Preparation and Active-Site Specific Properties of Sturgeon Muscle Glyceraldehyde-3-phoshate Dehydrogenase†

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ABSTRACT: Sturgeon muscle glyceraldehyde-3-phosphate dehydrogenase has been isolated and purified to maximal activity. The purified enzyme contains *four* unusually reactive cysteine sulfhydryls per 145,000 daltons. This highly selective reactivity is manifest in the reaction of enzyme with the sulfhydryl reagent, 2,2′-dithiobis(5-nitrobenzoate) (Nbs_2). Enzyme activity is directly proportional to the fraction of unreacted sulfhydryls. Enzyme samples of lower specific activity invariably give a *proportionately* lower stoichiometry of reaction. The highest purity enzyme has a specific activity in excess of any previously reported *muscle* enzyme preparation. Near pH 9, where the catalytic oxidative phosphorylation rate is nearly optimal for both the sturgeon *muscle* and the *yeast* enzyme, the two highly purified enzymes have virtually the same specific activity. The highly purified enzyme tetramer permits critical investigations of ligand binding affinity and active site stoichiometry. Evidence is presented for both precise "half-site reactivity" with β-(2-furyl)acryloyl phosphate (2 equiv/tetramer) and "full-site reactivity" (4 equiv/tetramer) with 1,3-diphosphoglycerate and Nbs_2. The binding of NAD^+ occurs at four sites per tetramer, but ligand affinity is heterogeneous; affinity decreases as the extent of bound NAD^+ increases ("negative cooperativity"). This heterogeneity in NAD^+-binding affinity is adequately described by assuming two independent *pairs* of binding sites in the enzyme tetramer. The purified apoenzyme has an absorbancy ratio, A_{280}/A_{260}, of 2.1 and an absorbance of 0.895 ml mg^{-1} cm^{-1} at 280 nm. Its specific activity according to the Ferdinand assay is 330 ± 10 units per mg.

The chemical (Velick and Furfine, 1963; Furfine and Velick, 1965; Harris, 1970), physical (Jaenicke, 1969; Watson *et al.* 1972; Gorjunov *et al.*, 1972), and allosteric (Conway and Koshland, 1968; Malhotra and Bernhard, 1968; Kirschner *et al.*, 1971; Trentham, 1971b; Boers *et al.*, 1971) properties of a variety of glyceraldehyde-3-phosphate dehydrogenases have been discussed in considerable detail. Enzymes obtained from a wide range of species have very similar structural properties. All enzymes are tetrameric with a molecular weight of approximately 145,000 daltons. In no case is there evidence for heterogeneity in the polypeptide composition (Harris, 1970; Jones and Harris, 1972). There is striking sequence homology among all the species thus far studied, and indeed a long sequence of amino acids in the region of the active-site cysteine residue appears identical. All Glyc-3-P-dehydrogenase[1] enzymes bind oxidized coenzyme (NAD^+) in a characteristic fashion. The resultant enzyme–coenzyme complex gives rise to an electronic absorption band in the 360- to 400-nm range, the "Racker band," atypical of either single component (Krimsky and Racker, 1955). All enzymes catalyze the reversible oxidative phosphorylation of glyceraldehyde 3-phosphate according to a common mechanism involving the formation of an intermediate acyl-enzyme (thiol ester) (eq 1a,b).

$$\underset{\text{D-glyceraldehyde 3-phosphate}}{RCHO} + ECH_2SH + NAD^+ \rightleftharpoons ECH_2SCOR + NADH + H^+ \quad (1a)$$

(acyl-enzyme)

$$ECH_2SCOR + HPO_4^{2-} \rightleftharpoons ECH_2SH + \underset{\text{1,3-diphosphoglycerate}}{RCOOPO_3^{2-}} \quad (1b)$$

Despite the common chemical catalytic mechanism, and the uniquely reactive cysteine residue and common adjacent sequence, Glyc-3-P-dehydrogenase enzymes obtained from different species are differently affected by effectors of the catalytic process, most notably the effector NAD^+. In a limited number of instances specific steps in the reaction sequence have been shown to be catalytically dependent on the presence of bound NAD^+ (Malhotra and Bernhard, 1968; De Vijlder *et al.*, 1969b; Trentham, 1971a; Kirschner, 1971). The concentration dependence and avidity of NAD^+ binding, and its specific effect on each of the catalyzed processes, are variable, depending on the enzyme species (Furfine and Velick, 1965).

Equilibrium binding studies of NAD^+ to rabbit *muscle* Glyc-3-P-dehydrogenase indicate a heterogeneity of binding sites. Apparent affinity constants spread over the range of $>10^9$–10^4 M^{-1} have been reported (Conway and Koshland, 1968; Boers *et al.*, 1971). Such results, wherein NAD^+ binding affinity decreases with increasing extent of site saturation, have been defined as *negative cooperativity* (Conway and Koshland, 1968). In contrast, the binding of NAD^+ to the *yeast* enzyme at 40° and pH 8.5 is demonstrably *positively cooperative* (Kirschner *et al.*, 1966, 1971): initial binding of NAD^+ facilitates further NAD^+ binding. At 20° the binding of NAD^+ to the *yeast* enzyme is also *positively cooperative* (Ellenrieder *et al.*, 1972), but to a lesser degree.

It is well to note in the case of rabbit muscle enzyme that the "tightly bound" NAD^+ is more tightly bound to the

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[1] Abbreviations used are: 1,3-P_2Glyc, 1,3-diphosphoglycerate; Nbs_2, 2,2′-dithiobis(5-nitrobenzoic acid); Glyc-3-P, glyceraldehyde 3-phosphate; Glyc-3-P-dehydrogenase, D-glyceraldehyde-3-phosphate dehydrogenase.

enzyme than is the NAD^+ bound to any of the sites of the yeast enzyme (Boers *et al.*, 1971; Ellenrieder *et al.*, 1972). Coenzyme so bound is extremely difficult to desorb from the rabbit muscle enzyme (Bloch, 1970) as well as from the lobster muscle enzyme (Trentham, 1968; De Vijlder *et al.*, 1969a). Removal of all the coenzyme from these enzyme species by prolonged treatment with activated charcoal results in a less stable *apoenzyme*. It is well known for all species investigated that the presence of NAD^+ stabilizes the enzyme in regard to both catalytic and structural stability. Consequently, it is difficult to quantitate the properties of the rabbit muscle apoenzyme. Moreover, the reported extreme avidity of initial NAD^+ binding makes it difficult or impossible to determine the binding constants precisely. These are important considerations since they bear directly on the mechanism of *negative cooperativity*, an apparent feature of all muscle Glyc-3-P-dehydrogenase enzymes.

In order to investigate the NAD^+ effector role in detail, we sought a Glyc-3-P-dehydrogenase enzyme which exhibited characteristic *negative cooperativity* in coenzyme binding, but in which the ligand–enzyme interactions were sufficiently weak so that equilibrium studies of the binding interactions could be carried out at accessible conditions of concentration. In this regard we examined readily obtainable sturgeon muscle enzyme which has already been described in some detail by the Molecular Enzymology Laboratory in Bristol[2] (Trentham, 1971a,b; Harrigan and Trentham, 1971) and by Allison and Kaplan (1964). Like the enzyme obtainable from yeast, this enzyme can be crystallized such that the crystals are colorless. This is indicative of the absence of a bound-NAD^+ "Racker band" in the enzyme crystals. Although all glyceraldehyde-3-phosphate dehydrogenases are stabilized by the presence of NAD^+, the relative stabilities of apoenzymes are highly variable. Indeed it has been found possible to prepare apo-(sturgeon muscle)enzyme free of bound nucleotides at the enzyme site, and to study the properties of this apoenzyme in some detail. The effector properties of NAD^+ on the activity of the sturgeon mucle enzyme are qualitatively similar to those already reported in detail for the rabbit muscle enzyme. However, the stability of the apoenzyme, coupled with a more accessible concentration range for the quantitative determination of ligand-binding processes, make this Glyc-3-P-dehydrogenase enzyme far more useful for the investigation of regulatory processes. Moreover, as we shall demonstrate, the enzyme activity and the active-site stoichiometry obtainable from preparations of this enzyme are the highest and most reproductible thus far reported for muscle Glyc-3-P-dehydrogenase. In this and subsequent papers, we shall report on regulatory and stoichiometric processes involving this sturgeon muscle enzyme. Therefore we believe it desirable, at this time, to describe in some detail the preparations of the sturgeon muscle enzyme and to report on some characteristic properties of the enzyme site.

