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FACTORS AFFECTING COENZYME BINDING AND SUBUNIT INTERACTIONS IN GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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Summary

There is no evidence, at pH 9.4, of negative cooperativity in the binding of NAD^+ or NADH to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phorphorylating), EC 1.2.1.12) nor in the binding of acetyl pyridine adenine dinucleotide at pH 7.6 and pH 9.4. The binding of NAD^+ to carboxymethylated enzyme at pH 7.6 and pH 9.4 also occurs without cooperativity. The possible implications of these findings for the involvement of ionising groups in the enzyme in the subunit interactions responsible for negative cooperativity, previously reported for coenzyme binding at pH 7.4—8.6, are discussed.

Introduction

Negative cooperativity in the binding of NAD^+ and NADH to glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase, phosphorylating, EC 1.2.1.12) is well established at pH 7.6—8.6 [1—5]. The affinities of the tetrameric enzyme molecule for the third and fourth molecules of NAD^+ are greater at pH 8.2 that at pH 7.6 [4]. In this paper we report studies of the binding of NAD^+ and NADH to native enzyme at pH 9.4 which show no evidence of negative cooperativity. For each coenzyme, the single apparent dissociation constant that describes the binding at this pH is

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intermediate between those estimated for the second and third molecules at lower pH values. Studies of the binding of acetyl pyridine-adenine dinucleotide to the native enzyme, and of NAD^+ and NADH to cysteine-149-carboxymethyl enzyme, are also described. The results at pH 7.6 and pH 9.4 are consistent with independent binding at identical sites.

Materials and Methods

Tri-ethanolamine hydrochloride, NAD^+, NADH (Grade 1) and yeast alcohol dehydrogenase were obtained from Boehringer-Mannheim. (London). Acetyl pyridine-adenine dinucleotide, adenosine diphosphoribose, dithiothreitol, *N*-acetyl tryptophan, Tris-HCl and DL-glyceraldehyde-3-phosphoric acid were from Sigma (London) Ltd. Other reagents were of the highest grade available commercially. Iodoacetate was recrystallised from petroleum ether [6]. Double glass-distilled water was used for all solutions.

Crystalline glyceraldehyde-3-phosphate dehydrogenase was prepared from rabbit muscle by the method of Ferdinand [7]. Traces of haem were removed by refractionation and washing of the crystals with ammonium sulphate. The specific activities of several preparations ranged from 180—215 units/mg, the higher values being obtained when 5—15 mM dithiothreitol was added to the 5 mM EDTA buffer (pH 7.5) in which the crude enzyme was dissolved for refractionation. The ratio of absorbancies at 280 nm and 260 nm was 1.1—1.2, corresponding to 3—4 mol nucleotide/mol enzyme.

The major part of the nucleotide was non-reducible, as is usual for holoenzyme prepared by this method. Bloch et al. [8] identified it as adenosine diphosphoribose, and suggested that it is formed from NAD^+ during the isolation procedure. In support of this, we found that when 25 mM nicotinamide was included in the tissue extraction medium to inhibit NAD^+ nucleosidase, or when NAD^+ was added to the crude enzyme before refractionation, the final product contained 3.5 mol NAD^+/mol enzyme and no significant amount of non-reducible nucleotide. The total enzyme-bound nucleotide was estimated from the absorbance at 260 nm after precipitation of the protein with 5% perchloric acid, using an extinction coefficient of 17 600 $M^{-1} \cdot cm^{-1}$. NAD^+ was estimated by dissolving the holoenzyme in 12 mM sodium arsenate buffer (pH 8.5) containing 1 mM EDTA, adding 2 mM DL-glyceraldehyde-3-phosphate and measuring the NADH formed by fluorimetry. It was assumed that 2 mol NADH/mol enzyme would remain bound and have a quantum yield of half that of free NADH [9].

Apoenzyme was prepared by treatment of a dialysed solution of holoenzyme with activated charcoal [10], as described previously [5], except that the buffer used was 0.1 M triethanolamine (pH 7.6) or 0.036 M sodium phosphate (pH 7.6) each containing 1 mM EDTA. The specific activities of the products were 170—190 U/mg when phosphate buffer was used and 140—170 U/mg when triethanolamine buffer was used. In the latter buffer, the apoenzyme is less stable and treatment with activated charcoal caused a decrease of pH. The ratio of absorbances of the apoenzyme at 280 nm and 260 nm was 1.78—1.92.

