Waxman, S., and Schreiber, C. (1974c), *Proc. Soc. Exp. Bio. Med. 147*, 760–764.
Waxman, S., and Schreiber, C. (1975), *Clin. Res. 23*, 284A.
Waxman, S., Schreiber, C., and Landowne, A. (1974), *Prog. Am. Soc. Hemat.*, 103.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406–4412.
Zacharius, R. M., Zell, T. E., Morrison, J. H., and Woodlock, J. J. (1969), *Anal Biochem. 30*, 148–152.

Subunit Interactions in Yeast Glyceraldehyde-3-Phosphate Dehydrogenase†

Stephen C. Mockrin, Larry D. Byers, and D. E. Koshland, Jr.*

ABSTRACT: The spontaneous inactivation of yeast glyceraldehyde-3-phosphate dehydrogenase was found to fit a simple two-state model at pH 8.5 and 25°. The first step is a relatively rapid dissociation of the tetramer to dimers with the equilibrium largely in favor of the tetramer. In the absence of NAD^+ the dimer inactivates irreversibly. The apoenzyme is quite stable with a half-life for complete activity loss proportional to the square root of the enzyme concentration. Perturbations of the protein structure (by pH, ionic strength, and specific salts), which have no effect on the tetrameric state of the molecule, result in an alteration of the cooperativity of NAD^+ binding, the reactivity of the active-site sulfhydryl group, and the catalytic activity of the enzyme. Covalent modification of two of the four active-site sulfhydryl groups has profound effects on the enzymic activity which are mediated by changes in the subunit interactions. Sedimentation analysis and hybridization studies indicate that the interaction between subunits remains strong after covalent modification. Under normal physiological and equilibrium dialysis conditions the protein is a tetramer. Equilibrium dialysis studies of NAD^+ binding to the enzyme at pH 8.5 and 25° reveal a mixed cooperativity pattern. A model consistent with these observations and the observed half-of-the-sites reactivity is that of ligand induced sequential conformational changes which are transferred across strongly interacting subunit domains. Methods for distinguishing negatively cooperative binding patterns from mixtures of denatured enzyme and multiple species are discussed.

Analysis of subunit interactions is an important tool for delineating the nature of protein conformational changes (Koshland, 1970). Although the nature of the communication between subunits is fundamentally similar to the communication between sites within the same peptide chain, there are some advantages to the study of intersubunit effects. It is difficult to directly relate conformational changes within a subunit to energy changes, but cooperativity can be expressed in terms of ligand binding constants and these in turn to the free energy of subunit interactions. Moreover, association–dissociation experiments can give further insight into the energetics of the subunit contacts.

A particularly attractive protein for these studies is glyceraldehyde-3-phosphate dehydrogenase since the crystallography of the enzyme (Watson et al., 1972; Buehner et al., 1973, 1974), its primary structure (Jones and Harris, 1972), its mechanism (Krimsky and Racker, 1963; Furfine and Velick, 1965; Trentham, 1971; Orsi and Cleland, 1972; Harrigan and Trentham, 1973), and its cooperativity patterns (Conway and Koshland, 1968; DeVijlder and Slater, 1968; Velick, 1970; Smith and Velick, 1972) have been investigated extensively. Studies on the acylation and alkylation of the yeast enzyme clearly reveal a half-of-the-sites reactivity pattern which has been described in terms of negatively cooperative interactions (Stallcup and Koshland, 1973a–c). The NAD^+ binding pattern is more ambiguous with evidence of both concerted and sequential binding patterns (Kirschner et al., 1966; Kirschner, 1971; Cook and Koshland, 1970; Sloan and Velick, 1973). Moreover the intersubunit communication in association–dissociation is not clear. Accordingly studies were initiated to focus on the subunit interactions with the view of clarifying their properties and of relating them to dehydrogenases from other sources.

Materials and Methods

GPD[1] from *Saccharomyces cerevisiae* was prepared as described previously (Stallcup et al., 1972) except that the cells were broken with a Manton Gaulin-APV homogenizer (Hetherington et al., 1971) instead of by grinding with alumina. This more rapid process reduced the time required for obtaining the crude extract (from 1 hr to 5 min) and resulted in a twofold increase in final yield of enzyme (~400

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received June 6, 1975.* This work was supported by research grants from the National Science Foundation (GB 7057) and the U.S. Public Health Service (AM-GM 90765), a Postdoctoral Fellowship (GM 49094-02) to L.D.B., and a U.S. Public Health Service Training Grant (5 T01- GM-31-14) and a National Science Foundation Traineeship Grant (GZ 1144) to S.C.M.

[1] Abbreviations used are: GPD, glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12]; G3P, glyceraldehyde 3-phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxylmethyl)-aminomethane; N_S, number of substrate molecules bound per mole of enzyme, e.g., $[NAD^+]_b/[E_t]$.

mg/lb of yeast). The enzyme preparation had no detectable aspartic β-semialdehyde dehydrogenase activity (see Holland and Westhead, 1973). The protein was homogeneous as judged by (1) isoelectric focussing (Stallcup et al., 1972), (2) sodium dodecyl sulfate and native gel electrophoresis (method of Ames, 1960), (3) sedimentation analysis (protein = 0.2–3.5 mg/ml in 0.01 *M* pyrophosphate or 0.05 *M* bicine and 1 m*M* EDTA (pH 8.5)), (4) titration with *p*-nitrophenyl acetate (Stallcup et al., 1972), 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959), and 4,4′-bis(dimethylamino)diphenylcarbinol (Rohrbach et al., 1973) all indicating 3.9 (±0.2) free sulfhydryl groups in the native enzyme and 7.8 (±0.3) sulfhydryl groups in the Gdn·HCl denatured protein, (5) NADH bursts (rapid transient release of 4.0 (±0.1) NADH molecules per mole of tetramer when NAD^+ (1 m*M*) and G3P (1 m*M*) are added to enzyme in 0.05 *M* bicine at pH 8.5), and (6) amino-terminal analysis (method of Weiner et al., 1972) indicating a single valine terminal (Velick and Udenfriend, 1953). The enzyme had a specific activity of 100–110 μmol of NADH/(min mg) and an A_{280}/A_{260} ratio of 2.08–2.12. The extinction coefficient of the apoenzyme (at 280 nm), corrected for the molecular weight of 145000, is $1.35 \times 10^5\ M^{-1}\ cm^{-1}$ (Krebs, 1955).

The tetracyanylated enzyme was prepared as described previously (Byers and Koshland, 1975). G3P was a racemic mixture prepared from the barium salt of the diethyl acetal obtained from Sigma Chemical Co. β-NAD^+ was obtained from Sigma Chemical Co. (either Grade III or V). [^{14}C]NAD^+ was prepared from [7-^{14}C]nicotinamide (Amersham/Searle) by enzymic exchange as described by Colowick and Kaplan (1957) with the following modifications: after the lyophilization step, the residue was dissolved in 5 ml of 0.01 *M* glycylglycine buffer (pH 7.5) and then placed on a P-2 column (40 × 2.2 cm, 200–400 mesh, Bio-Rad, 8 ml/hr under 36-cm pressure head). The NAD^+ containing fractions were pooled and placed on a DEAE-cellulose column (1.4 × 15 cm, Whatman DE 52, equilibrated with 0.01 *M* glycylglycine buffer (pH 7.5)). The NAD^+ was eluted in this same buffer system with a 0–0.4 *M* NaCl gradient of 1.4 l. total volume (700 ml in each reservoir). The NAD^+ containing fractions were pooled and acidified and the NAD^+ was then precipitated as originally described by Colowick and Kaplan (1957).

