PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

# Glyceraldehyde-3-Phosphate Dehydrogenase [and Discussion]

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# Glyceraldehyde-3-phosphate dehydrogenase

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Conflicting experimental evidence of the pathway of catalysis for the enzyme from rabbit, pig and lobster muscle tissues is reviewed. Transient kinetic studies with the enzyme from rabbit muscle are presented. The results are shown to be consistent with the double-displacement mechanism of catalysis originally proposed by Segal & Boyer (1953). The rate constant for combination of the aldehyde form of the substrate with the $NAD^+$ complex of the enzyme is about $3 \times 10^7$ $M^{-1}$ $s^{-1}$, and for all four subunits of the molecule the rate constant for hydride transfer in the ternary complex formed is greater than $10^3$ $s^{-1}$, consistent with their simultaneous participation in catalysis. Recent steady-state kinetic studies with the rabbit muscle enzyme, in contrast to earlier studies, also provide evidence to support the Segal–Boyer pathway if the kinetic effects of the negative cooperativity of $NAD^+$ binding are taken into account. Experimental data for the binding of $NAD^+$ to the enzyme from muscles and from *Bacillus stearothermophilus*, and their interpretations, are also briefly reviewed. The information currently available from X-ray crystallography regarding the structures of holoenzyme and apoenzyme from *B. stearothermophilus* and lobster muscle is outlined.

## 1. Introduction

D-Glyceraldehyde-3-phosphate dehydrogenase was first purified and crystallized from yeast by Warburg & Christian (1939) and from rabbit skeletal muscle by Cori *et al.* (1945) and Caputto & Dixon (1945). The properties of the enzymes from these two sources in particular have been studied in great detail, but the crystallized forms have not proved amenable to structural studies by X-ray diffraction techniques. High resolution structures have been computed for the crystalline enzyme from lobster tail muscle (Buehner *et al.* 1974; Moras *et al.* 1975) and *Bacillus stearothermophilus* (Biesecker *et al.* 1977). Complete amino acid sequences were established for the enzymes from these sources and from pig muscle, yeast and *Thermus aquaticus* by Harris and his coworkers. They are strictly homologous, with sequence identities of 50–60%, and the enzyme from each source appears to have four identical polypeptide chains in the molecule and a relative molecular mass of 146000 (for references, see Harris & Waters 1976).

In this contribution, the main emphases will be on the pathway of catalysis for the rabbit enzyme, which has been controversial, the negative cooperativity of NAD binding by the enzymes from muscles and *B. stearothermophilus*, and information from structural studies about the molecular basis of cooperativity. The yeast enzyme, which binds NAD less firmly than the muscle enzyme and with positive cooperativity under some conditions, will not be discussed.

## 2. Pathway of catalysis

Early studies of the rabbit muscle enzyme (Racker & Krimsky 1952; Segal & Boyer 1953) indicated an enzyme-substitution mechanism of catalysis (figure 1) involving the intermediate formation of 3-phosphoglyceryl-enzyme acylated at its iodoacetate-sensitive thiol groups. The

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other stable enzyme species in this double-displacement mechanism was assumed to be the binary complex of enzyme with $NAD^+$ (holoenzyme) because the recrystallized enzyme retains a complement of firmly bound $NAD^+$. The experimental basis for this mechanism included evidence that $NAD^+$ in the holoenzyme is rapidly reduced to NADH by glyceraldehyde-3-phosphate without the addition of inorganic phosphate, isolation and characterization of 3-phosphoglyceryl-enzyme, and facilitation of acyl-transfer to inorganic phosphate by $NAD^+$.

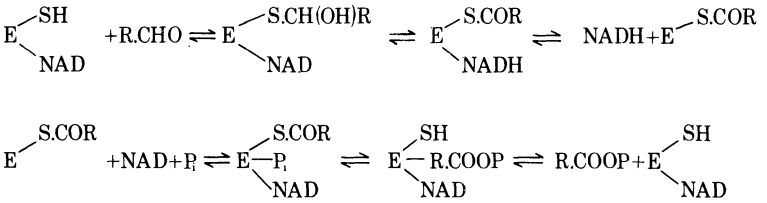


FIGURE 1. Proposed pathway of catalysis. E–SH represents one subunit of the enzyme (after Segal & Boyer 1953).

Furfine & Velick (1965) tested the validity of this scheme as a description of overall catalysis in the steady state by detailed initial rate measurements with the rabbit enzyme. They showed that the effects on the initial rate of changing the concentrations of the three substrates could not be accounted for by the rate equation for this mechanism, but were consistent with a rapid equilibrium random mechanism in which all three substrates combine with the enzyme before hydride transfer and release of NADH occur. Similar studies with the pig muscle enzyme by Keleti & Batke (1965) gave results similarly at variance with the Segal–Boyer pathway, and were thought to indicate a partly ordered mechanism with inorganic phosphate combining with the enzyme after hydride transfer but again before NADH release. Accepting that these studies precluded the enzyme-substitution pathway, Orsi & Cleland (1972) adduced evidence from inhibition studies that the kinetic mechanism for the rabbit muscle enzyme is of the ordered sequential type, $NAD^+$ being the first substrate to combine with, and NADH the last product to dissociate from, the apoenzyme.

In contrast, studies of transient and partial reaction kinetics with enzyme from lobster muscle (Trentham 1971) gave results that were entirely consistent with the pathway shown in figure 3, and established, *inter alia*, that the two half-reactions are fast enough to account for the maximum rate of overall catalysis, and that $NAD^+$ participates in efficient acyl transfer. However, the characteristic rate equation for the Segal–Boyer mechanism has not been tested by detailed initial rate measurements with the lobster enzyme. Only for the enzyme from pea seeds have steady-state kinetic measurements been found consistent with this pathway (Duggleby & Dennis 1974).