Experimental Section

Reagents. NAD^+, NADH (both of highest purity available), and the barium salt of DL-glyceraldehyde-3-phosphate diethyl acetal were purchased from Sigma Chemical Co. Free DL-GLYCERALDEHYDE 3-PHOSPHATE was prepared as described by Furfine and Velick (1965). NAD^+ and Glyc-3-P solutions were assayed by the method of Ferdinand (1964). NADH concentrations were determined by absorbance at 340 nm using a molar extinction coefficient of 6220 M^{-1} cm^{-1} (Horecker and Kornberg, 1948). The purity of NAD^+ and NADH in fresh solutions was found to be greater than 98% from comparisons of the absorbance at 260 and 340 nm. D-GLYC-3-P was prepared from D-ribose 5-phosphate according to Klybas *et al.* (1959) and was purified on a Dowex 1-X2 column equilibrated with 10 mM imidazole (pH 6.5). 1,3-DIPHOSPHOGLYCERATE was prepared, purified, assayed as previously described (Furfine and Velick, 1965), and stored frozen at −60° in standard imidazole buffer (pH 7.0) (see below) for no more than 3 weeks. β-(2-FURYL)ACRYLOYL PHOSPHATE was prepared as described by Malhotra and Bernhard (1968). Nbs_2 (Calbiochem) was recrystallized from glacial acetic acid. Urea (Baker Chemical Co.) was recrystallized from 95% ethanol. Ammonium sulfate was Special Enzyme Grade from Schwarz-Mann Co. All other chemicals used were analytical grade.

Isolation and Purification of Sturgeon Muscle Glyc-3-P-dehydrogenase. All steps in the isolation and purification procedure were carried out at 5°.

STEP I. EXTRACTION. Frozen sturgeon (*Acipenser transmontanus*) muscle (900 g) were cut up into small chunks and placed in a Waring blender. The enzyme activity was extracted with 500 ml of 50 mM EDTA (pH 8.5) (Ferdinand, 1964) and the homogenate was centrifuged at 16,000*g* for 45 min in a Sorvall RC2B centrifuge. The extraction was repeated twice more, 400 ml of EDTA buffer being used each time. The pink extraction liquor (1L) was filtered through glass wool to remove lipid particles.

STEP II. AMMONIUM SULFATE FRACTIONATION. Solid ammonium sulfate (428 g/l.) was slowly added to the extraction liquor until 65% saturation was reached (Green and Hughes, 1955). The pH of the solution was kept above 6.5 by the addition of concentrated aqueous NH_3. After 0.5 hr, the precipitate was removed by centrifugation at 16,000*g* for 40 min and discarded. The enzyme was then precipitated by the addition of solid ammonium sulfate (190 g/l., 90% saturation) to the yellow supernatant (1.2 l.) and stored overnight. The next morning the suspension was centrifuged at 16,000*g* for 50 min. The pale yellow active precipitate was resuspended in about 50 ml of 5 mM EDTA (pH 7.5) and the undissolved inactive material was removed by centrifugation at 31,000*g* for 20 min. The clear supernatant (80 ml) was desalted by filtration through a Bio-Gel-P-2 column (17 × 5.7 cm) equilibrated with 5 mM EDTA (pH 7.5).

STEP III. DEAE-SEPHADEX CHROMATOGRAPHY. The desalted enzyme solution (130 ml) was loaded onto a DEAE-Sephadex A50 (Pharmacia) column (42 × 4.5 cm) equilibrated with 5 mM EDTA (pH 7.5). Elution was carried out with the same buffer at a flow rate of 120 ml/hr. The enzyme was not retained in this column and was eluted immediately after the column void volume. Hemoproteins were eluted shortly after the active peak. The active fractions were pooled and the specific activity of this partially purified enzyme was between 200 and 250 units per mg depending on the starting material. A small absorption peak at 410 nm was observed, indicating some contamination of the enzyme by hemoproteins.

STEP IV. CM-CELLULOSE CHROMATOGRAPHY. Further purification of the enzyme was achieved by chromatography on a CM-cellulose CM52 (Whatman) column (33 × 4.5 cm) equilibrated with 5 mM EDTA (pH 6.6) (Bloch *et al.*, 1971). The pH of the enzyme solution (200 ml) was adjusted to 6.6 by careful addition of 0.5 N HCl and the enzyme was then applied at the top of the CM-cellulose column. The elution

[2] We are indebted to Dr. David Trentham for the original suggestion to study the sturgeon enzyme.

of the enzyme was carried out at a flow rate of 60 ml/hr with a linear salt gradient obtained by mixing 850 ml of 0.15 M KCl–5 mM EDTA (pH 6.6) with the same volume of the equilibration buffer. Under these conditions, *four* protein peaks were resolved. The first peak, coming off with the solvent front, was completely inactive but varying activity was found in the three following peaks (I, II, III, see Figure 1). Peak III, which contained the maximally active apoenzyme (about 800 mg, see Results), was pooled.

Storage of the Enzyme. The more concentrated fractions (up to 4 mg/ml) were converted to the holoenzyme by the addition of excess NAD^+ and were then precipitated by dialysis against saturated ammonium sulfate–1 mM EDTA (pH 7.0). A very fine yellow precipitate was obtained and was stored for several months at 0–5°. This precipitated holoenzyme lost about 7% of the initial activity/month.

More dilute apoenzyme fractions (from 0.5 to 1 mg per ml) were concentrated by dialysis against the appropriate buffer under vacuum. The concentrated apoenzyme (15–25 mg/ml) solutions was stored in standard ethylenediamine buffer for several weeks. The loss in activity of the apoenzyme was about 15%/month. Apoenzyme solutions became turbid within a few days. After 0.5 hr of incubation at room temperature with gentle occasional stirring, the precipitated material was removed by filtration through a Millipore filter (3 μ, SSWPO-1300). The soluble apoenzyme was as active as the corresponding holoenzyme and no further turbidity was detectable over a few days. Over longer periods this procedure could be repeated with only minor loss of insoluble enzyme, allowing for accurate spectrophotometric and spectrofluorometric titration of the apoenzyme.

Crystallization of the enzyme could be easily achieved even after step III, essentially as described by Allison and Kaplan (1964). Large colorless needles were obtained. Nevertheless, since recrystallization did not substantially improve the specific activity of the partially purified enzyme (Allison and Kaplan, 1964; Ferdinand, 1964), no attempt was made to recrystallize the enzyme as part of the purification procedure. The solubility of the enzyme in ammonium sulfate solution was greatly increased by NAD^+ and attempts to crystallize the holoenzyme were unsuccessful.

Isolation of Acylated Glyc-3-P-dehydrogenase. PHOSPHOGLYCEROYL-D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. A 10–100 μM solution of holoenzyme (0.5 ml) in standard imidazole buffer was incubated at 0° with a fivefold molar excess of purified 1,3-P_2Glyc for 1 min and filtered through a small Bio Gel-P-10 column (9 × 1.4 cm) equilibrated with the same buffer. Aliquots of the acyl-enzyme were immediately titrated with Nbs_2 and with NADH. Protein concentration was determined by absorbance at 280 nm assuming an extinction coefficient of 0.895 mg^{-1} ml^{-1}. In the absence of acceptors (NADH, HPO_4^{2-}) the spontaneous hydrolysis of the fully acylated, (nearly) NAD^+-free enzyme had a half-life of about 5 hr at 0° as monitored by the appearance of Nbs_2-reactive sulfhydryl.

FURYLACRYLOYL-D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. The holoenzyme (10–30 μM) was incubated with varying excessive concentrations of β-(2-furyl)acryloyl phosphate (0.5–5 mM) in ethylenediamine buffer at 25°, and the time-dependent absorbance at 360 nm was recorded. When the pseudo-first-order reaction was completed (in seconds to minutes) the reaction mixture was chilled at 0° and gel filtered as with the phosphoglyceroyl-enzyme. The uv spectrum of the β-(2-furyl)acryloyl phosphate free acylated enzyme was immediately recorded and the protein concentration was estimated by the Biuret method (Laynes, 1957). The acyl group content of the enzyme was determined from the maximal absorption at 345 nm: ϵ 3.0 × 10^4 M^{-1} cm^{-1} (Malhotra and Bernhard, 1968).

