BBA 66301

THE BINDING OF NAD+ TO RABBIT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE STUDIED BY PROTEIN FLUORESCENCE QUENCHING

N. C. PRICE AND G. K. RADDA
Department of Biochemistry, University of Oxford, Oxford (Great Britain)

(Received November 26, 1970)

SUMMARY

The binding of NAD+ to glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD+ oxidoreductase (phosphorylating), EC 1.2.1.12) from rabbit muscle has been studied by the technique of protein fluorescence quenching. The results are consistent with a model in which binding of the fourth molecule of coenzyme causes a quenching of the protein fluorescence, and the dissociation constants we obtain agree well with those derived by other techniques. The results are not consistent with the earlier proposal that the binding of the fourth molecule of coenzyme causes no overall change in protein fluorescence, and reasons for the differences between the conclusions are discussed.

RESULTS AND DISCUSSION

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD+ oxidoreductase (phosphorylating), EC 1.2.1.12) from rabbit muscle crystallises with about 3 moles of NAD+ bound per mole of enzyme; the coenzyme can be removed by treatment with charcoal[1]. The binding of NAD+ to glyceraldehyde-3-phosphate dehydrogenase has been studied by many techniques and has been the subject of considerable interest since the advancement of theories to account for allosteric regulation of enzyme activity[2,3]. "Direct separation" techniques for studying binding, like equilibrium dialysis[4] and ultracentrifugation[5] have shown the existence of four NAD+ binding sites per mole of the tetrameric enzyme[6], and that binding at these sites displays negative cooperativity. However, optical techniques, such as protein fluorescence quenching[7], absorption spectroscopy[5], and circular dichroism[8] have detected only three NAD+ sites per mole of enzyme, and it has been assumed that binding of the fourth NAD+ molecule causes no incremental change in these optical properties.

Abbreviation: $E(NAD^+)_x$, glyceraldehyde-3-phosphate dehydrogenase with x moles NAD+ bound/mole enzyme.

We wish to report a re-examination of this problem using the protein fluorescence quenching technique and to show that, using a greater concentration range of NAD^+, the binding of the fourth molecule of coenzyme can be detected.

Glyceraldehyde-3-phosphate dehydrogenase was either isolated according to the procedure of AMELUNXEN AND CARR[9], or purchased as an $(NH_4)_2SO_4$ suspension from Boehringer. There were no significant differences between these two preparations. NAD^+ was obtained from Sigma Chem. Co., St. Louis, Mo. Glycyl-L-tryptophan monohydrate was a product of Mann Research Laboratories, New York.

Apo glyceraldehyde-3-phosphate dehydrogenase was prepared by charcoal treatment of the holoenzyme[10]. Enzyme concentrations were determined using the published extinction coefficients[11].

Fluorescence titrations were performed using a Zeiss spectrofluorimeter, with a thermostatted cell compartment. Equal increments of NAD^+ solutions were added to two cuvettes, one containing Apoenzyme (approximately 0.4 mg/ml) and the other containing a glycyl-L-tryptophan solution with the same absorbance at the

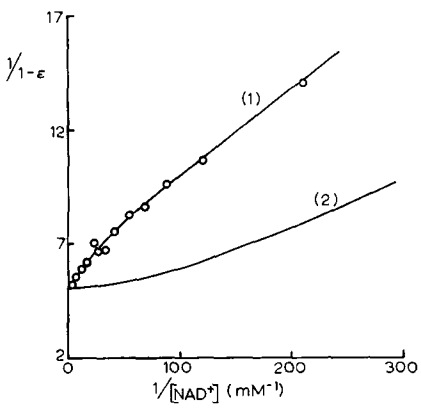
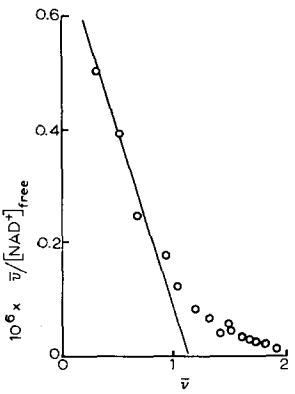


Fig. 1. Double reciprocal plot of fluorescence of glyceraldehyde-3-phosphate dehydrogenase on addition of NAD^+ (relative to $E(NAD)_2$ as 1.0) o experimental points. The solid Curves 1, 2 are calculated as described in the text. ($E(NAD)^+_2$ was at a concentration of 2.83 μM in 0.1 M Tris–HCl buffer (pH 8.2) containing 5 mM EDTA. Temp., 25°.

excitation wavelength as the apoenzyme. Excitation was at 300 nm and emission at 350 nm; the bandwidths were 7 and 10 nm, respectively. In the range of NAD^+ concentrations used (0–700 μM), formation of the charge transfer complex between NAD^+ and glycyl-L-tryptophan is negligible since the dissociation constant of this complex is approximately 0.3 M[12].