Another complicating factor is the negative cooperativity of $NAD^+$ binding to the enzyme, discovered by Conway & Koshland (1968) and de Vijlder & Slater (1968), which will be discussed in detail later. In general terms, these workers showed that the apoenzyme can bind 4 mol $NAD^+$ per mole, in accordance with the subunit composition, but only two are firmly bound, the other two dissociating fairly readily. There was, of course, no provision for this in the Segal–Boyer mechanism, and these findings raised questions about whether or not the four subunits are all equally and simultaneously active in catalysis.

Much of the accumulated information about the chemical properties and enzymic activities

of glyceraldehyde-3-phosphate dehydrogenase has come from experiments with the rabbit muscle enzyme and has been assumed valid for the enzyme from other sources. In 1974, it seemed to us worthwhile to try to resolve the uncertainties about the pathway of catalysis by transient and steady-state kinetic studies with apoenzyme from rabbit muscle, the more so because negative cooperativity of coenzyme binding is most marked with enzyme prepared from this source (see above) and its effects had not been detected in any previous kinetic studies.

## 3. Kinetic studies of rabbit muscle enzyme

### (a) *Transient kinetics*

#### (i) *Experimental*

Holoenzyme was freshly prepared as described by Reynolds & Dalziel (1979). Apoenzyme solutions were prepared immediately before use by treating holoenzyme in 0.1 M triethanolamine buffer, pH 7.6, and 1 mM EDTA with activated charcoal at 0 °C for 45 min (Krimsky & Racker 1963), and centrifuging. The ratio of absorbances at 280 and 260 nm was 1.85. Apoenzyme concentrations were calculated from the measured absorbance at 280 nm, by using an absorbance coefficient of 0.83 $mg^{-1}$ $cm^2$ (Fox & Dandliker 1956; Reynolds & Dalziel 1979) and a relative molecular mass of 146000. The turnover number at 25 °C in the assay described by Ferdinand (1964) was 400 $s^{-1}$.

DL-Glyceraldehyde-3-phosphate (GAP) was prepared by hydrolysis of the dicyclohexylammonium salt in 0.1 M $H_2SO_4$ at 50 °C for 3 h. The concentration of the active isomer was determined enzymatically (Meunier & Dalziel 1978). Freshly prepared 3 mM GAP contained 0.12 mM inorganic phosphate. To minimize reactant blanks, $NAD^+$ was purified by chromatography (Dalziel & Dickinson 1966), precipitated with acetone and assayed enzymatically.

The stopped-flow apparatus (Gibson & Milne 1953) had an optical path length of 2.13 cm, a time constant of 0.1 ms and dead-time of 2 ms. Changes of absorbance or transmittance at 340 nm were monitored with an oscilloscope, by using a Xenon lamp and monochromator, together with a beam splitter, compensating photomultiplier and voltage-divider circuitry to reduce the effects of lamp instability. A calibrated back-off potential allowed oscilloscope display with high amplification. Apoenzyme and $NAD^+$ solutions, in 0.1 M triethanolamine buffer, pH 7.6, containing 1 mM EDTA, were mixed and put in one syringe of the apparatus, and GAP in the same medium, with or without added inorganic phosphate, was put in the other. The absorbances of the two reactant solutions were measured independently immediately before and after stopped-flow measurements, which were made at 25 °C.

#### (ii) *Results and discussion*

A progress curve for oxidative phosphorylation with saturating concentrations of the three substrates and 3.6 μM enzyme (14.4 μM subunits) is shown in figure 2. The absorbance of the reaction mixture at the earliest time of observation, 0.31, is substantially greater than the sum of the reactant absorbances, 0.16. This fast burst of NADH formation in the dead-time of the apparatus is followed by slower progress to equilibrium. The initial rate is in satisfactory agreement with the steady-state rate for this large enzyme concentration estimated from ordinary initial rate measurements. Similar experiments with the same enzyme and GAP concentrations showed that with smaller $NAD^+$ or inorganic phosphate concentrations the initial rate of the slow phase was smaller, but the amount of NADH formed in the dead-time did not change significantly. These results suggest fast hydride transfer from GAP to $NAD^+$ in the transient

phase of catalysis, followed by slower steps that involve $NAD^+$ as well as inorganic phosphate and limit the maximum turnover rate.

Figure 3 shows a progress curve obtained with 7.4 μM enzyme and, again, effectively saturating concentrations of GAP and NAD but with no added inorganic phosphate. It was recorded directly as the absorbance of the reacting mixture, backed off to allow display with high amplification, and the reactant blank has been subtracted. An absorbance increase of 0.32 in the dead-time is again followed by turnover to equilibrium because of inorganic phosphate contaminant in the GAP solution.

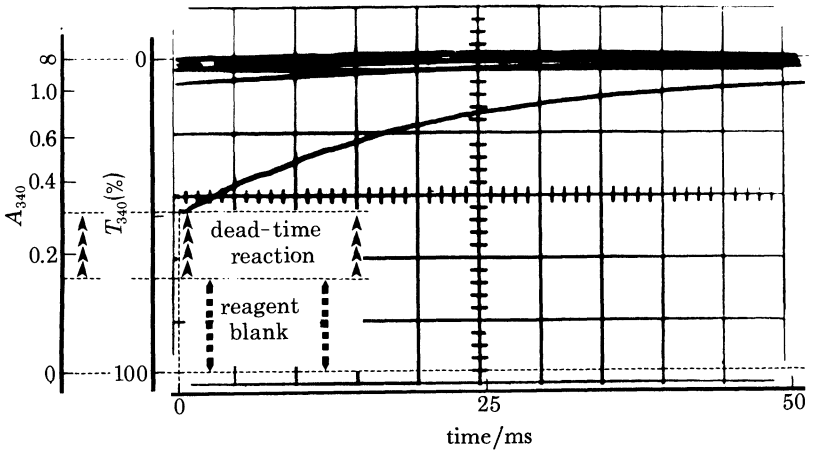


FIGURE 2. The time-course of NADH formation, monitored by decrease of light transmission at 340 nm, after mixing apoenzyme solution containing $NAD^+$ with GAP solution containing inorganic phosphate. The concentrations after mixing were 3.6 μM enzyme, 1 mM $NAD^+$, 3 mM D-GAP and 50 mM phosphate. The upper traces are continuations of the first curve to equilibrium, recorded by automatic repeated triggering of the oscilloscope.

