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The Investigation of Substrate-Induced Changes in Subunit Interactions in Glyceraldehyde 3-Phosphate Dehydrogenases by Measurement of the Kinetics and Thermodynamics of Subunit Exchange

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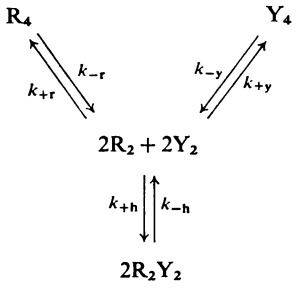
An investigation was made of changes in subunit interactions in glyceraldehyde 3-phosphate dehydrogenase on binding NAD^+, NADH and other substrates by using the previously developed method of measurement of rates and extent of subunit exchange between the rabbit enzyme (R_4), yeast enzyme (Y_4) and rabbit–yeast hybrid (R_2Y_2) [Osborne & Hollaway (1974) *Biochem. J.* **143**, 651–662]. The free energy of activation for the conversion of tetramer into dimer for the rabbit enzyme ($R_4 \rightarrow 2R_2$) is increased by at least 12kJ/mol in the presence of NAD^+. This increase is interpreted in terms of an NAD^+-induced 'tightening' of the tetrameric structure probably involving increased interaction at the subunit interfaces across the *QR* plane of the molecule [see Buehner *et al.* (1974) *J. Mol. Biol.* **82**, 563–585]. This tightening of the structure only occurs on binding the third NAD^+ molecule to a given enzyme molecule. Conversely, binding of NADH causes a decrease in the free energy of activation for the $R_4 \rightarrow 2R_2$ and $Y_4 \rightarrow 2Y_2$ conversions by at least 10kJ/mol. This is interpreted as a NADH-induced 'loosening' of the structures arising from decreased interactions across the subunit interfaces involving the *QR* dissociation plane. In the presence of NADH the increase in the rate of subunit exchange is such that it is not possible to separate the hybrid from the other species if electrophoresis is carried out with NADH in the separation media. In the presence of a mixture of NADH and NAD^+ the effect of NAD^+ on subunit exchange is dominant. The results are discussed in terms of the known co-operativity between binding sites in glyceraldehyde 3-phosphate dehydrogenases.

The homotetrameric yeast and rabbit skeletal muscle glyceraldehyde 3-phosphate dehydrogenases (EC 1.2.1.12) exhibit co-operativity towards the binding of NAD^+ and 'half-of-the-sites' reactivity towards certain electrophilic reagents (for example, see Malhotra & Bernhard, 1973; Stallcup & Koshland, 1974; Levitzki, 1974). Since the detailed X-ray-crystallographic structure of the closely similar lobster enzyme is available (Buehner *et al.*, 1974) these enzymes represent attractive subjects for the investigation of information transfer between subunits in oligomeric proteins.

The rabbit muscle and yeast enzymes, despite strong similarities in most chemical and physicochemical properties (for example, see Perham & Harris, 1963; Velick & Furfine, 1963; Harris & Perham, 1965, 1968; Jones & Harris, 1972), show considerable differences in the nature of the NAD^+-binding processes. The rabbit muscle enzyme shows strong negative co-operativity between the sites (Conway & Koshland, 1968; DeVijlder & Slater, 1968; Velick *et al.*, 1971; Price & Radda, 1971), with the dissociation constants for the first two molecules of NAD^+ bound being in the nanomolar range, whereas the third and fourth bind with average dissociation constants of 0.3 and 26μM respectively (Conway & Koshland, 1968). Binding of NAD^+ to the yeast enzyme differs in at least two ways. First, the binding is much weaker since, whichever set of data is accepted (Kirschner *et al.*, 1971; Kirschner, 1971; Cook & Koshland, 1970), the intrinsic dissociation constant for the tightest site in the yeast enzyme is in the region of 10μM. Second, the interactions between the sites show positive co-operativity at 40°C (Kirschner *et al.*, 1971; Kirschner, 1971) and a mixture of positive and negative co-operativity at lower temperatures (Cook & Koshland, 1970).

The binding of NADH to these enzymes also shows interesting differences. Thus binding of the reduced coenzyme to the yeast enzyme (von Ellenreider *et al.*, 1972) reveals an equivalence in the four sites for NADH binding, whereas the binding of NADH to the rabbit muscle enzyme is negatively co-operative with dissociation constants of 0.5, 0.5, 2.5 and 50μM respectively (Boers *et al.*, 1971). The values of these dissociation constants indicate a looser binding of



Scheme 1. *Kinetic model of dimer exchange between rabbit (R_4), yeast (Y_4) and hybrid (R_2Y_2) glyceraldehyde 3-phosphate dehydrogenases*

The exchange of subunits involves first-order dissociations of the tetramers to dimers, R_2 and Y_2, followed by second-order recombinations.

NADH than NAD^+, an inversion of the relative affinities of these ligands for most other dehydrogenases.

The aim of this work was to study how the subunit interactions changed in rabbit and yeast glyceraldehyde 3-phosphate dehydrogenases on binding NAD^+, NADH and other specific ligands. The method of investigation was based on the measurement of the kinetics and thermodynamics of the first level of hybridization of the yeast and rabbit enzymes (designated Y_4 and R_4) which proceeds according to Scheme 1 (Osborne & Hollaway, 1974).

It was shown by Osborne & Hollaway (1974) that the rate-limiting steps in the hybridization process were the dissociations of the tetramers to dimers, the kinetics of the system being such that the rate of hybridization was relatively insensitive to the second-order dimer-recombination rate constants (k_{+r}, k_{+y} and k_{+h}). Therefore ligands that bind to the native rabbit and yeast molecules and alter the free energy of interaction between subunits, would be expected to alter the rate, and possible extent, of hybrid formation. In this communication we present the results of the effects of NAD^+ and NADH and other substrate molecules on the hybridization process.

