Reactions of D-Glyceraldehyde 3-Phosphate Dehydrogenase with Chromophoric Thiol Reagents

By P. J. HARRIGAN AND D. R. TRENTHAM

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

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The kinetics of the reaction of D-glyceraldehyde 3-phosphate dehydrogenase with 5,5'-dithiobis-(2-nitrobenzoic acid) show that NAD^+ dissociates from the enzyme before the reaction. In contrast 2-chloromercuri-4-nitrophenol reacts with the holoenzyme without prior dissociation of NAD^+ . These studies and observations on the dissociation constant of NAD^+ to the lobster enzyme show that NAD^+ must dissociate from sites modified by substrates during the reductive dephosphorylation of 1,3-diphosphoglycerate. All four sites per tetramer of the apoenzyme are acylated by 1,3-diphosphoglycerate. Hydrolysis of the acyl-enzyme occurs at a significant rate even in the absence of NAD^+ , which may explain previous estimates that only two sites per tetramer can readily be acylated.

The facilitation by NAD^+ of reactions catalysed by D-glyceraldehyde 3-phosphate dehydrogenase is well established (Trentham, 1971a, and references therein). It is therefore noteworthy that in certain processes the holoenzyme is less reactive than the apoenzyme (Taylor, Meriwether & Park, 1963; Kirschner, 1967; Eisele & Wallenfels, 1969). This lower reactivity of the holoenzyme is an important aspect of reactions catalysed by D-glyceraldehyde 3-phosphate dehydrogenase and its origin should be elucidated.

The differential reactivity of the holoenzyme and apoenzyme is marked in the reactions of 5,5'-dithiobis-(2-nitrobenzoic acid) with yeast D-glyceraldehyde 3-phosphate dehydrogenase (Kirschner, 1967). Dr M. R. Hollaway (personal communication) has been able to remove NAD^+ from the rabbit muscle enzyme by treatment of the enzyme with 5,5'-dithiobis-(2-nitrobenzoic acid), so a plausible model is that 5,5'-dithiobis-(2-nitrobenzoic acid) reacts only at NAD^+ -free sites of the enzyme to liberate 3-carboxy-4-nitrothiophenolate. This hypothesis has been tested in a series of kinetic experiments. The influence of NAD^+ on the reactivity of D-glyceraldehyde 3-phosphate dehydrogenase with 2-chloromercuri-4-nitrophenol has also been investigated.

The increase of the NAD^+ dissociation constant to D-glyceraldehyde 3-phosphate dehydrogenase after modification of the active site by substrates has an important bearing on the enzyme mechanism (Trentham, 1971a). If it can be shown that the rate constant for NAD^+ dissociation from the holoenzyme is less than the catalytic-centre activity of

D-glyceraldehyde 3-phosphate dehydrogenase then this dissociation is not the major reaction pathway of the reversible oxidative phosphorylation of D-glyceraldehyde 3-phosphate, supporting the mechanism proposed by Trentham (1971b). Two approaches are used to determine this dissociation rate constant from D-glyceraldehyde 3-phosphate dehydrogenase. The first depends on finding a reagent that will displace NAD^+ from the holoenzyme with concomitant production of a chromophore, when the rate constant for NAD^+ dissociation can be determined. The second is to determine the equilibrium dissociation constant of NAD^+ to D-glyceraldehyde 3-phosphate dehydrogenase and, since the rate of NAD^+ association to the enzyme can be no faster than diffusion-controlled, an upper limit for the NAD^+ dissociation rate constant can be calculated.

Chromophoric reagents such as 5,5'-dithiobis-(2-nitrobenzoic acid) and 2-chloromercuri-4-nitrophenol provide an excellent way of monitoring the time-course of reactions in which a thiol group is produced or destroyed and so can be used to follow reactions of lobster muscle D-glyceraldehyde 3-phosphate dehydrogenase, which has an essential thiol group, cysteine-148 (Allison & Harris, 1965; Davidson, Sajgò, Noller & Harris, 1967). All four sites on the tetrameric enzyme are active simultaneously during the reversible oxidative phosphorylation of D-glyceraldehyde 3-phosphate (Trentham, 1971b). However, the experiments of MacQuarrie & Bernhard (1969) have led them to suggest that 1,3-diphosphoglycerate acylates only two out of four sites of the apoenzyme of rabbit

muscle D-glyceraldehyde 3-phosphate dehydrogenase. This acylation has to be monitored by back-titration techniques. 5,5'-Dithiobis-(2-nitrobenzoic acid) is used here to monitor the acylation and to investigate the lability of the acyl-enzyme. The advantage of using 5,5'-dithiobis-(2-nitrobenzoic acid), which reacts with the apoenzyme of D-glyceraldehyde 3-phosphate dehydrogenase several orders of magnitude faster than iodoacetic acid, the reagent used by MacQuarrie & Bernhard (1969), is that the time available for acyl decomposition is much less during the assay for acyl-enzyme.

