Aspects of the Chemistry of D-Glyceraldehyde 3-phosphate Dehydrogenase

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Crystalline D-glyceraldehyde 3-phosphate dehydrogenase from lobster tail contains 4 moles of NAD^+ bound and reacts specifically with 4 moles of iodoacetic acid/mole of tetramer. The essential thiol group of D-glyceraldehyde 3-phosphate dehydrogenase appears to react with iodoacetic acid with a rate constant for the overall process that is independent of the extent of carboxymethylation. The D-glyceraldehyde 3-phosphate dehydrogenase- NAD^+ absorption band has a variable molar extinction coefficient in the presence of phosphate that may be correlated with a proton dissociation of pK 6.86. The binding of NAD^+ to D-glyceraldehyde 3-phosphate dehydrogenase weakens as alkylating agents react with the enzyme, and NAD^+ promotes the reactivity of the essential thiol group. It is suggested that, on binding to D-glyceraldehyde 3-phosphate dehydrogenase, NAD^+ lowers the pK of the essential thiol group, resulting in a catalytic role of NAD^+ in the reaction catalysed by D-glyceraldehyde 3-phosphate dehydrogenase. If this theory is correct, then it is likely that a proton will be liberated during the phosphorylation of the acyl-enzyme rather than in the redox step.

The work described is part of a general programme for investigation of the dynamic properties of GPDH.* The enzyme from lobster tail was chosen since the amino acid sequence and molecular weight of the enzyme are known (Davidson, Sajgò, Noller & Harris, 1967), and work is in progress to determine the three-dimensional structure by using X-ray-diffraction analysis (Watson & Banaszak, 1964). A precise knowledge of the stoichiometry of the binding of substrates and the number of sites on the enzyme available for reaction is necessary in order to study the detailed kinetic parameters and single-turnover properties of the enzyme. Hence accurate measurements of the number of moles of NAD^+ bound and of the number of thiol groups alkylated by iodoacetic acid/mole of tetramer in the crystal were made. The specificity of the iodoacetic acid reaction with the enzyme had therefore to be examined. In GPDH isolated from rabbit muscle the thiol group alkylated is the same as that which is acylated by the substrates and is called the essential thiol group (Harris, Meriwether & Park, 1963). The essential thiol group and the amino acid sequence around it are conserved to a remarkable extent in GPDH from many species, including lobster (Allison & Harris, 1965). The broad absorption band from 320 to 450 $\text{m}\mu$ associated with enzyme-bound NAD^+ is called the Racker band

(Racker & Krimsky, 1952). The magnitude and rate of the extinction change of the Racker band resulting from the reaction of iodoacetic acid with the enzyme were studied to investigate relative reactivities of the essential thiol groups during the course of the reaction. The qualitative difference reported in the absorption bands remaining after the enzyme had reacted with iodoacetic acid and other alkylating agents (Boross & Cseke, 1967) was also examined. The molar extinction coefficient of the Racker band was determined in the presence of phosphate, a substrate for the enzyme. NAD^+ plays an important role apparently unconnected with its redox action in the reactions of GPDH with a variety of substrates (Harting & Velick, 1954; Krimsky & Racker, 1955; Park, Meriwether, Clodfelder & Cunningham, 1961; Malhotra & Bernhard, 1968). A study was made of the change of the dissociation constant of NAD^+ after alkylation of the enzyme. The rates of the reaction of iodoacetic acid with NAD^+ -bound and NAD^+ -free enzyme were compared. A simple theory is proposed to explain the behaviour and function of NAD^+ in its non-redox role, and a method to test the theory is indicated.

MATERIALS AND METHODS

Preparation and assay of GPDH. GPDH was isolated from lobster tails by essentially the method of Allison & Kaplan (1964), except that centrifugation at 15000g (MSE 18

* Abbreviation: GPDH, D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12).

centrifuge, 6×250 ml. angle rotor) for 30 min. replaced the filtration steps and the enzyme was crystallized only once. The enzyme was not used until 3 weeks after the beginning of crystallization.

NAD⁺-free enzyme was made by stirring 150 mg. of freshly activated charcoal (Norit OL; Hopkin and Williams Ltd., Chadwell Heath, Essex) with 1 ml. of GPDH (5.8 mg.) in EDTA (5 mM) and 2-mercaptoethanol (1 mM) for 5 min. at 5° (Velick, Hayes & Harting, 1953). The mixture was centrifuged at 150 000g (MSE 50 T.C. centrifuge, 10×10 ml. angle rotor) for 1 hr. at 5° and the supernatant removed. The recovery of enzyme activity was 4%.

The enzyme was assayed by a modification of Ferdinand's (1964) procedure. The assay medium contained D-glyceraldehyde 3-phosphate (0.28 mM), NAD⁺ (0.60 mM), EDTA (0.8 mM), 2-mercaptoethanol (0.75 mM), NaH₂PO₄ (39 mM) and triethanolamine (31 mM). The enzyme was added to a 1 cm. cell to initiate the reaction. The E_{340} change due to the formation of NADH was followed spectrophotometrically at 25°. The rate of increase of the first 0.2 E_{340} unit change was linear. The specific activity of the enzyme obtained from the dissolved crystals from all three preparations was consistently 160 ± 10 μ moles of NADH produced/min./mg. of enzyme. Where possible, the enzyme was preincubated at its final dilution immediately before the assay for 5 min. in phosphate buffer (50 mM), EDTA (1 mM) and 2-mercaptoethanol (1 mM) at pH 6.86 at 0° (Ferdinand, 1964). The molar extinction coefficient of NADH at 340 m μ was taken as 6220 (Horecker & Kornberg, 1948).