Materials and Methods

Yeast and rabbit muscle glyceraldehyde 3-phosphate dehydrogenases were prepared as described previously (Osborne & Hollaway, 1974). The specific activities of the yeast and rabbit preparations were between 140 and 170 units (μmol/min)/mg and 160 and 210 units/mg respectively as measured by the standard assay method described by Trentham (1968).

Grade 1 NAD^+ and NADH were purchased from Boehringer (Mannheim, Germany). Other materials were as described in Osborne & Hollaway (1974). Hybridization experiments were carried out in a phosphate buffer (10mM-NaH_2PO_4) containing 1mM-dithiothreitol and 1mM-EDTA adjusted to pH7.8 with NaOH, hereafter referred to as the pH7.8 phosphate buffer.

Before use a portion of each enzyme, as a crystalline suspension in $(NH_4)_2SO_4$ storage medium, was centrifuged for 30min at 38000g and the pellets were dissolved in the pH7.8 phosphate buffer. Removal of $(NH_4)_2SO_4$ was effected by passage of the enzyme solutions through columns (12cm×0.6cm) of Sephadex G-50 equilibrated in the pH7.8 phosphate buffer.

Yeast and rabbit apoenzymes were prepared as described by Osborne & Hollaway (1974). Enzyme containing 1, 2, 3 and 4mol of bound NAD^+/mol were prepared by addition of the calculated amounts of standard NAD^+ solutions to the apoenzymes. The protein concentrations and enzyme-bound NAD^+ were estimated from the absorptions at 260 and 280nm by using the values of Fox & Dandliker (1956) for the rabbit enzyme and those of Kirschner *et al.* (1971) for the yeast enzyme. The molecular weights of both enzymes were assumed to be 145000.

Hybrid formation

All reactions were carried out in 1ml plastic Eppendorf centrifuge tubes to which were added 0.04ml of ligand solution (or buffer for control reactions) and 0.2ml of each enzyme. The resulting mixtures were analysed immediately after incubation under the experimental conditions by starch-gel electrophoresis in a pH8.6 Tris–borate discontinuous system (Rosemeyer & Huehns, 1967). Photographic negatives of gels stained in Amido Black 128 and differentiated in a water–methanol–acetic acid (4:5:1, by vol.) solvent mixture were analysed by densitometry and the densitometric records evaluated as previously described (Osborne & Hollaway, 1974).

Hybrid reversion

The hybrid species R_2Y_2 was isolated as previously described (Osborne & Hollaway, 1974). Ligand solution or phosphate buffer for control reactions (0.02ml in either case) was added to 0.2ml of the isolated hybrid solution (6mg/ml). This was calculated by using an arbitrary value of $E^{1\%}_{1cm,280nm} = 9.50$, i.e.

$$\frac{E^{1\%}_{1cm\ rabbit} + E^{1\%}_{1cm\ yeast}}{2}$$

The resulting mixture was incubated under the experimental conditions for different times and then analysed by starch-gel electrophoresis as described above.

Equilibrium perturbation

Equimolar mixtures of yeast and rabbit muscle enzyme solutions (6mg/ml each) were incubated at 37° or 28°C for 3 and 6h respectively in which time (about three half-lives) all species in the reaction mixture are close to equilibrium. To 0.4ml of such equilibrated mixtures was added 0.04ml of ligand or buffer for controls. The resulting mixtures were incubated at various temperatures and the mixtures analysed by means of starch-gel electrophoresis and densitometry as described above.

Results

The effect of specific ligands on subunit-exchange reactions was investigated in three kinds of experiment, referred to respectively as 'hybrid formation', 'hybrid reversion' and 'equilibrium perturbation'. Before the effect of each ligand is described in detail the rationale of these experiments is given together with some typical results.

Hybrid-formation experiments

Equimolar concentrations of yeast and rabbit enzymes were incubated together for different times in the presence or absence of the specific ligand and the composition of the mixtures determined by electrophoretic analysis as described in the Materials and Methods section. Given that Scheme 1 is a valid model for the hybrid-forming reaction, a steady-state treatment for the amount of hybrid formed at time t ($[R_2Y_2]_t$) gives eqn. (1) (Osborne & Hollaway, 1974)

$$[R_2Y_2]_t = A(1-e^{-k_1^{obs.}\cdot t}) \qquad (1)$$

where the observed first-order rate constant for hybrid formation, $k_1^{obs.}$, is given by:

$$k_1^{obs.} = \frac{k_{-r}+\left(\frac{2k_{+r}}{k_{+h}}\cdot k_{-h}\right)}{1+\left(\frac{2k_{+r}}{k_{+h}}\right)}$$

and the concentration of hybrid formed when equilibrium is reached, A by:

$$A = \frac{2k_{-r}}{k_{-r}+\left(\frac{2k_{+r}}{k_{+h}}\cdot k_{-h}\right)}\cdot[R]_0$$

where $[R]_0$ is the initial concentration of the rabbit and yeast enzymes.

In deriving eqn. (1) the simplifying assumptions have been made that $k_{-r}\approx k_{-y}$ and $k_{+r}\approx k_{+y}$. Since it has been shown that the value of k_{-y} is about three times that of k_{-r} (Osborne & Hollaway, 1974), the first of these assumptions is not strictly valid. However, since the electrophoretic analysis used in this study makes it difficult to obtain detailed accurate time-courses, we have analysed the data using eqn. (1) and regard the present results as being of a semi-quantitative nature (estimated values of rate constants are nevertheless estimated to be within a factor of five of the true values).