Further Treatment of the Purified Enzyme. PREPARATION OF ENZYME SOLUTIONS. Suspensions of the enzyme in saturated ammonium sulfate were centrifuged for 40 min at 31,000*g* and the precipitate was dissolved in standard ethylenediamine buffer (10 mM ethylenediamine–0.1 M KCl–1 mM EDTA, pH 7.0). Ammonium sulfate and excess NAD^+ were removed either by dialysis against the same buffer or by Bio-Gel P-10 filtration. More reproducible reaction properties were noted in the presence of imidazole rather than ethylenediamine buffer. Standard imidazole buffer contained 10 mM imidazole–0.1 M KCl–1 mM EDTA (pH 7.0). This buffer was used *in acylation* and kinetic studies.

DETERMINATION OF THE ENZYME CONCENTRATION AND THE NAD^+ CONTENT OF THE ENZYME. Both enzyme and NAD^+ were assayed by absorbance measurements. The extinction coefficient of the apoenzyme at 280 nm ($E_{280}^{1\%,1cm}$ 8.95) was determined from the dry weight of protein solution as described by Ferdinand (1964). Protein extinction coefficients at 280 nm for enzyme solutions with various A_{280} to A_{260} ratios were determined from a graph of A_{280} *vs.* A_{280}/A_{260} obtained by adding known amounts of NAD^+ to the apoenzyme. Possible contamination of the apoenzyme with very small amounts of NAD^+ was detected by incubation of the enzyme (10 μM) with Glyc-3-P (0.5 mM) and arsenate (5 mM) in standard imidazole buffer. Any NADH formed could be estimated fluorimetrically. The sensitivity of this method permitted the detection of less than 0.1 μM NAD^+, as verified by addition of known amounts of NAD^+ to the incubation mixture.

CHARCOAL TREATMENT OF THE HOLOENZYME. Small amounts of apoenzyme were prepared from the holoenzyme by charcoal treatment, essentially as described by Krimsky and Racker (1963). A small column constructed from a Pasteur pipet and containing a fivefold excess by weight of charcoal (25–50 mg) over protein (5–10 mg in 1 ml) was used. The apoenzyme so prepared was indistinguishable from the apoenzyme prepared by CM-cellulose chromatography.

Titration of the Enzyme with Nbs_2. A small volume (20–100 μl) of the enzyme solution (20–100 μM) was added to 0.85 ml of 0.23 mM Nbs_2 in standard ethylenediamine buffer. The absorbance at 412 nm was recorded. The "burst" reaction was completed in less than 1 min. The normality of the enzyme was calculated from this burst using $\Delta\epsilon_{412}$ = 13,600 M^{-1} cm^{-1} (Ellman, 1959) after correction for a slow continual linear increase in absorbance (5 × 10^{-4} OD min^{-1} μM^{-1} (enzyme)) observed under these conditions. The accuracy of the measurement was about ±2%.

The extinction coefficient of thionitrobenzene is unchanged in 8 M urea (pH 7.0); thus the same titration procedure could be used. The complete reaction of urea denatured enzyme with Nbs_2 required approximately 5 min.

Titration of the Enzyme by Coenzyme and Substrate. Spectrophotometric titrations of the apoenzyme were carried out in a Cary 14 or 16 spectrophotometer at 25°. Small aliquots of titrant (5–20 μl) were added to the filtered enzyme solution (0.75 or 2 ml) in silica cells. Mixing was accomplished by means of a plastic plunger (Calbiochem). Fluorimetric titrations were carried out in a Hitachi Perkin-Elmer spectrofluorimeter equipped with a thermostated cell holder. The observed adsorption or fluorescence was corrected for dilution and absorption and fluorescence contributions from the titrant (Price and Radda, 1971; Franzen *et al.*, 1972).

TABLE I: Summary of the Preparation of Sturgeon Muscle Glyc-3-P-dehydrogenase.

Step	Vol (ml)	Estimated Total Protein (mg)[d]	Total Act.[g] (Units)	Sp Act. (Units/mg)	A_{280}/A_{280}	Yield (%)
I. Extraction[a]	1020	*e*	9.1×10^5	*e*	*e*	100
II. 90% precipitate[b]	130	3450[f]	4.85×10^5	145	1.19	53
III. DEAE-Sephadex chromatography	192	1650	4.03×10^5	246	1.55	44.3
IV. CM-cellulose[c] chromatography	290	815	2.77×10^5	340[h]	2.10[i]	30.4

[a] From 920 g of frozen muscle. [b] After Bio-Gel P-2 desalting. The total activity in the 65% ammonium sulfate supernatant was 7.2×10^5 units. [c] Peak III material only. [d] From the absorbance at 280 nm. [e] Not determined. [f] An extinction coefficient of 1 cm^{-1} mg^{-1} was assumed for this material. [g] Activity was assayed by the Ferdinand (1964) procedure. [h] The specific activities of peaks I and II material were 240 and 150 units per mg, respectively. [i] The absorbance ratios of peaks I and II material were 1.20 and 1.50, respectively.

Binding stoichiometries were evaluated by the initial tangent method (Velick, 1958; Holbrook, 1972a). Since this method estimates the site concentration *plus* the value of the corresponding dissociation constant, high concentrations of enzyme (about 10 μM) were used in most cases in order to keep the site concentration much larger than the presumed value of the dissociation constant. The reliability of the stoichiometries determined by this method was estimated at better than $\pm 5\%$.

Assay of the Enzyme Activity. The enzyme was assayed at pH 8.9 and 25° in a Cary 14 spectrophotometer by the Ferdinand (1964) procedure. The final enzyme concentration in the absorbance cell was about 10^{-4} mg/ml (7×10^{-10} M); dilution from the enzyme stock was carried out immediately prior to assay. Specific activities are reported in micromoles of NADH formed per minute per milligram of enzyme; these activities are uncorrected for suboptimal conditions of concentration (Ferdinand, 1964).

Steady state kinetic analyses of the oxidative phosphorylation of Glyc-3-P were performed by monitoring the NADH formation either by spectrophotometry at 340 nm, or fluorimetrically in a Hitachi Perkin-Elmer fluorimeter precalibrated with a standard NADH solution (excitation at 340 nm, emission at 460 nm).

Results

Isolation and Purification of Sturgeon Glyc-3-P-dehydrogenase. The sturgeon Glyc-3-P-dehydrogenase preparation procedure outlined in Table I was developed on the basis of previous observations on the rabbit Glyc-3-P-dehydrogenase isolation in this laboratory (Bloch *et al.*, 1971). Three active peaks (I, II, III) were resolved by CM-cellulose chromatography (Figure 1). These peaks are significantly different in specific activity and in their 280/260 nm absorbance ratio (Table I). Active site titration with Nbs_2 (see below) showed that peak III contained four Nbs_2-reactive equiv/145,000 daltons of protein material whereas peak I reacted with only 2.5 Nbs_2 equiv. Peaks I and II could not always be resolved as well as is indicated in Figure 1. Their amplitudes compared to that of the highly homogeneous peak III increase significantly with the age of the starting material and the period of time elapsed from the initial extraction. These observations suggest that the two first peaks represent partially inactivated Glyc-3-P-dehydrogenase rather than isoenzymes. The excellent reproducibility and the molecular properties of peak III material strongly indicate that this is the fully active native apo-Glyc-3-P-dehydrogenase. In consequence, the further results reported herein relate exclusively to this material. The amino acid composition[3] of the apoenzyme is in excellent agreement with that published by Allison and Kaplan (1964).

No detectable amounts of NAD^+ were found in the apoenzyme. The A_{280}/A_{260} ratios and the corresponding extinction coefficients for various NAD^+ to enzyme ratios are given in Figure 2. The absorbance ratio of the holoenzyme prepared as described in the Experimental Section was 1.13 ± 0.02, corresponding to 3.9 ± 0.2 mol of NAD^+/mol of enzyme.