Lobster D-glyceraldehyde 3-phosphate dehydrogenase, which crystallizes with 4mol of NAD⁺ bound/tetramer, and the sturgeon enzyme, which crystallizes as the apoenzyme, have been used for these studies. The functional identity of these two enzymes has been established and their complementary characteristics for studying the influence of NAD⁺ on D-glyceraldehyde 3-phosphate dehydrogenase discussed by Trentham (1971a).

MATERIALS AND METHODS

Enzymes. D-Glyceraldehyde 3-phosphate dehydrogenases from lobster tail and sturgeon muscle were isolated and assayed as described by Trentham (1971a). Before experiments with thiol reagents the enzyme solutions were treated with 2-mercaptoethanol (1mM) in EDTA (5mM) at pH7.0 and then chromatographed at 5°C through fine grade Sephadex G-25 with EDTA (5mM) at pH7.0 as eluting solvent. Protein concentrations, which were corrected for loss of bound NAD⁺, were determined as described previously (McMurray & Trentham, 1969; Trentham, 1971a). Experiments with the apoenzyme of sturgeon D-glyceraldehyde 3-phosphate dehydrogenase were performed on the same day that the crystals of enzyme were dissolved. The stoichiometry of the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with the apoenzyme of sturgeon D-glyceraldehyde 3-phosphate dehydrogenase was generally in the range of 2.5–3.5 mol of 3-carboxy-4-nitrothiophenolate released/mol of tetramer. The instability of sturgeon apoenzyme solutions (Trentham, 1971a) and the tendency of the active centre thiol to form oxidation products (Parker & Allison, 1969) probably explains why the stoichiometry of the sites per tetramer reacting with 5,5'-dithiobis-(2-nitrobenzoic acid) is less than 4. Loss of enzyme specific activity directly corresponded to the loss of thiol groups titrated, as Kirschner (1967) found with the yeast enzyme.

Reagents. NAD⁺ was obtained from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany, and 5,5'-dithiobis-(2-nitrobenzoic acid) from Sigma Chemical Co., St Louis, Mo., U.S.A. The synthesis of 2-chloromercuri-4-nitrophenol has been described (McMurray & Trentham, 1969). Water was glass-distilled and all other reagents were analytical reagent grade where possible and used without further purification.

Spectroscopic measurements. Spectroscopic methods including those using stopped-flow have been described previously (Trentham, 1971a). ϵ_{412} of 3-carboxy-4-

nitrothiophenolate is $13.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Ellman, 1959). Except where otherwise stated oscilloscope traces were analysed as one or more exponential traces that were well resolved on the time-axis. The rate constants were evaluated by plotting the logarithm of the appropriate concentration ordinate versus time.

RESULTS

Reactions of thiol reagents with the apoenzyme and holoenzyme of D-glyceraldehyde 3-phosphate dehydrogenase. The chromophoric organomercurial 2-chloromercuri-4-nitrophenol reacts at two sites per subunit of the holoenzyme of lobster D-glyceraldehyde 3-phosphate dehydrogenase. The rate profile of these two reactions is well resolved on the time-axis and the more rapidly reacting site is at the active site (McMurray & Trentham, 1969). The rate of reaction of the organomercurial with the active site was first order in organomercurial, the second-order rate constant being $2.2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, and was independent of NAD⁺ concentration (Figs. 1 and 2). The slower site of reaction on the holoenzyme was also first order in organomercurial concentration, the second-order rate constant being $3.1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$.