A progress curve obtained with an initial $NAD^+$ concentration equal to the enzyme subunit concentration is shown in figure 4. NADH formation, causing an absorbance increase of 0.128, occurs during the 2 ms dead-time, but there is no further increase of absorbance during the next 20 ms, nor after several seconds as shown by a repeat sweep of the oscilloscope. With an initial $NAD^+$ concentration equal to only half the enzyme subunit concentration, the progress curve was similar except that the amplitude of the dead-time reaction was smaller.

The main results from these and other experiments with several initial molar ratios of NAD to enzyme are summarized in table 1. The amounts of NADH formed in the transient phase were calculated from the absorbance at time 0, obtained by extrapolating the initial, linear parts of the progress curves to −2 ms. An absorbance coefficient of 5.24 $mM^{-1}\,cm^{-1}$ was assumed; this is the difference between the absorbance coefficient for free NADH, 6.22 $mM^{-1}\,cm^{-1}$, and that for enzyme-bound NAD, 0.98 $mM^{-1}\,cm^{-1}$. The following conclusions can be drawn from the results obtained with 3 mM D-GAP.

1. For each initial molar ratio of $NAD^+$ to apoenzyme, the molar ratio of NADH formed to apoenzyme used is constant. Thus, the amount of NADH formed in the fast burst is strictly proportional to the apoenzyme concentration, over a twofold range with excess NAD and over a fourfold range with either 2 or 4 mol NAD per mole of enzyme.

2. With 500 μM $NAD^+$, the amplitude of the burst of NADH formation is almost equal to the

enzyme subunit concentration, which shows that NAD bound to all four subunits is reduced within 2 ms, and that the rate constant for the hydride transfer step at each subunit is at least 1000 $s^{-1}$. It appears that all four subunits are active simultaneously, at least during the first turnover, and there is no evidence of a half-sites or reciprocating-subunit mechanism. It should be noted that if the product of this fast phase is enzyme-bound NADH and not free NADH, its concentration will have been overestimated only if its absorbance coefficient is greater than that of free NADH. There is no precedent for this among dehydrogenases. Further, if the apoenzyme preparation was not fully active, the molar ratios will be underestimates.

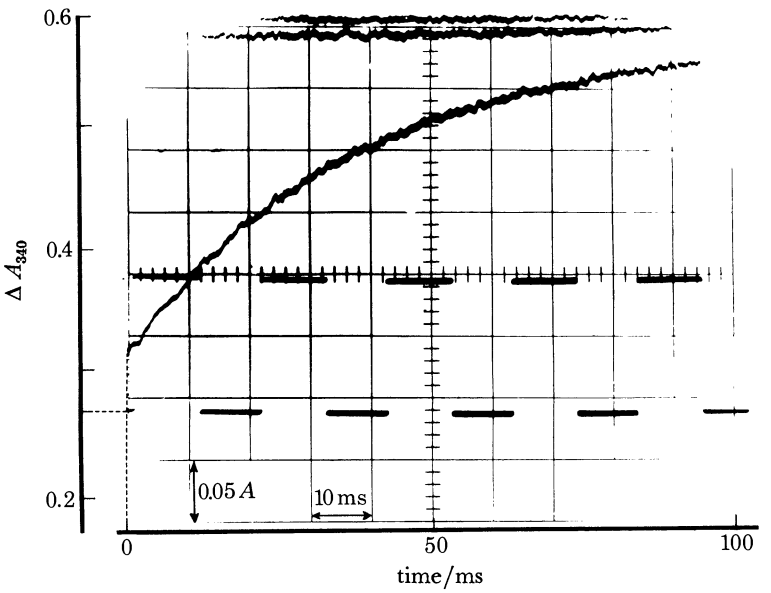


FIGURE 3. Progress curve for the reaction of 7.4 μM enzyme, 0.5 mM $NAD^+$ and 3 mM D-GAP monitored by increase of absorbance at 340 nm. The broken lines represent a square-wave potential and the lower level corresponds to the absorbance backed off, with the reactant blanks subtracted.

3. When the initial $NAD^+$ concentration is only four times the apoenzyme concentration, 80% is reduced within 2 ms after mixing with excess GAP, whereas with 2 mol $NAD^+$ per mole of apoenzyme more than 90% is reduced. This is consistent with tight and practically complete binding of $NAD^+$ to two subunits of the enzyme molecule and weaker, incomplete, binding to the other two, in accordance with the results of equilibrium binding studies (see below). Evidently $NAD^+$ reduction is not substantially slower in 'half-holoenzyme' than in holoenzyme. These results also indicate that the absorbance coefficient for NADH formed in the burst cannot be much smaller than the assumed value of 6.22 $mM^{-1}$ $cm^{-1}$. This speaks against an ordered mechanism (Orsi & Cleland 1972) with dissociation of the binary enzyme–NADH complex as the rate-limiting step because the absorbance coefficient for this complex is 5.0 $mM^{-1}$ $cm^{-1}$ (Pecson & Spivey 1972).