Reagents. *N*-(4-Iodophenyl)iodoacetamide and *N*-(4-methylphenyl)iodoacetamide were prepared similarly to related compounds (Clark & Hams, 1953). Crystalline chloroacetic acid anhydride was obtained as a distillate (b.p. 200–210° fraction) from a solution of chloroacetic acid (94 g.), acetic anhydride (250 ml.) and toluene (300 ml.). Chloro[2-¹⁴C]acetic acid anhydride was synthesized by incubating chloroacetic acid anhydride (100 mg.) with chloro[2-¹⁴C]acetic acid (0.05 mc) at 37° for 2 days in a sealed tube.

N-(4-Iodophenyl)iodo[2-¹⁴C]acetamide was synthesized as follows. 4-Iodoaniline (26 mg.) in dry dioxan (0.1 ml.) was treated with chloro[2-¹⁴C]acetic acid anhydride (24 mg., specific radioactivity 0.085 c/mole) at 0° for 5 min. Ice-cold water (3 ml.) was added to the product, which was centrifuged after a further 5 min. The precipitate was washed with water and dried *in vacuo*. The chloroacetamide derivative was dissolved in acetone (0.1 ml.) and treated with NaI (100 mg.) in acetone (0.38 ml.). The acetone had been passed through a column of molecular sieves (aluminium sodium silicate, 8–12-mesh beads). The reaction flask was stoppered, wrapped in aluminium foil and shaken gently at 35° for 24 hr. Acetone was removed by a stream of air and the product was washed with water to extract soluble material. *N*-(4-Iodophenyl)iodo[2-¹⁴C]acetamide was crystallized from ethanol.

N-(4-Methylphenyl)iodo[2-¹⁴C]acetamide was prepared in the same way. Non-radioactive analogues were synthesized on a larger scale (overall yield 60%).

N-(4-Iodophenyl)iodoacetamide has m.p. 202° (decomp.), $\lambda_{\max}$ in 95% ethanol 267 m μ (ϵ 14 300) (Found: C, 25.1; H, 1.9; I, 65.4; N, 3.6; C₈H₇I₂NO requires C, 24.8; H, 1.8; I, 65.6; N, 3.6%).

N-(4-Methylphenyl)iodoacetamide has m.p. 162–164°, $\lambda_{\max}$ in 95% ethanol 262 m μ (ϵ 8700). (Found: C, 39.2;

H, 3.7; I, 46.4; N, 5.05; C₉H₁₀INO requires C, 39.3; H, 3.6; I, 46.2; N, 5.1%). Melting points are uncorrected.

DL-Glyceraldehyde 3-phosphate (as the barium salt of the diethylacetal), NADH and NAD⁺ were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Solutions of DL-glyceraldehyde 3-phosphate were prepared by the method of Racker, Klybas & Schramm (1959). D-Glyceraldehyde 3-phosphate and NAD⁺ were assayed spectrophotometrically by the methods of Ferdinand (1964). The arsenate assay medium contained Na₂HAsO₄ (12 mM), EDTA (0.2 mM) and triethanolamine (200 mM) adjusted to pH 8.7 with HCl.

Triethanolamine was redistilled under reduced pressure and stored at –18°. HCl and aq. NH₃ were prepared by isopiestic distillation (Irving & Cox, 1958) and were used for all pH adjustments where appropriate. Water was glass-distilled. Iodoacetic acid and iodoacetamide were recrystallized. All other reagents were A.R. grade where possible and were used without further purification.

Iodo[2-¹⁴C]acetic acid and chloro[2-¹⁴C]acetic acid were supplied by The Radiochemical Centre (Amersham, Bucks.).

Radioactivity measurements. These were made with a Mk 1 model 6860 liquid-scintillation counter (Nuclear-Chicago Corp.). Standards of iodo[2-¹⁴C]acetic acid or of the synthesized amides were used as internal controls.

Spectroscopic measurements. These were made in the 1 cm. thermostatically controlled cuvette of a Uvispek H700 spectrophotometer (Hilger and Watts Ltd.) provided with a Gilford recording attachment and a Sunvic recorder (type 10s).

Microanalyses. These were performed by Dr Franz Pascher (Mikroanalytisches Laboratorium, Bonn, Germany).

RESULTS

The stoichiometry of the binding of NAD⁺ to the crystalline enzyme was determined. The enzyme crystals were washed four times at room temperature with saturated ammonium sulphate adjusted to pH 7 with ammonia. Between each wash the crystals were centrifuged gently in an MSE bench centrifuge to remove any amorphous material.

A portion of the final supernatant was kept as a control solution. The crystals were dissolved in 2.5 ml. of EDTA (5 mM) and 2-mercaptoethanol (1 mM) at pH 7.5. This enzyme solution (45.4 mg./ml.; see below) was also used to determine the extinction of a known weight of protein and the stoichiometry of the reaction with iodoacetic acid. The control solution had an active enzyme concentration 8×10^{-6} times that of the enzyme solution.

A 0.80 ml. portion of the enzyme solution was added to 1 ml. of a solution of 0.2 M-hydrochloric acid containing pepsin (1 mg.) to ensure breakdown of the protein. After incubation for 2 hr. at 37° the product was centrifuged and the supernatant and precipitate washings made up to 5.00 ml. with 0.2 M-phosphate buffer at pH 7.8. The solution was centrifuged for 1 hr. at 15° at 150 000g (MSE 50 T.C. centrifuge, 10×10 ml. angle rotor). A 4.00 ml.

portion of the solution was treated with 9.00 ml. of the arsenate assay medium. Three assays were made of the change in E_{340} when 3.00 ml. of the mixture was treated with GPDH (10 μ g.). The original enzyme solution (45.4 mg./ml.) contained 1.202 mM-NAD⁺.

