Rate-Determining Processes and the Number of Simultaneously Active Sites of D-Glyceraldehyde 3-Phosphate Dehydrogenase

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Transient kinetic studies of the reversible oxidative phosphorylation of D-glyceraldehyde 3-phosphate catalysed by D-glyceraldehyde 3-phosphate dehydrogenase show that all four sites of the tetrameric lobster enzyme are simultaneously active, apparently with equal reactivity. The rate-determining step of the oxidative phosphorylation is NADH release at high pH and phosphorolysis of the acyl-enzyme at low pH. For the reverse reaction the rate-determining step is a process associated with NADH binding, probably a conformation change, at high pH and D-glyceraldehyde 3-phosphate release at low pH. NADH has previously been shown to be a competitive inhibitor of the enzyme with respect to D-glyceraldehyde 3-phosphate and *vice versa*. This is consistent with the mechanism deduced from transient experiments given the additional proviso that 1-arseno-3-phosphoglycerate has a half-life of about 1 min or longer at pH 7. The dissociation constants of D-glyceraldehyde 3-phosphate and 1,3-diphosphoglycerate to the NAD⁺-bound enzyme are too large to measure but are nevertheless consistent with the low K_m values of these substrates.

In previous work (Trentham, 1971) various elementary processes of reactions of D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) have been studied. From this a model has been proposed that describes the preferred reaction pathway of the enzyme at physiological pH (Scheme 1). In the text reference to processes in Scheme 1 is made by use of Roman numerals (e.g. reaction II is the redox step). The introduction of the hemithioacetal enzyme into this scheme and the absence of Michaelis complexes between the enzyme and 1,3-diphosphoglycerate or glyceraldehyde 3-phosphate are discussed below. The aim of the work reported here is to establish the rate-determining process of the enzyme-catalysed reversible oxidative phosphorylation of glyceraldehyde 3-phosphate over a wide pH range and to find out how many sites of the tetrameric enzyme are simultaneously active. The model (Scheme 1) is discussed in the light of the extensive steady-state kinetic analyses by Furfine & Velick (1965) and Keleti & Batke (1965).

Lobster and sturgeon D-glyceraldehyde 3-phosphate dehydrogenases have been used for these studies. The two enzymes are functionally identical. Their special characteristic properties which complement each other for elucidating the mechanism have been described by Trentham (1971).

MATERIALS AND METHODS

The isolation and assay of glyceraldehyde 3-phosphate dehydrogenase from lobster and sturgeon, the synthesis

Scheme 1. R-CHO refers to D-glyceraldehyde 3-phosphate. Roman numerals refer to individual processes, k_{+1} , k_{-1} etc. are rate constants.

and source of reagents, except as indicated below, were described by Trentham (1971).

NAD⁺ was further purified for experiments in which it was used in high concentration (Figs. 6 and 7) by chromatography on a Dowex 1 (Cl⁻ form) column. NAD⁺ was eluted with 10 mM-HCl. The NAD⁺ solution was concentrated by freeze-drying and used within 48 h. The purification removed an impurity with a relatively high extinction at 340 nm. This procedure was developed by Dr R. A. Stinson in these laboratories.

Monodeuterated NADH was prepared by dithionite reduction of NAD⁺ in D₂O. This reaction has a low stereospecificity so that approx. 50% of the NADH molecules contain a deuterium atom that will be transferred during the enzyme-catalysed NADH oxidation (Fisher, Conn, Vennesland & Westheimer, 1953).

Spectroscopic methods including those with stopped-flow apparatus were as described by Trentham (1971).