In these titrations the $E(NAD^+)_2$ complex was formed by addition of stoichiometric amounts of NAD^+ to the apoenzyme[5]. We therefore consider only the titration of this complex with further NAD^+. The fluorescence data at pH 8.2 are shown in Fig. 1 in the form of a double reciprocal plot. The fluorescence intensities) (ε) were corrected for the attenuation of the incident light by added NAD^+ using the glycyl-L-tryotophan solution as a blank. Addition of 700 μM NAD^+ increased the absorbance at 300 nm by 0.18, and the decrease in fluorescence intensity of the blank was 32%. The overall dilution was less than 8%.



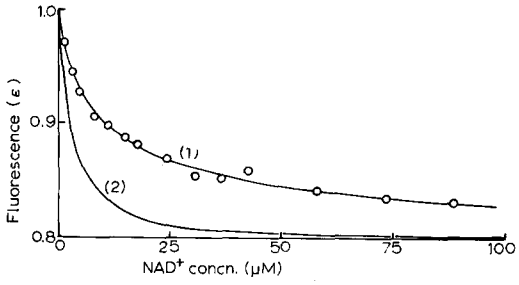


Fig. 2. Scatchard plot of data in Fig. 1. $\bar{\nu}$ is the average number of moles NAD^+ bound per mole of $E(NAD^+)_2$; $[NAD^+]_{free}$ is the concentration of free NAD^+. For conditions see Fig. 1.

Fig. 3. Protein fluorescence (ε) as a function of added NAD^+, relative to fluorescence of $E(NAD^+)_2 = 1.0$. o experimental points. The solid Curves 1, 2 are calculated as described in the text. For conditions see Fig. 1.

From the double reciprocal plot we obtain by extrapolation to infinite NAD^+, a limiting value of $\varepsilon = 0.8$. Assuming that this corresponds to the fluorescence of $E(NAD^+)_4$ we can plot the data according to the equation of SCATCHARD[13]; this is shown in Fig. 2. (ν is the average number of NAD^+ moles bound per mole of $E(NAD^+)_2$, and $[NAD^+]_f$ is the concentration of free NAD^+.)

From this plot K_3* is 1.70 μM and K_4 is approx. 37 μM. A better value of K_4 is determined from the fluorescence data by an iterative procedure in which the value of ε for $E(NAD^+)_3$ and K_4 are allowed to vary. The "best fit" values are $K_4 =$ 34 μM and ε for $E(NAD^+)_3 = 0.9$. Using these parameters we can calculate the shape of the double reciprocal plot as outlined in APPENDIX. This is shown as Curve 1 in Fig. 1. We can also calculate the expected double reciprocal plot assuming that the limiting fluorescence ($\varepsilon = 0.8$) represents the fluorescence of both the $E(NAD^+)_3$ and $E(NAD^+)_4$ complexes. (*i.e.* the model which assumes that binding of the fourth molecule of NAD^+ causes no change in the protein fluorescence). This is shown as Curve 2 in Fig. 1.

Fig. 3 shows the excellent agreement between the calculated values of ε as a function of total NAD^+ and the experimental points; Curve 1 is calculated assuming that the binding of the fourth mole of NAD^+ does cause fluorescence quenching; Curve 2 is calculated on the basis that it does not.

Titrations have also been performed at pH 7.6 (0.1 M triethanolamine buffer). A similar analysis of the data showed that NAD^+ binding was weaker at this pH with $K_3 = 20.5$ μM, $K_4 = 318$ μM. These higher dissociation constants are consistent with the increased K_m for NAD^+ at the lower pH[14].

Our results are clearly only compatible with a model which assumes that binding of the fourth molecule of NAD^+ to the enzyme causes a change in the protein fluorescence. The alternative model, in which the fourth coenzyme molecule causes

* K_3 refers to $\frac{[E(NAD^+)_2]\,[(NAD^+)]}{[E(NAD^+)_3]}$; K_4 to $\frac{[E(NAD^+)_3]\,[(NAD^+)]}{[E(NAD^+)_4]}$.