One site per subunit of the sturgeon enzyme reacted with 2-chloromercuri-4-nitrophenol. The rate of reaction of the organomercurial with the apoenzyme was first order in organomercurial, the second-order rate constant being $1.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 1). Under these conditions the rates of

Fig. 1. Observed first-order rates, k , for the reaction of D-glyceraldehyde 3-phosphate dehydrogenase and 2-chloromercuri-4-nitrophenol measured in the stopped-flow apparatus at 410 nm. The reaction mixture contained D-glyceraldehyde 3-phosphate dehydrogenase, 2-chloromercuri-4-nitrophenol, EDTA (2mM) and triethanolamine hydrochloride (100mM) adjusted to pH7.9 with NaOH. One syringe contained the enzyme and the other organomercurial. Triethanolamine and EDTA were present in both syringes. ▲, Lobster holoenzyme (0.0975 mg/ml); ●, sturgeon apoenzyme (0.159 mg/ml).

Fig. 2. Influence of NAD^+ concentration on the observed first-order rates, k , for the reaction of lobster D-glyceraldehyde 3-phosphate dehydrogenase with 2-chloromercuri-4-nitrophenol ($26.8 \mu\text{M}$) measured at 410 nm and with 5,5'-dithiobis-(2-nitrobenzoic acid) ($10 \mu\text{M}$) measured at 412 nm. The abscissa records the logarithm of the total NAD^+ concentration. ●, Reaction conditions were as for lobster enzyme in Fig. 1 except that the enzyme concentration was 0.082 mg/ml and added NAD^+ was in the enzyme syringe; ○, determined from the upper line of Fig. 1 at $26.8 \mu\text{M}$ -organomercurial; ▲, as ● with lobster enzyme (0.22 mg/ml) except that 5,5'-dithiobis-(2-nitrobenzoic acid) ($10 \mu\text{M}$) replaced the organomercurial.

Fig. 3. Spectrophotometric record at 412 nm of the reaction of sturgeon D-glyceraldehyde 3-phosphate dehydrogenase apoenzyme and 5,5'-dithiobis-(2-nitrobenzoic acid). The reaction mixture contained sturgeon apoenzyme (0.12 mg/ml), 5,5'-dithiobis-(2-nitrobenzoic acid) ($100 \mu\text{M}$), EDTA (1.0 mM) and inorganic phosphate (50 mM) at pH 6.9. One syringe contained apoenzyme and EDTA and the other 5,5'-dithiobis-(2-nitrobenzoic acid). Phosphate was in both syringes. The horizontal trace measures the extinction of the solution a few seconds after mixing the reactants.

reaction of the organomercurial with both the sturgeon apo- and holo-enzyme were similar and comparable with the reaction rate with the lobster

Fig. 4. Observed first-order rates, k , for the reaction of D-glyceraldehyde 3-phosphate dehydrogenase and 5,5'-dithiobis-(2-nitrobenzoic acid) measured in the stopped-flow apparatus at 412 nm. The reaction mixture contained D-glyceraldehyde 3-phosphate dehydrogenase, 5,5'-dithiobis-(2-nitrobenzoic acid), EDTA (2 mM) and triethanolamine hydrochloride (200 mM) adjusted to recorded pH with NaOH. One syringe contained enzyme and NAD^+ (when present) and the other 5,5'-dithiobis-(2-nitrobenzoic acid). Triethanolamine hydrochloride and EDTA were present in both syringes. ■, Sturgeon apoenzyme (0.20 mg/ml) at pH 7.9; ●, as ■ with NAD^+ ($5.6 \mu\text{M}$) at pH 7.9; ▲, as ■ with enzyme concentration at 0.17 mg/ml and NAD^+ ($4.6 \mu\text{M}$) at pH 7.2; ○, as ■ with lobster holoenzyme (0.22 mg/ml) but with no added NAD^+ at pH 7.9.

holoenzyme, showing that NAD^+ has little influence on the kinetics.

The reaction between 5,5'-dithiobis-(2-nitrobenzoic acid) and the sturgeon apoenzyme was studied (Fig. 3). Graphs of time versus the logarithm of the difference of extinction at the end and at various times during the reaction were only linear over 60–80% of the reaction amplitude. Rate constants were calculated from the linear portion of the graphs. The rate of reaction was first order in 5,5'-dithiobis-(2-nitrobenzoic acid), the second-order rate constant being $1.9 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 4). The true value will be slightly greater than this since the graphical approximation introduces a systematic error of up to 10%. Differential reactivity between thiols on the different subunits may explain why the reaction profiles (Fig. 3) are not exponential processes that can be described by a single rate constant.

