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SUBUNIT INTERACTIONS IN GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES

THEIR INVOLVEMENT IN NUCLEOTIDE BINDING AND COOPERATIVITY

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Summary

1. The hybridization of rabbit muscle and yeast glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) was used to study the involvement of subunit interactions in NAD^+ and NADH binding by these enzymes.

2. In the presence of 1 mM NAD^+ or NADH no hybrid formation was observed with our preparations of the two enzymes.

3. The inhibition by NADH of the hybrid formation is shown to be a consequence of an unfavourable equilibrium of the hybridization process in the presence of NADH.

4. The inhibition by NAD^+ of the hybrid formation is shown to be a consequence of both a shift in the equilibrium, as in the case of NADH, and a decrease in the rate of the dissociation of the enzymes.

5. The dimer of the yeast enzyme binds NAD^+ or NADH with equal affinity irrespective of whether it is combined with another yeast dimer in the yeast tetramer or with a rabbit muscle dimer in the hybrid.

6. The binding of NAD^+ and NADH to the dimer of the rabbit muscle enzyme is stronger in the rabbit muscle tetramer than in the hybrid; this explains the shift in the equilibrium of the hybridization process caused by these nucleotides.

7. Alkylation of the rabbit muscle enzyme with iodoacetate does not influence the hybridization process in the absence of nucleotides.

8. After alkylation of the rabbit muscle enzyme NADH cannot cause a large shift in the equilibrium of the hybridization process.

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9. In accordance with this it was found that the binding of NADH (and NAD^+) to the rabbit muscle enzyme is weakened by alkylation, whereas the binding of NADH to the alkylated rabbit muscle subunits is not affected strongly by the hybridization.

10. An attempt is made to combine the effects of nucleotides on the hybridization properties of the yeast enzyme and the alkylated or unalkylated rabbit muscle enzymes with the binding properties of all tetrameric species involved in the hybridization processes in a thermodynamic description of nucleotide binding and subunit interactions.

Introduction

Hollaway and coworkers [1—3] have shown how a study of the kinetics and thermodynamics of the hybridization of glyceraldehyde-3-phosphate dehydrogenases (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) from rabbit muscle and yeast can give valuable information about interactions between the four identical subunits of each of the proteins. They studied the effects of ligands bound by these enzymes on the hybridization process in an attempt to correlate the observed effects on NAD^+ and NADH on the rate and extent of hybrid formation with the cooperative binding properties of these nucleotides. However, the observation that NAD^+ and NADH have qualitatively different effects on the hybridization process could not be correlated in a straightforward manner with the 'negative cooperativity' observed in the binding of both nucleotides to their preparation of the rabbit muscle enzyme.

Since our preparation of the rabbit muscle enzyme was shown to bind NAD^+ and NADH in a more homogeneous manner, that is without the pronounced negative cooperativity [4,5] which has become the main feature of nucleotide binding by the muscle enzyme, it is interesting to repeat the hybridization experiments with our preparation of the rabbit muscle enzyme. The results are described in this paper. In order to be able properly to interpret these hybridization experiments, the binding properties of all tetrameric species involved have been determined.

Methods

The enzyme was isolated from rabbit skeletal muscle as described previously [4] and from commercial baker's yeast (*Saccharomyces cerevisiae*) as described by Kirschner and Voigt [6]. The specific activities were 180 and 117 μmol/min per mg protein, respectively, determined by the method of Ferdinand [7], not correcting for incomplete saturation with P_i.

The tetracarboxymethyl-enzyme was prepared by the addition of an excess (5 mol/mol enzyme) of iodoacetate to the holoenzyme, immediately before the enzyme solution was brought on the CM-Sephadex column to remove the bound NAD^+ [4]. The enzyme was completely inactivated by this procedure.

All experiments were carried out in a buffer (pH 7.8) containing 10 mM phosphate, 1 mM EDTA and 1 mM dithiothreitol, unless indicated otherwise.

The hybridizations were carried out in this buffer at 37°C; samples were taken during the incubation and stored at 5°C in the phosphate buffer, to which 1 mM NAD^+ and glycerol (20%, v/v) were added, in order to stop any further hybrid formation or reversion (see Results). Electrophoresis on 5% polyacrylamide gels [8] was carried out at 10°C with about 10 μg of protein in 50—100 μl in a buffer (pH 8.8) containing 0.1 M Tris, 0.063 M boric acid and 2 mM EDTA. The gels were stained with Coomassie brilliant blue and destained in CH_3OH/acetic acid. Scans were run spectrophotometrically at 500 nm on a Zeiss spectrophotometer and peak surfaces were determined with the aid of a Tektronix 4954 Graphics tablet. The hybrid was isolated as described by Osborne and Hollaway [1] and contained none of the other enzyme species as judged from polyacrylamide gel electrophoresis (see above). The concentration of the isolated hybrid was determined spectrophotometrically at 280 nm. Since no change occurs in the ultraviolet spectrum of an equimolar mixture of the rabbit muscle enzyme and the yeast enzyme during hybridization [3], the extinction coefficient of the hybrid equals the mean of those of the pure apoenzymes [2].

All fluorescence measurements were performed at 25°C on a Perkin Elmer spectrofluorimeter model MPF 2a, using thermostated cells with a light path of 1 cm. The inner-filter quenching coefficient was determined for each pair of wavelengths used from the decline of the fluorescence of the rabbit muscle enzyme after complete saturation with NAD^+ or NADH had been reached [9].

Thermodynamic description of subunit interactions and ligand binding. The results presented in this paper will be discussed in terms of a thermodynamic description of subunit interactions and ligand binding illustrated in Fig. 1.