Like the results of Trentham (1971) for lobster enzyme, and those obtained by Pecson & Spivey (1972) in less detailed experiments with rabbit enzyme, our results are consistent with the pathway of catalysis shown in figure 1 and indicate that steps for the partial dissociation of $NAD^+$ prosthetic group from the tetrameric holoenzyme should be added. The experiments

seem to establish clearly that all four subunits are active in the redox step ($k > 1000\ s^{-1}$). In previous work this was inferred either by extrapolation from results with commercial enzyme preparations of relatively low specific activity (Pecson & Spivey 1972) or from results at pH 5.4 with several $NAD^+$ concentrations, which gave bursts much smaller than the enzyme subunit

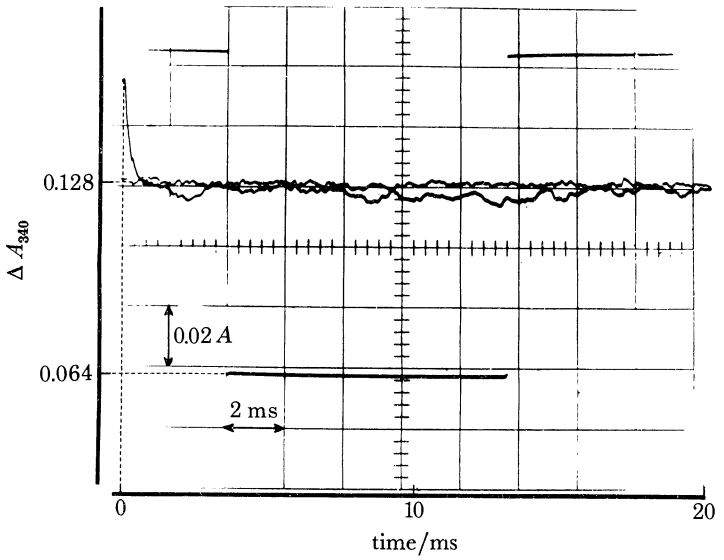


FIGURE 4. The time-course of NADH formation with 3.7 μM enzyme, 15 μM $NAD^+$ and 3 mM D-GAP.

TABLE 1. NADH FORMATION IN THE TRANSIENT PHASE

| | initial concentrations | | | | NADH formed | |
|---|---|---|---|---|---|---|
| | D-GAP mM | enzyme μM | $NAD^+$ μM | $NAD^+$/enzyme | NADH μM† | NADH/enzyme |
| (*a*) | 3.0 | 3.7 | 500 | 136 | 14.5 | 3.9 |
| | | 7.4 | 500 | 68 | 28.6 | 3.9 |
| | | 3.7 | 15 | 4.1 | 11.7 | 3.2 |
| | | 7.3 | 30 | 4.1 | 23.9 | 3.3 |
| | | 14.8 | 60 | 4.1 | 45.4 | 3.1 |
| | | 3.7 | 6.9 | 1.9 | 6.4 | 1.8 |
| | | 7.3 | 15 | 2.05 | 14.0 | 1.9 |
| | | 14.6 | 30 | 2.05 | 26.6 | 1.8 |
| (*b*) | 1.0 | 3.5 | 14.4 | 4.1 | 10.5 | 3.1 |
| | | 7.2 | 28 | 3.9 | 21.1 | 3.0 |
| | | 14.5 | 57 | 3.9 | 35.0 | 2.4 |
| | | 14.5 | 1000 | 70 | 35.0 | 2.4 |

† Calculated from an assumed absorbance coefficient of 5.24 $mM^{-1}\ cm^{-1}$ at 340 nm.

concentration, apparently because of ready dissociation of $NAD^+$ from the reactive ternary complex (Trentham 1971). Unlike Pecson & Spivey (1972), we could not detect dissociation of NADH from the acyl-enzyme–NADH complex as a separate step distinguishable from the preceding hydride transfer step.

A minimum estimate of $10^7\ M^{-1}\ s^{-1}$ for the rate constant for the combination of the aldehyde form of D-GAP with holoenzyme may also be inferred from these experiments, because the

pseudo-first-order rate constant for this reaction must also be at least 1000 $s^{-1}$; also, from the work of Trentham *et al.* (1969), the concentration of the aldehyde form in 3 mM D-GAP solution at pH 7.6 will be about 0.1 mM, the predominant diol form not being active as substrate. We confirmed this by recording progress curves with 1 mM D-GAP and several apoenzyme and $NAD^+$ concentrations (table 1). As is illustrated in figure 5, with this smaller substrate concentration the transient phase is not complete until about 8 ms after mixing, which indicates that combination of D-GAP with E.NAD is now rate-limiting for NADH formation in the transient phase, and allows a very approximate estimate of $3 \times 10^7$ $M^{-1}$ $s^{-1}$ for the rate constant, assuming

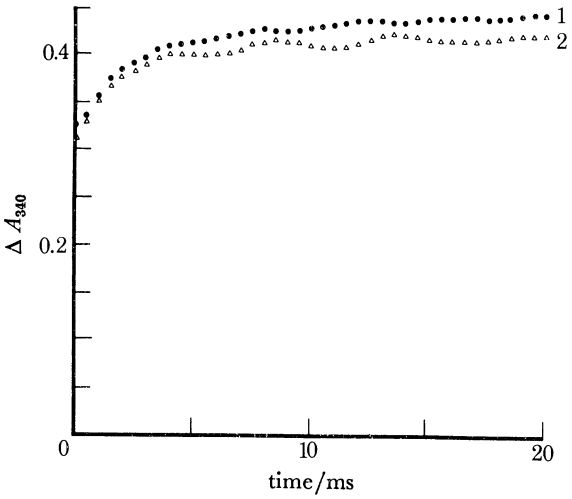


FIGURE 5. Progress curves for the reaction with 14.5 μM enzyme, 1 mM D-GAP and $NAD^+$: curve 1, 1 mM $NAD^+$; curve 2, 57 μM $NAD^+$.

