2 k_{+3}}{1+K_2} + \frac{k_{-1}}{1+K_2}} \\ &= k'_{+1}[\text{aldehyde}][\text{E}_{\text{NAD}^+}] \quad (10) \end{aligned}$$

This analysis is valid at low aldehyde concentrations such that the aldehyde-association reaction is rate-determining, that is, provided that:

$$k_{+1}[\text{aldehyde}] < 0.2 \left(\frac{K_2 k_{+3}}{1+K_2} + \frac{k_{-1}}{1+K_2} \right)$$

The terms $K_2 k_{+3}/(1+K_2)$ and $k_{-1}/(1+K_2)$ (see below) are respectively the catalytic constants for the forward reaction (130s^{-1}) and reverse reaction (80s^{-1}) catalysed by the enzyme (Trentham, 1971b). Thus:

$$k'_{+1} = 0.62k_{+1}$$

and a single exponential process will be seen for NADH formation. Thus taking the mean value of k'_{+1} at pH 8 and 21°C as $5 \times 10^7 \text{M}^{-1} \cdot \text{s}^{-1}$, in eqn. (1), the second-order rate constant, $k_{+1} = 8 \times 10^7 \text{M}^{-1} \cdot \text{s}^{-1}$. For the process to be a single exponential the model requires that the upper limit for the observed rate constant is about 40s^{-1} . For higher rates the profile will only approximate to a single exponential, ultimately resulting in a rate profile where the formation of $\text{E}_{\text{NADH}}^{\text{Acyl}}$ and NADH are well resolved. This is illustrated by Fig. 2 of Trentham (1971b).

The argument that the catalytic-centre activity for 1,3-diphosphoglycerate reduction, k'_{cat} , can be expressed as $k_{-1}/(1+K_2)$ is as follows. k'_{cat} can be related to eqns. (1)–(5). At pH 8 at saturating substrate concentrations and sufficient NAD^+ for full activation (a requirement discussed by Trentham, 1971b):

$$k'_{\text{cat}} = [1/k_{-2} + 1/k_{-4} + (1+K_2)/k_{-1}]^{-1} \quad (11)$$

Steps controlled by k_{-2} and k_{-4} were shown to be not rate-determining by Trentham (1971b) and Harrigan

& Trentham (1974), the former because there was no deuterium kinetic-isotope effect when $[2H]NADH$ was used as substrate and the latter based on temperature-jump kinetic studies. However, although k_{+2} and k_{-2} will be decreased, if deuteride rather than hydride transfer occurs, the equilibrium constant of the redox step K_2 , will be altered little if at all. So on the basis of eqns. (1)–(5) and (11)

$$k'_{cat} = k_{-1}/(1 + K_2) \quad (12)$$

The concentration of transient NAD^+ formed on mixing the acyl-enzyme with $NADH$ would then depend on the equilibrium constant of the rapid step and be given by $[E_0]/(1 + K_2)$, where $[E_0]$ corresponds to the total concentration of simultaneously active sites of the enzyme. In fact, no transient NAD^+ formation was observed (Trentham, 1971b). So for eqn. (12) to be valid, the ratio K_2 must be such that any transient NAD^+ would not have been detected. In practice this means that K_2 must be ≥ 5 . The experimental evidence that suggests that this is the case is as follows (Fig. 2 of Trentham, 1971b). When

the lobster holoenzyme, E^{NAD^+} , was mixed with D-glyceraldehyde 3-phosphate in the presence of 5.5 mM- NAD^+ at pH 5.4, a quantity of $NADH$ was formed at a rate that was too high to measure ($>400 s^{-1}$) and exceeded the rate of $NADH$ dissociation from E^{Acyl}_{NADH} . The amount of $NADH$ formed in this rapid phase was therefore associated with E^{Acyl}_{NADH} formation and indicated $K_2 \geq 5$.