In contrast the rate of reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with the sturgeon holoenzyme was not first order in 5,5'-dithiobis-(2-nitrobenzoic acid) since a tenfold increase in 5,5'-dithiobis-(2-nitrobenzoic acid) concentration only gave a 1.5-fold increase in reaction rate (Fig. 4). The reaction

Fig. 5. Spectrophotometric record at 412nm of the reaction of lobster D-glyceraldehyde 3-phosphate dehydrogenase holoenzyme and 5,5'-dithiobis-(2-nitrobenzoic acid). The reaction mixture contained lobster enzyme (0.22 mg/ml), 5,5'-dithiobis-(2-nitrobenzoic acid) (500 μ M), EDTA (2 mM) and triethanolamine hydrochloride (200 mM) adjusted to pH 7.9 with NaOH. One syringe contained enzyme and the other 5,5'-dithiobis-(2-nitrobenzoic acid). Triethanolamine hydrochloride and EDTA were present in both syringes.

was biphasic (similar to Fig. 5) and the rate constants (Fig. 4) are those of the more rapid phase.

The reaction of the lobster holoenzyme with 5,5'-dithiobis-(2-nitrobenzoic acid) was also not first order in 5,5'-dithiobis-(2-nitrobenzoic acid) and had a complex rate profile that could be described by two exponentials whose total amplitude was equivalent to the liberation of between 3 and 4 mol of 3-carboxy-4-nitrothiophenolate/mol of tetramer (Figs. 4 and 5). This was followed by a much slower process of large amplitude which reflected release of more thiol groups for reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) as the protein denatured and is not considered further here (Wassarman & Major, 1969).

If NAD^+ was added to the reaction medium the rate of 3-carboxy-4-nitrothiophenolate release was markedly decreased (Fig. 2) and high concentrations of NAD^+ protected the enzyme from attack by 5,5'-dithiobis-(2-nitrobenzoic acid). For example, in the presence of 500 μ M- NAD^+ less than 1 mol of 3-carboxy-4-nitrothiophenolate was released/mol of tetramer of lobster D-glyceraldehyde 3-phosphate dehydrogenase in the first 10 s after mixing the enzyme (0.18 mg/ml) with 10 μ M-5,5'-dithiobis-(2-nitrobenzoic acid) at pH 8.0.

After reaction with thiol reagents both lobster and sturgeon D-glyceraldehyde 3-phosphate dehydrogenases are inactive as catalysts for the oxidative phosphorylation of D-glyceraldehyde 3-phosphate in the absence of 2-mercaptoethanol.

Dissociation constant of NAD^+ to lobster D-glyceraldehyde 3-phosphate dehydrogenase. The routine preparation of solutions of lobster D-glyceraldehyde 3-phosphate dehydrogenase from the crystalline

enzyme either by dialysis or Sephadex chromatography gives information about the dissociation constant of NAD^+ . The crystalline enzyme contains 4 mol of NAD^+ /mol of tetramer. It has been shown that NAD^+ is not removed from the enzyme by dialysis of 1 ml of holoenzyme solution (45.4 mg/ml) against two changes of 3 litres of first 5 mM-EDTA and then 1 mM-EDTA at pH 7 and 5°C (Trentham, 1968). Each dialysis step was for 24 h. This result was reproducible and is striking since it indicates a very small dissociation constant for NAD^+ when four molecules of NAD^+ are bound per molecule of tetramer.

In a control experiment the rates of equilibration were measured of a series of samples of 3 ml of 5 mM- NAD^+ , which were each dialysed against 5 litres of 5 mM-EDTA at pH 7 and 5°C. The solutions and dialysis bags (Visking, from Scientific Instruments Centre) were kept in quite rapid motion by the use of magnetic 'fleas'. The concentration of NAD^+ in the dialysis bags decayed exponentially with time over 48 h. The half-time of the equilibration process was 9.7 h.

Freshly dissolved crystalline enzyme (at a concentration of 100 mg/5 ml) was also always filtered through a column of Sephadex G-25 (fine grade, 26 cm $\times$ 2.5 cm²) at pH 7 and 5°C. The extinction ratio, E_{276}/E_{260} , of the enzyme eluate is a measure of NAD^+ still bound and was 1.17 ± 0.01 . The enzyme had full catalytic activity after dialysis or Sephadex treatment. The specific activity of lobster D-glyceraldehyde 3-phosphate dehydrogenase was measured in similar conditions of pH, solvent and temperature to those used for the Sephadex treatment and was 30 ± 2 μ mol of NADH oxidized/min per mg of enzyme. The relevance of these results to the NAD^+ dissociation constant and mechanism of D-glyceraldehyde 3-phosphate dehydrogenase is discussed below.