If the value of the rate constant for hybrid formation from R_2 and Y_2 dimers is taken to be twice that of the R_4 species from R_2 molecules ($k_{+h}=2k_{+r}$) then eqn. (1) still holds but the expressions for $k_1^{obs.}$ and A simplify to those given in eqn. (2).

$$k_1^{obs.} = \frac{k_{-r}+k_{-h}}{2}$$

and

$$A = \frac{2k_{-r}}{k_{-r}+k_{-h}}\cdot[R]_0 \qquad (2)$$

This assumption is based on the expectation that the R_2–Y_2 collision frequency will be twice that for R_2–Y_2 collisions.

Plate 1(*a*)(i) demonstrates the effect of NAD^+ on the hybrid-forming reaction. Whereas the hybrid forms rapidly in the absence of NAD^+, when 6mM-NAD^+ is included in the reaction mixture there is no observable hybrid formation after 5h. By itself, this result cannot be interpreted unambiguously, since it could arise either from NAD^+ binding causing a greatly increased value of k_{-h} or greatly diminished value of k_{-r} (see eqns. 1 and 2). Further experiments (see below) have resolved these alternatives.

The effect of NADH on the hybrid-forming reaction is quite different [Plate 1(*a*)(ii)]. It is clear that the initial rate at which hybrid forms is greatly increased although the amount formed at equilibrium is less than that in the control experiment. From inspection of eqns. (1) and (2) it can be seen that to obtain this result the values of both k_{-r} and k_{-h} must be increased in the NADH-liganded species.

Hybrid-reversion experiments

In these experiments the rate of reversion of the isolated R_2Y_2 hybrid to an equilibrium mixture comprising species R_4, Y_4 and R_2Y_2 was followed in the presence and absence of different ligands. A steady-state treatment of Scheme 1, with the same assumptions as those used to obtain eqn. (1), leads to the expression given by eqn. (3) for the concentration of the rabbit (or yeast) species formed from the hybrid species after time t($[R_4]_t$):

$$[R_4]_t = B(1-e^{-k_1^{obs.}\cdot t}) \qquad (3)$$

where $k_1^{obs.}$ is given by the same expression as that given in eqn. (1) and the concentration of the rabbit (or yeast) species at equilibrium, B, by:

$$B = [R_2Y_2]_0\cdot\frac{k_{-h}}{2k_{-h}+\left(k_{-r}\cdot\frac{k_{+h}}{k_{+r}}\right)}$$

where $[R_2Y_2]_0$ is the initial concentration of hybrid.

An electrophoretogram of a typical hybrid reversion experiment is shown in Plate 1(*b*). Incubation of the hybrid with 20mM-D-glyceraldehyde 3-phosphate (iv) or 6mM-NAD^+ (ii) at 4°C for 5min has little observable effect on hybrid breakdown whereas 6mM-NADH (iii) leads to extensive reversion to the species R_4 and Y_4 within this period. It can be concluded from this result that the half-life of the hybrid in the presence of 6mM-NADH at 4°C is approx. 5min, corresponding to a $k_1^{obs.}$ value of $2\times10^{-3}s^{-1}$.

It is noteworthy that a mixture of NAD^+ and glyceraldehyde 3-phosphate in the incubation mixture, which also contains the third substrate P_i as a buffer [Plate 1(*b*)(vi)], only gives a slightly increased extent of degradation of the hybrid compared with the control. This result would seem to be inconsistent with the result of Plate 1(*b*)(iii) since NADH is formed rapidly under these conditions (Trentham, 1968), and this would be expected to accelerate the reversion of the hybrid. However, it is possible to interpret this result in terms of the higher affinity of the enzyme and/or acylenzyme for NAD^+ than for NADH. (Alternatively the acylated enzyme could have a 'tighter' structure even when NADH is bound.)

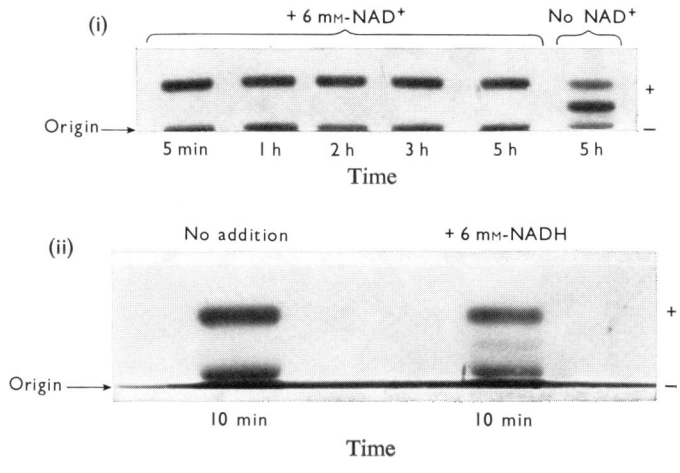
Equilibrium perturbation

This approach involves ligand-induced perturbation of pre-established equilibria and serves as a useful check on conclusions reached by application of the hybrid-formation and hybrid-reversion experiments. An electrophoretogram obtained in a typical experiment is shown in Plate 2(*a*). An equilibrium between species R_4, R_2Y_2 and Y_4 was established at 37°C by incubation of native rabbit and yeast enzymes for three reaction half-lives. Samples (0.4ml) were then incubated for either 12h or 20h at 0°C in the presence of NAD^+ or NADH. Whereas incubation with NAD^+ resulted in a slight diminution in the amount of the R_2Y_2 species, NADH caused a marked decrease to give a mixture of components in the same concentrations as those obtained in the hybrid-formation or -reversion experiments carried out in the presence of NADH. To understand the ligand-induced changes in subunit exchange we have found it essential to carry out hybrid-formation and -reversion experiments, and advantageous to confirm the results by use of equilibrium-perturbation experiments.