that the concentration of the reactive aldehyde form in 1 mM D-GAP is 35 μM. The correctness of this assumption is shown by the results in table 1. With 1 mM D-GAP, 14.5 μM apoenzyme (58 μM subunits) and either 1 mM $NAD^+$ or 57 μM $NAD^+$ (figure 5), only 35 μM NADH (2.4 mol per mole of enzyme) was formed in the transient phase. With smaller enzyme concentrations and 4 mol $NAD^+$ per mole of enzyme, however, 3 mol NADH per mole of apoenzyme were formed, as in the experiments with 3 mM D-GAP (table 1).

### (*b*) *Steady-state kinetics*

The initial-rate equation for the Segal–Boyer mechanism (figure 1) shows that Lineweaver–Burk plots with the $NAD^+$ concentration as variable and several constant concentrations of GAP should be linear and parallel. However, linear but intersecting plots were obtained by Furfine & Velick (1965) and by Keleti & Batke (1965). Duggleby & Dennis (1974) suggested that these results might have been distorted by unsuspected product inhibition. Using a recording fluorimeter of sufficient sensitivity to give practically linear progress curves with small concentrations of $NAD^+$ and GAP, and thus preclude product inhibition, Meunier & Dalziel (1978) found that Lineweaver–Burk plots are not linear, but concave downwards. These results are consistent with the pathway for catalysis in figure 1 if partial dissociation of $NAD^+$ from the tetrameric holoenzyme occurs. The behaviour of NADH and adenosine diphosphoribose (ADPR) as inhibitors of the oxidative phosphorylation reaction was also found to support this

pathway. Both these nucleotides show competitive kinetics when the $NAD^+$ concentration is varied, but when the $NAD^+$ concentration is small and the concentration of GAP is varied, NADH is a non-competitive inhibitor whereas ADPR is uncompetitive (Meunièr & Dalziel 1978). This is to be expected if both nucleotides compete with $NAD^+$ for acyl-enzyme (figure 1) because NADH can also reverse enzyme acylation by GAP, whereas ADPR can only form a dead-end complex. Competition with $NAD^+$ for apoenzyme in the sequential pathways proposed by Furfine & Velick (1965), Keleti & Batke (1965) and Orsi & Cleland (1972) would result in non-competitive inhibition by both nucleotides when the concentration of GAP is varied.

## 4. Negative cooperativity in coenzyme binding

After the molecular mass and subunit composition of the enzyme had been established (Harrington & Kerr 1965; Harris & Perham 1965, 1968) it became clear that the equilibrium between rabbit muscle apoenzyme and $NAD^+$ cannot be described by a single equilibrium

Table 2. Dissociation constants for the NAD complex of rabbit muscle enzyme at 20–25 °C

(The dissociation constants are defined by the equilibrium

$$E(NAD)_4 \overset{K_4}{\rightleftharpoons} E(NAD)_3 \overset{K_3}{\rightleftharpoons} E(NAD)_2 \overset{K_2}{\rightleftharpoons} E(NAD) \overset{K_1}{\rightleftharpoons} E.)$$

| pH | $K_4$ / μM | $K_3$ / μM | $K_2$ / μM | $K_1$ / μM | reference |
|---|---|---|---|---|---|
| 7.6 | 318 | 21 | — | — | Price & Radda (1971) |
| | 144–250 | 12–25 | 0.2 | 0.01 | Bell & Dalziel (1975) |
| 8.2 | 35 | 4 | < 0.05 | < 0.05 | De Vijlder & Slater (1968) |
| | 34 | 1.7 | — | — | Price & Radda (1971) |
| | 28 | 1.9 | < 0.1 | < 0.1 | Allen & Harris (1975) |
| 9.4 | 3.2 | 1.2 | 0.53 | 0.2 | Reynolds & Dalziel (1979) |

constant, and negative cooperativity between the four chemically identical subunits was proposed as the likeliest explanation (Conway & Koshland 1968; De Vijlder & Slater 1968). The equilibrium can be described in terms of four dissociation constants for the stepwise dissociation of $E(NAD)_4$, and values reported by several groups of workers for the rabbit enzyme are listed in table 2. From these, it seems clear that at pH 7.6 and 8.2, $K_1$ and $K_2$ are much smaller than $K_3$ and $K_4$, even when statistical factors are taken into account. There is also a consistent and significant difference between $K_4$ and $K_3$ at each of these pH values. At pH 9.4, however, the binding of four molecules of $NAD^+$ to the rabbit enzyme can be described by a single microscopic dissociation constant of 0.8 μM, meaning that the four dissociated constants are related by statistical factors (table 2) and that the subunits behave as independent binding sites with identical affinities. Increase of pH evidently weakens the binding of two $NAD^+$ molecules and strengthens the binding of two more.

Similar results were reported for the lobster enzyme (table 3), including a significantly greater $K_4$ than $K_3$. Simpler behaviour was observed with the enzymes from sturgeon muscle and *B. stearothermophilus* in that the binding of four equivalents of $NAD^+$ could be described adequately by only two dissociation constants. This simpler behaviour has also been reported recently for rabbit muscle enzyme purified by a new method (Scheek & Slater 1978), in which

ion-exchange chromatography replaces the repeated crystallizations from ammonium sulphate involved in the longer, classical procedure. The larger of the two dissociation constants reported for this preparation (table 3) is much smaller than $K_3$ and $K_4$ for the classical preparation (table 2), and is similar to those for the sturgeon and *B. stearothermophilus* enzymes. These new findings imply that enzyme purified from rabbit muscle by the classical procedure, and used for all kinetic studies as well as earlier binding studies, may have been heterogeneous or, alternatively, altered during isolation (Scheek & Slater 1978).