DEAE-cellulose glass plates (using Cellex D, Bio-Rad, as directed by the manufacturer) proved to be a rapid (30–45 min), sensitive way of separating nicotinamide, NAD^+, NADH, and ADP-ribose, using 0.1 *M* ammonium carbonate as an eluent. Good separation was also obtained with PEI plates (Levitzki and Koshland, 1971) and with DEAE-cellulose paper (Silverstein, 1970) but these systems were much slower. Radioactive samples were mixed with cold NAD^+, nicotinamide, and NADH. This mixture was then evaporated onto the plates (5 × 20 cm) and eluted. The spots were located under a uv lamp, removed with a razor blade, and placed into scintillation vials containing 1 ml of water plus 10 ml of Bray's solution for counting. The [^{14}C]NAD^+ was shown to be 99% pure by this method. The NAD^+ was also shown to be 99% pure by enzymatic conversion to NADH (Klingenberg, 1963) followed by chromatography in this system.

Sedimentation studies were performed with Spinco Model E ultracentrifuge equipped with a schlieren optical system and a photoelectric scanning optical system. Runs were carried out at 52000 rpm. Reacting enzyme sedimentation velocity experiments were carried out as described by Cohen and Mire (1971). A positive density gradient was maintained by the addition of 40% D_2O to the assay mixture (see Taylor et al., 1972). The assay mixture contained D-G3P (1.14 m*M*), NAD^+ (1 m*M*), arsenate (10 m*M*), Tris-HCl (0.022 *M*), EDTA (2.2 m*M*), and 40% D_2O (v/v) at pH 8.7, 19°. The enzyme concentration initially in the reacting band was 1.27×10^{-9} *M*. Under these conditions the presence of 40% D_2O had negligible effect on the specific activity of the enzyme.

For the equilibrium dialysis experiments, enzyme was centrifuged from an ammonium sulfate suspension immediately prior to use, and then quickly desalted using a small G-50 column (~8 ml) equilibrated with pyrophosphate buffer (0.05 *M* sodium pyrophosphate (pH 8.5, 25°)–1 m*M* EDTA–1 m*M* dithiothreitol). Equilibrium dialysis was routinely carried out in 0.3-ml cells. Denaturation was kept to a minimum by using membranes of high porosity (Type SM 11536, Sartorius Division, Brinckman Instruments, Westbury, N.Y.), which equilibrated within 4 hr, and by adding NAD^+ to both the enzyme side and to the ligand side of the membrane. This latter technique stabilized the enzyme (Stancel and Deal, 1969a; Velick, 1970; Fenselau, 1972) and further decreased the NAD^+ equilibration time. Assays of enzyme activity immediately after a binding study showed denaturation occurred only to a minor degree, i.e., less than 4%.

In a routine experiment, 150 μl of enzyme solution (18–25 mg/ml) was placed in the enzyme compartment, and 150 μl of pyrophosphate buffer was placed in the ligand compartment. Radioactive NAD^+ (50 μl, neutralized with NaOH) was then added to each compartment. After equilibration, 50-μl samples were withdrawn for counting, which was performed in a Packard TriCarb Model 3375 scintillation counter, using 10 ml of Bray's solution (Bray, 1960) and 1 ml of water. Microliter samples were pipetted with Hamilton syringes with chaney adapters.

Spontaneous inactivation of the enzyme was carried out by incubating the enzyme in buffer containing 10 m*M* dithiothreitol (or dithioerythritol)[2] at room temperature (24–26°) and periodically removing and assaying aliquots. Assays were carried out either in 0.05 *M* pyrophosphate or 0.5 *M* bicine [*N*,*N*-bis(hydroxyethyl)glycine] buffer (pH 8.5, 25°) containing 10 m*M* EDTA, 10 m*M* sodium arsenate, 2 m*M* glyceraldehyde 3-phosphate (1 m*M* D enantiomer), and 1 m*M* NAD^+. Thus, this method measures the loss of enzymic activity which is not rapidly (within 5 min) reactivated by NAD^+.

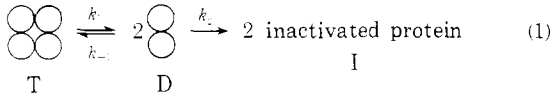
Results

Dissociation–Inactivation. Studies on the hybridization between yeast glyceraldehyde-3-phosphate dehydrogenase and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Spotorno and Holloway, 1970; Osborne and Holloway, 1974) and between two species of yeast GPD (Stallcup and Koshland, 1973b) reveal that a relatively rapid rearrangement of the subunits of the enzyme occurs. Dissociation does not occur further than dimers since only three molecular species are obtained after considerable lengths of time. Ultracentrifuge studies show that no detectable amount of dimer or monomer is present. A calculation using the detectable analytical accuracy indicated the yeast

[2] The rate of enzymic activity loss is at least ten times faster if sulfhydryl reagent is not present in the incubation buffer.

enzyme has a dissociation constant of less than 10^{-8} M [in agreement with previous studies (Jaenicke et al., 1968)] in contrast to the rabbit muscle enzyme which has a dissociation constant of approximately 10^{-6} M (Hoagland and Teller, 1969).

During these studies, it was observed that a slow, spontaneous inactivation of the apoenzyme occurs and a detailed kinetic analysis of the process was initiated. The rate of the activity loss was measured as a function of apoenzyme concentration. Since hybridization studies (Stallcup and Koshland, 1973b) of two species of yeast GPD indicate that formation of the hybrid species A_2B_2 occurs rapidly ($t_{1/2} <$ 1.5 hr at pH 8.5) relative to activity loss, it is reasonable to assume that equilibrium between tetramer and dimer is established rapidly relative to the slow irreversible inactivation step (k_2 of eq 1). The rate of loss of tetramer can then be represented by eq 2 where K_d ($=k_1/k_{-1}$) is the intrinsic dissociation constant of the tetramer to dimer.



(1)

$$-d[T]/dt = k_2K_d^{1/2}[T]^{1/2}/2 \quad (2)$$

Since, at even the highest enzyme concentrations investigated, the final value of enzymic activity was zero, any reasonable value of K_d ($<10^{-4}$ M) requires the additional k_2 step (with $k_2 \gg k_{-2}$). This follows from the fact that the simple dissociation of tetramer to an inactive dimer species cannot account for the enzyme concentration dependence of inactivation[3] or the final value of zero activity. The observed activity is proportional to the concentration of molecular species multiplied by their relative activity, but the contribution of dimer activity will be negligible if the dissociation constant of the tetramer is small, as is the case with the yeast enzyme. In this case the half-life of the active species can be obtained by integrating eq 2 to yield eq 3a and substituting in the rate expression to get eq 3b:

$$[T]^{1/2} = [T_0]^{1/2} - (k_2K_d^{1/2}/4)t \quad (3a)$$

$$t_{1/2} = 2(2 - 2^{1/2})[T_0]^{1/2}/k_2K_d^{1/2} \quad (3b)$$

A plot of the half-life vs. the square root of the total enzyme ($\approx [T_0]$ = concentration of tetramer at $t = 0$) should yield a straight line and this has been observed over a 2500-fold range in concentrations as shown in Figure 1. The rate of activity loss was unaffected by the addition of bovine serum albumin at a concentration of 6.25 mg/ml indicating that no extraneous absorption on the wall of the tube occurred.

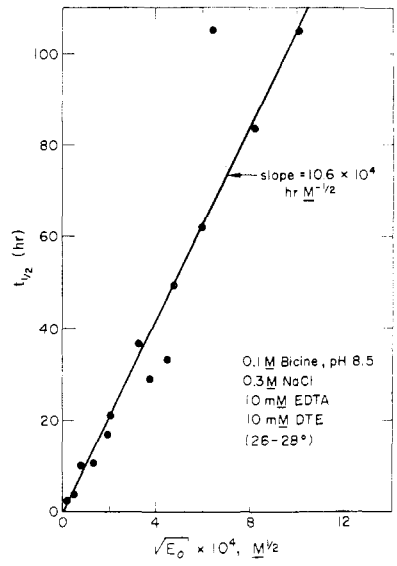


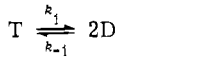
FIGURE 1: Dependence of the half-life for complete spontaneous inactivation as a function of the square root of total enzyme concentration ($[E]^{1/2}$). Initial velocities were determined as described in the text. Enzyme (at various concentrations) was incubated in buffered solutions (pH 8.5, 0.1 M bicine, 0.3 M NaCl, 10 mM EDTA, and 10 mM dithioerythritol (DTE) or 10 mM dithiothreitol, 26–28°).