In transient experiments burst amplitudes were determined as outlined by Gutfreund (1965). Since the burst amplitudes and the rates of transient formation critically affect discussion of the number of simultaneously active sites on the enzyme and of the relative reactivity of the sites, the analytical methods are summarized here. Let the production of P₁ from an enzyme-catalysed reaction be represented by the reaction scheme:

where E is the enzyme, A is the substrate present in large excess of E, P₁ and P₂ are products, EA and EB are enzyme-substrate intermediates and *k* and *k'* are rate constants. Under conditions where the bimolecular binding process is fast and the equilibrium of this process strongly favours EA, only rate constants *k* and *k'* and the enzyme concentration affect the rate of production of P₁. At time *t* after mixing E with A the concentration of P₁, *c_{P1}*, is given by:

$$c_{P_1} = nc_{E_0} \left(\frac{kk't}{k+k'} + \frac{k^2}{(k+k')^2} [1 - e^{-(k+k')t}] \right)$$

where *c_{E0}* is the total concentration of enzyme and *n* is the number of simultaneously active sites. An asymptote to the above function as *t* → ∞ is:

$$\pi = nc_{E_0} \left(\frac{kk'}{k+k'} + \frac{k^2}{(k+k')^2} \right)$$

At *t* = 0 this becomes:

$$\pi_0 = \frac{k^2}{(k+k')^2} nc_{E_0}$$

*π*₀ is the burst amplitude. For given values of *n* the rate constants *k* and *k'* can be evaluated from the steady-state rate of P₁ production, $\frac{kk'nc_{E_0}}{(k+k')}$, and the exponential function:

$$\pi - c_{P_1} = nc_{E_0} \left(\frac{k^2}{(k+k')^2} e^{-(k+k')t} \right)$$

RESULTS

Rate-limiting step of the reductive dephosphorylation of 1,3-diphosphoglycerate at pH 8. Previous

Fig. 1. Dependence of the rate of NADH production on glyceraldehyde 3-phosphate concentration. Stopped-flow traces were recorded at 340 nm. The reaction mixtures contained enzyme (0.503 mg/ml), NAD⁺ (5.5 mM), EDTA (76 mM), NaH₂PO₄ (47 mM), 2-mercaptoethanol (0.02 mM) at a final pH value of 5.4. In addition the solutions contained glyceraldehyde 3-phosphate [10.9 mM (A) and 1.09 mM (B) as indicated]. Associated with the upper traces are base-line traces which were coincident. The base-line traces were obtained with reaction mixtures containing all reagents except substrate. In each experiment one syringe contained enzyme, NAD⁺ and 2-mercaptoethanol and the other substrate. EDTA and phosphate were in both syringes. The 10.9 mM-glyceraldehyde 3-phosphate solution had extinction of 0.027 (i.e. a 6% transmission change at 100% transmission).

work has established that the rate-determining step of the reductive dephosphorylation of 1,3-diphosphoglycerate at pH 8 occurs after acyl-enzyme formation and subsequent NAD⁺ release (reactions V and IV) but concomitantly with the redox reaction (II) (Trentham, 1971).

Since a hydride ion is transferred from NADH to the acyl-enzyme in the redox step it is probable that the rate of the redox step will be slower if deuterated NADH replaces NADH. A deuterium isotope effect of 6 was observed for a transient of liver alcohol dehydrogenase identified as the hydride transfer step (Shore & Gutfreund, 1970). The steady-state rate of NADH oxidation was measured in the recording spectrophotometer with enzymes from both sturgeon and lobster. The assay medium contained NADH or deuterated NADH (0.12 mM), 1,3-diphosphoglycerate (0.12 mM), EDTA (1 mM), 2-mercaptoethanol (1 mM) and triethanolamine hydrochloride (0.18 M) adjusted to pH 8.5 with sodium hydroxide. The steady-state rate was identical within the accuracy of the experiment whichever form of NADH was substrate, so that a conformation change of the acyl-enzyme induced by NADH association is probably the rate-limiting step at pH 8.