Specific Activity and Steady-State Kinetic Investigations. Although the Ferdinand (1964) assay procedure was widely utilized to allow comparison with previously published specific activities of various Glyc-3-P-dehydrogenase near pH 9 (Allison and Kaplan, 1964; Trentham, 1968), it was found that this assay could also be carried out at pH 7.0 with the same substrates in standard imidazole buffer containing 50 mM phosphate as acceptor. The curvature of the absorbance *vs.* time recording was more pronounced at pH 7.0 than that observed in the Ferdinand assay. Sturgeon Glyc-3-P-dehydrogenase could also be assayed with purified 1,3-P_2Glyc and NADH at pH 7.0 under zero-order kinetic conditions. The instability of 1,3-P_2Glyc solutions precluded its use as a standard assay procedure, however.

Since the unusually high specific activity reported herein for the native sturgeon Glyc-3-P-dehydrogenase under the Ferdinand assay conditions was rather puzzling, the activity of the enzyme under various assay conditions was compared with that for highly purified yeast Glyc-3-P-dehydrogenase which has been reported to be more active than muscle Glyc-3-P-dehydrogenase (Allison and Kaplan, 1964). As shown in Table II, the relative specific activity of sturgeon to yeast Glyc-3-P-dehydrogenase (prepared according to Kirschner and Voigt, 1968) is dependent on the assay conditions. The enzymes have nearly equal specific activity in the Ferdinand assay, but the yeast enzyme is clearly more active than sturgeon Glyc-3-P-dehydrogenase at neutral pH. Thus no definitive conclusions can be drawn from such comparisons until a more thorough study of the influence of substrates and pH on the activity is carried out.

The initial rate of oxidative phosphorylation of Glyc-3-P at pH 7.0 at or near optimal levels of NAD^+ and Glyc-3-P is phosphate concentration dependent, exhibiting the same concentration-dependent complexity previously reported for rabbit Glyc-3-P-dehydrogenase (Ferdinand, 1964; Furfine and

[3] The amino acid analysis was performed by Worthington Biochemical Corp.

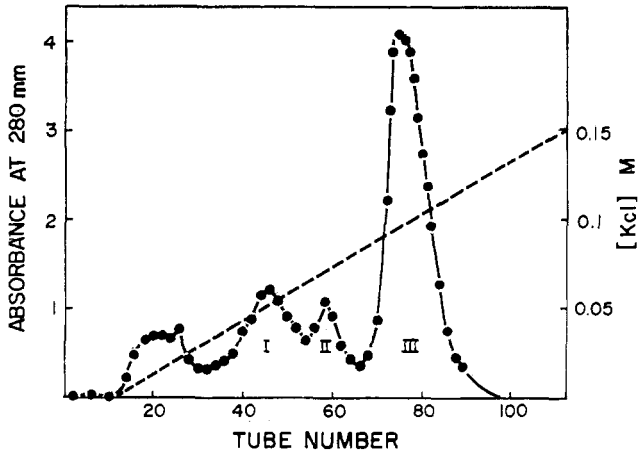

FIGURE 1: Elution profile of sturgeon Glyc-3-P-dehydrogenase on CM-cellulose (step IV, as given in Table I). Absorbances at 280 nm were measured in 0.5-cm pathlength cells. The volume of the fraction was 17 ml/tube. The broken line indicates the concentration of KCl (right-hand ordinate) in the elution buffer.

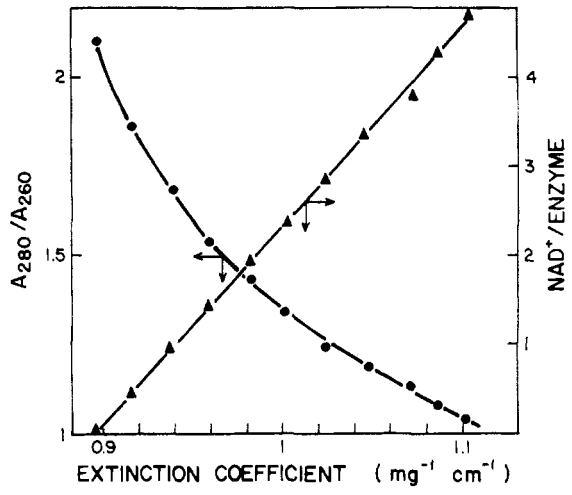

FIGURE 2: Absorbance ratio (A_{280}/A_{260}) and extinction coefficient of sturgeon Glyc-3-P-dehydrogenase as a function of the NAD^+ content of the enzyme.

Velick, 1965; Smith and Velick, 1972) (Figure 3). Double-reciprocal plots of initial rate *vs.* NAD^+ or Glyc-3-P concentration showed no significant deviation from linearity up to 1 mM for each substrate. K_m values of 72 and 83 μM were obtained for NAD^+ and D-Glyc-3-P, respectively, at 400 μM of the invariant substrate (Glyc-3-P or NAD^+) and at 25 mM phosphate. When optically pure D-Glyc-3-P was used instead of the commercially available racemic mixture, the same K_m for D-Glyc-3-P was obtained. Hence the L isomer is nonfunctional and noninhibitory compared to the D isomer. These kinetic parameters are in rough agreement with previously published values for the rabbit enzyme over a wide variety of experimental conditions (Furfine and Velick, 1965; Orsi and Cleland, 1972; Smith and Velick, 1972). The turnover number of the enzyme at pH 7.0, 25°, standard imidazole buffer and 25 mM phosphate as acceptor is 264 ± 24 sec^{-1}/mol of enzyme (145,000 daltons).

Active-Site Titration with Nbs_2. Since the various assay methods previously described permit only a *relative* comparison of specific activity among various enzyme preparations, a more reliable, *absolute* active-site titration was devised utilizing Nbs_2 (Ellman, 1959). This thiol-specific reagent was found to react rapidly with 4 equiv of cysteine/mol of native holo- or apoenzyme. After this fast burst reaction, monitored by absorbance at 412 nm (Figure 4), a complete inactivation of the enzyme was observed which could be totally reversed by immediate addition of 1 mM dithiothreitol. A linear absorbance increase followed the initial Nbs_2 burst reaction, as shown in Figure 4, but its rate (about $5 \times 10^{-4} \times$ OD min^{-1} μM^{-1} (enzyme)) was slow enough to allow a precise determination of the burst amplitude. This slow reaction, presumably by further reaction with other sulfhydryls, is in contrast to the very fast reaction with the active-site SH (see insert to Figure 4). The specific activity of variously aged Glyc-3-P-dehydrogenase preparations was found to be linearly related to the burst amplitude (Figure 5) indicating that this reaction occurs with catalytically active cysteines (Harris *et al.*, 1963) exclusively. In 8 M urea (pH 7) two other cysteines per protomer (*i.e.*, 11.8 ≈ 0.2 cysteines/mol of enzyme tetramer) were titrated. Preincubation of the holoenzyme in 5 mM dithiothreitol for 12 hr did not significantly affect the

TABLE II: Comparison of the Specific Activity of Yeast and Sturgeon Glyc-3-P-dehydrogenases under Various Assay Conditions at 25°.

Assay Conditions[a]	Sp Act. (Units/mg) Sturgeon[e]	Yeast[e]
0.84 mM Glyc-3-P, 0.84 mM NAD^+,[b] 42 mM phosphate (pH 8.9)	314	289
0.45 mM Glyc-3-P, 0.45 mM NAD^+,[c] 50 mM phosphate (pH 7.0)	80	137
0.08 mM 1,3-P_2Glyc, 0.09 mM NADH[c] (pH 7.0)	106	283
1,3-DPG generating system (pH 7.6)[d]	83.5	160

[a] Yeast enzyme was preincubated with 1 mM NAD^+ in standard ethylenediamine buffer. [b] Ferdinand assay procedure (1964), 120 mM triethanolamine. [c] In standard imidazole buffer. [d] Kirschner and Voigt (1968). [e] Active-site titration with Nbs_2 gave 3.75 ± 0.04 and 3.90 ± 0.04 equiv of trinitrobenzene per enzyme molecule for the sturgeon and yeast Glyc-3-P-dehydrogenases, respectively.

