An Investigation of the Nicotinamide–Adenine Dinucleotide-Induced ‘Tightening’ of the Structure of Glyceraldehyde 3-Phosphate Dehydrogenase

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An investigation was made of the effect of NAD^+ analogues on subunit interactions in yeast and rabbit muscle glyceraldehyde 3-phosphate dehydrogenases by using the subunit exchange (hybridization) method described previously [e.g. see Osborne & Hollaway (1975) *Biochem. J.* 151, 37–45]. The ligands ATP, ITP, ADP, AMP, cyclic AMP and ADP-ribose like NADH, all caused an apparent weakening of intramolecular subunit interactions, whereas NAD^+ caused an apparent increase in the stability of the tetrameric enzyme molecules. A mixture of NMN and AMP, although it did not simulate completely the NAD^+ -induced ‘tightening’ of the enzyme structure, did result in a more than 20-fold decrease in the rate of subunit exchange compared with that in the presence of AMP alone. These results show that occupancy of the NMN subsite of the enzyme NAD^+ -binding site is insufficient in itself to give the marked tightening of the enzyme structure induced by NAD^+ . The ‘tightening’ effect is specific in that it seems to require a phosphodiester link between NMN and ADP-ribose. These effects are discussed in terms of the detailed X-ray structure of the lobster holoenzyme [Buehner *et al.* (1974) *J. Mol. Biol.* 90, 25–49].

Previously (Osborne & Hollaway 1974, 1975) we described how an investigation of the kinetics and thermodynamics of dimer exchange between the homotetrameric yeast (Y_4), rabbit (R_4) and yeast–rabbit hybrid (R_2Y_2) glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) molecules could provide information about differences in subunit interactions in the different ligated states of these enzymes.

Scheme 1. Kinetic model for the process of subunit exchange between rabbit, yeast and hybrid glyceraldehyde 3-phosphate dehydrogenases

The symbol R_4 represents a tetrameric rabbit enzyme molecule which can dissociate to the dimeric species R_2 . There are similar dissociations for the yeast (Y_4) and hybrid (R_2Y_2) molecules. Under the conditions of the present experiments, the concentrations of dimeric species are sufficiently low for a steady-state treatment to be used in deriving the rate equations arising from this scheme.

The dimer-exchange process was shown to conform to Scheme 1.

A steady-state treatment of Scheme 1, with certain assumptions (Osborne & Hollaway, 1974) gives the expression for the concentration of hybrid formed at any time, t , $[(R_2Y_2)_t]$ given in eqn. (1).

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

where the observed first-order rate constant for the formation of hybrid from equimolar concentrations of the native enzymes, k_1^{obs} , and the concentration of hybrid formed at equilibrium, A , are given by eqns. (2) and (3):

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

$$A = \frac{2k_{-r}}{\left(k_{-r} + \frac{2k_{+r} \cdot k_{-h}}{k_{+h}}\right)} \cdot [R]_0 \quad (3)$$

where $[R]_0 = [R_4] + \frac{1}{2}[R_2Y_2]$.

It was shown that the binding of NAD^+ to the rabbit muscle enzyme resulted in a completely different change in subunit interactions from that induced on binding NADH. Thus binding of the third molecule of NAD^+ to R_4N_2 (i.e. a rabbit enzyme molecule containing two bound NAD^+ molecules) leads to a

'tightening' of the structure of the enzyme molecule, probably through increased interactions across the interface containing the *QR* plane. [The *P*, *Q* and *R* axes of the molecule are as described by Adams *et al.* (1970) for the lobster muscle enzyme.] Conversely, binding of NADH leads to a decreased interaction between dimers within the tetrametric molecule, giving a 'loosened' structure with more tenuous interactions across the *QR* plane.

The present work was carried out with the object of (a) further investigating the basis of the differences in subunit interactions elicited by NAD⁺ and NADH, and (b) extending the knowledge of the effect of other specific ligands on the hybridization process in an attempt to ascertain the contribution of binding at different subsites to the changes in subunit interactions.

Materials and Methods

The rabbit muscle and yeast glyceraldehyde 3-phosphate dehydrogenases were prepared as described previously (Osborne & Hollaway, 1974). Best available grades of NAD⁺, NADH, ATP, ADP, AMP, cyclic AMP, NMN and ADP-ribose were purchased from Boehringer (London) Corp., London W.5, U.K., and adenosine was from Sigma Chemical Co. Ltd. Kingston-upon-Thames, Surrey, U.K.