The specific extinction coefficient of the protein was measured. The enzyme solution (1 ml.) was dialysed against 3 l. of EDTA (5 mM) and 2-mercaptoethanol (1 mM) and then against 3 l. of EDTA (1 mM) and 2-mercaptoethanol (0.2 mM). The diffusate was used as a control solution. The enzyme solution was made up to 5.00 ml. with water. The dry weight of 2.75 ml. of this solution due to non-diffusible material was 20.45 mg. A 0.90 ml. portion of this solution was made up to 10.00 ml. with phosphate buffer (50 mM) at pH 6.86, and this had E_{276} 0.674 and E_{260} 0.614 in a 1 cm. cell. The E_{276}/E_{260} ratio was 1.10. This is the same as the E_{276}/E_{260} ratio of the enzyme before dialysis, namely 1.108. Hence no NAD⁺ was removed from the enzyme during dialysis, and so the specific extinction coefficient of the enzyme, $E_{1\text{cm}}^{1\%}$, was 10.1 at 276 $m\mu$.

On the basis of a molecular weight of 146 650 for the enzyme tetramer (Davidson *et al.* 1967) and the measured E_{276} value, the enzyme solution contained 45.4 mg./ml. and hence was 0.309 mM in

enzyme. Therefore the enzyme crystal has 3.9 moles of NAD⁺ bound/mole of tetramer.

The stoichiometry of the reaction of iodoacetic acid with the enzyme was determined. First the incorporation of iodo[2-¹⁴C]acetic acid was followed during and after the enzyme had been inhibited to test whether the reaction was selective. The rate of reaction of iodoacetic acid with GPDH may be linked with the rate of change of extinction of the Racker band and the rate of inhibition of the enzyme. The second-order rate constants for these two processes are shown below to be about 8 M⁻¹sec.⁻¹ at 5° at pH 7.5. A 100-fold excess of 2-mercaptoethanol over enzyme active-site concentration did not affect the inhibition rate. The enzyme was incubated with iodo[2-¹⁴C]acetic acid, and samples were removed at intervals and subjected to Sephadex chromatography so that the incorporation of radioactivity into the protein could be determined (Table 1). The E_{276}/E_{260} ratio

Table 1. Relative incorporation of ¹⁴C radioactivity into GPDH after various incubation times of iodo[2-¹⁴C]acetic acid with GPDH

A solution (1.0 ml.) containing iodo[2-¹⁴C]acetic acid (2.0 mM, specific radioactivity 0.6 c/mole), GPDH (18 mg.), phosphate buffer (30 mM), EDTA (2 mM) and 2-mercaptoethanol (0.4 mM) was incubated at pH 7.0 at 0°. Samples (0.2 ml.) were removed at intervals and passed through Sephadex G-25 (fine grade, packed in columns 50 cm. × 0.8 cm. internal diam.) with EDTA (5 mM) and 2-mercaptoethanol (1 mM) as the eluting solvent at pH 7.5 at 5°. The times of incubation do not include the approx. 15 min. period for the protein to separate from the free iodoacetic acid during adsorption on the column. After hydrolysis of the eluted protein fractions ¹⁴C incorporation was measured. Only background levels of radioactivity were found in fractions eluted between the protein fractions and free iodo[2-¹⁴C]acetic acid fractions. Results are expressed as the ratio of protein specific radioactivity after various incubation times to that after a 60 min. incubation. The enzyme activity measurements of the eluted protein fractions are relative to native enzyme activity.

Time of incubation (min.)	Relative ¹⁴ C incorporation	Relative enzyme activity
0.5	0.88	0.11
15	1.00	
30	1.01	
60	1.00	< 0.003

Fig. 1. Stoichiometry of the reaction of iodoacetic acid with GPDH. The ordinate represents enzyme activity relative to the native enzyme. A solution (1.00 ml.) containing GPDH (36.5 mg., 0.249 μ mole), EDTA (1 mM), 2-mercaptoethanol (1 mM) and phosphate buffer (7.5 mM) at pH 7.0 was treated at 5° with 10.0 μ l. portions of iodoacetic acid (16.7 mM) adjusted to pH 7. The residual enzyme activity was allowed to become constant between successive additions of iodoacetic acid. Corrections were made for volume changes.

Table 2. *Extinction of a solution of GPDH after various times of incubation with iodoacetic acid*

The $E_{1\text{cm.}}$ values of a solution of GPDH (8.0mg./ml.), iodoacetic acid (2.00mM), phosphate buffer (50mM), EDTA (5mM) and 2-mercaptoethanol (1mM) at pH 7.3 at 5° were measured at various times after the addition of iodoacetic acid that had been adjusted to pH 7.

Time of incubation

(hr.)	$E_{365}^{1\text{cm.}}$	$E_{390}^{1\text{cm.}}$	$E_{550}^{1\text{cm.}}$
0	0.117	0.071	0.001
0.5	0.040	0.0215	0.002
16	0.016	0.008	0.000

of the protein was 1.262 ± 0.003 for each Sephadex-column eluate. Hence the relative protein concentrations could be determined. Samples of the protein fractions were hydrolysed with 6M-hydrochloric acid for 24hr. at 110° before measurement of ^{14}C radioactivity. This analysis showed (Table 1) that the reaction of iodoacetic acid with GPDH was selective, and hence the stoichiometry of the reaction could be determined. The loss of activity of GPDH on adding portions of iodoacetic acid was then studied (Fig. 1). Control solutions showed that there was no loss of enzymic activity other than from iodoacetic acid inhibition. It was found that 3.8 moles of iodoacetic acid/mole of tetramer were necessary to inhibit the enzyme completely.