When the protein concentration is increased by the addition of heat-denatured GPD no increase in stability is observed indicating stabilization depends specifically on native GPD. From Figure 1 a value of $k_2K_d^{1/2} = 1.1(\pm 0.1) \times 10^{-5}$ $M^{1/2}$ hr^{-1} is obtained.

ATP increases the rate of activity loss yielding a value of $k_2K_d^{1/2}$ of 7.6×10^{-5} hr^{-1} $M^{1/2}$ at 10 mM ATP, but NAD^+ retards the activity loss giving in this case a $k_2K_d^{1/2}$ of 0.67×10^{-5} hr$^{-1}M^{1/2}$ at 1.2 mM NAD^+. Under normal assay conditions, which contain 1 mM NAD^+ at pH 8.5, the specific activity of yeast GPD is independent of enzyme concentration between 10^{-9} and 2×10^{-6} M. This indicates that either the tetramer does not dissociate directly to an inactive dimer, i.e., $K_d < 2 \times 10^{-10}$ M or that the specific activity of the dimer is equal to that of the tetramer. A recent report (Bartholmes and Jaenicke, 1975) indicates that the dimeric state of yeast GPD is catalytically inactive at pH 7.0.

There have been reports that the dimeric form of GPD from rabbit muscle (Fuller-Noel and Schumaker, 1973) and from rat muscle (Nagradova et al., 1974) is catalytically active. To determine the size of the catalytic unit(s) of the yeast enzyme a reacting enzyme sedimentation study (Cohen and Mire, 1971) was carried out by centrifuging the protein through an assay solution (see Materials and Methods). The results indicate that the only active form of the enzyme is the tetramer. If any active dimer were present, the K_d is calculated to be less than 2×10^{-10} M. These studies, therefore, are all consistent with an NAD^+ stabilization of the tetramer form and with only miniscule amounts of dimer present in the solution in the presence of NAD^+. The enzyme in the presence of NAD^+ is sufficiently stable so that negligible inactivation should occur in binding experiments requiring equilibrium dialysis. The fact that the spontaneous inactivation proceeds essentially via dimers makes this enzyme similar to pyruvate kinase (Kuczenski and Suelter, 1970, 1971) and rabbit muscle phosphofructokinase (Bock and Frieden, 1974). The stabili-

[3] If the observed inactivation were simply due to the dissociation of active tetramer to totally inactive dimer:



then the kinetics for the activity loss (assuming initially all enzyme is present as tetramer) is obtained as follows:

$$-dx/dt = k_{-1}D^2 - k_1([T_0] - x)$$

where $x = [T_0] - [T]$. Since $D = 2x$

$$-dx/dt = 4k_{-1}x^2 + k_1x - k_1[T_0]$$

integration yields

$$\ln\left[\frac{8k_{-1}([T_0] - [T]) + k_1 + (k_1^2 + 16k_1k_{-1}[T_0])^{1/2}}{8k_{-1}(T_0 - T) + k_1 - (k_1^2 + 16k_1k_{-1}[T_0])^{1/2}}\right] = (k_1^2 + 16k_1k_{-1}[T_0])^{1/2}t + \ln\left[\frac{k_1 + (k_1^2 + 16k_1k_{-1}[T_0])^{1/2}}{k_1 - (k_1^2 + 16k_1k_{-1}[T_0])^{1/2}}\right]$$

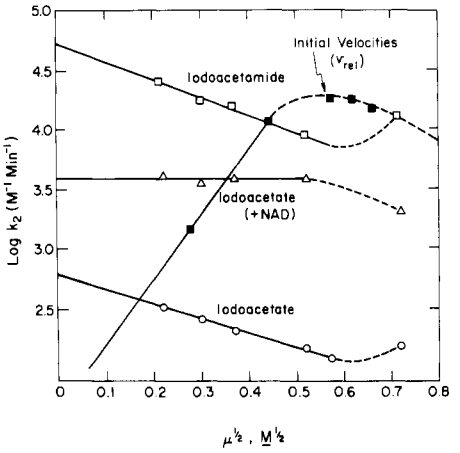


FIGURE 2: Brønsted-Bjerrum plot for various reactions of yeast GPD. The reactions were carried out at 25° and pH 8.5 (0.5 *M* bicine-0.5 m*M* EDTA) and the ionic strength (μ) was maintained with NaCl. The enzyme (9 μM) was incubated in buffer containing 5×10^{-5} *M* iodoacetamide (□) or 2.5×10^{-5} *M* iodoacetate in the presence of 1 m*M* NAD^+ (△) or in the absence of NAD^+ (○). Aliquots were removed and assayed as described in the text. The initial velocities (V_{rel}, in arbitrary units) were determined in the same buffer containing various amounts of NaCl, in the presence of substrates (1 m*M* G3P, 1 m*M* NAD^+, 10 m*M* AsO_4^{-3}). The enzyme concentration was 3.2×10^{-9} *M*. The apparent K_m of NAD^+ (at 1 m*M* G3P) and of G3P (at 1 m*M* NAD^+) increase with increasing NaCl concentration. At the highest salt concentration [G3P] = $0.7K_m^{app}$ (for G3P) and [NAD^+] = $0.5K_m^{app}$ (for NAD^+).

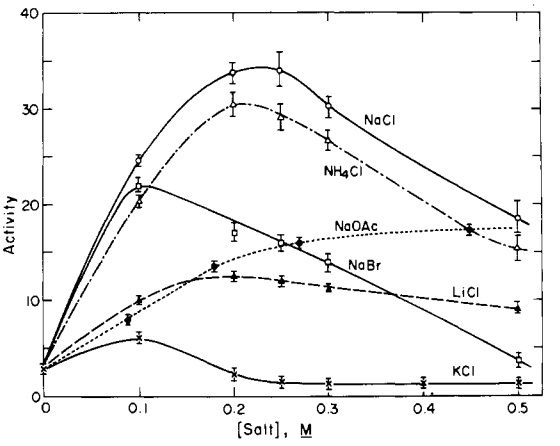


FIGURE 3: Effect of specific ions on the initial velocity (arbitrary units) of the dehydrogenase reaction catalyzed by GPD. Assays were carried out at 25° and pH 8.4 (±0.1) in buffer (0.05 *M* bicine-0.5 m*M* EDTA) containing NAD^+ (1 m*M*), G3P (1 m*M*), and sodium arsenate (10 m*M*).

zation of the tetramer form by NAD^+ is consistent with the observation that NAD^+ inhibits hybridization of two species of yeast GPD (Stallcup and Koshland, 1973b).

Effect of Salt Concentrations on Yeast GPD. Since 1.5 *M* phosphate (pH 7.5) causes partial dissociation of the enzyme to polydisperse species (Jaenicke et al., 1968) it was of interest to pursue this finding to see whether the effect was related to ionic strength. Low salt concentration (<0.3 *M* NaCl), however, has little effect on the gross structure and subunit interactions in yeast GPD as evidenced by no detectable change in $[\alpha]_{233}$ (Jaenicke, 1970) or in the $s_{20,w}$ value or the stability of the enzyme on standing at room temperature. Salt, nevertheless, does have profound effects on the activity of yeast GPD. This is illustrated in Figure 2. As the NaCl concentration is increased to about 0.3 *M*, the dehydrogenase activity is increased while the rate of reaction with iodoacetamide and iodoacetate (and with furylacryloyl phosphate) is decreased. In the presence of NAD^+ the reaction of the enzyme with iodoacetate is unaffected by increased salt concentrations.