In Fig. 1A are shown the Gibbs-energy levels for a single polypeptide chain

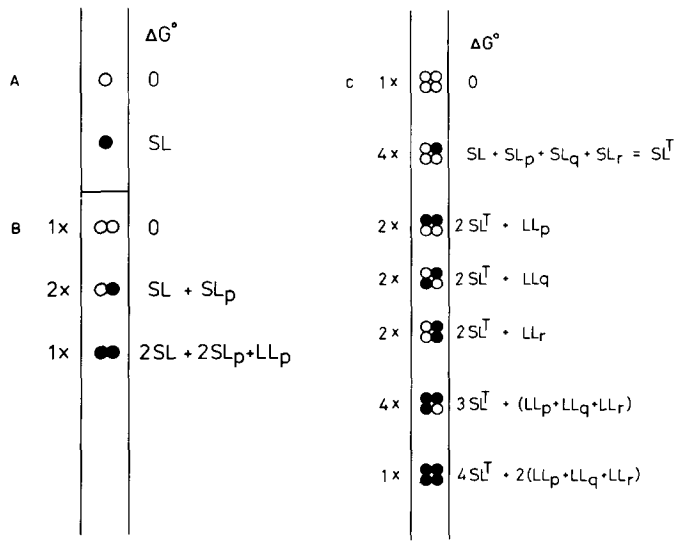


Fig. 1. Gibbs-energy levels for the different ligation states of a monomer (A), a dimer (B) and a tetramer (C) with one binding site for a ligand per monomer. ○, unliganded polypeptide chains; ●, liganded ones. For explanation of the terms: see text.

(represented by an open circle) with one binding site for a ligand. The energy level of the unliganded chain is set as zero, and SL represents the change in Gibbs energy occurring when one ligand molecule is bound by this isolated subunit (the closed circle).

The dimer, consisting of two identical monomers in contact via a P-boundary, is shown in Fig. 1B. Again the unliganded state was chosen as the zero level and the Gibbs-energy change upon binding of one ligand molecule is represented by $SL + SL_p$, where SL_p is the energy of interaction between the second monomer and the ligand, both of which can be considered as liganded to the first monomer. A negative value for SL_p means that the ligand binds more strongly to the dimer than to the monomer, but also that the second monomer binds more strongly to a liganded monomer than to an unliganded one, so the subunit interactions across the P-boundary are strengthened by the binding of a ligand molecule.

The second ligand, then, binds with a change in Gibbs energy equal to $SL + SL_p + LL_p$. If $LL_p = 0$, both ligands bind with equal strength and no cooperativity occurs. Negative cooperativity occurs when $LL_p > 0$ and positive cooperativity when $LL_p < 0$. This influence between the ligands may be exerted directly, for example by electrostatic or even direct steric interactions between the ligands on binding sites that are not too far apart, or indirectly, via disturbances of the energetic balance of the polypeptide chain in a common region, most likely in some part of the boundary region [10].

The tetrameric case is illustrated in Fig. 1C. X-ray crystallographic studies [11,12] have revealed that the four identical monomers of glyceraldehyde-3-phosphate dehydrogenase are combined in a tetraeder and three different kinds of dimers, each with its own type of boundary (designated P, Q and R) are present in such a structure. It is reasonable, therefore, to assume that three different kinds of interactions occur across these boundaries and these can be recognized in the Gibbs-energy levels of the tetramer as the P, Q and R terms, which describe the changes in the subunit interactions across the P, Q and R boundaries, respectively, that occur upon ligand binding. Again, as in the dimer case, the LL terms describe the cooperativity, while the terms SL_p, SL_q and SL_r, modulated by the corresponding LL terms, describe the effects of ligand binding on the subunit interactions.

This treatment of ligand binding to a tetramer is not as general as that given by Adair [13]. It predicts that the ratio of the first and second macroscopic dissociation constants is equal to the ratio of the third and fourth constants, i.e. $K_1/K_2 = K_3/K_4$. The reason for this symmetry is that in our description the assumption was made that the binding of ligand to one of the subunits only modulates the interactions between this subunit and the three other ones, leaving unchanged all interactions amongst these three other subunits. Since it is known that ligand-induced conformational changes are most extensive in the polypeptide chain to which the ligand is bound [14], this approximation is not unreasonable.

Our previous binding studies on rabbit muscle glyceraldehyde-3-phosphate dehydrogenase [5] gave information on the sum of the four SL terms and the sum of the three LL terms: $SL + (SL_p + SL_q + SL_r)$, calculated from the dissociation constant measured at low saturation levels, equals −39 kJ/mol and

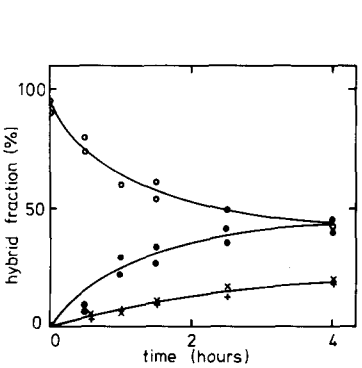
−35 kJ/mol for binding of NAD^+ and NADH, respectively, at 16°C. The total interaction energy ($LL_p + LL_q + LL_r$), calculated from the ratio of the dissociation constants measured at low and at high saturation levels, equals 2 kJ/mol for both NAD^+ and NADH.

From these binding data, however, we cannot decide whether the binding of NAD^+ or NADH causes a strengthening or a loosening of subunit interactions, because by studying the tetramer we can only quantitate the sum of all SL terms. The only way to resolve this sum into its individual terms is by studying the nucleotide binding by the three different dimers and the monomer, but this is not easily done directly because of the low concentrations of monomer and dimers. The approach we chose, following Osborne and Hollaway [2] and Hollaway et al. [3], is to change the character of two of the three boundaries and study the influence of this change on nucleotide binding and subunit interactions. This was achieved by hybridization of the rabbit muscle enzyme with the enzyme isolated from yeast.

Results

Hybridization experiments

In the absence of nucleotides hybrid formation in an equimolar mixture of the rabbit muscle enzyme (R_4) and the yeast enzyme (Y_4) in the phosphate buffer is complete within 4 h at 37°C (Fig. 2). In the equilibrium mixture about 40% of the protein is present as the hybrid (R_2Y_2), which was isolated and incubated again to study the reversion. Again it takes about 4 h to reach the same equilibrium situation (Fig. 2). This is in agreement with the results of Osborne and Holloway [1].



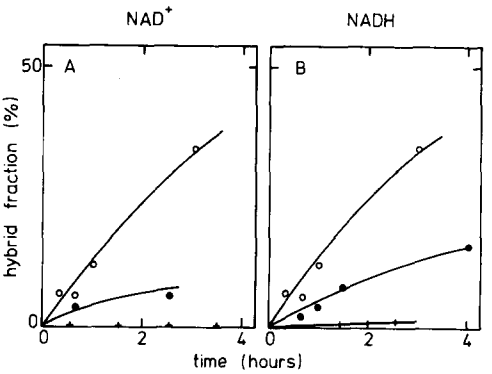


Fig. 2. Hybrid formation and reversion. Equal amounts of the rabbit muscle and the yeast enzyme (12 μM) (•) or unequal amounts of the two enzymes (16.4 μM R_4 and 3.3 μM Y_4 (X), 3.5 μM R_4 and 17.8 μM Y_4 (+)) were incubated in the phosphate buffer at 37°C. The hybrid was isolated as described [1] and the reversion of a 9 μM solution was followed under the same conditions (○). The amount of hybrid is given as the percentage of the total amount of protein used in the experiment.