Information about the relative reactivity of enzyme active sites was obtained by studying the change in absorption of the Racker band with time during the reaction of iodoacetic acid with GPDH. The rate of change of absorption followed biphasic kinetics (Table 2). Interpretation of the more rapid reaction (Fig. 2) showed that the overall process followed pseudo-first-order kinetics within the limits of measurement. The second-order rate constant for this reaction, $9.0\text{ M}^{-1}\text{sec.}^{-1}$, is similar to the second-order rate constant obtained from the inhibition studies at 0° (see below and Fig. 4). The half-time of the slower reaction varied between 10 and 50hr. (2000–10000 times that of the faster reaction). The E_{365}/E_{390} ratio of the native enzyme was 1.65 and rose to 1.85 during the more rapid reaction. The enzyme used in the experiment described had been passed through Sephadex G-100, so that about 20% of the NAD^+ had been removed as estimated by absorption of the Racker band, and by the fact that the E_{278}/E_{260} ratio was 1.23. However, the fast reaction could still be interpreted as an overall pseudo-first-order process when the enzyme had its full complement of NAD^+ .

The variation in the absorption of the Racker band with pH was studied at $365\text{m}\mu$ (Fig. 3). In the presence of phosphate E_{365} varies with pH. The continuous line (Fig. 3) shows that this

Fig. 2. (a) Spectrophotometric record of the change of E_{365} with time after the addition of iodoacetic acid to a solution of GPDH (for conditions see Table 2). (b) Description of the rate of change of $E_{365}^{1\text{cm.}}$ in (a) as an overall first-order process. X is the ratio of the extinction difference at various times and the end of the more rapid reaction to the extinction difference before addition of iodoacetic acid and the end of the more rapid reaction. The abnormal curvature of the trace during the first 10sec. was considered to be due to the recorder response time.

extinction change is probably associated with a proton dissociation of pK close to 6.86. The heat of reaction associated with the spectral change was determined by following the extinction change in a solution of the enzyme as the temperature was raised from 4° to 26° and fell back to 4°. No extinction change was observed. The solution contained GPDH (5.5mg./ml.), pyrophosphate (60mM), phosphate (25mM), potassium chloride (150mM) and Na^+ ions at pH 6.5. No extinction

Fig. 3. Variation with pH of $E_{365}^{1\text{cm}}$ at 10° of solutions of GPDH in various buffers. In addition to GPDH (4.5 mg./ml.), NaCl (67 mM) and Na^+ ions appropriate to the pH, the solutions contained: ●, phosphate (30 mM), pyrophosphate (30 mM), K^+ ions (15 mM), EDTA (0.67 mM); ○, pyrophosphate (45 mM), EDTA (0.67 mM); ▲, EDTA (33 mM); △, as ▲, with KCl (100 mM). pH values were measured at 25° . The line is that predicted for a material with two different molar extinction coefficients resulting from a proton dissociation with pK 6.86.

change was observed in a control solution when its temperature was raised from 4° to 11° at pH 8.5. Above 11° the solution at pH 8.5 became turbid. If the proton ionization step is assumed to have pK 6.86, then the heat of reaction differs from that of the reaction:

by less than $1.5 \text{ kcal. mole}^{-1}$ (Bernhard, 1956; *Stability Constants of Metal-Ion Complexes*, 1964).

The rates of extinction change after a rapid pH change by using stopped-flow techniques were studied in phosphate-pyrophosphate buffers. The shortest half-time that could be measured by this technique was 2 msec. The rates of extinction change, when the pH of the enzyme solution was changed from 7.7 to 5.6 and from 5.4 to 7.3, were too fast to measure. The apparatus and techniques for the stopped-flow equipment were those of Barman & Gutfreund (1966).

Studies on the variable dissociation constant of NAD^+ to GPDH arose from inhibition experiments of the enzyme with *N*-(4-iodophenyl)iodoacetamide and *N*-(4-methylphenyl)iodoacetamide. Since the iodoacetic acid reaction with the enzyme has been shown to be selective and the thioalkyl derivative so formed is well characterized, it was reasonable to assume that these compounds would provide an isomorphous pair of enzyme derivatives for X-ray analysis of the enzyme crystal.

The iodophenyl-enzyme derivative was prepared by adding a solution (0.5 ml.) of *N*-(4-iodophenyl)-iodo[2- ^{14}C]acetamide (3.1 mM) in acetone to a solution (15 ml.) of GPDH (4.55 mg./ml.) and EDTA (1 mM) at pH 7.2 and incubating the mixture at 5° for 16 hr. The product showed 10% of the native enzyme activity. The methylphenyl derivative was prepared in the same way and showed 5% of the native enzyme activity. Acetone alone did not affect the enzyme activity. The solution of inhibited enzyme was dialysed against a solution of EDTA (1 mM) and 2-mercaptoethanol (1 mM) at pH 7. The pH of the solution was adjusted to 5.9. Precipitate was removed by centrifugation. Crystallization of the enzyme was attempted at 5° by adding a saturated ammonium sulphate solution that had also been adjusted to pH 5.9. In the absence of NAD^+ precipitation of protein occurred at 50% saturation of ammonium sulphate. However, if ammonium sulphate was added to an inhibited enzyme solution containing NAD^+ (1 mM) and phosphate (10 mM) then crystallization of the enzyme occurred at 75% saturation of ammonium sulphate.

The crystals, which appeared with a dividing crack, were similar in shape to crystals of the enzyme grown in the presence of excess of *p*-chloromercuribenzoic acid (Watson & Banaszak, 1964). Measurement of ^{14}C incorporation against protein concentration, which was measured by a modified biuret procedure (Beisenherz *et al.* 1953), showed that 2–3 moles of inhibitor were bound/mole of tetramer in the crystals.