The value of K_2 at pH 5.4 has now to be related to K_2 in the pH range 7–8. Harrigan & Trentham's (1973) experiments suggested that no proton is released into the reaction medium during the redox step at pH 8, so that K_2 may well be independent of pH. Alternatively, if any protons are released during the redox step, the nature of the redox reaction is such that proton release is more likely to accompany NAD^+ reduction than $NADH$ oxidation,

evidence and so it was not included in Trentham's (1971b) mechanism. Subsequent attempts to obtain positive evidence for this process have failed; for example, no protein fluorescence change could be identified with a rate comparable with that of the turnover rate of the dehydrogenase when a complex of D-glyceraldehyde 3-phosphate and the sturgeon apoenzyme was mixed with $NADH$ (P. J. Harrigan & D. R. Trentham, unpublished work). This proposal of an $NADH$ -induced conformation change, which demands an extra intermediate in the mechanism, is less likely than the new hypothesis presented here.

Nature of the aldehyde-enzyme complex

The existence of a hemithioacetal enzyme-substrate complex as an intermediate in catalysis has been a long-standing postulate in the mechanism of this enzyme, and is supported by evidence such as the ability of the aldehyde to protect cysteine-149 from reaction with thiol reagents (Harrigan & Trentham, 1974) and the existence of the acyl-enzyme as a thioester involving cysteine-149. There has been no direct demonstration of such an intermediate. Given that the adduct between glyceraldehyde 3-phosphate and other thiols is a hemithioacetal, the favourable equilibrium observed for this process suggests that the formation of such a complex between the aldehyde and enzyme would also be thermodynamically favourable. If we assume that the aldehyde-enzyme complex is a hemithioacetal, an important question is raised: what is the location of protons in the structure? With small neutral thiols the structure of the hemithioacetal is believed to be:

If this is the structure of the enzyme-aldehyde intermediate, then the oxidation of this intermediate must proceed with the release of a proton:

making K_2 greater the higher the pH. For these reasons it is probable that $K_2 \geq 5$ in the pH range 7–8.

Formerly an $NADH$ conformation change of the acyl-enzyme was assigned as the rate-determining step at pH 8, because no deuterium kinetic-isotope effect was observed with $[2H]NADH$ as substrate when E^{Acyl}_{NADH} was the steady-state intermediate (Trentham, 1971b). Some caution was noted in assigning a process to a mechanism purely on the basis of negative

In the reductive dephosphorylation of 1,3-diphosphoglycerate by $NADH$ and the enzyme, the only proton-uptake step which was observed was associated with the initial acylation of the holoenzyme,

and there was no proton uptake associated with the

to the sturgeon acyl-enzyme shows no evidence of anti-co-operativity (Seydoux *et al.*, 1976) and it is this binding process, in contrast with NAD⁺ binding to the apoenzyme, that is most likely involved in the reversible oxidative phosphorylation of D-glyceraldehyde 3-phosphate (eqn. 4). Likewise, all four sites of the enzyme show equivalent reactivity in the acylation of holoenzyme by 1,3-diphosphoglycerate (Trentham, 1971b; Seydoux *et al.*, 1973). Similarly, all four sites show equivalent and

Table 6. *Comparative rates of oxidation and Michaelis constants for various aldehydes with glyceraldehyde 3-phosphate dehydrogenase*

This Table has been assembled from the cited references. $V_{rel.}$ is the relative maximum velocity; the only adjustment in $V_{rel.}$ for the various different pH values used by different workers is by setting relative rates for the same aldehyde at the same value. No adjustment has been made to K_m . The references were as follows: (a) Harting & Velick (1954); (b) Nygård & Sumner (1952); (c) Fife & Rikihisa (1970); (d) Velick & Hayes (1953); (e) Ferdinand (1964).