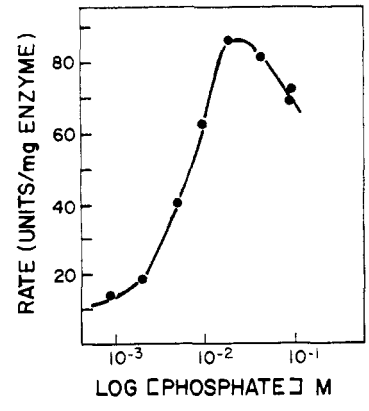

FIGURE 3: Initial rate of Glyc-3-P oxidation as a function of orthophosphate concentration. Conditions: 0.405 mM NAD^+, 0.411 mM Glyc-3-P, 0.47×10^{-3} μM holo-GDPH. Standard imidazole buffer (pH 7.0), 25°.

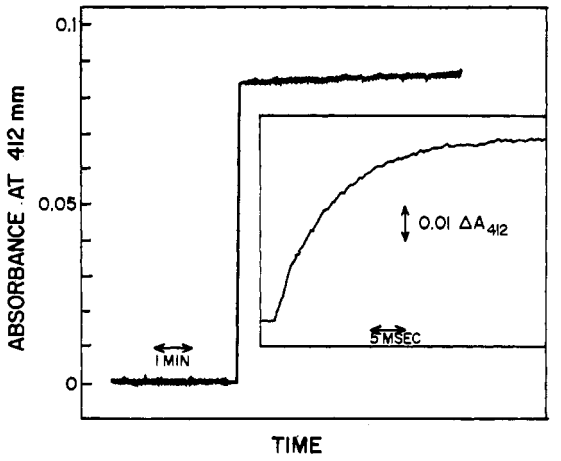

FIGURE 4: Nbs_2 reaction with sturgeon apo-Glyc-3-P-dehydrogenase. Reaction was initiated by the addition of 20 μl of apoenzyme to the reaction mixture (final volume 0.92 ml). Conditions: 0.33 mM Nbs_2 and 1.52 μM enzyme. Standard ethylenediamine buffer (pH 7.0), 25°. Insert: stopped-flow trace at 0.25 mM Nbs_2 and 1.2 μM apoenzyme in the same solvent.

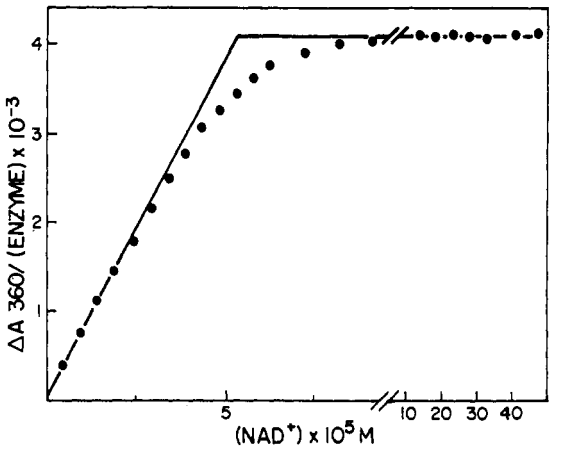

FIGURE 6: Racker band titration of sturgeon apo Glyc-3-P-dehydrogenase. Conditions: 13.9 μM apoenzyme. Standard ethylenediamine buffer (pH 7.0), 25°. Assuming stoichiometric binding to two of the four sites and equivalent extinctions at all four sites per molecule, a dissociation constant of 3.3 ± 0.4 μM is calculable for the two "looser" binding sites from the data in the figure. This value is in good agreement with that calculated from the more sensitive fluorescence data illustrated in Figure 7.

number of cysteines titrated in either the native or the urea-denatured enzyme.

Molecular Parameters of Sturgeon Glyc-3-P-dehydrogenase. The specific activity was 330 ± 10 units/mg as assayed by the method of Ferdinand (1964); 145 mg of enzyme reacted rapidly with 4.1 ± 0.1 μequiv of Nbs_2. The absorbancy ratio A_{280}/A_{260} was 2.10 ± 0.05 for the purified apoenzyme. These parameters are averaged over ten preparations. The optical density of a 1% protein solution at 280 nm in a 1-cm path-length cell was 8.95 ± 0.20.

Coenzyme Binding Studies. The NAD^+ binding to the apoenzyme was studied either by Racker band titration at 360 nm or by the resultant quenching of the enzyme (tryptophan) fluorescence emission (excitation at 290–295 nm and emission at 340 nm).

Racker-band titrations required high enzyme concentration (>10 μM) and were utilized primarily for stoichiometric determinations as shown in Figure 6. Fluorescence quenching studies permitted the use of enzyme concentrations as low as 5×10^{-8} M, and this method was found convenient for the determination of NAD^+-binding isotherms. Both methods gave stoichiometries of four NAD^+ binding sites per molecule and indicated a *strictly* equal contribution of each binding site to the total observed change. The coenzyme-binding isotherm obtained from fluorescence measurements at low enzyme concentrations showed heterogeneity among sites (*i.e.*, negative cooperativity) as is illustrated in the "Scatchard plot" of Figure 7, similar to the results already reported for various vertebrate glyceraldehyde-3-phosphate dehydrogenase (Conway and Koshland, 1968; Bloch, 1970; Boers *et al.*, 1971; Price and Radda, 1971; Velick *et al.*, 1971). The data of Figure 7 could be fitted to a simple model involving equal numbers of two different and independent NAD^+-binding sites (Bernhard and MacQuarrie, 1973), *viz.*, eq 2, where F is

$$F = F_0 - \frac{\Delta F_\infty}{2}\left[\frac{[NAD^+]_{free}}{([NAD^+]_{free} + K_1)} + \frac{[NAD^+]_{free}}{([NAD^+]_{free} + K_2)}\right] \quad (2)$$

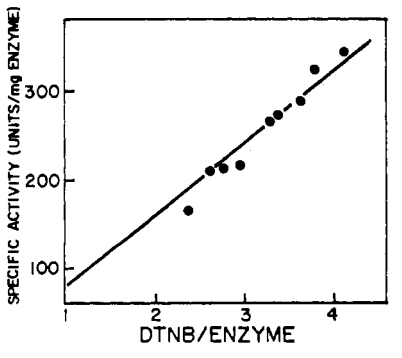

FIGURE 5: Relationship between the specific activity of various aged sturgeon Glyc-3-P-dehydrogenase preparations and the number of active cysteines per enzyme molecule as titrated with Nbs_2. Activity was measured under Ferdinand (1964) assay conditions. The lower activity data point is for 1-year-old holoenzyme.

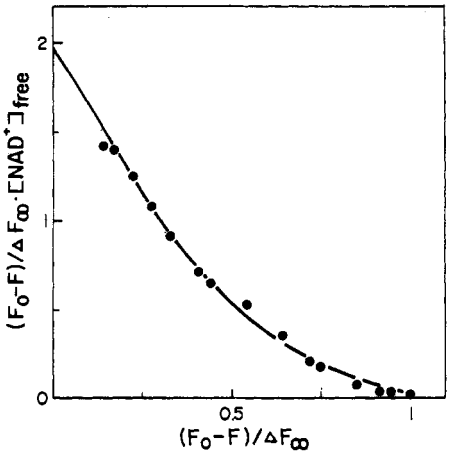

FIGURE 7: NAD^+ binding isotherm for sturgeon apo-Glyc-3-P-dehydrogenase. "Scatchard plot" of the enzyme fluorescence quenching data. Free NAD^+ concentrations are calculated by assuming a stoichiometry of *four* molecules of NAD^+ bound per enzyme molecule and an *equal* contribution of each site to the observed fluorescence quenching. Accordingly, the expression $(F_0 - F)/\Delta F_\infty$ is proportional to the extent of binding. The solid line is calculated from eq 2, as described in the text. Conditions: 0.03 μM enzyme and standard ethylenediamine buffer, 25°.