The crystallization experiment indicated that NAD^+ binds to the inhibited enzyme less strongly than to the native enzyme. This effect was examined more closely by treating the enzyme with portions of inhibitors and determining the number of moles of inhibitor and moles of NAD^+ bound/mole of tetramer. The protein was separated from low-molecular-weight species by using a small Sephadex column. Under similar conditions only a small amount of NAD^+ was removed from the native enzyme. The results (Table 3) show that the total number of moles of inhibitor that have reacted plus moles of NAD^+ bound/mole of tetramer is close to 4 except when excess of inhibitor has reacted. It was shown that the reaction of *N*-(4-

Table 3. *Stoichiometry of the moles of tightly bound NAD⁺ and moles of covalently bound inhibitors/mole of tetramer of GPDH*

Two solutions (0.8 ml.) of GPDH (13.2 mg.) in EDTA (5 mM) and 2-mercaptoethanol (1 mM) were treated with 10 μ l. of acetone solutions of *N*-(4-iodophenyl)iodo[2-¹⁴C]acetamide (26 mM) and *N*-(4-methylphenyl)iodo[2-¹⁴C]acetamide (20 mM) respectively at pH 7 at 25°. After 30 min. the enzyme activity relative to the initial activity was measured. Samples (0.4 ml.) of the solutions were chromatographed on Sephadex G-25 (fine grade, packed in columns 15 cm. $\times$ 0.8 cm. internal diam.) with EDTA (1 mM) and 2-mercaptoethanol (1 mM) as the eluting solvent at pH 7.5 at 5° to isolate the protein fractions. The remainder of the enzyme solutions was treated with further 10 μ l. portions of inhibitors. After a further 30 min. enzyme activity measurements and Sephadex chromatography were repeated. A control enzyme solution treated with acetone alone showed no loss of enzyme activity. The E_{276} and E_{280} values of the protein solution were measured. The amount of NAD⁺ bound to the enzyme was measured by Ferdinand's (1964) procedure, and the amount of inhibitor that had reacted by assay of ¹⁴C incorporation. In estimating the molar extinction coefficient at 276 m μ of the enzyme eluted from the columns, corrections were made for NAD⁺ bound and the amide incorporated. After allowance for variations of molar extinction coefficients, particularly of the amide in its protein environment, the estimated maximum error of the stoichiometry of moles of NAD⁺ bound plus moles of inhibitor incorporated/mole of tetramer was 20%.

Inhibitor	Moles bound/mole of enzyme tetramer		Relative enzyme activity
	NAD ⁺	Inhibitor	
<i>p</i> -I-C ₆ H ₄ ·NH·CO·CH ₂ ·I	2.4	1.2	0.55
<i>p</i> -CH ₃ ·C ₆ H ₄ ·NH·CO·CH ₂ ·I	1.9	1.9	0.45
<i>p</i> -I-C ₆ H ₄ ·NH·CO·CH ₂ ·I	0.9	2.9	0.04
<i>p</i> -CH ₃ ·C ₆ H ₄ ·NH·CO·CH ₂ ·I	0	5.4	0.01

Fig. 4. Relative rates of inhibition by iodoacetic acid of NAD⁺-bound and NAD⁺-free GPDH. *X* is the ratio of the initial enzyme activity to the enzyme activity after various incubation times with iodoacetic acid. The incubation mixture (0.5 ml.) contained: ●, charcoal-treated GPDH (equivalent to 4 μ g. of fully active enzyme), NaCl (600 mM), phosphate buffer (40 mM), 2-mercaptoethanol (50 μ M), iodoacetic acid (80 μ M); ▲, as ●, except NAD⁺ (8 μ M) and iodoacetic acid (73 μ M). Iodoacetic acid and NAD⁺ were adjusted to pH 7 before addition to the enzyme solutions. Addition of NAD⁺ did not alter the relative initial activity of the solutions. Incubation was carried out at 5° at pH 7.5. After 46 hr. both the NAD⁺-bound and the NAD⁺-free enzyme solutions had 1% of their initial activity and a control NAD⁺-free solution untreated with iodoacetic acid had 18% of its initial activity.

methylphenyl)iodo[2-¹⁴C]acetamide with GPDH was not specific to a single site in the same way that iodoacetic acid is, since, on treating the enzyme with 8 equiv. of inhibitor/sub-unit, all the radioactivity was found in the high-molecular-weight fraction after Sephadex chromatography.

Iodoacetic acid inactivates NAD⁺-free GPDH less rapidly than NAD⁺-bound GPDH (Racker & Krimsky, 1958). This effect was studied further, since it may reflect relative reactivities of the thiol group that forms the thioalkyl derivative.

The relative rates of inhibition of the NAD⁺-bound and NAD⁺-free forms of the enzyme by iodoacetic acid were 4.9 M⁻¹sec.⁻¹ and 0.62 M⁻¹sec.⁻¹ when expressed as second-order rate constants (Fig. 4). This method of presentation of the results is not intended to represent a proposal of the mechanism of the rate-determining step, but is merely a convenient way of expressing the overall pseudo-first-order processes by which the results can be described (NAD⁺ migration may be rate-determining). The experiment was repeated in imidazole buffer (60 mM) at pH 7.7, since phosphate is a substrate for the enzyme. The second-order rate constants observed were 5.9 M⁻¹sec.⁻¹ for NAD⁺-bound GPDH, 0.38 M⁻¹sec.⁻¹ for NAD⁺-free GPDH and 0.79 M⁻¹sec.⁻¹ for NADH-bound GPDH.