Fig. 3. Hybrid formation in the presence of nucleotides. (A) The effect of NAD^+ on the hybrid formation. Starting conditions: 16 μM R_4 and Y_4; [NAD^+]: ○, 0 μM; •, 16 μM; no hybrid formation was observed with 32 μM, 48 μM, 64 μM or 1 mM NAD^+ present (X). (B) The effect of NADH on the hybrid formation. Starting conditions: 16 μM R_4 and Y_4: [NADH]: ○, 0 μM; •, 16 μM; X, 32 μM; no hybrid formation was observed with 48 μM, 64 μM or 1 mM NADH present.

No difference was observed in the kinetics of hybrid formation or reversion when the proteins were diluted; lowering the concentrations of either of the two enzymes relative to the other gave the result presented in Fig. 2. This shows that the two enzymes used by us have a similar dissociation rate constants (contrast Ref. 1).

Hybrid formation is inhibited completely by excess of either NAD^+ or NADH (Fig. 3). Although at the lowest concentrations of NAD^+ and NADH used, maximally 25% of the binding sites in the rabbit muscle enzyme would be occupied, there are large effects on the hybrid formation. These could be due to a shift in the equilibrium or to a decrease in the rate(s) of dissociation of the enzyme(s) or to a combination of the two factors.

The reversion experiments shown in Fig. 4 seem to indicate that NAD^+ and NADH have fundamentally different effects on the hybrid. NADH promotes the dissociation of the hybrid, finally resulting in a complete reversion to the original tetramers, whereas NAD^+ seems to stabilize the hybrid, at least if added in excess.

However, in low concentrations NAD^+ has an effect similar to that of NADH. Thus the effect of NAD^+ in high concentration must be kinetic in origin. By inhibiting the dissociation, it freezes the mixture in its starting composition. There is no indication that NADH and NAD^+ have appreciably different effects on the position of the equilibrium finally reached.

Although it is clear that both NADH and NAD^+ shift the equilibrium from the hybrid to the symmetrical tetramers, these experiments give no information on which of the three tetrameric species is stabilized or destabilized in the thermodynamic sense. The binding studies which will now be described were carried out in an attempt to resolve this question.

Binding of NAD^+

The shifts in the hybridization equilibrium in the presence of NAD^+ and NADH show that both ligands bind more strongly to an equimolar mixture of the rabbit muscle enzyme (R_4) and the yeast enzyme (Y_4) than to the hybrid (R_2Y_2). We can describe these shifts by introducing an apparent equilibrium

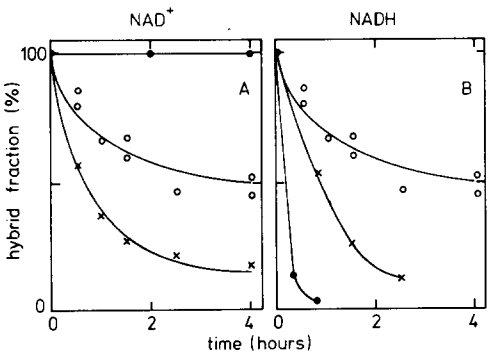


Fig. 4. Hybrid reversion in the presence of nucleotides. (A) The effect of NAD^+ on the hybrid reversion. Starting conditions; 9 μM R_2Y_2; [NAD^+]: ○, 0 μM; X, 9 μM, and ●, 1 mM. (B) The effect of NADH on the hybrid reversion. Starting conditions: 15 μM R_2Y_2; [NADH]: ○, 0 μM; X, 60 μM, and ●, 1 mM.

constant for the hybridization process:

$$K_{eq} = \frac{[R_2Y_2]^2}{[R_4] \cdot [Y_4]}$$

$$K_{eq}^{app} = K_{eq} \frac{(1 + pL/K_r)^4(1 + qL/K_y)^4}{(1 + L/K_r)^4(1 + L/K_y)^4} \quad (1)$$

in which K_r and K_y are the (mean) dissociation constants for ligand binding to the rabbit muscle and the yeast enzyme, respectively, L stands for the concentration of free ligand and K_r/p and K_y/q are the dissociation constants for ligand binding to R_2 (in R_2Y_2) and Y_2 (in R_2Y_2), respectively. At saturating concentrations of the ligand this expression simplifies to:

$$K_{eq}^{app} = K_{eq} \cdot (p \cdot q)^4$$

The assumption made in deriving these formulae is that ligand binding to both R_4 and Y_4 can be treated as binding to a homogeneous pool of binding sites, whereas two kinds of sites, on R_2 and Y_2, are assumed to be present in the hybrid tetramer. Thus, in principle, measuring the shifts in the hybridization equilibrium can give information, via the factors p and q in Eqn. 1, about the consequences for the affinity of the ligand of the changes in the interactions across the altered boundary regions in the hybrid tetramer. This information is more easily obtained, however, from binding studies.

For this purpose we made use of the quenching of the protein fluorescence by bound nucleotides. This technique was chosen instead of equilibrium dialysis, because a fluorimetric titration can be performed sufficiently rapidly so that hardly any change in the ratio of the three enzymes takes place during a titration carried out at 25° C.

The quenching of the fluorescence of the rabbit muscle enzyme by NAD^+ was measured at 25°C in the phosphate buffer and analysed as described before [5]. This binding can be described with a mean dissociation constant of 0.5 μM, thereby ignoring the slight negative cooperativity.

The weak binding of NAD^+ to the yeast enzyme poses problems. The titration given in Fig. 5 shows that complete saturation is not reached at 1 mM NAD^+. The initial part of the titration is characterized by positive cooperativity, but beyond about 50% quenching there is an apparent negative cooperativity. This combination of positive and negative cooperativity is a well-documented feature of the yeast enzyme [15—18]. Comparison of our titration of the yeast enzyme with published direct binding studies [18,19] indicates that saturation is probably almost complete at the highest concentration of NAD^+ used in our study, and it was assumed that 75% quenching corresponds to saturation. Assuming also that the degree of quenching is linearly related to the saturation of the binding sites, the concentration of free NAD^+ could be calculated to construct Fig. 5. A mean dissociation constant of 8 μM can be estimated from this titration curve to describe the initial part of the binding process displaying positive cooperativity. The last part of the titration probably reflects what Gennis called affinity heterogeneity [19].