Elementary processes of the oxidative phosphorylation of glyceraldehyde 3-phosphate at pH 5.4. The reaction [glyceraldehyde 3-phosphate] + [NAD⁺ + lobster enzyme] in the presence of phosphate had a

Fig. 2. Spectrophotometric record of the reaction of glyceraldehyde 3-phosphate with NAD^+ and lobster enzyme. The stopped-flow trace was recorded at 340 nm. The reaction mixture contained enzyme (0.503 mg/ml), NAD^+ (5.5 mM), glyceraldehyde 3-phosphate (10.9 mM), EDTA (0.12 M), 2-mercaptoethanol (0.02 mM) at a final pH of 5.4. One syringe contained enzyme, NAD^+ and 2-mercaptoethanol and the other substrate. Both syringes contained EDTA. The arrow indicates the percentage transmission of the solutions before mixing.

complicated rate profile at pH 5.4 (Fig. 1). (A stopped-flow experiment is described as $[A+B]+[C]$ when reactants A and B in one syringe are mixed with reactant C in the other syringe.) The rate of NADH production in the transient phase was biphasic. At high glyceraldehyde 3-phosphate concentration the major fraction of NADH in the transient was produced within the dead-time of the stopped-flow machine (3 ms) but a small fraction was produced at a measurable rate which was independent of glyceraldehyde 3-phosphate concentration. The rate of the process that was dependent on glyceraldehyde 3-phosphate concentration corresponds to the rate of aldehyde- NAD^+ -enzyme complex-formation, which is also dependent on glyceraldehyde 3-phosphate concentration (Trentham, 1971). The small fraction of the transient was still present when phosphate was left out of the reaction system [glyceraldehyde 3-phosphate] + [NAD^+ + lobster enzyme] (Fig. 2) so that this process is not related to phosphorolysis of the acyl-enzyme.

It follows that this process describes the rate of NADH dissociation from or NAD^+ association to the acyl-enzyme (reaction III or IV) and the rate-determining step of the oxidative phosphorylation of glyceraldehyde 3-phosphate at pH 5.4 is phosphorolysis of the acyl-enzyme (reaction V). The observed steady-state rate (Fig. 1) is a function of more than one rate constant if the enzyme is not saturated in phosphate, as is likely to be the case since the phosphate dianion is the substrate (S. A. Bernhard, personal communication).

Rate-determining step of the oxidative phosphorylation of glyceraldehyde 3-phosphate at pH 8. The catalytic-centre activity of enzyme increased

Fig. 3. Comparison of the rates of the catalytic-centre activity (▲) and of the slower phase of NADH production after reaction of enzyme, glyceraldehyde 3-phosphate and NAD^+ (Figs. 2 and 4) (●) at various pH values. The catalytic-centre activities at pH 5.4, 6.4 and 7.35 were determined in the stopped-flow apparatus. The medium at pH 5.4 was as described in Fig. 1. At pH 6.4 and 7.35 the medium contained lobster enzyme (0.214 mg/ml), glyceraldehyde 3-phosphate (7.1 mM), NAD^+ (2.1 mM), Na_2HPO_4 (4.3 mM), imidazole (0.13 M), EDTA (5 mM) and 2-mercaptoethanol (0.01 mM) adjusted to pH 6.4 or 7.35. At pH 8.7 the activity was determined in the normal assay medium used during enzyme purification (Trentham, 1971). The rates of the slow phase of NADH production were measured as described in Fig. 2 at pH 5.0, 5.4 and 6.4 and as described in Fig. 4 at pH 7.2 and 8.4.

rapidly in the pH range 5–7 and reached a plateau at higher pH values (Fig. 3). The catalytic-centre activities, which are not necessarily maximum possible values, especially at low pH, were determined from the specific activities on the basis of all four sites being simultaneously active.

The rate of the process assigned to NADH dissociation from or NAD^+ association to the acyl-enzyme was independent of pH and equal to the catalytic-centre activity at pH 8 (Fig. 3). Above pH 7 this process was monitored by preincubating sturgeon enzyme with glyceraldehyde 3-phosphate and then following the reaction [aldehyde-enzyme complex] + [NAD^+] (reaction VI *et seq.*) (Fig. 4). The reason for this was because phosphorolysis is rapid at high pH and the equilibrium of the oxidative phosphorylation favours 1,3-diphosphoglycerate formation so that more than a single turnover of the enzyme is observed by using conditions of Fig. 2 because of inorganic phosphate impurity in glyceraldehyde 3-phosphate. However, the concentration of glyceraldehyde 3-phosphate can be decreased 30-fold by preincubating sturgeon enzyme with glyceraldehyde 3-phosphate, because in this case no allowance has to be made for the fact that only 4% of glyceraldehyde 3-phosphate is in the catalytically active form since there is time during