The reaction of iodoacetamide and its derivatives with GPDH and the enzymic activity of the resulting partially inhibited enzyme show two marked differences from the analogous iodoacetic acid inhibitions. First, the rates of iodoacetamide

inhibition of NAD^+ -bound and NAD^+ -free GPDH were not markedly different. Racker & Krimsky (1958) have noted that the rate of inhibition of rabbit muscle GPDH by iodoacetamide is much lower than by iodoacetic acid. Secondly, the residual active sites of GPDH partially inhibited by *N*-(4-iodophenyl)iodoacetamide and *N*-(4-methylphenyl)iodoacetamide were less active than the active sites of the native enzyme. In particular, the rate of increase of E_{340} showed large deviations from linearity during the first 0.2 E_{340} unit increase in a 1 cm. cell. This is in contrast with the reactivity of the residual active sites in a normal assay of GPDH that has been partially inhibited by iodoacetic acid (Fig. 1 and Table 1).

DISCUSSION

For studies on elementary processes occurring during enzyme reactions it is essential to have pure enzyme, otherwise incorrect interpretation of experiments concerning the number of molecules of substrates reacting/molecule of enzyme and their rate of reaction in a single turnover of the enzyme is likely. Although GPDH is an enzyme ideally suited for a detailed study of its dynamic properties, the variable degree of purity of much of the enzyme used (Ferdinand, 1964) and the possibility that it may exist as more than one isoenzyme (Lebherz & Rutter, 1967) have meant that there is much uncertainty about its detailed chemistry.

The enzyme from lobster is well suited for a detailed study of its dynamic behaviour because the primary structure and molecular weight are known (Davidson *et al.* 1967), and work on the three-dimensional structure is in progress (Watson & Banaszak, 1964). It was found that 3.9 moles of NAD^+ were bound/mole of tetramer and 3.8 moles of iodoacetic acid were necessary to inhibit completely each mole of tetramer. Hence it would appear that the enzyme is both homogeneous and fully active with four active sites. The ^{14}C -incorporation experiment (Table 1) shows the specificity of the reaction of iodoacetic acid with GPDH.

The overall rate of change of absorption of the Racker band, during the reaction of iodoacetic acid with GPDH in the presence of a tenfold excess of acid, can be interpreted as a pseudo-first-order process. This indicates that the extinction of the GPDH- NAD^+ complex at one site and the rate of reaction of the enzyme with iodoacetic acid at that site are independent of the alkylation of the other essential thiol groups of the enzyme. However, this interpretation must be qualified by the fact that the sensitivity of the analysis is such that up to twofold differences in the rates of alkylation of the sites may be masked, and that a pseudo-first-order

process can be interpreted in terms of a slow alkylation step followed by a much more rapid one.

The crystal structure of carboxymethylated GPDH is isomorphous with that of the native enzyme (Wassarman & Watson, 1968). This evidence further suggests that carboxymethylation at one site is not likely to affect reactivity at another site. The X-ray-diffraction studies indicate that the tetramer could consist of structurally identical pairs of sub-units (Watson & Banaszak, 1964), and the four sub-units have identical protein chains (Davidson *et al.* 1967). The equivalence in reactivity of the four active sites is in contrast with the interpretation by Malhotra & Bernhard (1968) of their experiments on the reaction of β -(2-furyl)acryloyl phosphate with rabbit muscle GPDH.

The slower second phase of the reaction (Table 2) is of no consequence in the above discussion. It does, however, suggest that the differences noted in the absorptions of the GPDH- NAD^+ complexes after alkylation with iodoacetic acid or other alkylation reactions may be somewhat arbitrary (Boross & Cseke, 1967).

The variation in the absorption of the Racker band in the presence of phosphate is likely to be a direct consequence of the ionization of a phosphate group, in view of the similarity of the pK of the observed ionization and its heat of reaction with that of a phosphate group in a solution of ionic strength 0.2 (Bernhard, 1956; *Stability Constants of Metal-Ion Complexes*, 1964). There are three inferences from this. First, this suggests that tightly bound NAD^+ is close to the enzyme active site, since phosphate is also a substrate for the reaction. Secondly, since the absorption of the Racker band varies depending on the anionic species present, variable absorptions can be expected from the ternary GPDH- NAD^+ -anion complex. Moreover, as NAD^+ is added to a GPDH-anion complex the rate of absorption changes associated with this addition may be complicated, particularly if the binding constant of the anion varies with NAD^+ addition. The complex stoichiometry and kinetics of NAD^+ addition to the yeast enzyme in the presence of various anions may be reflecting just these effects (Chance & Park, 1967). Thirdly, a valuable parameter is available for measuring elementary steps during a single turnover of GPDH, particularly those relating to phosphate binding. Velick & Furfine (1963) have noted that parameters associated with phosphate in the GPDH-catalysed reaction are difficult to measure owing to the multiple binding sites of phosphate on the enzyme (Velick & Hayes, 1953). Preliminary studies of the relaxation times associated with phosphate binding by using temperature-jump

techniques indicate that this absorption band will yield much relevant information.

When *N*-(4-iodophenyl)iodoacetamide and *N*-(4-methylphenyl)iodoacetamide react with GPDH, it is probable that the essential thiol group is alkylated, since the enzyme becomes inactive and the NAD^+ dissociation constant increases. However, the site of alkylation is ambiguous; in particular there is another cysteine residue that is four amino acids removed from the essential thiol group (Davidson *et al.* 1967). The ability of the phenyl derivatives to diminish the activity of neighbouring sites and the contrasting behaviour of iodoacetamide and iodoacetic acid mean that inferences from amide derivatives should be treated cautiously.