Hybridization experiments were conducted at 0°C in a 0.1 M-Tris chloride buffer, pH 7.8, containing 1 mM-dithiothreitol and 1 mM-EDTA, and by using methods previously described (Osborne & Hollaway,

1974, 1975). In some experiments, a 0.01 M-sodium phosphate buffer, pH 7.8, containing 1 mM-dithiothreitol and 1 mM-EDTA was used. These buffers are referred to as the 'pH 7.8 Tris buffer' and 'pH 7.8 phosphate buffer' respectively.

Results

Effects of adenine nucleotides on the rate and extent of dimer exchange

The effects of AMP, cyclic AMP, ADP and ATP on the hybrid-formation reaction were investigated at 0°C in both the 0.1 M-Tris buffer (pH 7.8) and the 0.01 M-phosphate buffer (pH 7.8). Essentially the same results were obtained with either buffer system. Further, since each of the above adenine nucleotides gave indistinguishable results on the dimer-exchange reaction (see Table 1), only the results of the effect of ATP will be presented in detail.

It can be seen from Plate 1(a) that incubation of a mixture of R₄ and Y₄ for 5 min in the presence of 5 mM-ATP leads to mixture containing R₄, R₂Y₂ and Y₄ in the proportions R₄/R₂Y₂/Y₄ of 1:1:1. The composition of this mixture was found to be unchanged for incubation times up to 25 h, so that it can be concluded that the dimer-exchange reaction had reached equilibrium within 5 min. Since the half-life for the reaction must be less than 5 min, the minimum value for $k_1^{obs.}$ is about $2 \times 10^{-3} s^{-1}$ (see eqn. 1), whereas in the absence of ATP, $k_1^{obs.} = 7 \times 10^{-6} s^{-1}$ under the same reaction conditions (Osborne & Hollaway,

Table 1. *Equilibrium ratios and rate constants for subunit exchange between rabbit, yeast and hybrid glyceraldehyde 3-phosphate dehydrogenases at 4°C*

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 initial concentrations of yeast and rabbit enzyme (6 mg/ml each) and incubations were carried out in both the pH 7.8 phosphate and pH 7.8 Tris buffers, except where indicated by an asterisk (here the reaction was carried out only in the pH 7.8 phosphate buffer). The errors arising from the densitometric analysis of the stained gels are such that the standard deviations of the $[R_2Y_2]_{eq.}/[Y_4]_{eq.}$ values are 10% and those of the $k_1^{obs.}$ values are about 20%.

Ligand	$\frac{[R_2Y_2]_{eq.}}{[Y_4]_{eq.}}$	$10^5 \times k_1^{obs.} (s^{-1})$	$10^5 \times k (s^{-1})$		
			k_{-r}	k_{-y}	k_{-n}
Control (P _i buffer)	0.65	0.7	0.4	1.2	1.0
Control (Tris buffer)	0.62	0.7	0.4	1.2	1.0
5 mM-ATP	1.0	> 200	> 133	> 133	> 267
5 mM-ADP	1.0	> 200	> 133	> 133	> 267
5 mM-AMP	1.0	> 200	> 133	> 133	> 267
3 mM-cyclic AMP	1.0	> 200	> 133	> 133	> 267
1 mM-ADP-ribose	1.2	> 200	> 150	> 150	> 250
1 mM-ATP + 10 mM-MgCl ₂ *	0.7	> 200	> 110	> 110	> 290
3 mM-ITP*	0.3	> 200	> 51	> 51	> 349
3 mM-NMN	0.6	0.7	0.4	1.2	1.0
3 mM-NAD ⁺	< 0.05	< 0.2	< 0.2	< 0.2	< 0.2
3 mM-NADH	0.22	> 200	> 32	> 32	> 360
1.5 mM-NAD ⁺ + 5 mM-ATP	< 0.05	< 0.2	< 0.2	< 0.2	< 0.2
1.5 mM-NADH + 5 mM-ATP	0.2	> 200	> 32	> 32	> 360