The titration of an equimolar mixture of the rabbit muscle and the yeast enzyme is shown in Fig. 6. The relative contributions of the muscle enzyme

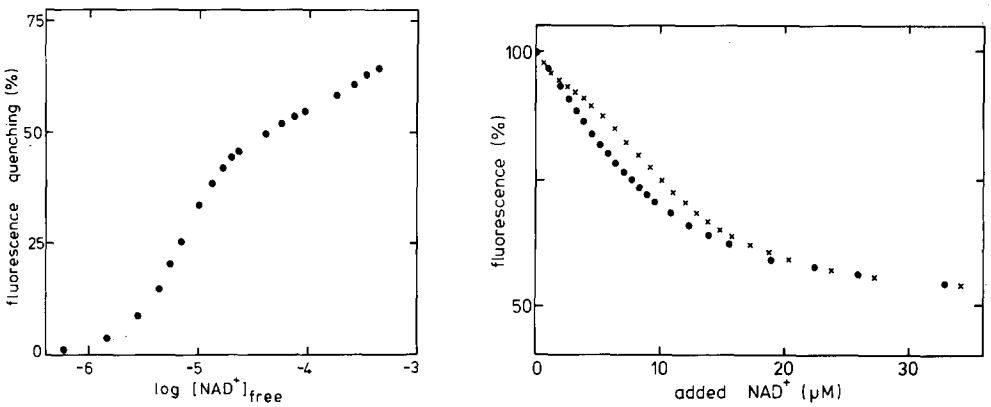
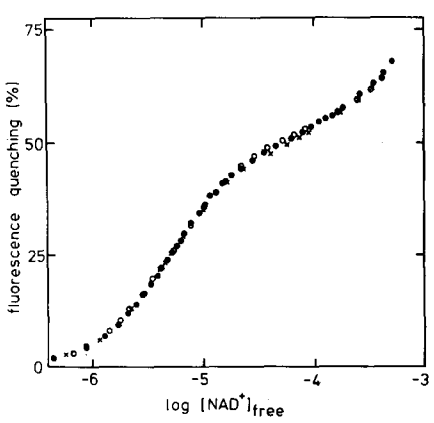


Fig. 5. Titration of the yeast enzyme with NAD^+ in the phosphate buffer at 25° C. Total concentration of binding sites: 3.3 μM. Protein fluorescence was excited at 280 nm (slit 4 nm) and measured at 340 nm (slit 40 nm). The points shown were corrected for inner-filter quenching (see Materials and Methods).

Fig. 6. Fluorimetric titration of an equimolar mixture of R_4 and Y_4 (X) and of R_2Y_2 (•); in both cases the total concentration of the binding sites was 6.6 μM. Other conditions as in Fig. 5.

and the yeast enzyme to the total fluorescence are 29% and 71%, respectively. It can be seen that the muscle enzyme is saturated preferentially, resulting in a small degree of fluorescence quenching, whereas the looser binding to the yeast enzyme results in a much larger signal. The typically biphasic appearance of the titration of the equimolar mixture of the two enzymes is absent from the titration of the hybrid (Fig. 6). This makes it likely that no tight binding occurs to sites on the rabbit muscle dimer in the hybrid. Indeed, the titration of the hybrid is very similar to the titration of the yeast enzyme (Fig. 5), indicating that the binding of NAD^+ to the yeast enzyme dimer in the hybrid is of the same strength as in the yeast enzyme tetramer.

The precise relation between the degree of quenching (the signal $\bar{S}$) and the saturation function $\bar{Y}$ can be found by carrying out the titration of the hybrid at different protein concentrations. To compare these different titrations we have to know the concentration of free ligand at every point of the titration which can be calculated only if we know this relation. The titrations were performed at three different concentrations of the hybrid and in Fig. 7 the degree of fluorescence quenching is plotted against the concentration of free NAD^+, which was calculated assuming a final amplitude of the signal of 75% and a linear relation between this signal and the saturation function. The coincidence of the three titrations is good, especially in the first part of the titration (up to about 50% quenching), which is most sensitive to mistakes in the assumed linear relation between $\bar{Y}$ and $\bar{S}$. This linearity implies that the two kinds of binding sites in the hybrid are saturated to the same extent during the titration, so no large difference in affinity exists between the two kinds of binding sites present in the hybrid (see Appendix). To investigate the sensitivity of this type of analysis for uncertainty in the final amplitude of the signal, we assumed a final amplitude of 90% and found that, in this case, the three titrations do not coincide as well if a linear relation between $\bar{Y}$ and $\bar{S}$ is still



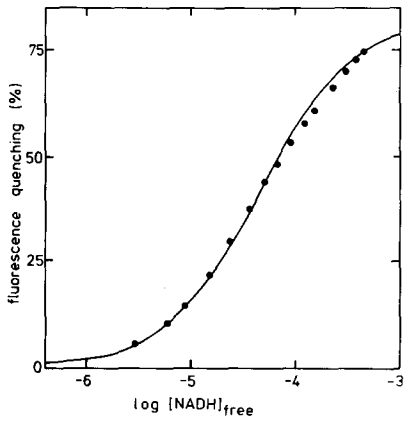


Fig. 7. Fluorimetric titrations of the isolated hybrid with NAD^+. Total concentration of binding sites: X, 1.4 μM; •, 6.6 μM, and ○, 17.7 μM. Other conditions as in Fig. 5.

Fig. 8. Fluorimetric titration of the yeast enzyme with NADH. Total concentration of binding sites: 2.4 μM. The protein fluorescence was excited at 290 nm (slit 5 nm) and measured at 380 nm (slit 40 nm). Other conditions as in Fig. 5. The continous line was calculated using a dissociation constant of 45 μM (final amplitude: 83%).

assumed. A smaller contribution of the first ligand molecules bound by the hybrid to the fluorescence quenching, due to preferential binding of the ligand by the muscle enzyme dimer concomitant with a smaller degree of quenching, can explain the poor coincidence observed in this case: a factor of two between the dissociation constants for binding to the two pools of sites already accounts for this non-linearity in the relation between $\bar{Y}$ and $\bar{S}$ (see Appendix).