The different rates of reaction of iodoacetic acid with the NAD^+ -free enzyme and the NAD^+ -bound enzyme are likely to arise from one of three reasons. First, a conformation change of the enzyme resulting from NAD^+ removal renders the thiol group less accessible to attack. Secondly, the positively charged nicotinamide ring forms an ion pair with iodoacetic acid anion, thus placing the thiol group and the iodoalkyl group in favourable proximity. Evans & Rabin (1968) have considered a similar

essential thiol group coupled with a metal ion. Only trace amounts of zinc could be found associated with GPDH from yeast and rabbit muscle (Vanderheiden, Meinhart, Dodson & Krebs, 1962; Ferdinand, 1964).

Cseke & Boross (1967) have shown that the absorption of the Racker band of pig muscle GPDH shows a variation with pH that they ascribe to an ionization with pK 5.4 at 25°. They observed an absorption spectrum associated with the ionization of 2-mercaptoethanol in the presence of NAD^+ . Boross & Cseke (1967) also showed that the rate of reaction of iodoacetic acid with pig muscle GPDH, as reflected by the change of absorption of the Racker band, varied with pH and could be interpreted in terms of the reactive species having pK 5.4. The evidence that a thiol anion is the active species in a reaction between a mercaptan and iodoacetic acid has been reviewed by Cecil (1963). They therefore postulated that the essential thiol group had pK 5.4. They determined the dissociation constant of NAD^+ by the method of Stockell (1959) and claimed that it was 2.4-fold greater at pH 5.6 than at pH 7.8.

These proton dissociation and NAD^+ dissociation data may be represented schematically as follows:

possibility in their inhibition studies with liver alcohol dehydrogenase. Thirdly, NAD^+ has promoted ionization of the thiol group, and the rate of alkylation depends on the thiol anion concentration. This concept is relevant if the nucleophilicity of the thiol anion is relatively insensitive to its basicity. It is this third possibility that is discussed here in detail.

Stockell (1959) measured the dissociation constant of NAD^+ to the yeast enzyme. Between pH 4 and 6.5 she used EDTA and acetate buffers in the solvent. She found that the dissociation constant was constant in the pH range 5.5–7.5 but became larger at lower pH values, so that she ascribed this change of dissociation constant to an ionization with pK 5.0 ± 0.3 at 26°. At 7° a similar result was obtained except that the ionization was now with pK 6.1 ± 0.3 , and thus a heat of reaction of $22.4 \pm 12 \text{ kcal.mole}^{-1}$ could be ascribed to this ionization. She suggested that this might be the ionization of the

where K_1 and K_2 represent the dissociation constants of NAD^+ to GPDH with the thiol group ionized and un-ionized respectively, and K'_1 and K'_2 represent the dissociation constants of the proton to NAD^+ -bound GPDH and NAD^+ -free GPDH. Since $K'_1 K_2 = K'_2 K_1$ and the experiments described show that K'_1 is less than K_2 , NAD^+ promotes ionization of the essential thiol group. That an ion pair is favoured over the single charged species is probably a contributory factor to this process.

A possible role of NAD^+ in the acyl transfer function of GPDH is now apparent. When NAD^+ is bound to GPDH the thiol group is ionized and so may form a thioacyl derivative with 3-phosphoglyceroyl phosphate, acetyl phosphate or β -(2-furyl)acryloyl phosphate (Harting & Velick, 1954; Krimsky & Racker, 1955; Malhotra & Bernhard, 1968). The rate of reaction of a thiol anion with the carbonyl function of an aldehyde or an acyl ester shows low sensitivity to the basicity of the thiol

(Ogilvie, Tildon & Strauch, 1964; Lienhard & Jencks, 1966). Hence a thiol group with a low pK will still be an efficient nucleophile to a carbonyl function, though not necessarily to an alkylating agent. Conversely, once the thioacyl derivative is formed then NAD^+ bound at that site labilizes the thiol ester linkage since a thiol anion is a good leaving group. Unfortunately there is a scarcity of data on the variation of the reactivity of thiol esters with the pK of the thiol. However, comparisons

with alcohols of low pK suggest that the proposed behaviour of the thioacyl derivatives is reasonable (Bender & Turnquest, 1957; Bruice, Fife, Bruno & Brandon, 1962).

The corollary of NAD^+ promoting the ionization of the thiol is that, when a thioacyl or thioalkyl derivative is formed, then the NAD^+ dissociation constant will increase since the thiol-nicotinamide ion pair no longer exists. The inhibition experiments described above (Table 3) tentatively support this. Furfine & Velick (1965) have estimated that the dissociation constant of NAD^+ to native GPDH differs from that during the time that the enzyme is turning over. The stability of the various thioacyl esters in the absence of NAD^+ can now be explained. Once the small amount of residual NAD^+ on the enzyme has promoted the formation of a thioacyl ester, then the NAD^+ dissociation constant at that site is increased. This results in the migration of the NAD^+ to other sites on the enzyme promoting the formation of more thioacyl ester. The thioacyl ester at a site free of NAD^+ will be relatively stable.

A conformational change in GPDH from rabbit muscle is reported to occur when the last molecule of NAD^+ /molecule of tetramer is removed. $NADH$ appears to reverse this change (Listowsky, Furfine, Bethel & England, 1965). Measurement of the rates of conformational changes is necessary for a critical discussion. If the turnover rate of the enzyme is more rapid than the rate of conformational change, then the mechanism of the reaction catalysed by GPDH will not involve the conformational change. The rate of the transformation between the T-state and R-state of the yeast enzyme is about 1000 times slower than the turnover rate, though it is difficult to judge the significance of this,

since the measurements were made under different experimental conditions (Kirschner, Eigen, Bittman & Voigt, 1966). Only the R-state was active in turning over glyceraldehyde 3-phosphate (Kirschner, 1967). Such conformational changes may possibly have a role in the enzyme catalytic function, but they are not considered further here.