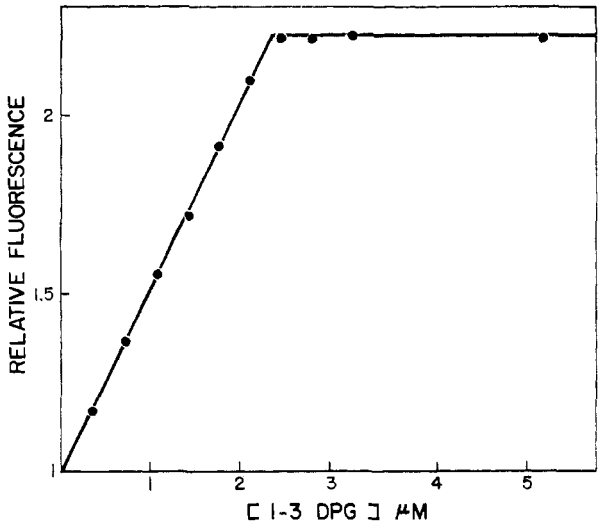

FIGURE 8: Titration of sturgeon holo-Glyc-3-P-dehydrogenase with 1,3-P_2Glyc. The enzyme fluorescence excitation was at 295 nm and the emission at 340 nm. Conditions: 98.3 μM NAD^+ and 0.62 μM enzyme. Standard imidazole buffer (pH 7.0), 25°.

the observed fluorescence, F_0 the initial fluorescence, and ΔF_∞ the total change in fluorescence. The two microscopic dissociation constants so defined were calculated to be 0.28 ± 0.02 and 3.10 ± 0.4 μM.

The titration of apo-Glyc-3-P-dehydrogenase by NADH was monitored by the difference in coenzyme at 340-nm absorbance between solvent and site environments. The coenzyme binding to the enzyme caused a hypochromicity in the dihydronicotinamide absorption (Fisher *et al.*, 1969). Titrations were carried out in the same range of high enzyme concentrations as for Racker-band titrations, and gave a stoichiometry of nearly 4 NADH/mol of enzyme (Table III). Quenching of the enzyme fluorescence by NADH could also be observed, but was geometrically rather than linearly related to the extent of bound NADH, as has been predicted and discussed by Holbrook *et al.* (1972a,b). Hence, further analysis of the quenching data was not attempted. Stoichiometries and optical parameters for coenzyme binding to the enzyme are summarized in Table III.

TABLE III: Coenzymes Binding to Sturgeon Glyc-3-P-dehydrogenase.[a]

Coenzyme	Method	Stoichiometry of Binding
NAD^+	Racker band (absorbance at 360 nm)[b]	4.1 ± 0.2
	Quenching of enzyme Fluorescence by NAD^+[c] Excitation at 290 nm Emission at 340 nm	3.9 ± 0.2
NADH	Absorbance at 340 nm[d]	3.8 ± 0.2

[a] Conditions: at pH 7.0, 25°, standard ethylenediamine buffer. [b] Extinction coefficient per site at 360 nm is 1000 ± 50 M^{-1} cm^{-1}. [c] The per cent quenching per site represents 12 ± 2% of the original apoenzyme fluorescence. [d] The difference extinction per site at 340 nm is −1200 ± 100 M^{-1} cm^{-1}. Enzyme concentration was 8.1 μM. At this concentration the NADH binding is virtually stoichiometric.

Acylation of the Enzyme by Physiological Substrate and by β-(2-Furyl)acryloyl Phosphate. The two half-reactions (eq 1) which yield acyl-enzyme were analyzed under a wide range of conditions in order to determine the acylation stoichiometries with substrates.

(1) ACYLATION BY Glyc-3-P + NAD^+ (*i.e.*, oxidative acylation) was monitored by spectrophotometric or fluorimetric titration of the NADH formed. Large excesses of NAD^+ and Glyc-3-P (up to 1 mM) were required to drive the equilibrium toward complete acylation of the enzyme at pH 7.0 (eq 1a). Moreover, the spontaneous hydrolysis of the acyl-enzyme increased at higher extents of acylation and was accompanied by an extrastoichiometric NADH production. These factors precluded detailed quantitative analysis of the titration curves. It was found that a maximum of 3.5 ± 0.5 mol of NADH was formed per mol of enzyme at high concentrations of reactants, in agreement with previous results (Peczon and Spivey, 1972). Attempts to isolate the acyl-enzyme by gel filtration of the reaction mixture were unsuccessful, indicative of both the unfavorable equilibrium conditions and strong NADH binding to the acyl-enzyme.

(2) ACYLATION BY 1,3-P_2Glyc. Preliminary experiments showed that incubation of the holoenzyme with stoichiometric amounts of purified 1,3-P_2Glyc at pH 7 nearly abolished its reaction with Nbs_2, suggesting full acylation of the enzyme under these conditions. The isolated acyl-enzyme (as described in the Experimental Section) has an A_{280}/A_{260} ratio greater than 1.8, indicating that less than 0.5 mol of NAD^+ is bound per mol of enzyme. This acyl-enzyme does not react with Nbs_2 but addition of 12 mM arsenate restored full reactivity of the enzyme instantaneously, as expected if complete deacylation of the fully acylated enzyme occurred; 4 mol of NADH/mol of enzyme was oxidized when a slight molar excess of NADH was added to the fully acylated enzyme. It was found that the extent of acylation of the NAD^+-saturated enzyme could also be monitored by the enzyme (tryptophan) fluorescence *increase* resulting from the NAD^+ release upon acylation of the enzyme. At 25°, acylation of the holoenzyme, in absence of phosphate, was stoichiometric with 4 mol of diphosphoglycerate/mol of enzyme, in excellent agreement with the isolated and characterized tetraacyl-enzyme prepared at 4° (Figure 8).

(3) ACYLATION BY β-(2-FURYL)ACRYLOYL PHOSPHATE. The furylacryloyl-enzyme isolated after incubation of the holoenzyme with a large excess of β-(2-furyl)acryloyl phosphate (Malhotra and Bernhard, 1968, 1973) had a uv ratio of about 1.5 indicating that 2 mol of NAD^+ were still strongly bound per enzyme molecule under these conditions. Two molecules of Nbs_2 reacted with the β-(2-furyl)acryloyl-acylated enzyme molecule with a rapidity characteristic of the *four* SH per tetramer in the nonacylated enzyme. These results are consistent with the acylation of only *two* of the *four* potentially acylatable sites of the enzyme, in excellent agreement with spectrophotometric determination of the acyl content, as found previously with the yeast and rabbit enzyme (Malhotra and Bernhard, 1968). Acylation data for the sturgeon Glyc-3-P-dehydrogenase are summarized in Table IV.

Discussion

Homogeneity of Enzyme Molecules and Allosteric Mechanisms. Muscle glyceraldehyde-3-phosphate dehydrogenase is known to have the following well-defined qualitative prop-

erties: (a) it contains four identical or apparently identical subunits; (b) its catalytic function involves the formation of a metastable acyl-enzyme intermediate; (c) this catalytic function involves one uniquely reactive cysteinyl residue per subunit; and (d) the *catalytic* function is affected by the oxidized coenzyme, NAD^+. The effector function of NAD^+ and its binding to otherwise unoccupied enzyme sites are not describable according to models involving homogeneous and independently functioning NAD^+-binding sites (Conway and Koshland, 1968; Malhotra and Bernhard, 1968; De Vijlder *et al.*, 1969; MacQuarrie and Bernhard, 1970; Bloch, 1970; Bernhard and MacQuarrie, 1973).

This oligomeric enzyme is one in which the catalytic mechanism has been investigated in considerable detail, and in which the individual chemical steps of catalysis (eq 1) can be investigated in regard to both molecular reaction mechanism and regulation by an effector (NAD^+). Previously, we and others have presented arguments concerning the symmetry (or lack of symmetry) in this oligomeric structure involving identical polypeptides (Hanson, 1968; MacQuarrie and Bernhard, 1971; Matthews and Bernhard, 1973; Bernhard and MacQuarrie, 1973). Our arguments have centered on two observable properties, perhaps intimately related, namely *negative cooperativity* (progressive decrease in ligand affinity with increasing extent of site saturation) and *half-site reactivity* (stoichiometric limitation of an irreversible reaction to one-half of the potential active sites of the molecule) (Levitzki *et al.*, 1971). Quantitative investigations of these two types of interactions appear to have the potential for distinguishing among a variety of models for regulatory function. All such analytical distinctions, however, rest on the foundation that the experiments have been carried out with enzymes whose sites are *homogeneous* within the limits that the regular subunit interactions will allow. Such measurements demand that prior to ligand interaction, all Glyc-3-P-dehydrogenase sites have the following properties: (a) each enzyme site contains a reactive sulfhydryl group (nonoxidized, noncomplexed with metal, etc.); (b) the active site is free of nucleotides and other inhibitors and effectors of coenzyme binding; and (c) all of the sites are functional, *i.e.*, all of the active-site sulfhydryl groups are located on polypeptides which are in their native active conformation, and hence, the specific activity of the enzyme is truly maximal. It is toward these goals that we have directed our efforts in the isolation of sturgeon muscle Glyc-3-P-dehydrogenase. We can now present a procedure for the preparation of an enzyme meeting all these criteria. It is our hope that such a preparation will serve as a basis for the investigation of the properties of this class of enzymes.