Effect of NADH on the rate of subunit interchange

The results of Plates 1(*a*), 1(*b*) and 2(*a*) show that NADH affects the hybridization in the following ways. (i) The rate of formation of the R_2Y_2 species from species R_4 and Y_4 is greatly increased over that of a control in the absence of NADH. (ii) The rate of reversion of the R_2Y_2 species is greatly increased in the presence of NADH. (iii) The amount of hybrid formed at equilibrium in the presence of NADH is less than that in the control experiment (see also Osborne & Hollaway, 1974), the equilibrium position reached being the same whether starting from native enzymes or from hybrid. Densitometric analysis of electrophoretograms of the type shown in Plate 1(*a*)(ii) gave the result that, at 28°C, the half-life for hybrid formation was less than 5min and the equilibrium ratio of hybrid ($A/[R]_0$, see eqn. 1) was 0.16. This sets a minimum value of $k_1^{obs.}=2\times10^{-3}s^{-1}$. The rapid formation of a small amount of hybrid is difficult to follow by the starch-gel-electrophoretic-analysis method used in this study. However, hybrid-reversion experiments at 4°C proved to be more definitive since greater changes in the hybrid concentration were involved, and this made it possible to follow the process [cf. Plates 1(*a*)(ii) and (1*b*)(iii)]. Thus it can be calculated from the electrophoretogram shown in Plate (1*b*)(iii) that the half-life for hybrid breakdown in the presence of NADH is about 5min at 4°C. This sets a minimum value for $k_1^{obs.}$ of about $2\times10^{-3}s^{-1}$ whereas the value of the rate constant in the absence of NADH is about $7.0\times10^{-6}s^{-1}$ (see Table 1) (calculated from the Arrhenius equation after substitution of a value of $E_a=84$kJ/mol; Osborne & Hollaway, 1974). To investigate whether NADH binding to the rabbit and yeast enzymes led to different effects on the values of k_{-r} and k_{-y}, experiments were conducted at 28°C in the presence of 6mM-NADH (*a*) with $[R_4]_0=5[Y_4]_0$ and (*b*) with $[R_4]_0=0.2[Y_4]_0$. It would be expected that if NADH binding selectively increased the value of k_{-r} then equilibrium would be reached more slowly under the condition of experiment (*a*). The results of these experiments indicated that in both (*a*) and (*b*) the equilibrium position had been reached within 5min so that it can be concluded that the values of both k_{-r} and k_{-y} are increased for the NADH-liganded enzymes. It should be noted that although most of the experiments involving NADH were conducted with a 6mM ligand concentration, essentially the same results were obtained with 0.6mM-NADH.

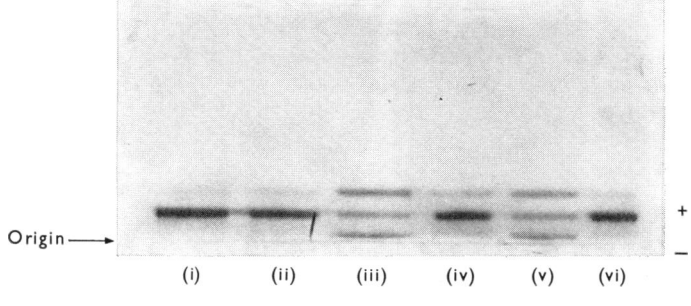
A large number of hybrid-formation, -reversion and equilibrium-perturbation experiments have been carried out in the presence and absence of NADH at 4° and 28°C and a summary of the results, together with calculated values of individual rate constants, is given in Table 1. It can be seen that, even at 4°C, the half-life for dimer exchange between the R_4, Y_4 and R_2Y_2 species is less than 5min in the presence of NADH. This time is shorter than the resolution time of the starch-gel electrophoresis so that incorporation of NADH in the gel buffer might be expected to result in a 'melting-out' of the R_2Y_2 species (see Osborne & Hollaway, 1974). To test this hypothesis an equilibrium mixture at 37°C ($[R_4]:[R_2Y_2]:[Y_4]$; 1:1.4:1) was applied to a starch gel made with a buffer containing 1mM-NADH. After electrophoreses at 4°C only two bands were observed [see Plate 2(*b*)(i)] corresponding



EXPLANATION OF PLATE 1(*a*)

Effect of NAD+ or NADH on the amount of hybrid formed

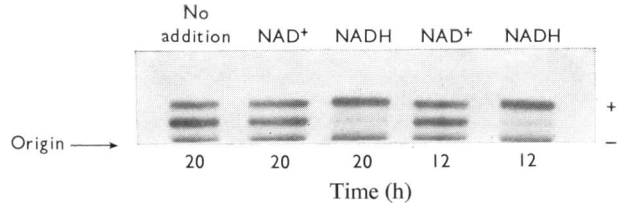
Electrophoretograms of mixtures of rabbit and yeast glyceraldehyde 3-phosphate dehydrogenases, each at 6mg/ml, in the presence and absence of different nucleotides. (i) Incubations, either in the absence or presence of 6mM-NAD^+, were at 37°C for the times shown. (ii) Incubation in the absence and presence of 6mM-NADH for 10min at 0°C. Incubations were in the pH7.8 phosphate buffer, and electrophoresis and protein staining were carried out as described in the Materials and Methods section.



EXPLANATION OF PLATE 1(*b*)

Effect of ligands on the stability of the isolated R_2Y_2 hybrid

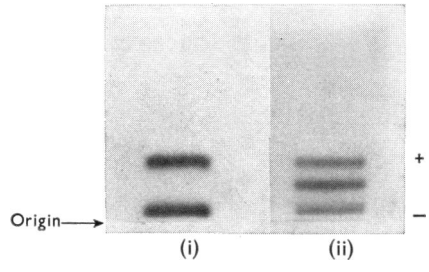
Electrophoretogram of the isolated hybrid species R_2Y_2 (6mg/ml) in the pH7.8 phosphate buffer at 4°C incubated for 5min with the additions shown: (i) No additions; (ii) 6mM-NAD^+; (iii) 6mM-NADH, (iv) 20mM-D-glyceraldehyde 3-phosphate; (v) 20mM-D-glyceraldehyde 3-phosphate+6mM-NADH; (vi) 20mM-D-glyceraldehyde 3-phosphate+6mM-NAD^+. Electrophoresis was carried out immediately after addition of the ligands as described in the Materials and Methods section.