The salt effect on these reactions cannot be simply attributed to an alteration in the pK_a of the active-site SH group. Thus, for the reaction of iodoacetamide with GPD an apparent pK_a of 8.45 and a $k_2^{lim} = 4.6 \times 10^4$ M^{-1} min^{-1} are observed at $\mu = 0.12$ *M* while at $\mu = 0.27$ *M* a $pK_a = 8.17$ and $k_2^{lim} = 1.7 \times 10^4$ M^{-1} min^{-1} are observed. This decrease in pK_a is about 0.2 unit larger than would be expected by the simple Debye-Hückel equation for an ionization of a thiol and, furthermore, the observed decreased in alkylation rate at pH 8.5 as μ is increased is opposite to that expected for reaction of iodoacetamide with the thiolate anion. In point of fact, the inherent reactivity of the thiolate anion with iodacetamide (k_2^{lim}) is decreased about 2.7-fold as the ionic strength is increased from 0.12 to 0.27 *M* with NaCl. This suggests that the altered reactivity of the enzyme is due to an effect of salt on the structure of the protein.

The Brønsted-Bjerrum (Brønsted, 1925; Bjerrum, 1924) relationship predicts that ionic strength should have no effect on an unassisted reaction of the active-site thiolate with the neutral iodoacetamide, and should increase the reaction rates of the anionic reagents iodoacetate and furylacryloyl phosphate. However, in all these cases the reaction rates are decreased as ionic strength is increased and this is in contrast both qualitatively and quantitatively to a simple electrostatic effect which predicts an activating effect of salt on reaction between two like-charged species.[4]

Consistent with the idea that the salt effects are not simply due to an electrostatic effect between the enzyme group and the reagent is the marked specificity of the effect on the nature of both the cation and anion on the activity of the enzyme. Figure 3 illustrates that both the nature and extent of the salt effect is dependent on the specific ions. It is interesting to note that the salt effect on the dehydrogenase activity of the yeast enzyme is distinct from that of GPD from other sources. Thus, in the region where salt inhibits the reaction the series of effectiveness for cations is: $K^+ > Li^+ > NH_4^+ \approx Na^+$ and for anions is $Br^- > {}^-OAc > Cl^-$. The enzyme isolated from *Clostridium acidi-urici* (W. B. Stallcup, unpublished observations) does not follow this pattern. For the pig or rabbit muscle enzyme, NaCl inhibits the catalytic activity even at low ionic strengths (Harrigan and Trentham, 1973).

When the ionic strength is increased to >0.25 *M* (with NaCl) the dehydrogenase activity of yeast GPD is decreased. This effect of salt is manifested by an alteration in the apparent kinetic constants (V_{max} and K_m) for both G3P and NAD^+. A break in the linear Brønsted-Bjerrum plot (Figure 3) is also apparent as NaCl is increased between 0.3 and 0.5 *M* for the reaction of the apoenzyme with iodoacetamide and of both the apo- and holoenzyme with iodoacetate. This is suggestive of an additional protein confor-

[4] It is unlikely that the high nucleophilic reactivity of Cys-149 toward alkylating and acylating reagents is due to an unassisted nucleophilic reaction [for the yeast enzyme factors of about 20 (for iodoacetamide) to about 200 (for *p*-nitrophenyl acetate) are obtained for the enhancement in the second-order rate constant for the thiolate anion relative to model compounds]. Indeed, Polgár (1975) recently presented evidence for the existence of an ion pair involving Cys-149 (and a histidine residue) in pig muscle GPD.

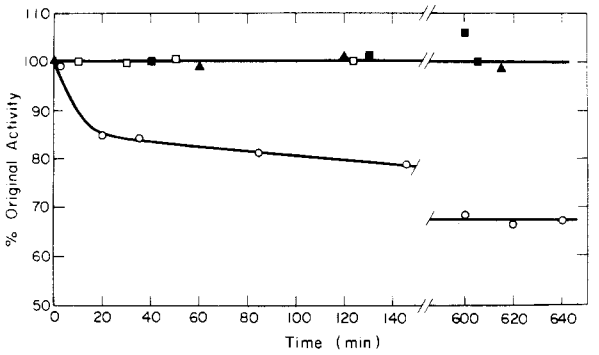


FIGURE 4: Hybridization of tetracyanylated and native GPD (at equal concentrations of each species). Conditions: 0.5 M bicine–1 mM EDTA, pH 8.5, 25°. The plot is percent original activity (normalized to control containing native enzyme only—total activity loss of control was 6% during the course of the experiment and corrected for reactivation of the cyanylated subunit). (○) 1.52 μM total protein (similar results were obtained with 5 μM total protein), (□) 1.5 μM total protein (0.05 M bicine), (▲) 1 μM native enzyme plus 1 μM heat-denatured protein, (■) 1.52 μM total protein plus 1 mM NAD^+.

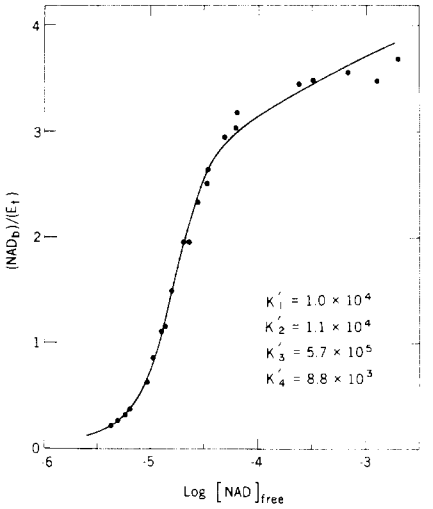
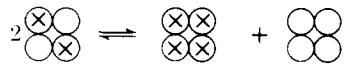


FIGURE 5: Binding of NAD^+ to yeast GPD at 25° as determined by equilibrium dialysis. Conditions: 0.05 M sodium pyrophosphate (pH 8.5), 1 mM EDTA, and 1 mM dithiothreitol. The circles are the experimental points and the solid line represents the best fit of the data to the general Adair equation. $[NAD_b]$ is the fraction of NAD^+ bound.

mation change. Nevertheless the enzyme remains a tetramer (as evidenced by sedimentation analysis) at $\mu = 0.5$ M. Some conformational changes must occur in the subunit structure which have substantial effects on the chemical and enzymic reactivity, but they are not sufficiently strong to destroy the intersubunit interactions. Similarly it was found that the enzymes which have been dimodified with the half-of-the-sites reagents remain as tetramers. Thus, the native enzyme, the dicarboxamidomethylated enzyme, the dicyanylated enzyme, the difurylacryloylated enzyme, and the tetracyanylated enzyme are all monodisperse tetramers [with $s_{20,w}{}^0 = 7.3$, pH 8.5, 0.05 M bicine–0.5 mM EDTA, 19°; cf. $s_{20,w}{}^0 = 7.7$, pH 8.5, pyrophosphate, 1 mM EDTA, 5 mM mercaptoethanol, ionic strength = 0.15 M, 20° for native yeast GPD (Jaenicke et al., 1968)].

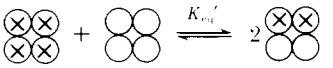
Hybridization Studies. The subunit attractions affecting K_d were shown to be comparable in the enzyme dimodified with half-of-the-sites reagents to those in the unmodified enzyme. Furthermore, the enzymically inactive dicarboxamidomethylated enzyme does not regain activity after standing at pH 8.5, 25° (0.05 M bicine), for several hours as would be expected if hybridization occurred according to eq 4 where unmodified tetramer is produced:



(4)

inactive inactive active

This result does not exclude all dissociations, but only those which could produce unmodified tetramer such as dissociation to monomers. This is consistent with hybridization studies on the native enzyme (Stallcup and Koshland, 1973b) which showed cleavage across only one subunit plane (cf. eq 5). Hybridization between the tetracyanylated enzyme (where each of the active site sulfhydryl groups is cyanylated) and the unmodified enzyme was followed by measuring the change in enzymic activity as a function of time. A decrease in the specific activity was observed as shown in Figure 4. Equilibrium appears to be reached after about 10 hr. In the same time period the tetracyanylated enzyme regains about 10% activity and the activities of the native enzyme alone, the holoenzyme (1 mM NAD^+), or a mixture of native enzyme and heat-denatured enzyme are unaltered, indicating that the loss of activity is due to interaction of the native subunits with the cyanylated subunits by a reshuffling of dimers as shown in eq 5.