Carboxymethyl-enzyme was prepared by adding NAD^+ and iodoacetate

(4 mol/mol enzyme) to holoenzyme. After 2 h, nucleotide was removed with activated charcoal.

Enzyme activities were measured at 25°C by the method of Ferdinand [7] and were not corrected for non-saturation by substrates. Protein concentrations were estimated from absorbance measurements at 280 nm and extinction coefficients of 0.92 $mg^{-1} \cdot cm^2$ for holoenzyme [7] and 0.83 $mg^{-1} \cdot cm^2$ for apoenzyme [11]. We confirmed the latter value by dry weight determinations.

Equilibrium dialyses were carried out for 16 h at 3°C with 0.5 ml enzyme solutions and 5 ml diffusate as described previously [12]. The sac contents and diffusate were analysed by absorbance measurements at 260 nm and 280 nm using an extinction coefficient of 15 400 $M^{-1} \cdot cm^{-1}$ for adenosine diphosphoribose [13]. We confirmed that the difference spectrum for enzyme and the nucleotide is negligible [14].

For ultrafiltration studies of the binding of NAD^+ and NADH, an apparatus similar to that described by Paulus [15] was used, with Amicon PM10 or UM, 10 membranes. Ultrafiltrates were analysed for NADH by absorbance measurements at 340 nm, and for NAD^+ by absorbance measurements at 260 nm, or at 340 nm after reduction by ethanol and yeast alcohol dehydrogenase [15].

Fluorescence titrations were made at 25°C in a Farrand spectrofluorimeter, MkI, essentially as described by Reynolds et al. [16]. Protein fluorescence was excited at 300 nm and measured at 350 nm. The fractional saturation of the protein with ligand was calculated as described previously [16], assuming that there are 4 binding sites per molecule which contribute equally to protein fluorescence quenching.

Results

Binding of NAD^+ and NADH to native apoenzyme was studied in 0.1 M Tris-HCl pH 9.4, by measurements of protein fluorescence. The maximum quenching with saturating coenzyme concentrations was estimated at 48% with NAD^+ and 60% with NADH. The results from the titrations are plotted as described by Scatchard et al. [17] in Fig. 1. The plots are linear, and the binding can be described by single dissociation constants of 0.8 μM for NAD^+ and 6 μM for NADH. The assumption that the maximum binding capacity is 4 mol coenzyme/mol enzyme, each of which causes the same degree of fluorescence quenching, was tested by fluorescence titrations with a large enzyme concentration of 5 μM, and by spectrophotometric titrations of 54 μM enzyme at 350 nm. The results indicated that 3—4 mol NAD^+/mol enzyme could bind at pH 9.4, as at pH 7.6 [5].

The binding interactions observed at pH 7.6—8.6 [1—5] might be lost at pH 9.4 because of dissociation of the enzyme into dimers or monomers in the very dilute solutions used for the fluorescence titrations. This possibility was tested by high-speed sedimentation equilibrium measurements [18] with an initial enzyme concentration of 0.15 mg/ml in the presence of 200 μM NAD^+. With native enzyme and carboxymethyl-enzyme at pH 7.7, there was no evidence of dissociation; apparent molecular weights of 131 000 and 142 000 were obtained. With native enzyme at pH 9.5, there was evidence of polydispersity. The apparent molecular weight varied from 142 000 to about 113 000 at the