Acyl-enzyme of sturgeon D-glyceraldehyde 3-phosphate dehydrogenase. When the apoenzyme is preincubated with excess of 1,3-diphosphoglycerate the acyl-enzyme is formed (Trentham, 1971a) and no thiol groups are available for reaction with 5,5'-dithiobis-(2-nitrobenzoic acid).

MacQuarrie & Bernhard (1969) claim that only two out of four sites of rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase are acylated after reaction of the apoenzyme with 1,3-diphosphoglycerate. Hence it may be that the acylation of one site prevents not one but two sites from reacting with 5,5'-dithiobis-(2-nitrobenzoic acid). This hypothesis can be tested by treating the sturgeon enzyme with known concentrations of 1,3-diphosphoglycerate and adding excess of 5,5'-dithiobis-(2-nitrobenzoic acid) to the resulting solution. In this way the stoichiometry between 1,3-diphosphoglycerate added and thiol groups

Fig. 6. Titration of available thiol groups of sturgeon D-glyceraldehyde 3-phosphate dehydrogenase to monitor the hydrolysis of the acyl-enzyme. The stopped-flow traces were recorded at 412nm. One syringe contained 1,3-diphosphoglycerate ($6.0\mu\text{M}$) and an equal active-site concentration of sturgeon D-glyceraldehyde 3-phosphate dehydrogenase in triethanolamine, EDTA (1.0mM) and triethanolamine hydrochloride (80mM) adjusted to pH 7.9 with NaOH. The other syringe contained 5,5'-dithiobis-(2-nitrobenzoic acid) ($20\mu\text{M}$) and triethanolamine (100mM) at pH 7.9. The solutions were mixed at various times and the reaction chamber concentrations of the reactants were half those in the syringes. The age of the acyl-enzyme solution before reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) was as follows (min): (A) 0.7; (B) 5; (C) 10; (D) 20; (E) 40.

available for reaction can be measured. It was not possible to perform this experiment at 25°C in a conventional spectrophotometer at pH 7.9 because of the susceptibility of the acyl-enzyme to hydrolysis and consequent drift in extinction of the solution as more thiol groups became available. However, the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with available thiol groups was complete in 1s and was much faster than the hydrolysis of acyl-enzyme so that the experiment could be performed in the stopped-flow apparatus. The reaction of sturgeon apoenzyme (0.30mg/ml) with $10\mu\text{M}$ -5,5'-dithiobis-(2-nitrobenzoic acid) in 90mM -triethanolamine hydrochloride and 0.5mM -EDTA was carried out at pH 7.9. Successive increments of 1,3-diphosphoglycerate ($1.95\mu\text{M}$, syringe concentration) were mixed with the enzyme. The reaction of the partially acylated enzyme with 5,5'-dithiobis-(2-nitrobenzoic acid) was monitored within 1min of adding the acyl phosphate. The molar ratio of 1,3-diphosphoglycerate added to thiol groups removed was 1.0 initially. As the acyl phosphate concentration approached that of the enzyme concentration the ratio increased to 1.4. This is the opposite sense to that expected if one site acylated is preventing two sites reacting with 5,5'-dithiobis-(2-nitrobenzoic acid).

The time-course of the acyl-enzyme breakdown was monitored over 40min by observing the increment of available thiol for reaction with 5,5'-

dithiobis-(2-nitrobenzoic acid) (Fig. 6). Within the limits of experimental sensitivity hydrolysis of the acyl-enzyme followed a simple exponential decay with half-time of 7min. This increment of available thiol groups with time was not caused by denaturation of the acyl enzyme, since no thiol groups became available for reaction after incubation of the sturgeon acyl-enzyme with excess of 1,3-diphosphoglycerate. Care was taken to exclude NAD^+ from the solutions and the stopped-flow apparatus.

DISCUSSION

A reaction scheme which predicts that NAD^+ must dissociate from D-glyceraldehyde 3-phosphate dehydrogenase before reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) is described by eqns. (1) and (2):

E is one subunit of the tetramer, RSSR is 5,5'-dithiobis-(2-nitrobenzoic acid), RS^- is the 3-carboxy-4-nitrothiophenolate ion, E-SR is the product of the reaction between 5,5'-dithiobis-(2-nitrobenzoic acid) and E, and k_{+1} , k_{-1} and k_{+2} are rate constants. For the purposes of describing the model k_{+1} , k_{-1} and k_{+2} will be treated as being independent of the degree of NAD^+ association or 5,5'-dithiobis-(2-nitrobenzoic acid) reaction on the other subunits. This in general will not be true and appropriate modifications will be considered where necessary.