(5)

ER_1 E ER_2

Cleavage occurs only at the qq and rr domains and not between the pp domains as shown by the previous studies. Hence, an estimation of the dissociation constant of the dimodified enzyme can be obtained from the activities of the cyanylated enzyme species (Byers and Koshland, 1975) and the equilibrium constants, K_{eq}'.

It is known that the di-, tri- and tetracyanylated enzyme species are inactive from previous modification studies (Byers and Koshland, 1975). The K_{eq}' of eq 5 can be seen to be a component of K_N, the dissociation constant of the native enzyme (eq 6a) K_C, the dissociation constant of the tetracyanylated enzyme (eq 6b), and K_H, the dissociation constant of the hybrid enzyme (eq 6c):

$$E_4 \overset{K_N}{\rightleftharpoons} 2E_2 \qquad (6a)$$

$$(ER)_4 \overset{K_C}{\rightleftharpoons} 2(ER)_2 \qquad (6b)$$

$$E_2(ER)_2 \overset{K_H}{\rightleftharpoons} (ER)_2 + E_2 \qquad (6c)$$

where $K_{eq}' = K_N K_C / K_H{}^2$.

The final equilibrium activity value of Figure 5 (ca. 67%) depends both on K_H and the specific activity of the hybrid (see calculations of Table I). Since the enzymic activity decreases when native and tetracyanylated enzymes are incubated it is clear that the resulting product (i.e., the dicyanylated hybrid) must have less than 50% of the native enzyme specific activity. If the specific activity of the hybrid was half the original specific activity then there would be no loss in enzymic activity in the hybridization experiment regardless of the value of K_{eq}'. The decrease in activity (by ca. 33%) which occurs when native and tetracyanylated enzymes are hybridized can be rationalized by a K_{eq}' of 1.0 if the dicyanylated species (eq 5) has an activity of zero. A

Table I: Equilibrium Activity Value in Hybridization Experiment as a Function of K_{eq}' and Specific Activity of the Hybrid.

K_{eq}[a]	Activity of Hybrid[b]	Final % Activity
1	0	67
<1	0	>67
1	0.5	100
2	0.3	67

[a] Equilibrium constant as defined in the text. [b] Specific activity of hybrid (dicyanylated enzyme) as a fraction of the original specific activity.

maximum value for K_{eq}' would be ~2 (assuming a maximum relative specific activity of ER_2 of ~0.3) indicating little change in K_C (relative to K_N) as a result of modification.

The Binding of NAD+. The binding of NAD^+ to yeast GPD determined by equilibrium dialysis is plotted in several ways. Figure 5 reports the data with the best fit to the general Adair equation with constants calculated using the computer program described previously (Cornish-Bowden and Koshland, 1970). The mechanism giving the best fit to the data is the ligand-induced model (Koshland et al., 1966; Koshland, 1970) in which a mixture of positive and negative cooperativity occurs. The first ligand bound induces a conformation change improving the binding of the second ligand which in turn increases the affinity of the third. The third, however, induces a conformation change which causes a decreased binding of the fourth ligand. The same data plotted on a Scatchard plot show the typical bell-shape curve of positive cooperativity but as the x axis is approached the curve tails off, indicative of negative cooperativity. Other investigators were unable to detect negative cooperativity at high NAD^+ concentrations (Von Ellenrieder et al., 1972; Sloan and Velick, 1973). The Hill plot of the same data shows a slope of greater than 1 at low values of NAD^+ (n_H = 1.88) and a decrease to a slope of less than 1 at high concentrations of NAD^+ (n_H = 0.28, cf. Cornish-Bowden and Koshland (1975) for interpretation of "broken" Hill plots). Furthermore, Hill plots (based on saturation kinetics) indicate no significant alteration in cooperativity as the total protein concentration is increased (from 10^{-9} to 10^{-6} M). This indicates that only one species (e.g., the tetramer) is significant and that cooperativity is due exclusively to subunit interactions within the tetramer (see, for example, Levitzki and Schlessinger, 1974). Plots based on models such as the symmetry model with nonexclusive binding, a preexisting asymmetry model (MacQuarrie and Bernhard, 1971; Seydoux et. al., 1974), and Michaelis-Menten binding do not fit as well. Representative curves are shown in Figure 6.

The Problem of Denatured Enzyme. Any binding curve showing apparent negative cooperativity, particularly in the last ligand bound, poses problems. It can be argued that a certain amount of denatured protein is present which fails to bind ligand. Moreover, since one of the biological advantages of negative cooperativity may well be that it makes it very difficult to saturate an enzyme, this situation may turn out to be very general. There are at least two ways of approaching this problem. The first is to develop a computer fit of a binding curve by assuming that one fraction of the enzyme molecules is denatured and no longer binds substrate, while a second fraction binds according to the model to be tested (for example, nonexclusive symmetry model or

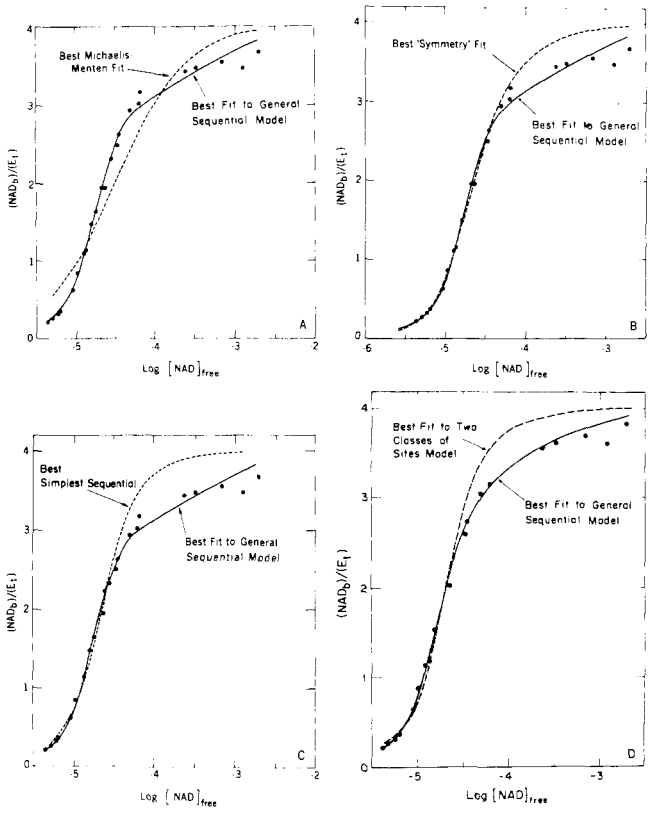


FIGURE 6: Comparison of theoretical curves and experimental data for binding of NAD^+ to yeast GPD. In each case, the computer program was designed to give the best fit using equations for a general sequential model (intrinsic constants can assume any value) and for (A) a Michaelis-Menten model ($K_1' = K_2' = K_3' = K_4'$), (B) a symmetry model with nonexclusive binding ($K_1' < K_2' < K_3' < K_4'$), (C) a sequential tetrahedral model ($K_2'/K_1' = K_3'/K_2' = K_4'/K_3'$), (D) a model having only two kinds of binding sites ($K_1' = K_2'; K_3' = K_4'$).

Michaelis-Menten). This combined model can then be fit to the data. Such assumptions appreciably increase the number of parameters in the curve fitting procedure. The symmetry model which does not fit the original data can now give a good fit to this combined model if one assumes 8% denatured protein which does not bind NAD^+ at all. However, it is then essential to test the assumptions that lead to this fit. As will be discussed below, the activity data make it highly improbable that 8% of the protein is denatured. Furthermore, the assumption that denatured protein binds no NAD^+ is incorrect.