EXPLANATION OF PLATE I

Effect of ATP on subunit exchange between species R_4 , R_2Y_2 and Y_4

(a) Effect of ATP on hybrid formation. Starch-gel electrophoretograms and densitometric analyses of mixtures of rabbit and yeast glyceraldehyde 3-phosphate dehydrogenases, each at 6 mg/ml, incubated for 5 min at 0°C in the pH 7.8 phosphate buffer in (i) the absence, and (ii) the presence, of 5 mM-ATP. The amounts of the individual species are given as percentages. The vertical arrows indicate the origin, and the horizontal arrows the direction of electrophoretic migration. (b) Effect of ATP on hybrid formation in mixtures containing different concentrations of rabbit and yeast enzyme. A similar analysis of a 5 min reaction mixture containing 5 mM-ATP but with (i) $[Y_4] = 12$ mg/ml and $[R_4] = 4$ mg/ml, and (ii) $[Y_4] = 4$ mg/ml and $[R_4] = 12$ mg/ml. In each case, only the amount of hybrid formed relative to the species at the lowest concentration is given, since the very dark band corresponding to the protein present at the greatest concentration falls outside the range of the densitometric analysis. (c) Hybrid reversion in the presence of ATP. Samples of the isolated R_2Y_2 hybrid at a concentration of 6 mg/ml in the pH 7.8 phosphate buffer were incubated for 5 min at 0°C in the presence (i) or absence (ii) of 3 mM-ATP as indicated. (d) Electrophoresis in the presence of ATP. The photograph shows an electrophoretogram of a mixture containing species R_4 , R_2Y_2 and Y_4 in a ratio $R_4/R_2Y_2/Y_4$ of 1.0:1.4:1.0, separated as described in the Materials and Methods section, but with 1 mM-ATP in both electrode and gel buffers. The central hybrid band is not present because of a 'melting' process on electrophoresis [see, e.g., Guidotti *et al.* (1963) and Huehns & Shooter (1966)].

EXPLANATION OF PLATE 2

Ligand-competition experiments

Mixtures of R_4 and Y_4 enzymes, each at 6 mg/ml, were incubated in the pH 7.8 phosphate buffer for 21 h at 0°C with the following additions: (i) 5 mM-ATP; (ii) 5 mM-ATP+1.5 mM-NADH; (iii) 5 mM-ATP+1.5 mM-NAD⁺; (iv) 1.5 mM-NAD⁺+1.5 mM-NADH. Electrophoresis and protein staining was carried out as described in the Materials and Methods section.

1974), i.e. ATP binding causes a change of at least 10 kJ/mol in the free energy of activation. Concentrations of ATP as low as 0.1 mM gave indistinguishable results from that with 5 mM-ATP.

The presence of MgATP^{2-} also caused equilibrium to be established within 5 min, but the equilibrium ratio $R_4/R_2Y_2/Y_4$ of 1.0:0.7:1.0 was slightly different from that with ATP alone (see Table 1).

The ATP-induced increase in the rate of subunit interchange results from increased values of both k_{-r} and k_{-y} as can be seen from the results of Plate 1(b) (i.e. about the same amount of hybrid forms in a given time with $[R_4]_0 = 3[Y_4]_0$ as with $[Y_4]_0 = 3[R_4]_0$).

Other confirmatory evidence for the rapid dimer exchange was provided by the observation of an ATP-induced increase in the rate of hybrid reversion ($2R_2Y_2 \rightarrow R_4 + Y_4$; see Plate 1c), and from the observation that incorporation of ATP in the electrophoresis medium for separating R_4 , R_2Y_2 and Y_4 led to a 'melting out' of the hybrid species so that only the R_4 and Y_4 bands were observed (Plate 1d). To determine whether binding of the nucleoside part of the nucleotides or of the phosphate moiety caused the 'loosening' of the tetrameric structure, three separate hybridization experiments were conducted at 0°C in the presence of 3 mM-adenosine, 10 mM-sodium phosphate, and 3 mM-adenosine plus 10 mM-sodium phosphate. In each case, as with the control, there was no observable hybrid formation after a 1 h incubation, whereas in the presence of 3 mM-AMP the hybridization-equilibrium position was reached within 5 min. The experiments were not extended for periods longer than 1 h, owing to slow precipitation of the adenosine. Further experiments were conducted at 37°C at which temperature the adenosine/phosphate mixture influenced the equilibrium position in the same way as either ligand alone. These results suggest that the 'loosening' effect of adenine nucleotides depends on the presence of the nucleoside moiety and the phosphate group in the same molecule.

Effect of other NAD^+ moieties on the subunit-exchange reaction

Since NAD^+ binding gives an enzyme molecule with a 'tightened' structure (the rate of dimer exchange was greatly decreased) and NADH binding gives a 'loosening' of the dimer-dimer interactions (Osborne & Hollaway, 1975), we decided to investigate which parts of the nucleotide molecules contributed to these effects. Thus a number of hybrid-forming reactions were carried out in the presence of ligands representing different parts of the NAD^+ molecule. The results, which are given in Table 1 and Fig. 1 and Plate 2, are consistent with the following conclusions: (a) the adenine-ribose-phosphate combination gives rise to a weakening of subunit interactions. It was shown that adenosine itself has no effect (see above).