The behaviour common to all these enzyme preparations is tight binding of two equivalents of $NAD^+$ and weaker binding of two more. On the assumption that heterogeneity of all the

TABLE 3. DISSOCIATION CONSTANTS FOR NAD COMPLEXES OF ENZYME FROM SEVERAL SOURCES AT 25 °C

(The dissociation constants are defined in table 2.)

| source of enzyme | pH | $K_4$ / μM | $K_3$ / μM | $K_2$ / μM | $K_1$ / μM | reference |
|---|---|---|---|---|---|---|
| lobster muscle | 8.2 | 13 | 0.6 | < 0.005 | | De Vijlder *et al.* (1969) |
| sturgeon muscle | 7.0 | 1.1 | | 0.09 | | Keleman *et al.* (1975) |
| | 7.0 | 1.4 | | 0.18 | | Long & Dahlquist (1977) |
| *B. stearothermophilus* | 8.2 | 2.0 | | < 0.1 | | Allen & Harris (1975) |
| rabbit muscle | 7.0 | 0.9 | | — | | Scheek & Slater (1978) |
| | 7.0 | 0.27† | | 0.028† | | Scheek & Slater (1978) |

† At 15 °C.

preparations and chemical differences between the subunits can be ruled out, two hypotheses have been suggested to explain both this behaviour and the 'half-sites' reactivity of the enzyme towards certain thiol alkylating and acylating reagents. One is pre-existing asymmetry of the apoenzyme molecule, one pair of subunits having a different conformation, and therefore different intrinsic affinities for $NAD^+$, from the other pair (Bernhard & MacQuarrie 1973). The alternative hypothesis is that the four subunits of the apoenzyme are entirely identical and have the same intrinsic affinity for $NAD^+$, and binding of $NAD^+$ to two subunits induces a conformation change in the other two which decreases their affinity for $NAD^+$ (Levitzki & Koshland 1976). To obtain evidence for the molecular basis of negative cooperativity has been one of the main objectives of X-ray crystallographic studies of the enzymes from lobster muscle and *B. stearothermophilus*.

## 5. STRUCTURE OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

The subunit of the enzyme is composed of two domains; the coenzyme-binding domain closely resembles those of lactate and alcohol dehydrogenases (Rossmann *et al.* 1975). The active site is in a cleft between the coenzyme-binding domain and the catalytic domain, with the essential thiol group of Cys 149 close to the nicotinamide C-4 atom of $NAD^+$ (figure 6). The unique feature of this enzyme is an S-shaped loop in the catalytic domain comprising residues 178–201 (Moras *et al.* 1975; Biesecker *et al.* 1977).

The subunits of the tetrameric enzyme are related by three orthogonal twofold axes, designated *P*, *Q* and *R* (Rossmann *et al.* 1973). The arrangement of subunits brings the S-loop regions




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into close contact at the centre of the tetramer, with pairs of active sites related by the *R*-axis twofold in close proximity.

Figure 6 is a diagrammatic representation of the relation between the S-loop of one subunit and the coenzyme molecule bound to the *R*-axis related subunit. The probable mode of binding of the substrate, inferred from model building, is by thiohemiacetal formation with Cys 149, and binding of the phosphate group by one of the two anion binding sites present in the subunit. It can be seen that the S-loop of this subunit interacts with the active centre through Thr 179,

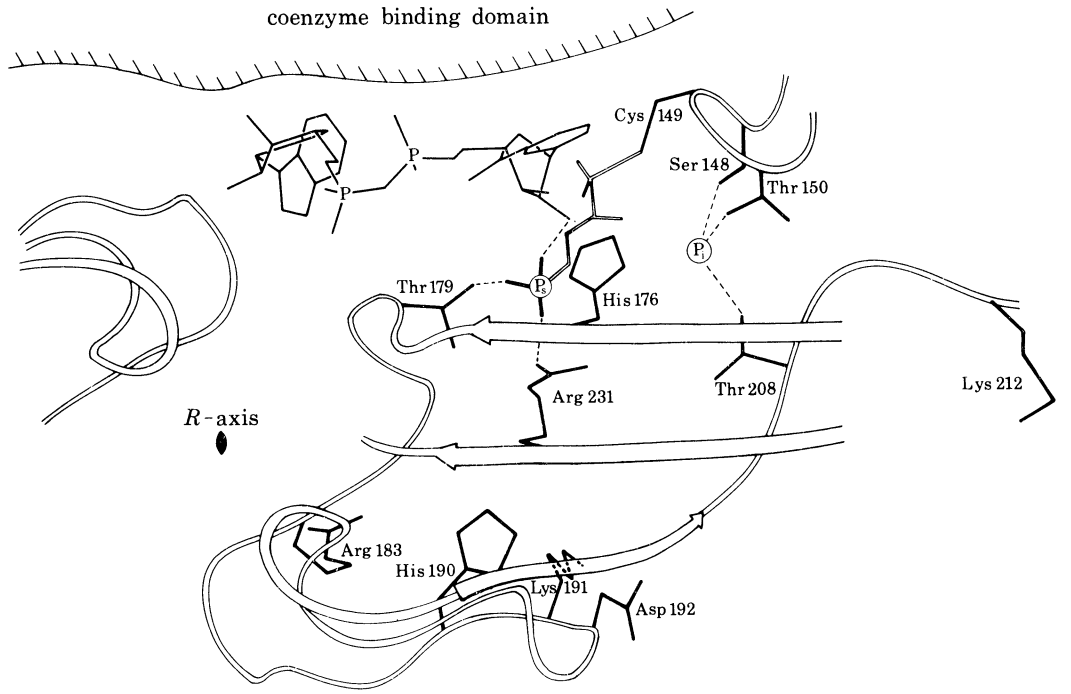


FIGURE 6. Diagrammatic representation of the active centre region of the tetrameric molecule of the enzyme from *B. stearothermophilus* showing the binding of $NAD^+$ and the disposition of the S-loop regions relative to the *R*-axis. In the lobster muscle enzyme, Arg 183 is replaced by a lysine residue.