A second way to test the hypothesis that this denatured, nonbinding protein is present is to alter the conditions of the experiment and, hence, the cooperativity pattern. For example, subunit interaction or equilibrium between conformation states are susceptible to alteration by pH. Therefore, a change in pH might allow one to saturate the enzyme surface. This in fact was done, as shown in Figure 7. At pH 7.5, 4 mol of NAD^+ is bound per mol of protein at saturation without increasing NAD^+ to excessive concentrations.

It might finally be argued that the preparation used in the binding curve of pH 7.5 was somehow different from that of pH 8.5, but this point was checked by taking protein which was used for equilibrium dialysis at pH 8.5 and shifting the pH to 7.5. The degree of saturation was then determined after allowing suitable time for reequilibration. Similarly, protein equilibrated with NAD^+ at pH 7.5 was altered to 8.5. The degree of saturation depended only on the pH at the time of measurement and not on the previous his-

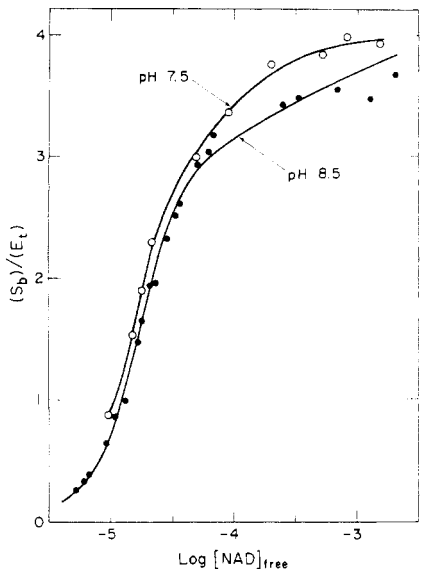


FIGURE 7: Equilibrium dialysis study of NAD^+ binding to yeast GPD at pH 7.5 and 8.5, at 25°. The enzyme sample used at pH 7.5 was the same as that used at pH 8.5 (N_S vs. log $[NAD]_{free}$).

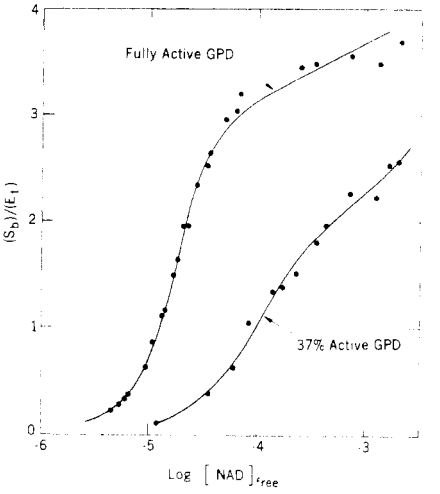


FIGURE 8: Comparison of NAD^+ binding curves of fully active and 37% active yeast GPD. Conditions are the same as described in Figure 5.

tory of the sample. At pH 7.5 a Hill plot was obtained indicating no cooperativity (n_H = 1.06). Thus, all four sites of the enzyme can be saturated and the difficulty in saturating at pH 8.5 is not due to denatured protein.

Binding of NAD^+ to Denatured Protein. To further check whether binding can occur to denatured protein, a sample of yeast GPD was deliberately denatured by long standing in ammonium sulfate solution at 4°. The protein suffered a gradual loss in activity over a period of several months and this protein was then assayed by equilibrium dialysis. The results shown in Figure 8 demonstrate that 37% active protein still binds NAD^+ very effectively and the protein is considerably more than 37% saturated. The partially denatured enzyme does not bind NAD^+ as well as the native enzyme. If the loss of binding activity were proportional to the loss of catalytic activity one would expect the binding curve to level off at 1.48 mol of NAD^+ per tetramer, whereas in fact, the curve is still rising at 2.6 mol of NAD^+ per tetramer. This indicates a nonparallel relationship between denaturation in terms of catalytic activity and denaturation in terms of binding. The loss of catalytic activity in this case is greater than loss of binding capacity.

Slight modification of protein structure can lead to alteration in activity and binding. In some proteins, cooperativity may be more sensitive than binding to these modifications. The effects are particularly easy to observe in the binding of NAD^+ to yeast glyceraldehyde-3-phosphate dehydrogenase because the equilibrium constants for each step in binding are rather similar. Minor effects, therefore, will shift the cooperativity pattern more dramatically than in a highly positively cooperative protein such as CTP synthetase (Long and Pardee, 1967; Levitzki and Koshland, 1972) or hemoglobin (Perutz, 1970).

Comparison with Other Models. The results reported above are most consistent with a general sequential model of the type shown in Figure 9. Curve fittings give intrinsic constants of $1.0 \times 10^4\ M^{-1}$, $1.0 \times 10^4\ M^{-1}$, $57 \times 10^4\ M^{-1}$, and $0.9 \times 10^4\ M^{-1}$ for K_1', K_2', K_3', and K_4', respectively, where K' are the association constants corrected for statistical effects as described previously (Cornish-Bowden and Koshland, 1970). The data do not fit the concerted symmetry model readily, as described above. Simple modifications of that theory, such as assuming that a certain fraction of denatured enzyme does not bind NAD^+, allow a fit to the saturation curve but fail other tests. The same is true for a preexisting asymmetry hypothesis. If one assumes a complex mixture of species, e.g., partially denatured protein, some protein undergoing Michaelis–Menten type binding, and other protein exhibiting cooperative effects, the binding data can be reconciled because many parameters are introduced. Hence it cannot be said that binding data alone establish a unique model. However, if one couples the information obtained here with the half-of-the-sites reactivity studies (Stallcup and Koshland, 1973a,c) by far the simplest explanation for all of the facts is that ligand-induced changes are imposed on a protein which is initially largely in one form. The ligand-induced effects depend on the reagent (iodoacetate vs. iodoacetamide), or the type of reaction (NAD^+ binding vs. acylation), and the state of saturation (the first NAD^+ bound vs. the third bound). This suggests a general picture of a deformable protein whose state of deformation is dependent on the nature of the ligand and the pH of the medium.

Discussion

This report emphasizes features of the strength of subunit interactions and subunit organization which are particularly pertinent to yeast glyceraldehyde-3-phosphate dehydrogenase but apply to any multisubunit protein.

Firstly, the strength of the subunit interactions across various binding domains differs appreciably. However, there is no necessary parallelism between the strength of the association–dissociation of bonds and the cooperativity. The dissociation constant of the yeast enzyme is appreciably less than that of the rabbit muscle enzyme ($<10^{-9}$ vs. $10^{-6}\ M$) indicating stronger bonding between the subunits of the two dimers in the yeast enzyme (by >4 kcal/mol) in regard to dissociation; yet the conformational changes induced across these subunits by the binding of NAD^+ are less for yeast enzyme than for the muscle enzyme. The yeast enzyme is only mildly cooperative. The Michaelis–Menten curve is a rather good approximation of the actual binding constants even though it is quite clear that it is not the best fit. On the other hand, the rabbit muscle enzyme shows strong negative cooperativity in which the binding of NAD^+ affects the binding of each subsequent NAD^+ by factors of 20–100.

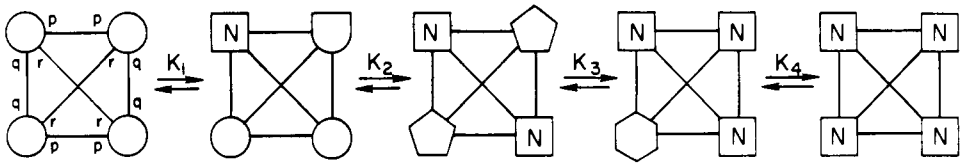


FIGURE 9: The conformational changes induced by NAD^+ binding to yeast GPD. The native enzyme consists of four identical subunits with the binding domains between subunits given as previously described (Cornish-Bowden and Koshland, 1970). The K's are the NAD^+ association constants. The first molecule of NAD^+ bound induces a conformational change which has little influence on the next molecule. The binding of the second molecule of NAD^+ increases the affinity of the third molecule of NAD^+ which, in turn, induces a change that causes a decreased affinity for the fourth NAD^+ molecule.