Thus, in spite of the uncertainty about the maximal degree of quenching of the hybrid fluorescence by NAD^+ we can conclude that binding of NAD^+ to the muscle and to the yeast enzyme dimer in the hybrid tetramer occurs with similar strength, thus resulting in a linear relation between the degree of quenching of the protein fluorescence and the saturation of the binding sites with NAD^+. The close similarity of the binding isotherms of the hybrid and the yeast enzyme suggests that the binding of NAD^+ to the yeast enzyme dimer is hardly affected by combining this dimer with a muscle enzyme dimer in the hybrid, whereas the binding of NAD^+ to the dimer of the rabbit muscle enzyme is much stronger in the muscle enzyme tetramer than in the hybrid.

Binding of NADH

The binding of NADH to the rabbit muscle enzyme, measured at 25°C in the phosphate buffer, can be described with a mean dissociation constant of about 2 μM, thereby ignoring the slight negative cooperativity [5].

The binding of NADH to the yeast enzyme was measured under the same conditions (Fig. 8). The final amplitude was not reached in this titration, but since it is known that NADH binding by the yeast enzyme occurs without cooperativity [20], we can estimate the final amplitude of the signal: the curve can be simulated assuming a dissociation constant of 45 μM (maximal degree of quenching: 83%).

A titration of the hybrid with NADH is shown in Fig. 9. The similarity of

this titration and the titration of the yeast enzyme (Fig. 8) is taken to indicate that, as in the case of NAD^+ binding, the binding of NADH to the dimer of the yeast enzyme is of similar strength in the yeast enzyme tetramer and in the hybrid. Strong binding sites are not apparent, so, as in the case of NAD^+, the binding of NADH to the dimer of the rabbit muscle enzyme is stronger in the muscle enzyme tetramer than in the hybrid.

Knowing that the binding strength of both NAD^+ and NADH to the dimer of the yeast enzyme is similar in the yeast enzyme tetramer and in the hybrid, we can simplify Eqn. 1, by putting $q = 1$, to:

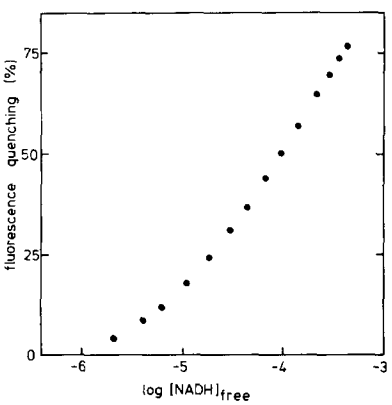
$$K_{eq}^{app} = K_{eq} \frac{(1 + pL/K_r)^4}{(1 + L/K_r)^4}$$

This expresses the finding that the shift in the hybridization equilibrium in the presence of NAD^+ or NADH can be fully ascribed to the tighter binding of these nucleotides to the rabbit muscle enzyme, compared with their binding to the dimer of the muscle enzyme in the hybrid.

Hybridization of alkylated enzyme

Alkylation of the rabbit muscle enzyme with iodoacetate is known to result in a complete inhibition of the dehydrogenase activity and this inactivation proceeds linearly with the extent of alkylation of the four Cys-149 residues present in the tetramer [21]. Hybrid formation and reversion with this fully alkylated enzyme and an equimolar concentration of untreated yeast enzyme are presented in Fig. 10. They proceed at about the same rate and reach the same equilibrium as with the unalkylated rabbit muscle enzyme, showing that the stabilities of the rabbit muscle enzyme and the hybrid are not affected by the alkylation of the subunits of the muscle enzyme.

In the presence of NADH, however, a completely different hybridization behaviour is observed (Fig. 11): only a small shift in the equilibrium can be



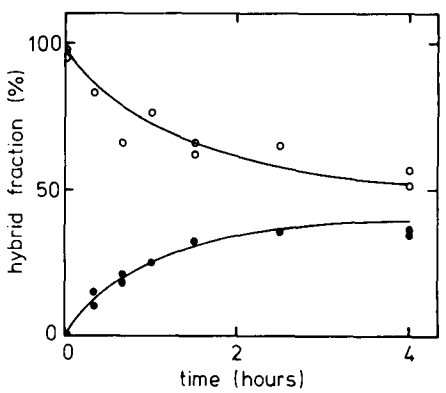
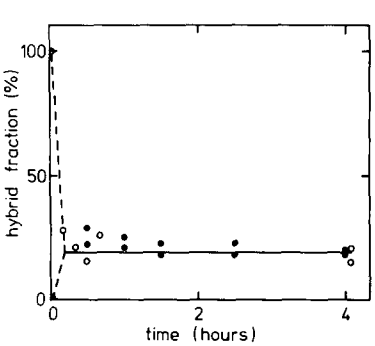


Fig. 9. Fluorimetric titration of the isolated hybrid with NADH. Total concentration of binding sites: 6.6 μM. Other conditions as in Fig. 8.

Fig. 10. Hybrid formation and reversion with the alkylated rabbit muscle enzyme. Formation (•): 10.4 μM R_4^{alk} and Y_4; reversion (○): 5.4 μM $R_2^{alk}Y_2$. Other conditions as in Fig. 2.



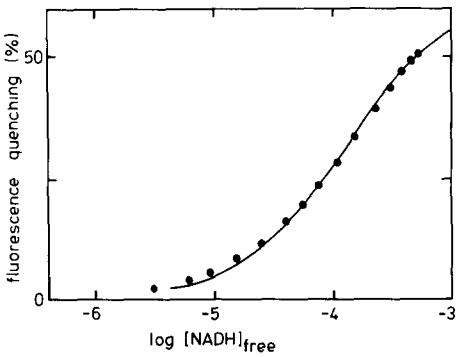


Fig. 11. Hybrid formation and reversion with alkylated rabbit muscle enzyme in the presence of 1 mM NADH. Formation (•): 12.3 μM R_4^{alk} and Y_4; reversion (○): 5.9 μM $R_2^{alk}Y_2$. Other conditions as in Fig. 2.