Aldehyde	R	$V_{rel.}$	K_m (M)	Reference
Acetaldehyde	CH ₃ -	1	0.1	(a), (b)
Glycolaldehyde	HOCH ₂ -	34.9	0.41	(c)
Propionaldehyde	CH ₃ CH ₂ -	1.2	0.093	(a), (c)
D-Glyceraldehyde	HOCH ₂ CH(OH)-	50	0.010	(a), (b)
D-Glyceraldehyde 3-phosphate	CH ₂ (OPO ₃ ²⁻)CH(OH)-	85 000	4×10^{-5}	(b), (d), (e)
Butyraldehyde	CH ₃ CH ₂ CH ₂ -	2.5	0.11	(a), (c)
Isobutyraldehyde	(CH ₃) ₂ CH-	4.4	0.18	(c)
Isovaleraldehyde	(CH ₃) ₂ CHCH ₂ -	1.3	0.22	(c)
Pivaldehyde	(CH ₃) ₃ C-	0.6	0.017	(c)
3,3-Dimethylbutyraldehyde	(CH ₃) ₃ CCH ₂ -	0.9	0.01	(c)
Phenylacetaldehyde	C ₆ H ₅ CH ₂ -	7.1	0.19	(c)

only 5% of the value of k'_{+1} at pH 8, instead of 17% as required for a single dissociating group of pK 6.1.

The value for pK_2 of D-glyceraldehyde 3-phosphate obtained by us is considerably lower than that reported by Kiessling (1934). For the preparation of the aldehyde that he used, the titration curve was essentially identical with that for $H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$. However, our estimate of the pK is similar to values obtained for other phosphomonoesters (Johnson, 1960), and is therefore more likely to be correct.

Nonetheless, other evidence makes it clear that the anionic character of D-glyceraldehyde 3-phosphate is important in the reaction of this substance with the enzyme. The enzyme does not have absolute specificity towards D-glyceraldehyde 3-phosphate, and will catalyse the oxidation of many other aldehydes. Table 6 summarizes K_m values and relative maximum velocities of oxidation for a series of aldehydes, and it is obvious that the enzyme shows a very marked preference for D-glyceraldehyde 3-phosphate. For D-glyceraldehyde the rate of oxidation is 0.06% of that for the phosphorylated aldehyde, the K_m is increased 250 times. For other uncharged aldehydes the differences are even more striking. This shows clearly that the phosphate group is necessary for rapid reaction with the enzyme. This is also suggested by the inhibition constant for D-threose 2,4-diphosphate (approx. $2 \times 10^{-7} M$; Fluharty & Ballou, 1959).

Fife & Rikihisa (1970) found that electron-withdrawing substituents increased the rate of oxidation of aldehydes markedly, as may be seen by comparing $V_{rel.}$ for the pairs acetaldehyde/glycolaldehyde and propionaldehyde/D-glyceraldehyde. Such behaviour would be consistent with interaction

between the carbonyl group and the strongly nucleophilic thiol group.

There is also clear evidence that the active site has steric requirements for reaction with aldehydes. This is best seen in the failure of the enzyme to oxidize L-glyceraldehyde 3-phosphate. Not only is this isomer not a substrate, but it also fails to inhibit the oxidation of the D isomer, since racemic glyceraldehyde 3-phosphate is oxidized at the same rate as the equivalent concentration of the D isomer.

In summary, the active site shows marked preference towards negatively charged ions, and the reactivity of aldehydes is increased by electron-withdrawing groups. The first may well reflect the location of the active site in a crevice. If there are favourable coulombic interactions between charged substrate molecules and the active site, these will act to attract and correctly orient the substrate as it approaches the enzyme. The inhibitory effects of ionic strength on aldehyde-association phosphorolysis and acylation of the enzyme are consistent with such an interpretation. Similarly, coulombic interactions will be much greater for di- and tri-anions than for monoanions, which is consistent with the pH-rate profiles for these reactions.