These results emphasize that ligand-induced changes within a tetramer are a different process than dissociation–association and hence have different energetic requirements.

The strength of the binding domain in regard to dissociation is most readily explained by assuming that the pp domains are far stronger, with regard to dissociation, relative to the qq and rr domains. If this were not true there would be evidence for monomers in the dissociation pattern or for more than one type of hybrid. Hence, this structure quite accurately fits the dimer of dimers classification quite frequently mentioned in regard to tetrahedral proteins.

It might next be assumed that the half-of-the-sites reactivity should be reflected predominantly in the turning off of the adjacent dimer. This is true, but the hybridization experiment with the tetracyanylated GPD and the native GDP shows that the induced changes also extend across the qq and rr domains. However, the cyanylated protein has a dissociation constant to dimers which is little, if at all, affected by the modification. The cooperativity depends on the induced change transmitted across the subunit contacts to the neighboring subunit, but does not depend on the absolute strength of these bonds. Although fairly obvious in retrospect, there is a fairly large number of studies which deduce that investigation of the dissociation–association patterns of proteins will establish new understanding of cooperative interaction. Such studies help but a whole new set of parameters are introduced and energetics within a tetramer can be quite distinct from dissociation behavior.

Although dissociation–association patterns will not per se establish the cooperative interactions, they indicate an important caution. The short time required for hybrids to form between the tetracyanylated and native enzyme indicates that such rearrangements of the protein should occur quite readily (see also, Smith and Schachman, 1971). It is not sufficient, therefore, to estimate activity of mono-, di-, etc., modified enzymes from heterogeneous mixtures without an evaluation of the kinetics of reshuffling of subunits. The studies outlined above show that there is never an appreciable amount of dimer present in the solution during the period of the modification studies and yet reassociation can occur sufficiently rapidly so that in any study of the enzyme over a period of time, this factor must be taken into account. There is no problem in calculating the contribution of such rearrangements with knowledge of the association constants of the protein; but ignoring them, particularly in well-studied tetramers or larger enzymes, could lead to quite incorrect results.

The hybridization experiments (with native and tetracyanylated enzyme) provide a convenient method for estimating the relative energetics of subunit interactions in native, di-, and tetramodified enzyme. Perturbation of the protein structure by covalent modification and by H^+, ionic strength, and specific ions, under conditions where the protein remains predominately a tetramer, has profound effects on the enzymic activity, the reactivity of the SH groups, and the cooperativity of NAD^+ binding. These perturbations are manifested as intra- and intersubunit conformational alterations.

Cooperativity of NAD^+ Binding. The finding that NAD^+ binding to yeast GPD involves a mixture of positive and negative cooperativity supports previous findings from this laboratory (Cook and Koshland, 1970). This cooperativity pattern is due to different induced conformational states of the subunit transmitted across subunit domains. A number of the most obvious alternatives have been eliminated such as irreversibly denatured enzyme, a concerted change in subunits, protein aggregation, etc. From the crystallographic structure of the lobster enzyme (Buehner et al., 1974) it is apparent that there is only one nucleotide binding site per subunit and these sites do not sterically interfere with each other. Dissociation of the tetrameric enzyme to the dimeric state, which could have an altered affinity for NAD^+, is not the explanation for the observed cooperativity pattern either, since the enzyme is completely (>99.99%) in the tetrameric state as evidenced by the magnitude of the dissociation constant ($<10^{-9}$ M).

It is always difficult to eliminate entirely more specialized ad hoc mechanisms. For example the pH 8.5 results might also be explained by the presence of 7–10% reversibly "denatured" enzyme provided that this fraction can bind NAD^+ (but not as well as the native enzyme) and has catalytic activity indistinguishable from the native enzyme when NAD^+ is bound. Nevertheless, to be consistent with the binding studies at pH 7.5 (where 4.0 mol of NAD^+ are bound to 1 mol of enzyme) this hypothesis would require that the fraction of weakly binding enzyme present at pH 8.5 is not manifested at pH 7.5. This ad hoc hypothesis seems unlikely but it cannot be excluded rigorously.

NAD^+ increases the subunit interaction energy as suggested by its inhibition of hybridization and by its protection against spontaneous denaturation. The cooperativity of NAD^+ binding is of a subtle sort and the differences between the successive intrinsic binding constants (K_1', K_2', K_3', and K_4') indicate that the enzyme is neither highly positively cooperative nor highly negatively cooperative. The conclusion that this protein has mixed positive and negative cooperativity appears to disagree with the conclusion of Kirschner et al. (1971) but actually there is no conflict with the experimental data. Kirschner et al. (1971) tried to fit their data to the simplest Koshland et al. (1966) model which utilizes only the two independent parameters (i.e., only two types of subunit conformation, exclusive binding, and conformational changes in the subunit to which the ligand is bound). Their data do not fit this model. However, their data also do not fit the simplest Monod et al. (1965) model with the same number of independent parameters, i.e., the exclusive binding model. By making additional assumptions it was possible to fit their data to a more complex

Monod, Wyman, and Changeux model, but the same data were never applied to a more complex induced fit model. In fact it has been shown that the T-jump data of Kirschner et al. (1971) are compatible with such a ligand-induced model (Loudon and Koshland, 1972) and analysis of the other data such as those obtained in the stopped-flow experiments (Kirschner, 1971) shows that they agree with the general ligand-induced model. Thus there is no inconsistency of the data with the sequential model. The new data reported here make a symmetry model for NAD^+ binding extremely unlikely. Additional assumptions such as more than one enzyme species and/or no binding to denatured protein, are necessary to rationalize such a model and many of them have been excluded experimentally.

The *simplest* symmetry model and the *simplest* sequential model have the same number of independent parameters. When these two cases do not fit the data, more complex models need to be considered and when positive cooperativity alone is observed it might still be argued that the symmetry model is "simpler" provided both models are consistent with the evidence. However, when both negative and positive cooperativity are observed for any ligand it seems clear that a ligand induced model is "simpler" since it does not require different mechanisms for different ligands.

This conclusion suggests an important experimental approach to distinguish between models. Since it is very difficult to distinguish between the symmetry and the ligand-induced models in a positively cooperative situation (not because of theoretical differences but because of the need for highly accurate data) it may turn out to be of greater experimental simplicity to study a second ligand or a second experimental condition, i.e., altered pH or temperature. If the second substrate, or altered condition, gives negative cooperativity a general induced fit model seems likely. For the yeast enzyme such negative cooperativity is apparent in the acylation and alkylation studies (Stallcup and Koshland, 1973a,c). The symmetry model is based on the fundamental assumption that the strength between subunits is sufficiently strong that symmetry is maintained during conformational alterations. If these forces were indeed so strong it seems unlikely (although not impossible) that one substrate would so easily destroy them whereas another substrate would not. Several enzymes show positive cooperativity for one substrate and negative cooperativity for another (see review by Levitzki and Koshland, 1975). These results indicate that the protein is programmed so that the transmission of the conformational effects to neighboring subunits depends on the structure and properties of the ligand. The simplest hypothesis then becomes one in which induced conformational changes are determining the positively cooperative phase as well as the negatively cooperative one.

Denaturation. The observation that the protein denatures by prior dissociation to dimers is of particular interest. This has been found for a number of other enzymes. The pattern is certainly not universal, but it appears to make physiological sense. Since the dissociation constant is rather slight, only small amounts of the protein are lost by denaturation at any one time. As discussed by Schimke (1970), the regulation of enzyme levels can be controlled by denaturation as well as by biosynthesis. If so, it is reasonable that when less substrate is present, less enzyme is being used and it will tend to dissociate to dimers and denature.