Fig. 12. Fluorimetric titration of the alkylated rabbit muscle enzyme with NADH. Total concentration of binding sites, 2.43 μM. Other conditions as in Fig. 8. The continous lines was calculated using a dissociation constant of 120 μM (final amplitude: 62%).

detected, but this equilibrium is reached very quickly compared with the unalkylated enzyme in the presence of NADH (cf. Fig. 3B).

The picture in the presence of NAD^+ is somewhat more complex: hybrid formation occurs at low concentrations of NAD^+, but with excess of NAD^+ the starting mixture is 'frozen'. Reversion also proceeds at an appreciable rate in the presence of low concentrations of NAD^+, while excess of NAD^+ only slightly hinders the dissociation of the semialkylated hybrid.

Binding of NAD^+ and NADH to the alkylated species

The quenching of the fluorescence of the alkylated rabbit muscle enzyme by NAD^+ is weak, probably due to the absence of the Racker band which overlaps the tryptophan emission spectrum in the unalkylated holoenzyme and may be involved in an energy-transfer mechanism of quenching [22]. The quenching of the fluorescence of the alkylated muscle enzyme by NADH is even stronger than in the unalkylated case.

The larger signal observed makes binding of NADH easier to study. The titration of the fully alkylated muscle enzyme with NADH is shown in Fig. 12. A dissociation constant of about 120 μM can be estimated, assuming a linear relationship between the degree of quenching and the saturation function and no negative cooperativity; this result is in good agreement with that of Reynolds and Dalziel [23].

Since hybridization also leads to a looser binding of NADH (and NAD^+) to the muscle enzyme dimer, it was of interest to investigate whether alkylation and hybridization produce their effects on nucleotide binding independently. A titration of the semialkylated hybrid with NADH showed that the dissociation constant for NADH binding to the alkylated rabbit muscle subunits (120 μM) is not further increased by the hybridization. We conclude that alkylation and hybridization produce their effects on the affinity of the rabbit muscle enzyme for NADH via at least partly similar mechanisms involving the interactions

across the Q- and R-boundaries in the tetramer. This would explain why the hybridization equilibrium reached with the alkylated muscle enzyme and the untreated yeast enzyme is not much affected by NADH, while the equilibrium reached with the untreated muscle enzyme and the yeast enzyme was shown to shift strongly in the presence of NADH, due to the tight binding of NADH to the untreated rabbit muscle enzyme; evidently this tight binding is prevented by alkylation (as it is by hydribization).

The binding of NAD^+ to the alkylated rabbit muscle enzyme measured fluorimetrically can be described with a dissociation constant of 7 μM, in agreement with the result of Reynolds and Dalziel [23].

Due to the stronger fluorescence quenching by the NAD^+ molecules bound to the yeast enzyme dimer in the semialkylated hybrid only conclusions about the strength of NAD^+ binding to this dimer can be drawn: it binds NAD^+ with similar strength in the semialkylated hybrid and in the yeast enzyme tetramer.

Discussion

Glyceraldehyde-3-phosphate dehydrogenase isolated from yeast differs in important respects from the enzyme isolated from muscle sources and some bacteria. Most of the attention of enzymologists has been drawn to the differences in cooperativity accompanying nucleotide binding. Whereas the binding of NAD^+ and NADH to preparations of this enzyme from most sources occurs with pronounced negative cooperativity [24—29], the binding of NAD^+ to the yeast enzyme exhibits no cooperativity at neutral pH [30], and a mixture of positive and negative cooperativity [16—18] or only positive cooperativity at higher pH [19]. Binding of NADH to the yeast enzyme occurs without cooperativity [20]. The negative cooperativity that was observed in nucleotide binding to the enzymes from muscle sources, bacteria and yeast was explained in several ways. True negative cooperativity [18], preexisting asymmetry [31, 32] and microheterogeneity [33] (affinity heterogeneity [19]) cannot, in fact, be discriminated by simply studying the binding properties of a single preparation. Recently, however, the apparent negative cooperativity in the binding of NAD^+ to the enzymes from rabbit muscle and yeast has been shown to be a reflection of differences in the preparation of the enzymes. In both cases preparations have been described in which the weak binding previously observed at high saturation levels is not detectable, and weakly negative [5] or only positive cooperativity [19] remained.

Another important difference between the preparations from rabbit muscle and yeast is the difference in average binding affinity for NAD^+ and NADH. Both nucleotides are bound more strongly by the muscle enzyme, the differences corresponding to about 7 kJ/mol in Gibbs energy. The subunits of the muscle enzyme lose their ability to bind nucleotides that strongly when they are combined with subunits of the yeast enzyme to form the hybrid. It was shown in this study that about 6.5 kJ/mol of the Gibbs-energy change upon binding of NAD^+ to the muscle dimer is lost on forming the hybrid, whereas the binding to the P-dimer of the yeast enzyme is scarcely affected. The most plausible explanation is that the change in the Q- and R-boundaries occurring upon hybrid formation hinders the favourable SL_q- and SL_r-interactions that

are responsible for the tight binding of the nucleotides by the muscle enzyme *. According to this view the differences in nucleotide binding affinity between the enzymes from the two sources is absent in the P-dimer of the two enzymes, and arises from the combination of the dimers to form the tetramer. In the muscle enzyme both NAD^+ and NADH produce more tightening of the interactions across the QR-plane of dissociation than in the yeast enzyme, thus causing the difference in binding affinity.

It is tempting to ascribe also the differences in cooperativity in nucleotide binding between the enzymes from the two sources to differences in the interactions across the same boundaries (the LL_q- and LL_r-terms), but in our view such conclusions are premature until direct binding studies on the hybrid have been made. Furthermore, our preparations of the yeast enzyme and the hybrid both exhibit apparent negative cooperativity at high saturation levels, which is not yet completely understood. We would like to stress, however, that the stabilizing effect of NAD^+ on the tetrameric structures is probably accompanied by negative cooperativity in the muscle enzyme, but by positive cooperativity in the yeast enzyme. To explain this it is only necessary to assume that the tightening effect on the interactions across a boundary by NAD^+ bound to one of the subunits is slightly more effective when a liganded subunit is present on the other side of the boundary in the case of the yeast enzyme, but with an unliganded subunit in the muscle enzyme. In this way positive and negative cooperativity are fully explained and there is no need to postulate additional destabilizing interactions across other boundaries in the yeast enzyme.