EXPLANATION OF PLATE 2(*a*)

Perturbation of the 37°C equilibrium mixture of species R_4, R_2Y_2 and Y_4 by NAD$^+$ and NADH

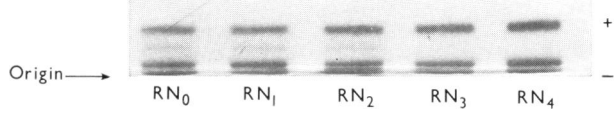
A mixture of yeast and rabbit enzymes each at 6mg/ml was left for sufficient time to reach equilibrium with the R_2Y_2 hybrid in the pH7.8 phosphate buffer at 37°C (over three half-lives). The photograph shows an electrophoretogram of the equilibrated mixtures incubated at 0°C with the additions shown. All ligand concentrations were 6mM. Electrophoresis and protein staining were carried out as described in the Materials and Methods section.



EXPLANATION OF PLATE 2(*b*)

Effect of NADH in the electrophoretic medium during the resolution of a mixture containing species R_4, R_2Y_2 and Y_4

Electrophoretograms of a mixture of R_4 and Y_4 each at 6mg/ml after incubation for 3h at 37°C in the pH7.8 phosphate buffer and separated (i) by electrophoresis as described in the Materials and Methods section but with 1mM-NADH in both the electrode and gel buffers or (ii) by electrophoresis as described in the Materials and Methods section.



EXPLANATION OF PLATE 2(*c*)

Ability to form species R_2Y_2 in mixtures of yeast apoenzyme and rabbit enzyme containing different amounts of bound NAD$^+$

Electrophoretograms of experiments in which yeast apoenzyme and rabbit enzyme, each at 6mg/ml, were mixed for 21h at 4°C in the pH7.8 phosphate buffer. Yeast apoenzyme (designated YN_0) was hybridized with samples of the rabbit enzyme containing different amounts of bound NAD$^+$ designated RN_0, RN_1, RN_2, RN_3, RN_4 according to the number of NAD$^+$ molecules bound. The dissociation constant for species RN_1, RN_2 and RN_3 are all lower than the dissociation constant for YN_1 (see the text). Electrophoresis and protein straining were carried out as described in the Materials and Methods section.

Table 1. *Equilibrium ratios and rate constants for subunit exchange between rabbit, yeast and hybrid glyceraldehyde 3-phosphate dehydrogenases*

The ratio $[R_2Y_2]_{eq.}/[Y_4]_{eq.}$ represents the ratio of the concentrations of hybrid to yeast enzymes at equilibrium. All experiments were conducted with equal amounts of rabbit and yeast subunits so that this ratio is the square root of the equilibrium constant. The headings Formation, Reversion and Perturbation refer to the three types of experiment respectively involving (i) formation of species R_2Y_2 from species R_4 and Y_4 (ii) reversion of the isolated (6mg/ml) to native species and (iii) perturbation of equilibria previously established at 37°C, at which temperature the $[R_2Y_2]_{eq.}/[Y_4]_{eq.}$ ratio has a value of 1.4. All reactions were carried out in the pH7.8 phosphate buffer. No corrections were made for pH variation between the different temperatures.

Temperature		$\frac{[R_2Y_2]_{eq.}}{[Y_4]_{eq.}}$			$10^5 \times k_1^{obs.}$ (s^{-1})			$10^5 \times k$ (s^{-1})		
		Formation	Reversion	Perturbation	Formation	Reversion	Perturbation	k_{-r}	k_{-y}	k_{-h}
4°C	Control	0.65	—	—	0.7	—	—	0.4	1.2	1.0
	+6mM-NAD^+	—	—	—	<0.2	<0.2	—	<0.2	<0.2	1.0
	+6mM-NADH	0.17	0.17	0.17	200	200	200	>32	>32	>360
28°C	Control	1.0	1.0	—	9.0	9.0	—	8.0	24	10
	+6mM-NAD^+	0.05*	—	—	<0.2	~9.0	<0.2	<0.2	<0.2	<0.2
	+6mM-NADH	0.22	0.17	0.32	>200	>200	>200	>40	>40	>360
37°C	Control	1.4	—	—	22	—	—	18	54	26
	+6mM-NAD^+	0.0†	—	—	<0.2	—	—	<0.2	<0.2	~26‡
	+6mM-NADH	0.22	—	—	200	—	—	>40	>40	>360

* Reaction still proceeding after 15h.
† No hybrid observed at this temperature after incubation for 12h.
‡ From the reversion time-course (k_{-h} is unchanged in the presence of NAD^+).

to the native yeast and rabbit enzymes. This is in accordance with the behaviour predicted from the kinetics (Table 1).

Two further observations were made during the experiments with NADH. First, that the enzymes migrated about 12% further after pretreatment with NADH (e.g. see Plate 2*a*). Second, that long periods of incubation (more than 3h) with NADH gives rise to more diffuse electrophoretic bands for each species, although the effect is most marked with the rabbit enzyme. This suggested that incubation with NADH was bringing about some structural changes in the enzymes, eventually leading to denaturation. Since this could affect the interpretation of the hybridization studies an investigation was made of the stability of the enzymes in the presence and absence of the perturbing ligands. The results (Fig. 1) showed that incubation with NADH under the experimental conditions used for the hybridization experiments caused a loss of activity in both rabbit and yeast enzymes over the period of hours. However, there was little change in activity in the time-range of minutes, during which time subunit exchange was complete in the hybridization experiments reported here.