Three special cases will be considered to describe the rate of 3-carboxy-4-nitrothiophenolate production in terms of the rate constants. In the first, if $k_{-1} \ll k_{+2}[\text{RSSR}]$ and $\text{E} \ll \text{total enzyme concentration} \ll [\text{NAD}^+]$ and $[\text{RSSR}]$, then the steady-state approximation holds (Frost & Pearson, 1961) and:

$$k_{\text{obs.}} = \frac{k_{-1}k_{+2}[\text{RSSR}]}{k_{+1}[\text{NAD}^+] + k_{+2}[\text{RSSR}]} \quad (3)$$

where $k_{\text{obs.}}$ is the observed rate constant of an exponential process describing the rate of 3-carboxy-4-nitrothiophenolate production. If, in addition, $k_{+1}[\text{NAD}^+] \ll k_{+2}[\text{RSSR}]$, then:

$$k_{\text{obs.}} = k_{-1} \quad (4)$$

In the second, if the equilibrium of eqn. (1) is maintained through the course of 3-carboxy-4-nitrothiophenolate production so that the concentration of unchanged enzyme equals:

$$\text{E} \left(\frac{k_{-1} + k_{+1}[\text{NAD}^+]}{k_{-1}} \right)$$

because $k_{+2}[\text{RSSR}] \ll k_{-1}$ and $k_{+1}[\text{NAD}^+]$, then:

$$k_{\text{obs.}} = \frac{k_{-1}k_{+2}[\text{RSSR}]}{k_{-1} + k_{+1}[\text{NAD}^+]} \quad (5)$$

In the third, if the total NAD^+ concentration is zero, then:

$$k_{\text{obs.}} = k_{+2}[\text{RSSR}] \quad (6)$$

$k_{\text{obs.}}$ for the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with the sturgeon apoenzyme is first-order in 5,5'-dithiobis-(2-nitrobenzoic acid) concentration in accord with eqn. (6). Qualitatively $k_{\text{obs.}}$ (Figs. 2, 4 and 5) for the holoenzyme reaction is predicted by eqn. (3). Quantitatively, however, the description is only approximate for several reasons; the concentrations of NAD^+ and the enzyme are often similar, a wide range of NAD^+ dissociation constants to the four sites of the tetramer is probable (Conway & Koshland, 1968; Velick, Baggot & Sturtevant, 1969) and the reversible character of the disulphide-exchange reaction has not been considered in eqn. (2). However, when 5,5'-dithiobis-(2-nitrobenzoic acid) is in large excess of NAD^+ then $k_{\text{obs.}}$ will tend to k_{-1} (eqn. 4), so that the experiments do allow an estimate of the rate constant of NAD^+ dissociation from the most weakly bound enzyme site. This rate is 9s^{-1} for the lobster enzyme, which has a specific activity of $80\mu\text{mol}$ of NADH oxidized/min per mg at pH 7.9. This corresponds to a catalytic centre activity of 50s^{-1} since all four sites are active simultaneously (Trentham, 1971b). In the lobster enzyme catalysed reductive dephosphorylation of 1,3-diphosphoglycerate NAD^+ must dissociate from sites modified by other reactants because the NAD^+ dissociation rate from the holoenzyme is less than the catalytic centre activity. The same conclusion holds for sturgeon D-glyceraldehyde 3-phosphate dehydrogenase at pH 7 and 8 from a comparison of the NAD^+ dissociation rate constants and enzyme activities.

Kirschner (1967) has carried out a detailed study of the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with yeast D-glyceraldehyde 3-phosphate dehydrogenase. His results are valuable because the rate constants k_{+1} and k_{-1} have been determined independently. He investigated the reaction over a range of conditions in which k_{-1} was greater than $k_{+2}[\text{RSSR}]$, in contrast with the conditions we have used. This means that if the reaction scheme can be described by eqns. (1) and (2) then the observed rate constants are described by eqns. (5) and (6). As the scheme predicts, Kirschner (1967) observed a first-order dependence on 5,5'-dithiobis-(2-nitrobenzoic acid) both in the presence and the absence of NAD^+ . The reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with the 'T' state of D-glyceraldehyde 3-phosphate dehydrogenase in the