The affinity of glyceraldehyde-3-phosphate dehydrogenase for the oxidized coenzyme is larger than that for the reduced coenzyme, which is exceptional among NAD-dependent dehydrogenases [34]. In our preparations this differences in $\Delta G^{0'}$ is about 4 kJ/mol in favour of NAD^+ binding, both for the muscle enzyme and for the yeast enzyme, if we neglect the apparent negative cooperativity accompanying NAD^+ binding to the yeast enzyme at high saturation levels. Hybridization studies and many other studies have revealed the stabilizing effect of NAD^+ on the tetrameric structure, but NADH shows a less consistent behaviour: it prevents dissociation of the rat skeletal muscle enzyme [35] by preferential binding to the tetramer, but promotes the dissociation of the yeast enzyme and the hybrid (Figs. 11 and 4B). NADH also promotes the dissociation of the alkylated rabbit muscle enzyme (Fig. 11), but probably inhibits the dissociation of the native rabbit muscle enzyme (Fig. 3B). These effects may be explained (at least semiquantitatively) by assuming that the 4 kJ/mol difference in binding Gibbs energy between NAD^+ and NADH bound to glyceraldehyde-3-phosphate dehydrogenase arises largely from differences in the effects of these nucleotides on the interactions across the QR-plane of dissociation in the tetramers. In this view, which has to be refined, NAD^+ and NADH bind with about equal strength to the P-dimer of the enzyme from rabbit muscle and yeast.

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* We are aware of the fact that the plane of dissociation in the tetramers may not be identical with the QR-plane defined by the crystallographers [11,12]. However, most workers make this assumption as a working hypothesis, although the question cannot be solved from the available crystallographic data on the holoenzyme.

In some details this picture deviates from recently developed views on this matter. In the first place, Hollaway et al. showed with their preparations of the two enzymes [3] that the first two molecules of NAD^+ bound to the rabbit muscle enzyme do not cause any inhibition of the hybrid formation. With our preparations, however, the inhibition starts with the first equivalent of NAD^+ or NADH added and is virtually complete when two or more molecules of NAD^+ or NADH are bound by the rabbit muscle enzyme (Fig. 3A and B). This observation and the fact that the binding of NAD^+ or NADH to our preparation of the rabbit muscle enzyme occurs without pronounced negative cooperativity lead us to the conclusion that the effects of both NAD^+ and NADH on the stability of the tetramer is the same for all four molecules bound, apart from the modulations by LL-interactions, which may be exerted across the QR-plane of dissociation, thus causing the slight negative cooperativity (see above).

In the second place, Hollaway et al. observed a small shift in the hybridization equilibrium in the presence of NADH and this equilibrium was reached very quickly [3]. However, we observed no hybrid formation at all and complete reversion in the presence of 1 mM NADH (Figs. 3B and 4B). Furthermore, with a low concentration of NADH hybrid formation was slower than in the absence of nucleotides (Fig. 3B), probably due to a stabilization of the muscle enzyme by NADH, as in the case of NAD^+. The smaller shift in the equilibrium and the more rapid kinetics of hybrid formation in the presence of NADH found by Hollaway and coworkers with the native enzyme were found by us only after alkylation of the rabbit muscle enzyme. We showed that this alkylation prevents the stabilization by bound NADH of interactions across the QR-plane in the muscle enzyme making possible a very rapid hybrid formation, which is also promoted by the destabilization by NADH of the yeast enzyme (Fig. 11).

These observations may shed light on the possible cause of the differences between the classical preparation of the rabbit muscle enzyme and ours (see also Ref. 4). The extremely strong binding of NAD^+ and NADH by the muscle enzyme is caused by extra, favourable interactions across the Q- and R-boundaries in this enzyme (the SL_q- and SL_r-terms). When, by alkylation or hybridization, these interactions are hindered, weaker binding and even destabilization by NADH results, as in the yeast enzyme and in the classical preparation of the rabbit muscle enzyme [2,3]. Furthermore, the SL_q- and SL_r-terms are responsible for the large shift in the hybridization equilibrium caused by NADH in our study, and changes in the muscle enzyme that prevent these favourable interactions will also prevent the large shift in the hybridization equilibrium induced by NADH, as was observed in the experiments with the classical preparation of the enzyme [5] and in our experiments with the alkylated muscle enzyme. Thus, some differences, at least, between the two preparations of the rabbit muscle enzyme may be caused by a change in the protein that hinders favourable SL_q- and SL_r-interactions. Differences in the parameters of spectroscopic signals associated with the nicotinamide part both of bound NAD^+ (the Racker band) [4,36] and bound NADH (NADH fluorescence quenching) [37,5] suggest that this change in the protein may have affected the nicotinamide-binding region of the coenzyme-binding site.

Alkylation of the rabbit muscle enzyme has no influence on the stability of

the apoenzyme (Fig. 10). However, it promotes the dissociation of this enzyme in the presence of NADH (Fig. 11). NAD^+ inhibits the dissociation of the hybrid tetramer less effectively when the muscle enzyme subunits in the hybrid are alkylated. Thus alkylation (and probably acylation) of the muscle enzyme destabilizes the tetramer only when it contains bound nucleotides.

It is not surprising, then, that the differences in nucleotide-binding properties between the classical preparation of the rabbit muscle enzyme and ours are largely abolished upon alkylation, which prevents the intersubunit interactions induced by nucleotides that are at least partly responsible for the differences between the two preparations. Indeed, the binding parameters for NAD^+ and NADH of our preparation after alkylation are similar to those obtained by Reynolds and Dalziel [23]. The surprising similarity of the kinetic parameters of the two preparations, in spite of the differences in their nucleotide-binding properties [5], can now be explained if we accept the view that these kinetic parameters are a measure of the properties of the acylated enzyme, which for the two preparations are probably similar.

Our findings suggest that the SL-interactions across the QR-plane in glyceraldehyde-3-phosphate dehydrogenases, which are very easily modulated by external factors, play an important role for the proper functioning of the enzyme in vivo.

Appendix

To describe the relationship between the normalized signal ($\bar{S}$) and the saturation function ($\bar{Y}$) the formalism described previously [5], which is based on a Koshland type of interaction model [38], can be used. The following equations for the saturation function and the signal apply:

$$\bar{Y} = (\alpha + p\alpha^2)/(1 + 2\alpha + p\alpha^2) \quad (1)$$

$$\bar{S} = (2\alpha\epsilon + p\alpha^2)/(1 + 2\alpha + p\alpha^2) \quad (2)$$

in which

$$\alpha = [\text{ligand}]_{\text{free}}/K_{\text{tight}}$$

and $p = K_{\text{tight}}/K_{\text{loose}}$, where K_{tight} and K_{loose} are the microscopic dissociation constants derived from the asymptotes of a Hill plot and ϵ is the relative contribution of the tightly bound ligands to the normalized signal.