Effect of NAD^+ on the rate of subunit interchange

The results of Plates 1(*a*), 1(*b*), 2(*a*) and 2(*c*) demonstrate that the presence of 6mM-NAD^+ affects the subunit-exchange processes in the following ways. (i) There is no observable hybrid formation from the R_4 and Y_4 species after incubation for 5h at 37°C in the presence of 6mM-NAD^+ [Plate 1(*a*)(i)]. In the absence of NAD^+ the half-life of hybrid formation at this temperature is less than 1h (Osborne & Hollaway, 1974). (ii) The presence of NAD^+ does not greatly enhance the rate of hybrid reversion (Plate 1*b*) or equilibrium perturbation (Plate 2*a*) so that failure to form hybrids in the presence of NAD^+ [Plate 1(*a*)(i)] must result from a large decrease in the rate constant(s) for tetramer-into-dimer conversions (k_{-r} and/or k_{-y}). It was decided to find out at which state of NAD^+ ligation the glyceraldehyde 3-phosphate molecule underwent the 'tightening' of structure which leads to a diminished rate of tetramer-to-dimer dissociation. To this end an experiment was carried out in which the yeast apo-enzyme was hybridized with different samples of the rabbit enzyme containing respectively 0, 1, 2, 3 and 4 bound NAD^+ molecules. The dissociation constants for NAD^+ binding to the four sites in the rabbit enzyme molecule are in the order of 10nM, 10nM, 0.3μM and 26μM (Velick *et al.*, 1971; Conway & Koshland, 1968), whereas for the yeast enzyme the corresponding dissociation constants are 220, 7, 13 and 280μM (Cook & Koshland, 1970). Thus addition of 3mol of NAD^+ to a solution containing equal numbers of molecules of the rabbit and yeast enzyme will result in a solution containing almost exclusively the triliganded rabbit enzymes (designated RN_3), and yeast apoenzyme (YN_0).

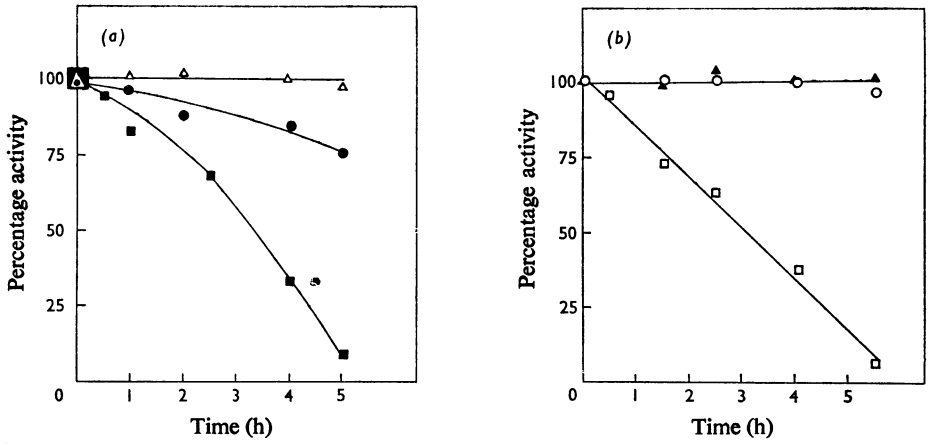


Fig. 1. *Activity of rabbit and yeast glyceraldehyde 3-phosphate dehydrogenase after incubation with NAD⁺ and NADH*

Samples of the yeast and rabbit enzymes, each at 3 mg/ml, were incubated separately in the pH 7.8 phosphate buffer at 28°C in the presence or absence of 6 mM nucleotides. Samples (□) of each mixture were assayed for enzyme activity at different times as described in the Materials and Methods section. (*a*) △, ■, Percentage of R_4 activity remaining in the presence of NAD^+ and NADH respectively. ●, Control reaction. (*b*) ▲, □, Percentage of Y_4 activity remaining in the presence and absence of NAD^+ and NADH respectively. ○, Control reaction.

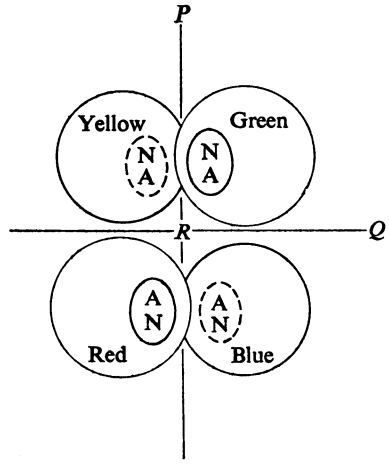
The experiment, the results of which are shown in Plate 2(*c*), was designed to exploit this property. It can be seen that mixtures containing yeast apoenzyme (YN_0) and species RN_0, RN_1 and RN_2 form significant amounts of the R_2Y_2 hybrid after a 21 h incubation at 4°C whereas hybridization of YN_0 with RN_3 does not give any detectable hybrid formation in this time. The incubation of a mixture containing four equivalents of NAD^+ and equimolar amounts of the rabbit and yeast enzymes also fails to yield hybrid. Thus it can be concluded that the third molecule of NAD^+ bound to a rabbit enzyme molecule causes a 'tightening' of the structure such that hybrid formation proceeds at a much lower rate. The binding of the first two molecules does not significantly affect the rate of hybrid formation (Plate 2*c*).

It is noteworthy that the NAD^+ effect on 'tightening' the structure dominated the NADH 'loosening' effect since incubation of equal amounts of species R_4 and Y_4 (each at 40 μM) in the presence of a mixture containing 0.3 mM-NAD^+ and 6 mM-NADH for 21 h at 4°C gave no observed hybrid formation.