Active-Site Titration with Nbs_2. Active-site sulfhydryl titrations with Nbs_2 clearly demonstrate that *four* sulfhydryl groups are especially reactive per enzyme tetramer. The very great enhancement of reactivity of these four over the other sulfhydryl groups of the molecule makes this distinction clear (Figure 4). In this light it is relevant to investigate the relationship between reactive sulfhydryl groups (according to the Nbs_2 "burst" reaction criterion) and *specific activity* of the enzyme. Such a comparison has been carried out, as illustrated in Figure 5. There is an absolute correlation between the number of reactive cysteinyl residues and the specific activity. It is hence our conclusion that the Nbs_2 burst reaction is a measure of both the number of available sulfhydryl groups and the number of available sulfhydryl groups which reside in catalytically functional sites. It is clear from the data presented that the purified isolated Glyc-3-P-dehydrogenase contains precisely *four* such reactive SH groups per enzyme tetramer.[4] It is of interest to note that this burst reaction of one Nbs_2 per subunit is not so distinct in lobster muscle Glyc-3-P-dehydrogenase (which contains five cysteines per subunit, Wassarman and Major, 1969) as is the case with sturgeon muscle Glyc-3-P-dehydrogenase (three cysteines per subunit).

TABLE IV: Acylation Data for Sturgeon Glyc-3-P-dehydrogenase at pH 7.0, 25°.

Acylating Agent	Act. Cysteines/ Tetramer[a]	No. of Acyl Group/ Tetramer
Glyc-3-P + NAD^+	*b*	3.5 ± 0.5
1,3-P_2Glyc	0.08 ± 0.02	3.70 ± 0.1[d]
	3.80 ± 0.1[c]	
β-(2-Furyl)acryloyl phosphate	2.06 ± 0.1	1.98 ± 0.1[e]
Untreated	3.86 ± 0.1	

[a] Equivalents of Nbs_2 reacting per mole of enzyme tetramer. [b] A nearly complete protection of the enzyme against Nbs_2 reaction was observed at 0.9 mM NAD^+ and 0.4 mM Glyc-3-P. [c] Nbs_2 equivalents after the addition of 12 mM arsenate to the acylated enzyme. [d] Moles of NADH (22 μM) oxidized per mole of enzyme (2 μM). [e] The acyl content was determined by absorbance at 345 nm using ϵ 3.0×10^4 M^{-1} cm^{-1}.

NAD^+ Binding to the Enzyme Active Site. Despite highly homologous structures and a common molecular mechanism (eq 1), glyceraldehyde-3-phosphate dehydrogenases from diverse species differ in the way they are affected as a result of binding oxidized coenzyme (NAD^+). Presumably this diversity in effector role has an influence on the peculiar metabolic flux within the particular organism. The interaction of this enzyme with NAD^+ has moreover played an important "anthropomorphic" role in the analysis of the validity of particular allosteric mechanisms of regulation (Conway and Koshland, 1968; Cook and Koshland, 1970; Kirschner *et al.*, 1968, 1971a,b). It is therefore of special significance to examine the way in which NAD^+ binds to the purified enzyme molecule we describe. It is important to emphasize various reports in the literature stating, or alluding to, nonintegral ratios of NAD^+ binding sites to protomers (usually more than two and less than four per tetramer), and to frequent reports of gross disymmetry in the apparent affinity of enzyme for NAD^+ at the ultimate binding site (Conway and Koshland, 1968; Boers *et al.*, 1971). Such reports if valid demand rather complex structural models for the tetrameric enzyme. In particular they demand *quaternary* conformational structures which (at equilibrium) are dependent on both the state and the extent of ligation. The existence of such structures is a controversial issue which distinguishes among currently favored

[4] It is noteworthy that the *kinetic* homogeneity of the rapid burst reaction of the active-site sulfhydryl with a large molar excess of Nbs_2 is dependent on the concentration of oxidized coenzyme (NAD^+). At intermediate extents of NAD^+ saturation the reaction rate is biphasic. However, even the slower phases of this reaction are very much faster than the reaction of Nbs_2 with any other sulfhydryl residue. We believe that this heterogeneity in rate arises as a consequence of the asymmetry of the subunit arrangement in the oligomeric enzyme molecule and the preferential reactivity at NAD^+-free sites, and shall comment further on this point elsewhere (Seydoux *et al.*, manuscript in preparation).

allosteric mechanisms (Monod *et al.*, 1965; Koshland *et al.*, 1966).

Rabbit muscle enzyme, in distinction to yeast enzyme, exhibits "negative cooperativity" in the binding of NAD^+, at pH 8.5 and 3°. Similar effects have been inferred for the NAD^+ binding to rabbit muscle enzyme at higher temperatures on the basis of the NAD^+ concentration dependence of kinetic phenomena (Conway and Koshland, 1968; De Vijlder *et al.*, 1969). Preliminary fast reaction studies have indicated that at room temperature the rabbit muscle enzyme also exhibits this feature of negative cooperativity (Bloch, 1971).

The fluorescence quenching of the constituent tryptophan chromophores of the sturgeon muscle enzyme by NAD^+ are illustrated by the "Scatchard plot" of Figure 7. The "negative cooperativity" in the isotherm is apparent from this plot. These data *suggest* that there are *two* types of NAD^+ binding sites; the solid line in Figure 7 is the theoretical expectancy for a molecule containing equal numbers of two types of NAD^+ binding sites. No better statistical fit is obtainable by assuming a greater heterogeneity in binding sites. Other experiments in this laboratory indicate that the extent of fluorescence quenching by NAD^+ (a nonradiative process) is linearly related to the extent of binding. Consistent with this finding is the fact that the formation of the enzyme–NAD^+ "Racker band" shows equal absorbance contribution for each site. Hence this binding isotherm suggests that the binding of NAD^+ occurs at two classes of sites with equal numbers of sites of each class. The stoichiometry of NAD^+ binding is readily obtainable at high enzyme concentrations. From such data we conclude that four NAD^+ molecules are bound per molecule of enzyme. Since we find a twofold asymmetry in physical properties for a tetrameric molecule with identical subunits, we must conclude that the tetramer has maximal twofold symmetry. We, and others, have commented on the implications of such an asymmetric structure previously (Hanson, 1968; Watson *et al.*, 1972; Matthews and Bernhard, 1973).

In equilibrium binding studies in which either heterogeneity or allosterism plays a role, it is essential that all enzyme sites be not only functional, but uninhibited by the occupancy of other ligands. Previously, tight binding of other adenine nucleotides to the enzyme site has been reported (Pfleiderer and Stock, 1962; Celliers *et al.*, 1963; Bloch *et al.*, 1971). Since such nucleotides are spectrophotometrically similar to NAD^+, the extent of NAD^+ occupancy is confused by their presence. In some preparative procedures, removal of bound nucleotides results in an instability of enzyme protein. Our results demonstrate that sturgeon muscle Glyc-3-P-dehydrogenase isolated according to the above experimental procedure can be totally freed of bound nucleotides without conformational destabilization or partial inactivation of the enzyme protein. Thus we can prepare true apoenzyme devoid of other adenine nucleotides, perform experiments of long duration, and store the enzyme sample for long periods of time.