and that the S-loop of the *R*-axis related subunit makes contact with the adenosine moiety of the coenzyme (figure 6). Thus, the S-loop is a potential means of information transfer between the two subunits.

From the earlier crystallographic studies of human muscle holoenzyme by Watson *et al.* (1972), it was established that the *Q*-axis in these crystals is an exact twofold symmetry axis. The lobster enzyme and the bacterial enzyme crystallize with the whole tetramer in the asymmetric unit so that *a priori* only approximate 222 symmetry could be inferred. While the high-resolution structures of the two enzymes are in good agreement as regards the main features of the subunit conformation, there is disagreement about the extent of asymmetry between subunits in a tetramer. For the lobster holoenzyme, a number of differences between the two pairs of subunits related by the *R*-axis have been described (Moras *et al.* 1975). These include differences of coenzyme conformation and different orientations of some amino-acid side chains in the active centre region. In a subsequent paper Olsen *et al.* (1976) showed that a bromine-labelled $NAD^+$ analogue was bound with the same conformation to all four subunits.

On the other hand, it has been concluded that in the bacterial holoenzyme molecule, the four subunits and their bound coenzyme molecules are all identical, to within the limits of error (Biesecker *et al.* 1977). Until these structures are refined, it is not possible to be sure whether the holoenzyme molecule is asymmetric or not.

There is both chemical and crystallographic evidence to suggest that binding of $NAD^+$ to the apoenzyme is accompanied by structural changes, especially in the S-loop region. The chemical evidence is from experiments with enzyme from rabbit and pig muscle, in which residue 183 is lysine (figure 6). Acetylation of apoenzyme with *p*-nitrophenylacetate or acetyl phosphate at pH 8.5 results in acetylation of this group by acyl transfer from Cys 149 (Park *et al.*

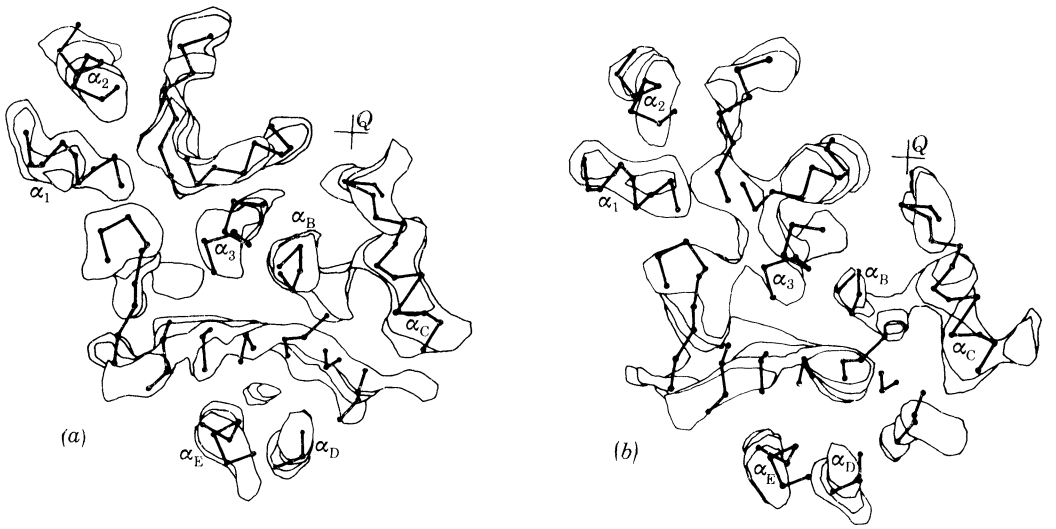


FIGURE 7. Superimposed sections of equivalent regions of the low-resolution electron density maps of (*a*) holoenzyme and (*b*) apoenzyme from *B. stearothermophilus* with the α-carbon backbone of the holoenzyme overlaid. α-Helices are labelled according to Biesecker *et al.* (1977).

1966; Harris & Polgar 1965). This does not occur with holoenzyme, in the structure of which these residues are about 20 Å apart. It can be inferred that either in the apoenzyme or after acylation at Cys 149, the residues are closer to one another than in the holoenzyme. The fact that pyridoxal phosphate reacts specifically with Lys 191 and Lys 212 in the holoenzyme, but only with Lys 212 in the apoenzyme, also suggests that the orientation of S-loop relative to the active-centre region may be different in the two enzyme forms (Forcina *et al.* 1973; Zapponi *et al.* 1973). Pyridoxal phosphate inhibits the enzyme competitively with GAP, and model building with the holoenzyme structure shows that if the phosphate group of pyridoxal phosphate binds to the anion binding site in place of the substrate, the aldehyde group is well positioned to form a Schiff's base with Lys 191 as well as Lys 212 (figure 6).