presence of NAD^+ was complete in 50–100 ms. By using the values of $3.2 \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$, 800s^{-1} and $7.1 \times 10^5 \text{M}^{-1} \cdot \text{s}^{-1}$ for k_{+1} , k_{-1} and k_{+2} respectively (Kirschner, 1967; Kirschner & Schuster, 1969), the predicted rate of 3-carboxy-4-nitrophenolate production would be between 142s^{-1} and 47s^{-1} in the range from zero to 5mM-NAD^+ at $0.2 \text{mM-5,5'-dithiobis-(2-nitrobenzoic acid)}$. This means that 99% of the reaction would be over within 100 ms as was observed. The reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with the 'R' state of D-glyceraldehyde 3-phosphate dehydrogenase in the presence of NAD^+ was much slower. By using the values of $1.1 \times 10^7 \text{M}^{-1} \cdot \text{s}^{-1}$, 1100s^{-1} for k_{+1} , k_{-1} (Kirschner & Schuster, 1969) and the same value of k_{+2} , the predicted second-order rate of 3-carboxy-4-nitrophenolate production is $1.4 \times 10^4 \text{M}^{-1} \cdot \text{s}^{-1}$. The observed second-order rate of $9 \times 10^3 \text{M}^{-1} \cdot \text{s}^{-1}$ is in good agreement. Kirschner (1967) presents some evidence that k_{+2} is slower for the 'R' state of the enzyme, based on the observation of a process of small amplitude when the apoenzyme reacts with 5,5'-dithiobis-(2-nitrobenzoic acid). If his interpretation is correct then the quantitative agreement is not so satisfactory. Overall the kinetics of the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with yeast, lobster and sturgeon D-glyceraldehyde 3-phosphate dehydrogenase are consistent with the scheme represented by eqns. (1) and (2) and the value of second-order rate constant deduced by Kirschner (1967) for the reaction of the holoenzyme with 5,5'-dithiobis-(2-nitrobenzoic acid) determines $(k_{-1}k_{+2})/(k_{-1} + k_{+1}[\text{NAD}^+])$.

Since the rate of reaction of 2-chloromercuri-4-nitrophenol with the apoenzyme and holoenzyme is first order in mercurial concentration and is unaffected by the addition of NAD^+ , the mercurial reacts without prior displacement of NAD^+ , in contrast with 5,5'-dithiobis-(2-nitrobenzoic acid). The origin of this difference probably arises from the different orientation of the thiol reagents when bound at the active site of D-glyceraldehyde 3-phosphate dehydrogenase (Gutfreund & McMurray, 1970). However, the possible proximity of cysteine-152 to the active site cysteine-148 introduces ambiguity over the site of attack of thiol reagents. At high NAD^+ concentrations the equilibrium of the 5,5'-dithiobis-(2-nitrobenzoic acid) reaction with the holoenzyme does not favour the disulphide-exchange reaction until protein denaturation begins.

The esterase activity of D-glyceraldehyde 3-phosphate dehydrogenase towards 4-nitrophenyl acetate is inhibited by NAD^+ (Park, Meriwether, Clodfelder & Cunningham, 1961; Taylor *et al.* 1963). There is a transient production of 4-nitrophenol in the first turnover of D-glyceraldehyde 3-phosphate dehydrogenase, which disappears in the presence of NAD^+ , showing that there is a change

of rate-determining step. It seems likely that, as in the reaction with 5,5'-dithiobis-(2-nitrobenzoic acid), NAD^+ must be displaced from the enzyme before its acylation by 4-nitrophenyl acetate.

Certain alkylating agents react more rapidly with the apoenzyme than with the holoenzyme (Eisele & Wallenfels, 1969). Whereas the differential reactivity is probably steric in origin the reaction rate of, for example, the D- α -iodopropionamide and yeast D-glyceraldehyde 3-phosphate dehydrogenase in the presence of 20 mM- NAD^+ is too fast for NAD^+ dissociation to precede alkylation.