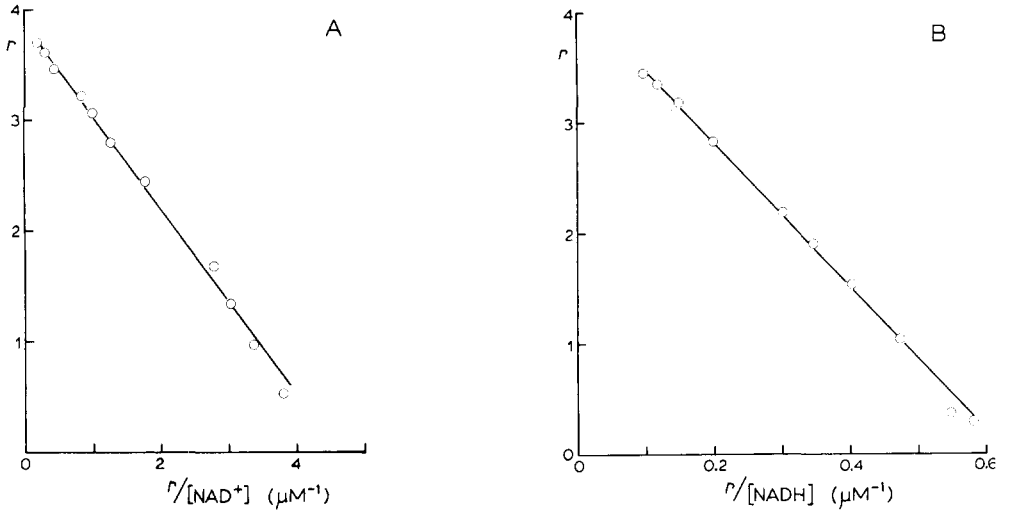

Fig. 1. Scatchard plots for the binding of NAD^+ (A) and NADH (B) to the apoenzyme. The ratio of the concentration of bound ligand to the enzyme concentration, r, is plotted against the ratio of r to the free ligand concentration. The data were calculated from protein fluorescence titrations in 0.1 M Tris-HCl buffer (pH 9.4), 1 mM EDTA. The enzyme concentration was 0.142 μM in A and 0.62 μM in B.

lowest concentration that could be measured accurately (0.35 fringe shift, 8 μg/ml). The degree of dissociation indicated is thus insufficient to explain the complete absence of subunit interactions at the enzyme concentrations of 22 μg/ml and 0.1 mg/ml used in the fluorescence titrations (Fig. 1).

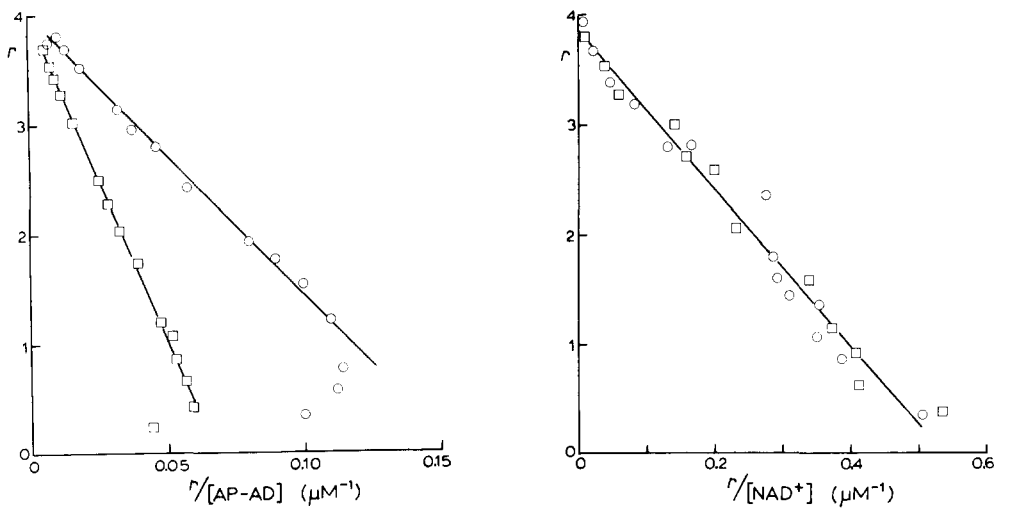

Fig. 2. Scatchard plots of data from protein fluorescence titrations of apoenzyme with acetyl pyridine-adenine dinucleotide (AP-AD) at pH 7.6 (○) and Ph 9.4 (□). The enzyme concentrations were 0.69 μM in 0.1 M triethanolamine-HCl buffer (pH 7.6) and 0.56 μM in 0.1 M Tris-HCl buffer (pH 9.4), 1 mM EDTA.

Fig. 3. Scatchard plots for the binding of NAD^+ to carboxymethyl-enzyme at pH 7.6, with 0.67 μM enzyme (○), and at pH 9.4 with 0.58 μM enzyme (□). Other conditions were as described in the legend to Fig. 2.