Fig. 1. Effect of specific ligands on the rate of hybrid formation

Mixtures of the native enzymes, each at 6 mg/ml, were incubated in the pH 7.8 phosphate buffer for given times, both in the presence and absence of the ligands. The reaction mixtures were then subjected to electrophoretic analyses as described in the Materials and Methods section. $\circ$ and Δ denote the relative amount of R_2Y_2 hybrid formed (expressed as $[R_2Y_2]/[Y_4]_0$) in the presence of the combinations 1 mM-AMP+3 mM-NMN and 1 mM-ATP+3 mM-NMN respectively. $\square$, Amount of R_2Y_2 in mixtures containing either 1 mM-ATP or 1 mM-AMP. ----, Amount of hybrid formed in mixtures to which no ligand was added as well as that in mixtures containing 1 mM-NMN.

(b) ADP-ribose also weakens the dimer-dimer interaction. However, the nature of the changes in subunit interaction must differ from those produced by ADP, since the hybridization-equilibrium positions are different for the two ligands (see Table 1). (c) NMN, like adenosine, has no observable effect on the rate of hybrid formation but greatly decreases the extent of weakening of the subunit interactions brought about by AMP (see Fig. 1). It is noteworthy that this combination of NMN and AMP gives a rate much greater than the control, i.e. it does not give the marked inhibition of dimer exchange produced by NAD^+ . (d) A mixture of NMN and ATP behaves similarly to the NMN/AMP mixture in that the presence of NMN also decreased the rate of hybrid formation from that observed with ATP alone (see Fig. 1). (e) The effects of the dinucleotides, NAD^+ and NADH dominate the effect of ATP in equimolar mixtures, i.e. of NAD^+ +ATP and NADH+ATP (see Plate 2).

Discussion

There are a number of reports in the literature of the effects of NAD^+ and NAD^+ analogues on the

conformation of the glyceraldehyde 3-phosphate dehydrogenase molecule. In these studies a variety of techniques have been used, including thermal inactivation (Vas & Boross, 1972; Eby & Kirtley, 1971; Yang & Deal, 1969a; Constantinides & Deal, 1969), susceptibility to proteolytic digestion (Cantau *et al.*, 1970; Polgar, 1966; Tucker & Grisolia, 1962; Fenselau, 1972; Foucault *et al.*, 1974; Yang & Deal, 1969b), ultracentrifugation (Constantinides & Deal, 1969; Stancel & Deal, 1969; Hoagland & Teller, 1969; Smith & Schachman, 1972, 1973), optical rotatory dispersion and circular dichroism (Listowsky *et al.*, 1965; Bolotina *et al.*, 1967; Jaenicke, 1970). In comparing these results with those of the present study, which involve measurement of the effects of ligands on the rate of subunit exchange, it should be noted that in principle each method measures different aspects of the changes in protein structure induced by the bound ligand, e.g. a change in conformation giving a large change in optical rotatory dispersion, need not involve a significantly changed tendency to dissociate.

From a steady-state kinetic study into the effects of NAD^+ analogues on the catalytic activity of the yeast enzyme as well as on enzyme stability (Yang & Deal, 1969a) it was suggested that the two nucleotide moieties in the nicotinamide-adenine dinucleotide molecule have separate functions. Yang & Deal (1969a) proposed that the adenine dinucleotide moiety was responsible mainly for binding, and the nicotinamide moiety for catalysis. In a study of the effects of NAD^+ and NADH on subunit exchange between the yeast and rabbit muscle enzymes (Osborne & Hollaway, 1975), NAD^+ was found to 'tighten' and NADH to 'weaken' sub unit interactions so that, in addition to its catalytic function, the nicotinamide moiety must affect sub unit interactions. The results of the present study further support this conclusion. The nicotinamide moiety is the crucial factor in dictating the 'tightening' effect of NAD^+ , since the binding of any of the adenine nucleotides ATP, ADP, AMP, cyclic AMP or ADP-ribose causes an apparent weakening of subunit interactions. It can be concluded that the nucleotides bind at the same site where presumably the same interactions will be made. This conclusion is consistent with reports that adenine nucleotides cause enzyme dissociation (Stancel & Deal, 1969; Constantinides & Deal, 1969) and increase the enzyme's susceptibility both to proteolytic digestion and thermal denaturation (Yang & Deal, 1969b).