The dissociation constants of NAD^+ to native lobster D-glyceraldehyde 3-phosphate dehydrogenase require careful consideration. Their values set an upper limit to the NAD^+ -off constant since the binding process of NAD^+ to the enzyme can be no faster than diffusion-controlled. The spectrum of NAD^+ -bound enzyme will be approximately the sum of the NAD^+ absorption spectrum plus the apoenzyme absorption spectrum in the 260–280 nm region. For the apoenzyme E_{280}/E_{260} is 1.99 (De Vijlder, Boers & Slater, 1969) whereas for the NAD^+ -bound holoenzyme E_{276}/E_{260} is 1.10 (Trentham, 1968). Since E_{276}/E_{260} for NAD^+ is 0.40, E_{276}/E_{260} for E_{NAD_1} , E_{NAD_2} and E_{NAD_3} will be about 1.61, 1.37 and 1.21 respectively. After dialysis the enzyme retains its full complement of 4 mol of NAD^+ /tetramer since E_{276}/E_{260} is still 1.10 (Trentham, 1968). As an upper limit to the extent of NAD^+ release suppose that the final state of the enzyme was $E_{\text{NAD}_{3.5}}$ (i.e. the fourth site has lost half its NAD^+), then the dissociation constant for this site, K_4 , would be 2×10^{-8} M. Although this value is in theory an upper limit for K_4 , in practice we have reservations about accepting it in view of the slow rate of equilibrium of NAD^+ across the dialysis membranes indicated by the control experiments. The Sephadex elution experiments show independently that the dissociation constants for the other sites, K_1 , K_2 and K_3 , must be very small. It would also appear that Sephadex chromatography fails to remove even 1 mol of NAD^+ /tetramer completely since the mean value of E_{276}/E_{260} is 1.17. These results conflict with the larger value for K_4 of 1.3×10^{-5} M obtained by De Vijlder *et al.* (1969) by using equilibrium dialysis. However, their value is extremely sensitive to the measurement of concentration of active enzyme and free NAD^+ in the diffusate since the concentration of enzyme used was six times their estimate of K_4 . No value is quoted for the enzyme activity after equilibrium dialysis studies. The rates of diffusion-controlled processes of substrates combining with enzymes are about $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Eigen & Hammes, 1963). De Vijlder *et al.* (1969) have shown that the rate of NAD^+ binding to the apoenzyme of lobster D-glyceraldehyde 3-phosphate dehydrogenase is

greater than $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (not $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ as they calculated). The rate of NAD^+ binding to the 'R' state of yeast D-glyceraldehyde 3-phosphate dehydrogenase is $7.0 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7.5 (Kirschner & Schuster, 1969). K_4 is probably less than or approximately equal to 2×10^{-8} M, so that (allowing for a statistical factor of 4) the maximum intrinsic rate constant of NAD^+ release from E_{NAD_4} to form E_{NAD_3} is about 0.5 s^{-1} at 5°C . The rate of NAD^+ release would be less from more tightly bound sites. The specific activity of lobster D-glyceraldehyde 3-phosphate dehydrogenase at 5°C is $30 \mu\text{mol}$ of NADH oxidized/min per mg of enzyme. This corresponds to a catalytic centre activity of 18 s^{-1} if all four sites per tetramer are active (and 72 s^{-1} if only one site is active). These results support the conclusion of the 5,5'-dithiobis-(2-nitrobenzoic acid) displacement experiments. Namely, NAD^+ only dissociates from sites modified by substrates in the lobster D-glyceraldehyde 3-phosphate dehydrogenase-catalysed reductive dephosphorylation of 1,3-diphosphoglycerate as Trentham's (1971a) transient experiments indicated. However, we would stress that whereas these experiments do not conclusively establish this aspect of the enzyme mechanism, they do positively indicate that the value of 1.3×10^{-5} M for K_4 determined by de Vijlder *et al.* (1969) is at least an order of magnitude too large. It is important to establish this latter point since otherwise the kinetics of the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) with the lobster holoenzyme (Fig. 4) are difficult to interpret.

The experiments on the acylation of the apoenzyme of sturgeon D-glyceraldehyde 3-phosphate dehydrogenase indicate that all four sites of the tetramer react. Indeed this is to be expected in view of the range of studies with transients and partial reactions of single turnovers of the enzyme which show that all four sites on the enzyme are active simultaneously (Trentham, 1971b). MacQuarrie & Bernhard (1969) have presented evidence to show that only two out of four sites of the apoenzyme of rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase are acylated by 1,3-diphosphoglycerate. Their results depend on back-titration of the free sites with iodoacetic acid after passing the acyl-enzyme through Bio-Gel. The sturgeon acyl-enzyme has been shown here to be fairly labile, so that the rate of rabbit acyl-enzyme hydrolysis must be clearly established. We conclude that present evidence does not show convincingly that the four sites of the rabbit apoenzyme react with 1,3-diphosphoglycerate at markedly different rates.

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