Fig. 4. Spectrophotometric record of the reaction of NAD^+ and sturgeon enzyme preincubated with glyceraldehyde 3-phosphate. The stopped-flow trace was recorded at 340 nm. The reaction mixture contained enzyme (1.73 mg/ml), glyceraldehyde 3-phosphate (0.1 mM), NAD^+ (2.8 mM), EDTA (1 mM) and triethanolamine hydrochloride (130 mM) adjusted to pH 8.4 with NaOH. One syringe contained enzyme that had been preincubated with substrate to ensure aldehyde-enzyme-complex formation and EDTA, and the other NAD^+ . Both syringes contained the triethanolamine buffer. The percentage transmission of the solutions before mixing was greater than 100% and is off scale.

preincubation for the inactive diol-aldehyde transformation to take place (Trentham, McMurray & Pogson, 1969). The observed process (Fig. 4) which follows a larger rapid burst of NADH production cannot be attributed to the NAD^+ -enzyme absorption band since no process of measurable rate was observed at 365 nm when NAD^+ was mixed with the same concentration of sturgeon enzyme in the absence of glyceraldehyde 3-phosphate. The rate-determining step of the oxidative phosphorylation at pH 8 is either NADH dissociation from or NAD^+ association to the acyl enzyme (reaction III or IV). Phosphate apparently has little effect on the rate of this process.

Number of simultaneously active sites per tetramer of enzyme. The rate of NAD^+ production during the reductive dephosphorylation of 1,3-diphosphoglycerate is biphasic at low pH (Trentham, 1971, Figs. 1 and 2a). The concentration of simultaneously active sites of the enzyme that is related to the concentration of NAD^+ produced in the transient phase is determined most accurately when the transient rate and the steady-state rate are well resolved. The reaction [1,3-diphosphoglycerate + lobster enzyme] + [NADH + glyceraldehyde 3-phosphate] (Fig. 5) had a well-resolved transient because the presence of glyceraldehyde 3-phosphate lowered the steady-state rate but not the transient rate. The transient rate (75 s^{-1}) was 25 times the steady-

state rate (3 s^{-1}) and so the factor $\frac{k^2}{(k+k')^2}$ was 0.92.

Fig. 5. Spectrophotometric record of the reaction of 1,3-diphosphoglycerate and lobster enzyme with NADH in the presence of glyceraldehyde 3-phosphate. The stopped-flow trace was recorded at 340 nm. The reaction mixture contained enzyme (0.34 mg/ml), 1,3-diphosphoglycerate (0.25 mM), NADH (0.12 mM), glyceraldehyde 3-phosphate (3.0 mM), 2-mercaptoethanol (0.01 mM) and EDTA (150 mM) adjusted to pH 5.2 with NaOH. One syringe contained enzyme, 1,3-diphosphoglycerate and 2-mercaptoethanol and the other NADH and glyceraldehyde 3-phosphate. EDTA was in both syringes.

In the transient phase 3.7 mol of NAD^+ was produced/mol of tetramer.

It was also possible to determine the number of simultaneously active sites by studying the reaction [NAD^+ + lobster enzyme] + [glyceraldehyde 3-phosphate] (Fig. 6). The equilibrium of this reaction only favoured acyl-enzyme formation at high NAD^+ concentration because then NAD^+ dissociation from the aldehyde-enzyme complex (reaction VI) was prevented and NADH, a reaction product, was displaced from the acyl enzyme by NAD^+ (reactions III and IV) (Fig. 7). The amount of NADH produced was calculated by measuring the extinction change, adding to this the extinction of the NAD^+ -enzyme absorption band and by using $E_{340} 6.22 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ to convert the extinction value into NADH concentration. The E_{340}/E_{365} ratio of the NAD^+ -enzyme extinction band is 0.9. If the model used to determine the number of sites acylated is incorrect because the non-acylated sites absorb (the distribution between aldehyde-enzyme and NAD^+ -enzyme is unknown) then the error will be large at low NAD^+ concentration but small at high NAD^+ concentration. The number of sites reacting per tetramer would be between 0.3 and 0.85 at 0.01 mM- NAD^+ and between 3.1 and 3.2 at 13.6 mM- NAD^+ .