The binding of acetyl pyridine-adenine dinucleotide to native enzyme was studied by protein fluorescence titrations at pH 7.6 (maximal quenching 28%) and at pH 9.4 (maximal quenching 48%). The results (Fig. 2) are again consistent with independent binding at equivalent sites with dissociation constants of 26 μM at pH 7.6 and 60 μM at pH 9.4.

With carboxy-methyl-enzyme, the protein fluorescence quenching accompanying the binding of NAD^+ was smaller than with native enzyme, amounting maximally to 18% at pH 7.6 and 20% at pH 9.4. Titration data were therefore less precise than with native enzyme, but Scatchard plots were linear within the experimental error (Fig. 3) and the dissociation constant from several titrations was 6 ± 2 μM at both pH values. The stoichiometry of binding was tested at pH 7.6 by ultrafiltration experiments, which showed that 4 mol NAD^+/mol carboxymethyl enzyme were bound at high NAD^+ concentrations. Quenching of the protein fluorescence of carboxymethyl enzyme by NADH was too small for useful titrations. Ultrafiltration experiments showed that NADH binding was very weak, the dissociation constant being about 100 μM.

Contrary to an earlier report by Bell and Dalziel [5], binding of adenosine diphosphoribose to the apoenzyme at pH 7.6 could not be detected by protein fluorescence quenching. Binding was detected and quantified by equilibrium dialysis at 3°C in 0.036 M sodium phosphate buffer (pH 7.6) containing 1 mM EDTA. The results indicated the binding of 2 mol NAD^+/mol enzyme with a dissociation constant of 7 μM, and weaker binding of a third molecule. Unfortunately, the apoenzyme was too unstable to allow binding studies in thriethanolamine buffer and at room temperature to be made by this method.

Discussion

The results of binding studies with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase at pH 9.4 are consistent with the presence of four identical and independent binding sites in the molecule for NAD^+ and NADH, with dissociation constants of 0.8 μM and 6 μM respectively. At pH 7.4—8.6, however, results from several laboratories have shown that the binding of coenzyme is negatively cooperative [1—5]. In this pH range, the tetrameric enzyme molecule binds two molecules of NAD^+ or NADH very firmly, with dissociation constants smaller than 0.05 μM. For the thrid and fourth molecules, the dissociation constants for NAD^+, according to Price and Radda [4], are 1.7 μM and 34 μM respectively at pH 8.2, and at pH 7.6, 21 μM and 318 μM. Other workers (1—3,5] have obtained similar values for NAD^+ and also for NADH [5]. Thus, the effect of a change from pH 9.4 to pH 7.4 is to increase the affinity off the apoenzyme for two molecules of NAD^+ or NADH, and to decrease the affinity of the two remaining sites for coenzyme.

From the dissociation constant for the independent binding at pH 9.4 and 25°C, the total free energy of binding of 4 mol NAD^+ to 1 mol enzyme is —139 kJ mol^{-1} (—35 kJ per site), and for NADH —119 kJ mol^{-1}. At pH 7.6, the four dissociation constants describing the negatively cooperative binding of NAD^+, obtained from the slopes of Scatchard plots reported by Bell and Dalziel [5] by corrections for statistical factors, are 0.01, 0.2, 12 and 144 μM, and correspond to free energies of binding for the four successive molecules of NAD^+ of —46,

−37, −28 and −22kJ mol^{-1}, and a total free energy for saturation of the enzyme with NAD^+ of −133 kJ mol^{-1}. Similar values are obtained for NADH binding [5]. Although the dissociation constants at pH 7.6 are subject to considerable experimental error, it appears that the overall binding energy is similar to that at pH 9.4, and that the binding energies for the first two molecules of NAD^+ are smaller and those for the binding of the last two molecules of NAD^+ are larger, than that for independent binding at a single site at pH 9.4.