However, if we treat the hybrid as a protein with preexisting asymmetry [31,32], the parameters have a physical meaning that is more easily understood: let K_1 and K_2 be the microscopic dissociation constants for ligand binding to the two kinds of binding sites present (designated 1 and 2) and e the relative contribution of a ligand molecule, bound to a type 1 site, to the normalized signal. With the following substitutions:

$$K_{\text{tight}} = 2\,K_1K_2/(K_1 + K_2)\ [39]$$

$$K_{\text{loose}} = (K_1 + K_2)/2\ [39]$$

$$\epsilon = K_2e/(K_1 + K_2) + K_1\,(1 - e)/(K_1 + K_2)$$

Eqns. 1 and 2 may be transformed into

$$\bar{Y} = \tfrac{1}{2}\alpha'/(1+\alpha') + \tfrac{1}{2}p'\alpha'/(1+p'\alpha') \quad (3)$$

$$\bar{S} = e\alpha'/(1+\alpha') + (1-e)p'\alpha'/(1+p'\alpha') \quad (4)$$

in which

$$\alpha' = [\text{ligand}]_{\text{free}}/K_1$$

and $p' = K_1/K_2$

In Eqns. 3 and 4 the two kinds of binding sites are more easily recognized. Putting $p' = 1$ the expressions for Y and $\bar{S}$ become identical, meaning that the signal is linearly related to the saturation function, when the two kinds of binding sites have equal affinities. This is also the case when $e = 0.5$, that is ligand bound to each type of binding site contributes equally to the signal.

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References

1 Osborne, H.H. and Hollaway, M.R. (1974) Biochem. J. 143, 651—662
2 Osborne, H.H. and Hollaway, M.R. (1975) Biochem. J. 151, 37—45
3 Hollaway, M.R., Osborne, H.H. and Spotorno, G.M.L. (1977) in Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H., ed.), pp. 101—117, Walter de Gruyter, Berlin, New York
4 Scheek, R.M. and Slater, E.C. (1978) Biochim. Biophys. Acta 526, 13—24
5 Scheek, R.M., Berden, J.A., Hooghiemstra, R. and Slater, E.C. (1979) Biochim. Biophys. Acta 569, 124—134
6 Kirschner, K. and Voigt, B. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 632—644
7 Ferdinand, W. (1964) Biochem. J. 92, 578—585
8 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
9 Henis, Y.I. and Levitzki, A. (1977) J. Mol. Biol. 117, 699—716
10 Weber, G. (1972) Biochemistry 11, 864—878
11 Buehner, M., Ford, G.C., Moras, D., Olsen, K.W. and Rossmann, M.G. (1974) J. Mol. Biol. 90, 25—49
12 Biesecker, G., Harris, J.I., Thierry, J.C., Walker, J.E. and Wonacott, A.J. (1977) Nature 266, 328—333
13 Adair, G.S. (1935) J. Biol. Chem. 63, 529—545
14 Wonacott, A.J. and Biesecker, G. (1977) in Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H., ed.), pp. 140—156, Walter de Gruyter, Berlin, New York
15 Cook, R.A. and Koshland, D.E., Jr. (1970) Biochemistry 9, 3337—3342
16 Kirschner, K., Gallego, E., Schuster, I. and Goodall, D. (1971) J. Mol. Biol. 58, 29—50
17 Sloan, D.L. and Velick, S.F. (1973) J. Biol. Chem. 248, 5419—5423
18 Mockrin, S.C., Byers, L.D. and Koshland, D.E., Jr. (1975) Biochemistry 14, 5428—5437
19 Gennis, L.S. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3928—3932
20 Von Ellenrieder, G., Kirschner, K. and Schuster, I. (1972) Eur. J. Biochem. 26, 220—236
21 MacQuarrie, R.A. and Bernhard, S.A. (1971) Biochemistry 10, 2456—2466
22 Brand, L. and Witholt, B. (1967) Methods Enzymol. 11, 776—856
23 Reynolds, C.H. and Dalziel, K. (1979) Biochim. Biophys. Acta 567, 287—294
24 De Vijlder, J.J.M. and Slater, E.C. (1968) Biochim. Biophys. Acta 167, 23—34
25 Conway, A. and Koshland, D.E., Jr. (1968) Biochemistry 7, 4011—4023
26 De Vijlder, J.J.M., Boers, W. and Slater, E.C. (1969) Biochim. Biophys. Acta 191, 214—220
27 Schlessinger, J. and Levitzki, A. (1974) J. Mol. Biol. 82, 547—561

28 Bell, J.E. and Dalziel, K. (1975) Biochim. Biophys. Acta 391, 249—258
29 Allen, G. and Harris, J.I. (1975) Biochem. J. 151, 747—749
30 Velick, S.F., Baggot, J.P. and Sturtevant, J.M. (1971) Biochemistry 10, 779—786
31 Matthews, B.W. and Bernhard, S.A. (1973) Annu. Rev. Biophys. Bioeng. 2, 257—317
32 Seydoux, F., Malhotra, O.P. and Bernhard, S.A. (1974) CRC Crit. Rev. Biochem. 2, 227—257
33 Kelemen, N., Kellershohn, N. and Seydoux, F. (1975) Eur. J. Biochem. 57, 69—78
34 Subramanian, S. and Ross, P.D. (1978) Biochemistry 17, 2193—2197
35 Nagradova, N.K., Muronetz, V.I., Grozdova, I.D. and Golovina, T.O. (1975) Biochim. Biophys. Acta 377, 15—25
36 Boers, W., Oosthuizen, C. and Slater, E.C. (1971) Biochim. Biophys. Acta 250, 35—46
37 Bell, J.E. and Dalziel, K. (1975) Biochim. Biophys. Acta 410, 243—251
38 Cornish-Bowden, A.J. and Koshland, D.E., Jr. (1970) J. Biol. Chem. 245, 6241—6250
39 Long, J.W. and Dahlquist, F.W. (1977) Biochemistry 16, 3792—3797