Both the rate of NADH disappearance in the transient phase (Fig. 5) and of NADH production (Fig. 6) were clean first-order processes that could be described by one rate constant, showing that no single site or pair of sites was more reactive than the others.

Fig. 6. Reaction of lobster enzyme and NAD^+ with glyceraldehyde 3-phosphate. Stopped-flow traces were recorded at 340nm. The reaction mixtures contained enzyme (0.376mg/ml), purified NAD^+ (13.6mM) glyceraldehyde 3-phosphate (0.85mM), citrate (1.1M), EDTA (0.03mM) and 2-mercaptoethanol (0.03mM). The pH of the reaction mixture was 5.2. One syringe contained enzyme, NAD^+ , EDTA and 2-mercaptoethanol and the other glyceraldehyde 3-phosphate. Citrate was present in both syringes.

Fig. 7. Relationship between the concentration of NAD^+ and the number of sites per tetramer of enzyme at which NADH is formed after reaction of the enzyme with glyceraldehyde 3-phosphate and NAD^+ . The numbers of sites reacting were determined in stopped-flow experiments. The reaction conditions were as described in Fig. 6 except: ●, the concentration of purified NAD^+ was as indicated; ▲, NADH (0.039mM) was present as well as NAD^+ (1.02mM). The abscissa axis has a logarithmic scale. The line drawn through the points was not based on any theoretical model.

DISCUSSION

Scheme 1 is proposed as a model for the preferred reaction pathway of D-glyceraldehyde 3-phosphate dehydrogenase at physiological pH and

is supported by the NAD^+ -facilitation experiments described by Trentham (1971). A hemithioacetal is proposed as the aldehyde-enzyme complex. Segal & Boyer (1953) have proposed the hemithioacetal as an intermediate because glyceraldehyde 3-phosphate protects the enzyme from carboxymethylation by iodoacetic acid. The slow rate of the reaction of glyceraldehyde 3-phosphate and the apoenzyme is also consistent with hemithioacetal formation (Trentham, 1971). The close parallel between the influence of the acyl and aldehyde groups on the dissociation constant of NAD^+ also argues in favour of a hemithioacetal because, apart from the change of hybridization of the carbonyl carbon atom from sp_2 to sp_3 orbitals, there is little stereochemical distinction between the acyl- and hemithioacetal-enzyme. The aldehyde- NAD^+ -enzyme complex is also expected to be a hemithioacetal as indicated in Scheme 1. However, such an intermediate is at present hypothetical since there is no evidence that it occupies a trough on the potential-energy profile of the reaction pathway. The elementary processes presented in Scheme 1 are necessary but probably not sufficient. For example, the identical rate of the reduction of the acyl-enzyme by NADH and by deuterated NADH is suggestive that there is a substrate-induced conformation change on NADH binding. However the available experimental evidence is not sufficient to postulate this as a discrete process.

One aim of these and earlier studies has been to establish the rate-determining steps of the reaction pathway. There are two reasons why it is difficult to be definite about this. First the observed rates are often a function of a number of rate constants. Secondly, it is difficult to prove that an observed process can be assigned to a particular step in the pathway on kinetic evidence alone. Rate-determining steps are therefore postulated on the basis of their being the most reasonable physically and because they fit into the simplest kinetic pattern consistent with the results.