TABLE I

VALUES OF K_3 AND K_4 BY DIFFERENT METHODS

	Protein fluorescence quenching	*Ultracentrifugation*[5]	*Equilibrium dialysis*[4]
K_3 (μM)	1.7	4	0.3
K_4 (μM)	34	35	20

no such change, leads to a calculated double reciprocal plot (the solid Curve 2 in Fig. 1) which has curvature opposite to that experimentally observed at high NAD^+ concentrations. The dissociation constants for the 3rd and 4th coenzyme molecules obtained by our analysis are consistent with the values reported using "direct separation" techniques (Table I).

The earlier results using protein fluorescence quenching[7] can be explained on the basis of inadequate coverage of the titration range, since the highest NAD^+ concentration used by Velick was 1.5 μM, which is considerably lower than the measured fourth dissociation constant for NAD (refs 4, 5). VELICK[7] also reported a "limiting" fluorescence quenching of 27% relative to the apoenzyme: this is close to our value for the $E(NAD^+)_3$ complex (28%) but considerably less than our value for $E(NAD^+)_4$ (37%) (both relative to the apoenzyme). Explanations for the lack of effect of the fourth NAD^+ molecule on the optical properties of the enzyme (see for instance VELICK *et al.*[15]) based on coupled interactions between the subunits would seem to be unnecessary. A recent re-examination of the binding of NAD^+ to the enzyme by absorption spectroscopy[16] suggests that binding of the fourth molecule of NAD^+ does have an effect on the "charge transfer" absorption band at 360 nm.

APPENDIX

For the equilibria

$$EX_2 + X \rightleftharpoons EX_3$$

$$EX_3 + X \rightleftharpoons EX_4$$

where X is any ligand we have

$$[EX_3] = \frac{[EX_2]\,[X_{free}]}{K_3} \text{ and } [EX_4] = \frac{EX_2]\,[X_{free}]^2}{K_3K_4}$$

Now

$$[E_{total}] = [EX_2] + [EX_3] + [EX_4]$$

and

$$[X_{total}] = [X_{free}] + [EX_3] + 2[EX_4]$$

By eliminating $[EX]_2$ between these equations, we obtain

$$\frac{([X_{total}] - [X_{free}])}{\dfrac{[X_{free}]}{K_3} + \dfrac{2[X_{free}]^2}{K_3K_4}} = \frac{[E_{total}]}{1 + \dfrac{[X_{free}]}{K_3} + \dfrac{[X_{free}]^2}{K_3K_4}}$$

knowing $[E_{total}]$, K_3, K_4; at any $[X_{total}]$, we can solve the cubic equation in $[X_{free}]$ and hence calculate the concentrations of EX_2, EX_3 and EX_4. Assuming values for the fluorescence of these species, the overall fluorescence can be calculated.

ACKNOWLEDGEMENTS

We wish to thank D. J. Birkett for many stimulating discussions, and the Science Research Council for financial support.

REFERENCES

1 J. B. Fox, Jr. and W. B. Dandliker, *J. Biol. Chem.*, 221 (1956) 1005.
2 J. Monod, J. Wyman and J. P. Changeux, *J. Mol. Biol.*, 12 (1965) 88.
3 D. E. Koshland, Jr., G. Nümethy and D. Filmer, *Biochemistry*, 5 (1966) 365.
4 A. Conway and D. E. Koshland, Jr., *Biochemistry*, 7 (1968) 4011.
5 J. J. M. De Vijlder and E. C. Slater, *Biochim. Biophys. Acta*, 167 (1968) 23.
6 J. I. Harris and R. N. Perham, *J. Mol. Biol.*, 13 (1965) 876.
7 S. F. Velick, *J. Biol. Chem.*, 233 (1958) 1455.
8 J. J. M. De Vijlder and B. J. M. Harmsen, *Biochim. Biophys. Acta*, 178 (1969) 434.
9 R. E. Amelunxen and D. O. Carr, *Biochim. Biophys. Acta*, 132 (1967) 256.
10 J. J. M. De Vijlder and E. C. Slater, *Biochim. Biophys. Acta*, 132 (1967) 207.
11 A. L. Murdock and O. J. Koeppe, *J. Biol. Chem.*, 239 (1964) 1983.
12 G. Cilento and P. Giusti, *J. Am. Chem. Soc.*, 81 (1959) 3801.
13 G. Scatchard, J. S. Coleman and A. L. Shen, *J. Am. Chem. Soc.*, 79 (1957) 12.
14 C. S. Furfine and S. F. Velick, *J. Biol. Chem.*, 240 (1965) 844.
15 S. F. Velick, J. P. Baggott and J. M. Sturtevant, in H. Sund, *Pyridine Nucleotide-Dependent Dehydrogenases*, Springer, Berlin, 1970, p. 229.
16 E. C. Slater, personal communication.