Discussion

The amino acid sequences of the lobster (Davidson *et al.*, 1967), rabbit (J. I. Harris, personal communication) and yeast (Jones & Harris, 1972) glyceraldehyde 3-phosphate dehydrogenases are closely homologous. Also it is possible to form molecular hybrids between glyceraldehyde 3-phosphate dehydrogenase molecules from a wide variety of species (Chilson *et al.*, 1966; Spotorno & Hollaway, 1970; Lebherz *et al.*, 1973; Kochman *et al.*, 1974). Therefore it seems likely that the three-dimensional molecular structures of these enzymes are similar. In this case it seems reasonable to discuss subunit interactions in species R_4, Y_4 and R_2Y_2 in terms of the 0.3 nm resolution X-ray-crystallographic structure of the lobster enzyme. A schematic representation of this crystallographic structure is given in Scheme 2.

In the NAD^+ holoenzyme structure (Buehner *et al.*, 1974) there are 35 amino acid residues per subunit involved in the red–blue (and yellow–green) intersubunit interactions across the *P* axes, whereas the red–green and red–yellow interactions across the *R* and *Q* axes respectively involve only 14 and 8 residues. The strong red–blue intersubunit interactions involve the extensive pleated-sheet regions of the catalytic domains of the red and blue subunits. Thus, on first consideration, it seems most likely that the preferential plane of dissociation for the tetrameric molecule would be that containing the *Q* and *R* axes. In this case the dissociation processes studied in the present work, e.g. $R_4 \rightarrow 2R_2$ (Scheme 1), would involve predominantly the formation of the red–blue and green–yellow, *P*-axis-related dimers. It should be noted that this conclusion could be invalid since the X-ray-crystallographic structure is of the NAD^+ holoenzyme, which shows a decreased tendency to dissociate compared with the apoenzyme [Plate 1(*a*)(i)]. It is possible that this NAD^+-induced 'tightening' of



Scheme 2. *Diagrammatic representation of the arrangement of the subunits in glyceraldehyde 3-phosphate dehydrogenase*

The labelling of axes and assignment of colours to the four subunits is that given by Buehner *et al.* (1974) from X-ray-crystallographic studies on the lobster enzyme. The letters A and N refer to the adenine and nicotinamide moieties of the NAD^+. The dashed lines indicate that two of the NAD^+-binding sites lie beneath the molecule as drawn.

$$\begin{array}{ccc} E{\cdot}N_2 & \rightleftharpoons & E^*{\cdot}N_2 \\ \pm N \downarrow\uparrow & & \pm N \downarrow\uparrow \\ E{\cdot}N_3 & \rightleftharpoons & E^*{\cdot}N_3 \end{array}$$

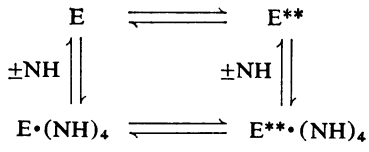
Scheme 3. *Schematic representation of the binding of the third NAD^+ molecule to a rabbit glyceraldehyde 3-phosphate dehydrogenase molecule containing two NAD^+ molecules*

The NAD^+ molecules are represented as N and the enzyme species as E and E*, where E* represents a 'tightened' conformation of the enzyme with increased interactions across the *QR* plane of the molecule.

the tetrameric structure could involve any combination of increases or decreases in interactions across the *P*, *Q* and *R* axes, including an increase across the *P* axis and a decrease across the *Q* and *R* axes. However, with this qualification in mind it will be assumed as a working hypothesis that the dimerization processes ($R_4 \rightarrow 2R_2$ etc.) each involve dissociation about the *QR* plane.

The ligand-induced changes in the rates of subunit exchange observed in this study could be attributed to a change in the free energies of activation of the reactions (ΔG^*) arising predominantly from either ground-state or transition-state effects or from an effect on both. It seems most likely that the major effect of NAD^+ binding is to change the standard free energy of the ground state, since a number of studies have provided evidence that the structure of the tetrameric glyceraldehyde 3-phosphate dehydrogenase molecule is altered on binding NAD^+. Thus NAD^+ binding to skeletal muscle glyceraldehyde 3-phosphate dehydrogenase, under conditions where there is no significant change in aggregation state, leads to a decrease in susceptibility to proteolytic digestion (Fenselau, 1972; Foucault *et al.*, 1974), a 1–2% decrease in hydrodynamic volume (Smith & Schachman, 1974) and a contraction in volume as measured by low-angle X-ray scattering (Simon, 1973). Therefore the present results suggest that binding of NAD^+ to glyceraldehyde 3-phosphate dehydrogenase leads to a 'tighter' structure and that this involves increased interactions across the *QR* dissociation plane. The experimental results shown in Plate 2(*c*) indicate that this structural change only takes place in binding the third NAD^+ molecule to the rabbit muscle enzyme. Clearly this structural change should be reflected in the thermodynamic parameters for the binding process and this is borne out by a number of NAD^+-binding studies (Conway & Koshland, 1968; DeVijlder & Slater, 1968; Velick *et al.*, 1971), which indicated that at 3–5°C there are two pairs of NAD^+-binding sites, one pair with a high affinity ($K_{diss.} \leqslant 1$ nM) and the other pair with a lower affinity ($K_{diss.} = 0.3\,\mu$M and $40\,\mu$M respectively). Velick *et al.* (1971) found that most of the difference between the free energies of binding of the second and third NAD^+ molecules ($\Delta\Delta G_0 = 14$ kJ/mol) was due to a difference in the entropy terms ($-T\Delta\Delta S_0 = 11$ kJ/mol). The results of the present study correlate this increase in order of the system on binding the third NAD^+ molecule with a 'tightening' of the enzyme structure to give increased subunit interaction across the surface in the *QR* plane. This process corresponds to the E to E* transition represented in Scheme 3. Owing to limitations in the dynamic range of the hybridization-rate measurements made in this study, it is not possible to obtain an absolute measure of the NAD^+-induced increase in the value of the activation energy for the $R_4 \rightarrow 2R_2$ process (i.e. $\Delta\Delta G^*$). However, a minimum value of $\Delta\Delta G^*$ of more than 12 kJ/mol can be calculated from the values of the rate constants at 37°C (Table 1).