The Michaelis complexes of glyceraldehyde 3-phosphate and 1,3-diphosphoglycerate to the enzyme have been omitted from Scheme 1. The rates of aldehyde and acyl-enzyme formation in the presence of NAD^+ have a first-order dependence on the concentrations of glyceraldehyde 3-phosphate and 1,3-diphosphoglycerate respectively, indicating that the Michaelis complexes have a negligible concentration (Trentham, 1971).

Features of the steady-state kinetic analysis are the low K_m values of glyceraldehyde 3-phosphate and 1,3-diphosphoglycerate at pH 7.4 and 8.5 (Furfine & Velick, 1965). Both substrates show a first-order dependence on the rate of their reactions with the enzyme at millimolar concentrations at pH 5.4, suggesting large dissociation constants (Trentham,

1971). At pH 7.4 and 8.4, however, both of these reactions occur before the rate-limiting processes, probably NADH dissociation (see below) and NADH association respectively. A model reaction scheme of the type:

has:

$$K_m = \left(\frac{k_{-a} + k_b}{k_{+a}} \right) \left(\frac{k_c}{k_b + k_c} \right).$$

When:

$$k_{-a} \gg k_b \gg k_c, K_m = \frac{k_c K_s}{k_b}$$

and hence $K_m \ll K_s$, the dissociation constant of the substrate. The low K_m values of glyceraldehyde 3-phosphate and 1,3-phosphoglycerate are therefore consistent with the proposed mechanism (Scheme 1).

A noteworthy result from the steady-state kinetic analysis is that glyceraldehyde 3-phosphate is a competitive inhibitor of the enzyme with respect to NADH and vice versa at pH 7.4 (Furfine & Velick, 1965). At first sight this suggests a random-order mechanism for the reaction. However, if glyceraldehyde 3-phosphate is competing with NADH by way of rapidly equilibrating intermediates, then it will be a competitive inhibitor of NADH and vice versa. This requirement shows that NADH release (reaction III) and not NAD⁺ association (reaction IV) is the rate-determining step of the oxidative phosphorylation of glyceraldehyde 3-phosphate above pH 7. Scheme 1 identifies the holoenzyme and the acyl-enzyme as the two rapidly equilibrating forms for which glyceraldehyde 3-phosphate and NADH are competing. Furfine & Velick (1965) used arsenate as the acyl acceptor in their studies of NADH inhibition of glyceraldehyde 3-phosphate. It would be of interest to see if the acyl arsenate analogue of 1,3-diphosphoglycerate has a half-life of the order of 1 min as is implied since step V must be reversible. The experiments of Teipel & Koshland (1970) led them to propose a relatively long half-life of 1-arseno-3-phosphoglycerate at pH 7. The steady-state kinetic results relating to reciprocal plots of velocity versus substrate at fixed concentrations of a second substrate imply random-order kinetics (Furfine & Velick, 1965). However, the complex steady-state equations associated with the reaction scheme make a critical analysis difficult without further direct comparison of transient experiments and more extensive steady-state information. Random-order binding between phosphate and either NAD⁺ or glyceraldehyde 3-phosphate is not in conflict with the scheme.

Keleti & Batke (1965) have also carried out an extensive steady-state kinetic analysis of the oxidative phosphorylation of glyceraldehyde 3-

phosphate catalysed by pig muscle enzyme at pH 8.8. They conclude that under their conditions NAD⁺ and glyceraldehyde 3-phosphate bind to the apoenzyme in a random order. At this high pH reactions associated with hemithioacetal formation and dissociation would be rapid for a typical alkyl thiol of pK 9 (Barnett & Jencks, 1969), so that the rate constants of the reversible reaction of glyceraldehyde 3-phosphate and the apoenzyme may be larger than the catalytic-centre activity. This serves to illustrate that the reaction scheme is only designed to represent the major reaction pathway of the glyceraldehyde 3-phosphate dehydrogenase mechanism especially at physiological pH. At high pH values for enzyme sources with weaker NAD⁺ binding to the apoenzyme such alternative pathways are to be expected.