In terms of the model for negative cooperativity proposed by Bell and Dalziel [5], by analogy with that of Perutz [19] for haemoglobin, the apoenzyme has a 'relaxed' structure, and combination of two NAD^+ molecules, through an induced fit change of tertiary structure of two subunits, allows the creation of new intersubunit bonds and causes a change in quaternary structure to a 'tense' form. The energy of formation of the intersubunit bonds contributes to the affinity of binding of the first two molecules of NAD^+, and the combination of the last two molecules of NAD^+ with the 'tense' structure occurs with smaller affinity. The absence of negative interactions at pH 9.4 suggests that a group or groups in the enzyme molecule with pK_a 8—9 must be protonated for these intersubunit bonds to be formed. The similarity of the overall free energy change accompanying saturation with NAD^+ at pH 9.4 and pH 7.6 further suggests that combination of the last two molecules of NAD^+ may cause dissociation of the intersubunit bonds formed during combination of the first two molecules, and reversion of the 'tense' structure of the half-saturated enzyme to a 'relaxed' structure in the holoenzyme, so that there is no overall interaction energy.

This suggestion involves the assumption that the affinity of each subunit in the tetramer at pH 9.4 is equal to that of the isolated subunits at both pH 9.4 and at lower pH, that is, that the effect of pH on coenzyme binding is solely due to subunit interactions and the formation and dissociation of intersubunit bonds at the lower pH values. There may, however, be an additional direct effect of pH upon coenzyme binding at each site, not related to subunit interactions, in which case there would be no justification for the deduction, from the findings at pH 9.4, that intersubunit bonds are broken again when the last two molecules of NAD^+ combine at pH 7.6. The question might be resolved by studies of coenzyme binding to isolated subunits at both pH values, as has been done with haemoglobin [20], or by high resolution crystal structures for apoenzyme and enzyme combined with two molecules of NAD^+, as well as holoenzyme [24].

The enzyme groups with pK 8—9 responsible for the pH effects on coenzyme binding may be present in subunit contacts or in the active centres, which are close to subunit contacts [21,22]. Our finding that after carboxymethylation of cysteine-149 the enzyme binds NAD^+ without cooperativity, and with the same affinity at pH 7.6 and pH 9.4, suggests that this group might be responsible. By contrast, it has been reported [23] that binding of NAD^+ to the enzyme in the presence of excess iodoacetate is negatively cooperative, and that after modification of cysteine-149 by trifluorobromoacetone negative cooperativity is retained. An effect of ionisation of cysteine-149 on the affinity for NAD^+ would also be inconsistent with the evidence that pK_a for the residue is the same in apoenzyme and holoenzyme [25,26].

Structural studies of the holoenzyme from lobster muscle [22] and *Bacillus stearothermophilus* [21] have revealed a flexible polpeptide loop in each subunit that interacts with two other subunits and makes contact with NAD^+ bound to one of them, and it has been suggested that this S sloop mediates the structural changes that give rise to negative cooperativity. The sequence of the loop is highly conserved in the enzymes from lobster, pig and yeast, but is substantially different in the enzyme from *B. stearothermophilus* [21]. Lysine-183 is conserved in the mesophile enzymes. Chemical modification of this loop residue in the yeast enzyme eliminates the half-of-the-sites reactivity of cysteine-149 [27], and in the muscle enzymes, including the rabbit muscle enzyme, this lysine is involved in an acyltransfer reaction with cysteine-149 [28,29]. It is therefore a possible candidate for the group with pK 8—9, deprotonation of which eliminates negative cooperativity of the rabbit muscle enzyme. In the enzyme from *B. stearothermophilus*, lysine-183 is replaced by arginine [21], which would be expected to remain protonated at pH 9.4. In preliminary experiments with the therophile enzyme, unfortunately with a preparation of low specific activity, we found that negative cooperativity in NAD^+ binding, demonstrated by Allen and Harris [30] at pH 8.2, is retained at pH 9.4. This provides some support for the suggestion that a protonated basic group in position 183 in the loop is needed for negative cooperativity, but these preliminary results require confirmation with fully active enzyme from *B. stearothermophilus*.

Binding studies with the acetyl-pyridine analogue of NAD^+ at pH 7.6 and pH 9.4 showed no evidence of negative cooperativity. The dissociation constants of 26 μM and 60 μM respectively are of the same order as those reported by others [31,32], but Eby and Kirtley [32] obtained some evidence of negative cooperativity. These authors also studied the binding of several other coenzyme analogues, but the structural features of the coenzyme needed to elicit negative cooperativity are not yet clear.