The structure of the crystalline apoenzyme from *B. stearothermophilus* has been determined at low resolution (Wonacott & Biesecker 1977). In figure 7, 6 Å electron density maps for apoenzyme and holoenzyme are compared, and show that binding of $NAD^+$ is accompanied by a rotation of the whole coenzyme-binding domain away from the active site. Movements of 3–5 Å by helices $\alpha_D$ and $\alpha_E$ in the coenzyme-binding domain are clearly visible. The S-loop conformation is likely to be influenced by this rotation since the coenzyme-binding domain




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forms an interface with the S-loop from the *R*-axis related subunit (figure 6). For the human enzyme, also, a 6 Å resolution difference Fourier synthesis for the almost isomorphous holoenzyme and apoenzyme shows that the loss of $NAD^+$ is associated with concerted movements of the polypeptide chain, particularly in the S-loop region (H. C. Watson, personal communication). However, it has been reported recently by Murthy *et al.* (1980) that the structure of lobster apoenzyme at 3 Å resolution shows no significant difference from that of the holoenzyme. The authors suggest that conformation changes may be inhibited by the low pH of crystallization (pH 6.2), the apoenzyme being unstable at higher pH values. No statement is made regarding the presence or absence of symmetry.

## 6. Concluding remarks

The pathway of catalysis shown in figure 1 accounts for the kinetics of the enzyme from rabbit muscle, as well as from some other sources, if allowance is made for the relatively ready dissociation of two molecules of $NAD^+$ from the tetrameric holoenzyme. It seems that this negative cooperativity may be exaggerated by modification of the rabbit muscle enzyme during isolation, but the enzymes from all sources (except yeast) studied so far do appear to bind two molecules of $NAD^+$ tightly and two more weakly. The molecular basis of this negative cooperativity is not yet clear. Asymmetry of the apoenzyme tetramer, implied by the close similarity of high-resolution structures of apoenzyme and holoenzyme from lobster muscle and the reported asymmetry of the latter, could provide a sufficient explanation. Alternatively, the substantial conformation changes that accompany the binding of $NAD^+$ to apoenzyme from *B. stearothermophilus* may include relatively small changes of subunit interaction responsible for negative cooperativity. The S-loop remains a likely locus for such interaction, which need be no more than the making and breaking of a salt linkage, for example.

It has been speculated that the change from negatively cooperative binding at pH 7.0–8.2 to apparently independent binding at pH 9.4, observed with the rabbit muscle enzyme, might be associated with deprotonation of Lys 183 in the S-loop and breakage of an inter-subunit salt linkage (Reynolds & Dalziel 1979). Preliminary experiments with the bacterial enzyme, in which residue 183 is arginine ($pK \approx 12$) indicated that negative cooperativity persisted at pH 9.4. In view of the large heats of ionization of such cationic acids, the decrease of negative cooperativity with increase of temperature reported for the enzyme from rabbit muscle (Velick *et al.* 1971) and sturgeon (Kelemann *et al.* 1975) might also be associated with deprotonation of such a residue. For the rabbit muscle enzyme, the absence of negative cooperativity at 37 °C (Velick *et al.* 1971), together with the small dissociation constants for $NAD^+$ from the 'weak' sites reported for a recent preparation, make it improbable that the phenomenon has any relevance to metabolic control.

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## *Discussion*

T. Keleti (*Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary*). I should like to comment on only one point of the very interesting and exhaustive presentation by Dr Dalziel. The negative cooperativity can also be interpreted in another way. Conway & Koshland (1968), when elaborating the concept of negative cooperativity, implicitly assumed that the oligomeric enzyme involved does not dissociate. However, it is well known that glyceraldehyde-3-phosphate (GAP) dehydrogenase is a very easily dissociable tetramer (Hoagland & Teller 1969; Lakatos *et al.* 1972; Lakatos & Závodszky 1976; Ouádi *et al.* 1979). The different oligomeric forms are enzymatically active (Ovádi *et al.* 1971; Nagradova 1974), but their specific activities are different (Ovádi *et al.* 1979). The substrate saturation curves and binding

functions of associable–dissociable systems may differ from Michaelis–Menten–type hyperbolic behaviour and may imitate allosteric, cooperative systems (Kurganov 1967; Nichol *et al.* 1967; Frieden 1967; Batke 1972). If in a dissociable oligomeric system the higher oligomeric form is less active than the lower one(s), in certain conditions one can observe negative cooperativity-like behaviour (Keleti *et al.* 1977). In this case the ligand-binding affinities of active centres in the tetramer are identical but differ from those in the dimer and monomer. The apparent negative cooperativity imitated by the dissociation of an oligomeric protein can be distinguished from genuine negative cooperativity by analysing the enzyme–ligand dissociation constants in the function of enzyme concentration. These will be concentration-invariant in real negative cooperativity but will depend on protein concentration in an apparent one (Keleti *et al.* 1977). Indeed, in GAP dehydrogenase the dissociation constant is of ligands depend on protein concentration (Keleti *et al.* 1977; Kálmán *et al.* 1980), i.e. the apparent negative cooperativity of coenzyme binding is due to the dissociation–association of the enzyme.

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K. Dalziel. I agree that dissociation of an oligomeric enzyme could provide the basis for apparent negative cooperativity, and also that there is good evidence that glyceraldehyde-3-phosphate dehydrogenase does dissociate into dimers and monomers, especially at low temperatures. However, there does not seem to be conclusive evidence that this explains the apparent negative cooperativity of $NAD^+$ binding, which occurs at temperatures and with enzyme concentrations such that all the enzyme is in the tetrameric form, and which some workers (e.g. Scheek & Slater 1978) found to be unaffected by change of enzyme concentration. Moreover, the conclusion that the tetramer is the inactive form of the enzyme (Ovádi *et al.* 1971) does seem to be at variance with our results and those of many other workers.


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