Considerable interest has been focused as to the number of sites that are active at any instant in a polymeric enzyme (Heck, McMurray & Gutfreund, 1968; Bernhard, Dunn, Luisi & Schack, 1970, and references therein; Shore & Gutfreund, 1970). In studies of the reductive dephosphorylation of 1,3-diphosphoglycerate 3.7 mol of NAD⁺ are produced/mol of tetramer in the transient phase (Fig. 5). The transient and steady-state rates are not well resolved in the absence of added glyceraldehyde 3-phosphate. In such cases the number of simultaneously active sites per tetramer cannot be calculated so accurately from the transient amplitude because of the large sensitivity of the proportionality factor to the rate constants. However, the transient amplitude has consistently corresponded to 3.7 ± 0.3 active sites/tetramer (Trentham, 1971, Figs. 1 and 2a). For the oxidative phosphorylation of glyceraldehyde 3-phosphate at pH 5.4 (Fig. 6) the number of mol of NADH/mol of tetramer produced in the pre-steady-state phase depends on the equilibrium between acyl and non-acylated enzyme. This equilibrium is shifted in favour of acyl enzyme by added NAD⁺ (Fig. 7). The concentrations of NAD⁺ required to shift the equilibrium may be larger than those expected if all four sites were completely identical, although a detailed analysis of the composition of the non-acylated sites is required before this point is established. Viel (1969) has observed that 4 mol of NADH are produced/mol of yeast enzyme after reaction of the enzyme with glyceraldehyde 3-phosphate and NAD⁺ at pH 8.5. The clean first-order processes associated with the reactions (e.g. Figs. 5 and 6; Trentham, 1971, Figs. 1, 2a and 9) show that no single site or pair of sites per tetramer are reacting more rapidly than the others and is consistent with all four sites having equal reactivity. The acylation reaction also indicates that all four sites have equal reactivity (Trentham, 1971, Fig. 5). Mechanisms have been proposed that suggest that only one or two sites per

tetramer are active at any one time. Two of these mechanisms have considered primarily the dissociation constant of NAD^+ to the apoenzyme when it is difficult to envisage all four sites being simultaneously active in view of the negative cooperativity of NAD^+ binding (Conway & Koshland, 1968; De Vijlder, Hilvers, Van Lis & Slater, 1969). Malhotra & Bernhard (1968) and MacQuarrie & Bernhard (1969) have proposed that only two sites per tetramer of rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase are active at any one time. There may be differences between rabbit and lobster enzyme. However, their experiments were not so directly related to the biologically important reaction in that they used pseudo-substrates. They carried out reactions over a much longer time-scale, which inherently allows time for secondary effects to occur.

In summary, although X-ray-diffraction analysis of NAD^+ -bound lobster enzyme suggests that the four subunits are related in pairs to form a tetramer of the $\alpha_2\alpha_2'$ type (Watson & Banaszak, 1964), the subunits appear to act independently of each other when catalysing the reversible oxidative phosphorylation of glyceraldehyde 3-phosphate.

Conclusions as to which are the rate-determining steps of the mechanism are summarized as follows. The rate-limiting process of the oxidative phosphorylation of glyceraldehyde 3-phosphate is phosphorolysis of the acyl-enzyme at low pH and NADH dissociation at high pH. The rate-limiting process of the reverse reaction is glyceraldehyde 3-phosphate release at low pH and a process associated with NADH binding at high pH. The observed rates are not necessarily described by a single rate constant of the reaction scheme. For example, the rate of glyceraldehyde 3-phosphate release at pH 5.2 is approximately equal to:

$$\frac{k_{-1}k_{-6}[\text{NAD}^+]}{k_{+6} + k_{-6}[\text{NAD}^+]}$$

and this function is more complicated if NADH binds to the aldehyde-enzyme complex (Trentham, 1971). It is noteworthy that the rate-determining

steps are associated with two types of process, chemical-bond cleavage and reactant binding or release, which are delicately balanced at physiological pH. This illustrates that a principle of enzyme evolution is to enhance the rate of the slowest step rather than any other.

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