An earlier report from this laboratory [5] that the enzyme binds two molecules of adenosine disphophoribose almost as firmly as NAD^+, and with negative cooperativity, as shown by protein fluorescence measurements, must now be withdrawn. Inhibition studies with this coenzyme analogue [33], and attempts to use it to displace NAD^+ from the enzyme were inconsistent with those results, and we were unable to confirm protein fluroescence quenching by an authenticated sample of ADPR. Equilibrium dialysis at 3°C showed that the enzyme does bind two molecules of adenosine diphosphoribose with a dissociation constant of 7 μM, and a third molecule more weakly.

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References

1 Conway, A. and Koshland, D.E. (1968) Biochemistry 1, 4011—4022
2 De Vijlder, J.J.M. and Slater, E.C. (1968) Biochim. Biophys. Acta 167, 23—34

3 De Vijlder, J.J.M., Boers, W. and Slater, E.C. (1969) Biochim. Biophys. Acta 191, 214—220
4 Price, N.C. and Radda, G.K. (1971) Biochim. Biophys. Acta 235, 27—31
5 Bell, J.E. and Dalziel, K. (1975) Biochim. Biophys. Acta. 391, 249—258
6 Reynolds, C.H. and McKinley-McKee, J.S. (1969) Eur. J. Biochem. 10, 474—478
7 Ferdinand, W. (1964) Biochem. J. 92, 578—585
8 Bloch, W., MacQuarrie, R.A. and Bernhard, S. (1971) J. Biol. Chem. 246, 780—790
9 Bell, J.E. (1974) D. Phil. Thesis, Oxford University
10 Krimsky, I. and Racker, E. (1963) Biochemistry 2, 512—518
11 Fox, J.B. and Dandliker, W.B. (1956) J. Biol. Chem. 221, 1105—1017
12 Dalziel, K. and Egan, R.R. (1972) Biochem. J. 126, 975—984
13 P-L. Biochemicals (1956) Circular OR-10
14 Clarke-Walker, G.D. (1965) D. Phil. Thesis, Oxford University
15 Ciotti, M.M. and Kaplan, N.O. (1957) Methods Enzymol. 3, 890—899
16 Reynolds, C.H., Kuchel, P.W. and Dalziel, K. (1978) Biochem. J. 171, 733—742
17 Scatchard, G. Coleman, J.S. and Shen, A.L. (1957) J. Amer. Chem. Soc. 79, 12—20
18 Yphantis, D.A. (1964) Biochemistry 3, 297—317
19 Perutz, M.F. (1972) Nature 237, 495—499
20 Antonini, E. and Brunori, M. (1971) Haemoglobin and Myoglobin and their Reactions with Ligands North Holland, Amsterdam
21 Biesecker, G., Harris, J.I., Thierry, J.C., Walker, E.J. and Wonacott, A.J. (1977) Nature 266, 328—333
22 Moras, D., Olsen, K.W., Sabesan, M.N., Buehner, M., Ford, G.C. and Rossmann, M.G. (1975) J. Biol. Chem. 250, 9137—9162
23 Boers, W. and Slater, E.C. (1973) Biochim. Biophys. Acta 315, 272—284
24 Bode, J., Blumenstein, M. and Raftery, M.A. (1975) Biochemistry 14, 1153—1160
25 MacQuarrie, R.A. and Bernhard, S. (1971) Biochemistry 10, 2456—2466
26 Behme, M.T.A. and Cordes, E.H. (1967) J. Biol. Chem. 242, 5500—5509
27 Stallarp, W.B. and Koshland, D.E. (1973) J. Mol. Biol. 80, 63—75
28 Harris, J.I. and Polgar, L. (1965) J. Mol. Biol. 14, 630—633
29 Park, J.H., Agnello, C.F. and Mathew, E.J. (1966) J. Biol Chem. 241, 769—771
30 Allen, G. and Harris, J.I. (1975) Biochem. J. 151, 747—749
31 Furfine, C.S. and Velick, S.F. (1965) J. Biol. Chem. 240, 844—855
32 Eby, D. and Kirtley, M. (1971) Biochemistry 10, 2677—2682
33 Meunier, J.-C. and Dalziel, K. (1978) Eur. J. Biochem. 82, 483—492