The simplified scheme for the reaction of GPDH during a single turnover near physiological pH is designed to bring out the ideas put forward:

where $R \cdot CHO$ represents D-glyceraldehyde 3-phosphate.

Smith (1966) concluded from his experiments that phosphorolysis of the acyl-enzyme occurs when $NADH$ and not NAD^+ is bound to the enzyme. However, he presents no clear evidence that NAD^+ would not displace $NADH$ from the acyl-enzyme when phosphate is bound to the enzyme.

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REFERENCES

- Allison, W. S. & Harris, J. I. (1965). *Abstr. 2nd Meet. Fed. Europ. biochem. Soc., Vienna*, A.205, p. 140.
- Allison, W. S. & Kaplan, N. O. (1964). *J. biol. Chem.* **239**, 2140.
- Barman, T. E. & Gutfreund, H. (1966). *Biochem. J.* **101**, 460.
- Beisenherz, G., Boltze, H. J., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E. & Pfeleiderer, G. (1953). *Z. Naturf.* **8b**, 555.
- Bender, M. L. & Turnquest, B. W. (1957). *J. Amer. chem. Soc.* **79**, 1656.
- Bernhard, S. A. (1956). *J. biol. Chem.* **218**, 961.
- Boross, L. & Cseke, E. (1967). *Acta biochim. biophys. Acad. sci. hung.* **2**, 47.
- Bruice, T. C., Fife, T. H., Bruno, J. J. & Brandon, N. E. (1962). *Biochemistry*, **1**, 7.
- Cecil, R. (1963). In *The Proteins*, vol. 1, p. 379. Ed. by Neurath, H. New York: Academic Press Inc.
- Chance, B. & Park, J. H. (1967). *J. biol. Chem.* **242**, 5093.
- Clark, N. G. & Hams, A. F. (1953). *Biochem. J.* **55**, 839.
- Cseke, E. & Boross, L. (1967). *Acta biochim. biophys. Acad. sci. hung.* **2**, 39.
- Davidson, B. E., Sajgó, M., Noller, H. F. & Harris, J. I. (1967). *Nature, Lond.*, **216**, 1181.

- Evans, N. & Rabin, B. R. (1968). *Europ. J. Biochem.* **4**, 548.
- Ferdinand, W. (1964). *Biochem. J.* **92**, 578.
- Furfine, C. S. & Velick, S. F. (1965). *J. biol. Chem.* **240**, 844.
- Harris, I., Meriwether, B. P. & Park, J. H. (1963). *Nature, Lond.*, **198**, 154.
- Harting, J. & Velick, S. F. (1954). *J. biol. Chem.* **207**, 867.
- Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
- Irving, H. & Cox, J. J. (1958). *Analyst*, **83**, 526.
- Kirschner, K. (1967). In *Fed. Europ. biochem. Soc. Colloq.: Regulation of Enzyme Activity and Allosteric Interactions, Oslo*, p. 39. Ed. by Kvamme, E. & Pihl, A. Oslo University Press (in the Press).
- Kirschner, K., Eigen, M., Bittman, R. & Voigt, B. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 1661.
- Krimsky, I. & Racker, E. (1955). *Science*, **122**, 319.
- Lebherz, H. G. & Rutter, W. J. (1967). *Science*, **157**, 1198.
- Lienhard, G. E. & Jencks, W. P. (1966). *J. Amer. chem. Soc.* **88**, 3982.
- Listowsky, I., Furfine, C. S., Bethel, J. J. & England, S. (1965). *J. biol. Chem.* **240**, 4253.
- Malhotra, O. P. & Bernhard, S. A. (1968). *J. biol. Chem.* **243**, 1243.
- Ogilvie, J. W., Tildon, J. T. & Strauch, B. S. (1964). *Biochemistry*, **3**, 754.
- Park, J. H., Meriwether, B. P., Clodfelder, P. & Cunningham, L. W. (1961). *J. biol. Chem.* **236**, 136.
- Racker, E., Klybas, V. & Schramm, M. (1959). *J. biol. Chem.* **234**, 2510.
- Racker, E. & Krimsky, I. (1952). *J. biol. Chem.* **198**, 731.
- Racker, E. & Krimsky, I. (1958). *Fed. Proc.* **17**, 1135.
- Smith, T. E. (1966). *Biochemistry*, **5**, 2919.
- Stability Constants of Metal-Ion Complexes* (1964). *Spec. Publ. chem. Soc., Lond.*, 2nd ed., no. 17, pp. 180, 192.
- Stockell, A. (1959). *J. biol. Chem.* **234**, 1286.
- Vanderheiden, B. S., Meinhardt, J. O., Dodson, R. G. & Krebs, E. G. (1962). *J. biol. Chem.* **237**, 2095.
- Velick, S. F. & Furfine, C. (1963). In *The Enzymes*, 2nd ed., vol. 7, p. 243. Ed. by Boyer, P. D., Lardy, H. & Mybäck, K. New York and London: Academic Press Inc.
- Velick, S. F. & Hayes, J. E. (1953). *J. biol. Chem.* **203**, 545.
- Velick, S. F., Hayes, J. E. & Harting, J. (1953). *J. biol. Chem.* **203**, 527.
- Wassarman, P. M. & Watson, H. C. (1968). *Abstr. 5th Meet. Fed. Europ. biochem. Soc., Prague*, p. 193.
- Watson, H. C. & Banaszak, L. J. (1964). *Nature, Lond.*, **204**, 918.