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References

- Barnett, R. E. & Jencks, W. P. (1969) *J. Am. Chem. Soc.* **91**, 6758-6785
- Boross, L. & Cseke, E. (1967) *Acta Biochim. Biophys. Acad. Sci. Hung.* **2**, 47-57

- Buehner, M., Ford, G. C., Moras, D., Olsen, K. W. & Rossman, M. G. (1974) *J. Mol. Biol.* **90**, 25–49
- Cseke, E. & Boross, L. (1967) *Acta Biochim. Biophys. Acad. Sci. Hung.* **2**, 39–46
- Elliot, J. S., Sharp, R. F. & Lewis, L. (1958) *J. Phys. Chem.* **62**, 686–689
- Ellman, G. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Ferdinand, W. (1964) *Biochem. J.* **92**, 578–585
- Fife, T. H. & Rikihisa, T. (1970) *Biochemistry* **9**, 4064–4067
- Fluharty, A. L. & Ballou, C. E. (1959) *J. Biol. Chem.* **234**, 2517–2522
- Frost, A. A. & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd edn., p. 172, John Wiley and Sons, New York and London
- Gutfreund, H. (1972) *Enzymes: Physical Principles*, p. 180, Wiley-Interscience, New York and London
- Harrigan, P. J. & Trentham, D. R. (1973) *Biochem. J.* **135**, 695–703
- Harrigan, P. J. & Trentham, D. R. (1974) *Biochem. J.* **143**, 353–363
- Harting, J. & Velick, S. F. (1954) *J. Biol. Chem.* **207**, 857–865
- Horecker, B. L. & Kornberg, A. (1948) *J. Biol. Chem.* **175**, 385–390
- Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 182–199, McGraw-Hill, New York
- Johnson, M. J. (1960) *Enzymes 2nd Ed.* **3**, 407–441
- Kiessling, W. (1934) *Biochem. Z.* **273**, 103–108
- Lazdunski, M. (1972) *Curr. Top. Cell. Regul.* **6**, 290–291
- Levitzki, A. (1974) *J. Mol. Biol.* **90**, 451–458
- Lienhard, G. E. & Jencks, W. P. (1966) *J. Am. Chem. Soc.* **88**, 3982–3995
- Nygård, A. P. & Sumner, J. B. (1952) *Arch. Biochem. Biophys.* **39**, 119–128
- Peczon, B. D. & Spivey, H. O. (1972) *Biochemistry* **11**, 2209–2217
- Schwarzenbach, G. & Geier, G. (1963) *Helv. Chim. Acta* **46**, 906–926
- Seydoux, F., Bernhard, S. A., Pfenninger, O., Payne, M. & Malhotra, O. P. (1973) *Biochemistry* **12**, 4290–4300
- Seydoux, F. J., Kelemen, N., Kellershohn, N. & Roucous, C. (1976) *Eur. J. Biochem.* **64**, 481–489
- Smith, C. M. & Velick, S. F. (1972) *J. Biol. Chem.* **247**, 273–284
- Stinson, R. A. & Holbrook, J. J. (1973) *Biochem. J.* **131**, 719–728
- Szewczuk, A., Wolny, E., Wolny, M. & Baranowski, T. (1961) *Acta Biochim. Pol.* **8**, 201–207
- Trentham, D. R. (1971a) *Biochem. J.* **122**, 59–69
- Trentham, D. R. (1971b) *Biochem. J.* **122**, 71–77
- Trentham, D. R., McMurray, C. H. & Pogson, C. I. (1969) *Biochem. J.* **114**, 19–24
- Velick, S. F. & Hayes, J. E. (1953) *J. Biol. Chem.* **203**, 545–562
- Watson, H. C. & Banaszak, L. J. (1964) *Nature (London)* **204**, 918–920
- Watson, H. C., Duée, E. & Mercer, W. D. (1972) *Nature (London) New Biol.* **240**, 130–133
- Weischet, W. O. & Kirschner, K. (1976) *Eur. J. Biochem.* **65**, 365–373