Active-Site Acylation. Whereas the disulfide formation reaction with Nbs_2 is a reaction which occurs with model cysteinyl compounds as well as with the enzyme site, the acylation of this specific cysteine thiol with acyl phosphate is a reaction peculiar (under the conditions of pH neutrality and temperature) to the enzyme site. Although the reaction of Nbs_2 with the active site thiol is very much faster than its reaction with other cysteines of the native enzyme, it still may not reflect on the total catalytic functionality of the site. Moreover, the heterogeneity of the NAD^+ interactions with the sites may lead one to question the functional significance of both the "tight" and "weak" NAD^+-binding sites. For these reasons we have studied the acylation reaction in detail. Toward this end we have employed the natural substrate 1,3-diphosphoglycerate and the more convenient chromophoric acylating reagent β-(2-furyl)acryloyl phosphate. Previously, we have reported on the reaction of β-(2-furyl)acryloyl phosphate with the rabbit and sturgeon muscle enzymes (Malhotra and Bernhard, 1968, 1973; MacQuarrie and Bernhard, 1971). Despite the four identical protomers, the reaction with β-(2-furyl)acryloyl phosphate leads quasi-irreversibly to the formation of a diacyl enzyme tetramer. In correspondence with these earlier reports, an enzyme molecule will form acyl-enzyme in an amount equivalent to *one-half* the amount of Nbs_2 with which it will react. Hence the maximal amount of acyl-enzyme obtainable from an excess of β-(2-furyl)acryloyl phosphate and enzyme at pH neutrality is equal to one-half the number of covalently identical protomers.

This half-site reactivity is in contrast to the readily obtainable *tetraacylated* enzyme by reaction with 1,3-P_2Glyc. Direct measurements of 1,3-P_2Glyc acylation by monitoring the equivalents of NAD^+ formed in the reaction of NADH with the acylated enzyme show that there are four acyl groups per enzyme. This acyl-enzyme does not react with Nbs_2 whereas prior hydrolysis *via* arsenate leads to an enzyme reaction with four equivalents of Nbs_2. In this regard it is of special note that the difurylacryloyl-enzyme still reacts with 2 equiv of the large Nbs_2 molecule (Table IV). This argues against mechanisms involving steric constraint for the stoichiometric limitation in β-(2-furyl)acryloyl reactivity.

The consequences of the acylation reaction with 1,3-P_2Glyc can be conveniently measured by some indirect techniques, most notably, by the protection which acylation (with 1,3-P_2Glyc affords against NAD^+ quenching of the protein fluorescence. Hence in the absence of alternative sulfhydryl modification, the extent of acylation can be followed by the resultant increase in total fluorescence in the presence of NAD^+. All chemical modifications of the active-site thiol destroy the peculiar E–NAD^+ electronic absorption band. Hence, in the absence of any alternative modification of the active-site thiol, the extent of acylation can also be ascertained (in the presence of NAD^+) by the extent of Racker band formation at the empty sites. By these phenomena, we have noted an extreme rapidity in the acylation reaction with natural substrate. We believe this reaction velocity to be nearly diffusion controlled. Hence under the conditions of the acylation experiments described herein we are unable to examine for differences in rates of reaction at the various sites. Elsewhere we shall examine the acylation reaction for potential heterogeneity in 1,3-P_2Glyc acylation sites *via* the nondiffusive rates of the reverse (phosphorolysis) reaction. At this time we can conclude from our acylation studies that reaction with true substrate (1,3-P_2Glyc) differs from reaction with pseudosubstrate in the stoichiometric limitation of reaction. The two acylation reactions also differ very significantly in equilibrium constant for acylation. Although quantitative details are not available at this time, the difference in equilibrium constants (for acylation by acyl phosphate at pH 7) appears to be of the order of 10^3-fold in favor of the true acyl-enzyme over the pseudo-furylacryloyl-enzyme. A thermodynamic comparison of equilibrium constants is complicated by the difference in stoichiometric limitation for acylation in the two cases. However, the preciseness of the half-site stoichiometry coupled with the crystallographic indications that the four sites in Glyc-3-P-hydrogenase are very distant from one another

once again indicates that the half-site reactivity is not a result of steric constraints imposed by the size of the furylacryloyl group. This is further substantiated by the fact that modification of the essential thiol by even larger residues, such as that produced in the Nbs_2 reaction, can still lead to full-site reactivity. It seems more likely to us that the explanation for the difference in stoichiometric limitation between 3-phosphoglyceroyl-enzyme and furylacryloyl-enzyme must lie in the *special* properties of the true substrate acyl-enzyme, rather than in the furylacryloyl-induced properties of the pseudo-acyl-enzyme. Therefore it remains more plausible to us that the furylacryloyldiacyl-enzyme reflects an asymmetry inherent in the enzyme molecule. In any event, the reaction of our isolated enzyme with either acylating reagent shows a clean reaction with either *all* or *half* of the identical polypeptide chains in every enzyme molecule.

The very fast acylation reaction with 1,3-P_2Glyc is dependent on the presence of enzyme-bound NAD^+. Although we report no quantitation herein, it has been demonstrated that the apoenzyme is catalytically inert toward acylation with 1,3-P_2Glyc (Hilvers and Weenen, 1962; Trentham, 1971a). We have noted a similar dependence of the acylation reaction on NAD^+ with β-(2-furyl)acryloyl phosphate (Malhotra and Bernhard, 1968).

A Comparison of the Sturgeon Muscle and Yeast Enzyme. The demonstration of virtually fully functional sites in our sturgeon muscle enzyme preparation prompted a comparison with another Glyc-3-P-dehydrogenase enzyme of demonstrated high purity, that of Baker's yeast Glyc-3-P-dehydrogenase. The relative activity of sturgeon *vs.* yeast enzymes is pH dependent. At pH 8.9, where activity is apparently optimal for both (Trentham, 1971b), the two enzymes have very similar specific activities (provided that the yeast enzyme is preincubated with NAD^+). On the other hand, the yeast enzyme is more active than sturgeon Glyc-3-P-dehydrogenase at neutral (suboptimal) pH (see Table II), indicating different activity–pH profiles for the two enzymes.

Apo-sturgeon Glyc-3-P-dehydrogenase, when mixed with NAD^+, immediately reaches its maximum activity (Trentham, 1971a) (*i.e.*, in less than 3 msec). In contrast, a slow NAD^+-dependent isomerization is observed with the yeast enzyme at pH 8.5 (Kirschner *et al.*, 1971b; Ellenrieder *et al.*, 1972). This slow interconversion from an inactive to an active form is related to the positive cooperativity in NAD^+ binding observed with this enzyme (Kirschner *et al.*, 1966). Although these phenomena clearly differentiate the yeast enzyme from the sturgeon Glyc-3-P-dehydrogenase, both molecules do require NAD^+ for the fast acylation by 1,3-P_2Glyc (Trentham, 1971a; Ellenrieder *et al.*, 1972). Thus although the role of NAD^+ as effector in the two enzymes is obviously different, the catalytic mechanism has common aspects.

Summary

We have detailed the procedure for isolation of highly purified sturgeon muscle Glyc-3-P-dehydrogenase such that virtually every polypeptide contains a functional site.

We have demonstrated that a variety of thiol-specific active-site reactions can take place at each of these sites but that the reactions are sometimes heterogeneous in ligand affinity or in rate. Despite the heterogeneity in the equilibrium binding affinity for NAD^+ to enzyme sites, the extent of fluorescence quenching and the Racker band extinction coefficient are the same at every site in the molecule. The heterogeneity can be accounted for on the basis of two classes of sites of equal number.

The reaction of a chromophoric pseudosubstrate acyl phosphate leads to half-site reactivity whereas the reaction of the true substrate acyl phosphate leads to acylation at every site in the enzyme molecule. Our current state of information suggests that the half-site reactivity is not the consequence of a steric constraint imposed by the pseudosubstrate acyl structure.

It now appears virtually certain that NAD^+ plays at least three roles in the overall enzyme reaction. (a) It is a participant in the oxidation of glyceraldehyde 3-phosphate. (b) It is an activator of the acyl bond in (at least) some of the acyl-enzyme reactions. (c) It regulates the activity of the enzyme by acting as an effector of subunit interactions, a property which is variable with the particular tetrameric enzyme species.

Detailed preparative procedures now exist for the large-scale and high-purity isolation and characterization of two Glyc-3-P-dehydrogenases with qualitatively different NAD^+-binding properties. Both the yeast and the sturgeon muscle enzyme can be prepared free of any adenine nucleotide. The interaction of these two enzymes with coenzyme and other adenine nucleotides can now be carefully compared. Likewise, the catalytic reaction pathways and the role of coenzymes and nucleotides in its regulation can now be investigated and compared.

Acknowledgments

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