There are a number of other reports in the literature consistent with a structural change in the rabbit muscle enzyme on binding the third NAD^+ molecule. First, Eisele & Wallenfels (1970) showed that between the binding of the second and third molecules of NAD^+ the reactivity of the enzyme thiol groups towards the D and L enantiomers of α-iodopropionate was inverted. Second, the binding of NAD^+ to the



Scheme 4. *Schematic representation of the binding of NADH to glyceraldehyde 3-phosphate dehydrogenases*

The NADH molecules are designated NH and two enzyme conformations by E and E**, where E** represents the conformation with an increased tendency to dissociate to dimers, probably through decreased interactions across the *QR* plane of the molecule (see Scheme 2).

two high-affinity sites of the enzyme does not significantly alter the rate of inhibition by maleic anhydride, whereas occupancy of the third and fourth sites gives considerable protection (Ovadi *et al.*, 1971). Third, Malhotra & Bernhard (1973) observed that the spectrum of furylacryloyl glyceraldehyde 3-phosphate dehydrogenase was perturbed on binding the third NAD^+ molecule.

Since the rabbit muscle enzyme has a much higher affinity for NAD^+ than the yeast enzyme, it is not possible to determine from the present studies whether NAD^+ binding also leads to a tightening of the structure of the yeast enzyme. However, Stallcup & Koshland (1974) observed that the presence of 1 mM-NAD^+ prevented hybrid formation between glyceraldehyde 3-phosphate dehydrogenases from two different strains of yeast. These authors concluded that the coenzyme either decreased the rate of dissociation to dimers or altered the equilibrium constant for the process. Taken in conjunction with the present work it seems most likely that binding of NAD^+ to both yeast and rabbit muscle enzymes leads to a tightening of the structures leading to decreases in the values of the rate constants for the conversions of tetramer into dimer.

In marked contrast with the effect of NAD^+, NADH binding causes an increase in all the values of the rate constants for the conversions of tetramer into dimer (k_{-r}, k_{-y} and k_{-h}; see Table 1). In particular, the values of k_{-r} and k_{-y} at 4°C increase by about 100-fold on NADH binding and the increase in the value of k_{-h} is even greater. These increases are such that the rate of subunit exchange is so rapid that it is impossible to resolve mixtures of the species R_4, R_2Y_2 and Y_4 on electrophoresis (Fig. 1). The effect of NADH almost certainly results from a change in enzyme conformation since the migratory behaviour of the enzymes on electrophoresis was changed after pretreatment with NADH (Plate 2*a*) and the susceptibility to heat denaturation was increased in the presence of NADH (Fig. 1). In addition, optical-rotatory-dispersion studies (Listowsky *et al.*, 1965), increased susceptibility to proteolytic digestion in the presence of NADH (Tucker & Grisolia, 1962) as well as kinetic studies (Trentham, 1971) indicate that NADH binding brings about a structural transition in glyceraldehyde 3-phosphate dehydrogenase. The results of the present study suggest that this structural transition involves a decrease in the free energy of interaction at the interface involving the plane containing the *Q* and *R* axes. Given that the ligand predominantly affects the ground state of the hybridization reactions, this decrease in free energy of interaction can be estimated as about 10kJ/mol. It is noteworthy that NADH binding also exhibits negative co-operativity (Boers *et al.*, 1971). The first two molecules bind to the rabbit muscle enzyme molecule with a dissociation constant of less than 0.5 μM at 5°C, but the next two exhibit dissociation constants of 2.5 and 50 μM respectively. This negative co-operativity can be interpreted in terms of intersubunit 'strain' induced on binding NADH, which by analogy with the NAD^+ binding could be elicited at the binding of the third NADH molecule (e.g. see Scheme 4). In general, dehydrogenases show a higher affinity for NADH than for NAD^+ but for glyceraldehyde 3-phosphate dehydrogenases this order of affinities is reversed (e.g. compare Boers *et al.*, 1971; Velick *et al.*, 1971; von Ellenreider *et al.*, 1972). The present work suggests that the cause of this inversion lies partly in the NADH-induced strain between the two *P*-axis dimers across the *QR* plane of the molecule.

Hybridization studies have been widely used to gain useful information about quaternary protein structure. In addition the kinetics of hybridization could prove to be a powerful general method for probing subunit interactions. The method has the advantage that changes in subunit interaction energy can be measured in solutions where oligomeric proteins are at concentrations at which they are in their native associated state (e.g. in principle less than 0.1% dissociated.) Under these conditions it would not be possible to use the available direct physical techniques for measuring dissociation. However, like all kinetic techniques, the method suffers the disadvantage that it is not always possible to ascribe a change in rate to a change in the ground state or the transition state for the reaction. In addition the use of electrophoresis to determine the composition of reaction mixtures imposes severe limitations on the time-scale for following the reaction. It can be estimated that rate constants can only be measured if they fall in the range from about $2\times10^{-3}s^{-1}$ (limited by 'spotting time') to $2\times10^{-5}s^{-1}$ (i.e. a half-life of 4 days). However, present investigations are being carried out with the object of developing continuous monitoring techniques, which will greatly increase the dynamic range of the method.

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