Acknowledgments

We thank Dr. Barry L. Taylor for assistance in carrying out and interpretation of the reacting enzyme sedimentation velocity experiments and Amy Hollander for valuable technical assistance in some of these studies. We also thank Dr. Geoffrey Howlett and Dr. C. Suelter for helpful suggestions concerning the manuscript.

References

Ames, G. F.-L. (1960), *Anal. Biochem. 1,* 279.
Bartholmes, P., and Jaenicke, R. (1975), *Biochem. Biophys. Res. Commun. 64,* 485.
Bjerrum, N. (1924), *Z. Phys. Chem. 108,* 82.
Bock, P. E., and Frieden, C. (1974), *Biochemistry 13,* 4191.
Bray, G. A. (1960), *Anal. Biochem. 1,* 279.
Brønsted, J. N. (1925), *Z. Phys. Chem. 115,* 337.
Buehner, J., Ford, G. C., Moras, D., Olsen, K. W., and Rossmann, M. G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3052.
Buehner, M., Ford, G. C., Moras, D., Olsen, K. W., and Rossmann, M. G. (1974), *J. Mol. Biol. 90,* 25.
Byers, L. D., and Koshland, D. E., Jr. (1975), *Biochemistry 14,* 3661.
Cohen, R., and Mire, M. (1971), *Eur. J. Biochem. 23,* 267.
Colowick, S. P., and Kaplan, N. O. (1957), *Methods Enzymol. 4,* 848.
Conway, A., and Koshland, D. E., Jr. (1968), *Biochemistry 7,* 4011.
Cook, R. A., and Koshland, D. E., Jr. (1970), *Biochemistry 9,* 3337.
Cornish-Bowden, A. J., and Koshland, D. E., Jr. (1970), *J. Biol. Chem. 245,* 6241.
Cornish-Bowden, A. J., and Koshland, D. E., Jr. (1975), *J. Mol. Biol. 95,* 201.
DeVijlder, J. J. M., and Slater, E. C. (1968), *Biochim. Biophys. Acta 167,* 23.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 74,* 443.
Fenselau, A. (1970), *J. Biol. Chem. 245,* 1239.
Fenselau, A. (1972), *J. Biol. Chem. 247,* 1074.
Fuller-Noel, J. K., and Schumaker, V. N. (1973), *Anal. Biochem. 54,* 586.
Furfine, C. S., and Velick, S. F. (1965), *J. Biol. Chem. 240,* 844.
Harrigan, P. G., and Trentham, D. R. (1973), *Biochem. J. 135,* 695.
Harrigan, P. G., and Trentham, D. R. (1974), *Biochem. J. 143,* 353.
Hetherington, P. J., Follows, M., Dunnill, P., and Lilly, M. D. (1971), *Trans. Inst. Chem. Eng. 49,* 142.
Hoagland, V. D., and Teller, D. C. (1969), *Biochemistry 8,* 594.
Holland, M. J., and Westhead, E. W. (1973), *Biochemistry 12,* 2276.
Jaenicke, R., (1970), in Pyridine Nucleotide Dependent Dehydrogenases, Sund, H., Ed., New York, N.Y., Springer-Verlag, p 209.
Jaenicke, R., Schmid, D., and Knof, S. (1968), *Biochemistry 7,* 919.
Jones, G. M., and Harris, J. I. (1972), *FEBS Lett. 22,* 185.
Kirschner, K. (1971), *J. Mol. Biol. 58,* 51.
Kirschner, K., Eigen, M., Bittman, R. B., and Voight, B. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 1661.
Kirschner, K., Gallego, E., Schuster, I., and Goodall, D. (1971), *J. Mol. Biol. 58,* 29.
Klingenberg, M. (1963), in Methods of Enzymic Analyses, Bergmyer, H. V., Ed., New York, N.Y., Academic Press, p 528.

Koshland, D. E., Jr. (1970), *Enzymes, 3rd Ed. 1,* 341.
Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5,* 365.
Krebs, E. G., (1955), *Methods Enzymol 1,* 407.
Krimsky, I., and Racker, E. (1963), *Biochemistry 2,* 512.
Kuczenski, R. T., and Suelter, C. H. (1970), *Biochemistry 9,* 939.
Kuczenski, R. T., and Suelter, C. H. (1971), *Biochemistry 10,* 2867.
Levitzki, A., and Koshland, D. E., Jr. (1971), *Biochemistry 10,* 3365.
Levitzki, A., and Koshland, D. E., Jr. (1972), *Biochemistry 11,* 247.
Levitzki, A., and Koshland, D. E., Jr. (1975), *Adv. Cell. Regul.* (in press).
Levitzki, A., and Schlessinger, J. (1974), *Biochemistry 13,* 5214.
Long, C. W., and Pardee, A. B. (1967), *J. Biol. Chem. 242,* 4715.
Loudon, G. M., and Koshland, D. E., Jr. (1972), *Biochemistry 11,* 229.
MacQuarrie, R. A., and Bernhard, S. A. (1971), *J. Mol. Biol. 55,* 181.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12,* 88.
Nagradova, N. K., Golovina, T. O., and Mevka, A. T. (1974), *FEBS Lett. 49,* 242.
Orsi, B. A., and Cleland, W. W. (1972), *Biochemistry 11,* 102.
Osborne, H. H., and Holloway, M. (1974), *Biochem. J. 143,* 651.
Perutz, M. F. (1970), *Nature (London) 228,* 726.
Polgár, L. (1975), *Eur. J. Biochem. 51,* 63.
Rohrbach, M. S., Humphries, B. A., Yost, F. J., Jr., Rhodes, W. G., Boatman, S., Hiskey, R. G., and Harrison, J. H. (1973), *Anal. Biochem. 52,* 127.
Schimke, R. T. (1970), *Acc. Chem. Res. 3,* 113.
Seydoux, F., Malhotra, O., and Bernhard, S. A. (1974), *Crit. Rev. Biochem. 2,* 227.
Silverstein, E. (1970), *Biochim. Biophys. Acta 215,* 205.
Sloan, D. L., and Velick, S/ F. (1973), *J. Biol. Chem. 248,* 5423.
Smith, G. D., and Schachman, H. K. (1971), *Biochemistry 10,* 4576.
Smith, C. M., and Velick, S. F. (1972), *J. Biol. Chem. 247,* 273.
Spotorno, G., and Holloway, M. (1970), *Nature (London) 226,* 756.
Stallcup, W. B., and Koshland, D. E., Jr. (1973a), *J. Mol. Biol. 80,* 41.
Stallcup, W. B., and Koshland, D. E., Jr. (1973b), *J. Mol. Biol. 80,* 63.
Stallcup, W. B., and Koshland, D. E., Jr. (1973c), *J. Mol. Biol. 80,* 77.
Stallcup, W. B., Mockrin, S. C., and Koshland, D. E., Jr. (1972), *J. Biol. Chem. 247,* 6277.
Stancel, G. M., and Deal, W. C., Jr. (1969a), *Biochemistry 8,* 4505.
Stancel, G. M., and Deal, W. C., Jr. (1969b), *Biochemistry 8,* 4005.
Taylor, B. L., Barden, R. E., and Utter, M. F. (1972), *J. Biol. Chem. 247,* 7783.
Trentham, D. R. (1971), *Biochem. J. 122,* 59.
Velick, S. F. (1970), in Pyridine Nucleotide Dependent Dehydrogenases, Sund, H., Ed., Berlin, Springer-Verlag, p 216.
Velick, S. F., and Udenfriend, S. (1953), *J. Biol. Chem. 203,* 575.
Von Ellenrieder, G., Kirschner, K., and Schuster, I. (1972), *Eur. J. Biochem. 26,* 220.
Watson, H. C., Duee, E., and Mercer, W. D. (1972), *Nature (London), New Biol. 240,* 130.
Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247,* 3242.