Interestingly, adenine and adenosine do not affect the rate of dimer exchange, although both molecules have been shown to bind to the enzymes (Yang & Deal, 1969a). Moreover, the combination of adenosine plus phosphate did not exhibit the same effects on dimer exchange as AMP. These results are consistent with the view that the adenosine-linked 5'-

phosphate is essential for the 'loosening'-effect behaviour of the adenine nucleotides.

In addition, the 6-amino group of the adenine moiety cannot be an important structural feature, since ITP has little effect on the k_2^{app} value for hybridization, and only changes the equilibrium constant by a factor of about three. It is noteworthy that Stancel & Deal (1969) did not detect dissociation of the yeast enzyme in the presence of AMP or cyclic AMP. The dimer-exchange experiments reported here give a more sensitive measure of changes in dimer-dimer interactions than that provided by either the activity-loss approach used by Stancel & Deal (1969) or by direct physical measurements, such as ultracentrifugation, and enable us to conclude that AMP and cyclic AMP must give some weakening of the inter-dimeric interactions in both yeast and rabbit enzyme molecules.

It is possible that the effect of the adenine nucleotides could result from binding of the nucleotide either in (a portion of) the NAD^+ binding site or at a separate site, occupancy of which leads to dissociation. Thus there have been several reports that ATP is a non-competitive inhibitor (Sapag-Hagar, 1969) or a mixed inhibitor (Oguchi *et al.*, 1973) in the enzymic reaction; other reports have shown mixed inhibition between ADP and AMP (Nagradova *et al.*, 1969) and cyclic AMP and NAD^+ (Kirtley & Koshland, 1970). These observations suggest that there are distinct binding sites for NAD^+ and ATP, although in view of the complex nature of the kinetics of the enzymic reaction this conclusion cannot be regarded as unequivocal.

The effect of the combination of ATP and NAD^+ in the hybridization process can be interpreted in at least two ways.

(a) NAD^+ and ATP bind at the same site, but the NAD^+ 'tightening' effect dominates the ATP effect simply because NAD^+ binds more strongly. The possibility that NAD^+ and ATP bind at the active site has been suggested by Yang & Deal (1969a) on the basis of the competitive nature of inhibition.

(b) NAD^+ and ATP can bind simultaneously to separate sites. This possibility indicated in the above-cited reports cannot be excluded. Detailed binding data for the combination of ligands is required to establish whether separate sites exist.

Similar possibilities could explain the dominant effect of NADH in the NADH , ATP combination hybridization experiment.

Given that occupancy of the adenine-ribose-phosphate-phosphate-ribose part of the NAD^+ -binding site complementary to ADP-ribose (e.g. by AMP, ADP or ADP-ribose) leads to a weakening of inter-dimer interactions, the 'tightening' effect of NAD^+ must be ascribed to interactions involving the nicotinamide moiety. The experiment designed to test this postulate did not give unequivocal support since, although a combination of AMP and NMN

gave a much lower rate of subunit exchange than that with AMP alone, the rate was still greater than in the absence of ligand. The result could be accounted for if the conformation change brought about by NAD⁺ binding could not take place with NMN and AMP bound in the same site, either because the combination of ligands bears two extra negative charges or because it contains an extra oxygen atom.

Other workers have found that the combination of NMN and AMP does not reproduce the effect of NAD⁺ (Eby & Kirtley, 1971; Fenselau, 1970; Francis *et al.*, 1971*a,b*). For example, Eby & Kirtley (1971) found that, whereas NAD⁺ completely protected the enzyme from heat denaturation, the protective effect of AMP and NMN was no greater than these ligands individually. Fenselau (1970) reported that although the rate of alkylation of cysteine-149 by iodoacetic acid was increased in the presence of NAD⁺, a combination of NMN+AMP or ADP-ribose with *N*-methylnicotinamide iodide had no effect.

It is tempting to try to interpret the basis of the nicotinamide 'tightening' effect in the terms of the detailed X-ray structure of the lobster holoenzyme presented by Buehner *et al.* (1974). Thus the thiol group of cysteine-149 lies sufficiently close to the nicotinamide ring in the X-ray crystallographic structure to form either a covalent bond or a charge-transfer interaction. Alternatively, an interaction with the phenol ring of tyrosine-317 could be the basis of the effect, since this group lies 0.55 nm away from the nicotinamide group with the two ring systems co-planar. Either of these interactions could involve changes in subunit interactions, since these groups are close to the interface between the more tightly associated subunits across the *P* axes. It would be expected that changing from an oxidized form of the nicotinamide ring in NAD⁺ to the reduced form in NADH would decrease the magnitude of these interactions.

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