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Interaction of Pigeon Liver Nicotinamide–Adenine Dinucleotide Kinase with Cibacron Blue F3GA

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The interaction of pigeon liver NAD kinase with Cibacron Blue F3GA was investigated. By using steady-state rate measurements, spectrophotometric titration and chromatography of the enzyme on immobilized dye, it was shown that binding occurs at two nucleotide sites with different affinities, and also at a site distinct from the substrate-binding region.

The sulphonated aromatic dye Cibacron Blue F3GA has long been recognized as a ligand for various enzymes with nucleotide substrates (Staal *et al.*, 1971; Blume *et al.*, 1971); it has been claimed that the dye binds specifically to the dinucleotide fold in proteins (Thompson *et al.*, 1975; Thompson & Stellwagen, 1976).

NAD kinase (EC 2.7.1.23) from pigeon liver appears to have independent binding sites for NAD⁺ (or NADH) and for MgATP²⁻ (or ADP³⁻) (Apps, 1968; Apps & Marsh, 1972); we therefore decided to investigate the interaction of the dye with this enzyme.

Materials and Methods

For kinetic and spectral work, NAD kinase was purified and assayed as described previously (Apps, 1968, 1975). Before affinity chromatography, the enzyme was partially purified by protamine precipitation and adsorption on Ca₃(PO₄)₂ (Apps, 1968). Spectrophotometric titrations were performed in a Unicam SP.1800 recording spectrophotometer, at 22°C. Cibacron Blue F3GA (a generous gift from CIBA-GEIGY, Manchester, U.K.) was purified by precipitation from methanol solution with dry diethyl ether, and the extinction coefficient in aqueous solution found to be 12.3 × 10³ litre · mol⁻¹ · cm⁻¹ at 610 nm. It was coupled to cross-linked desulphated Sepharose 4B (Porath *et al.*, 1971) by the method of Böhme *et al.* (1972). Alternatively, the dye was linked to carboxyhexyl-Sepharose by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Blue Dextran was coupled to Sepharose 4B by the method of Ryan & Vestling (1974). Affinity chromatography was performed on 6 cm × 0.8 cm columns, in 0.02 M-potassium phosphate buffer, pH 7.4, containing 0.1 M-KCl and 1 mM-EDTA, at 6°C. The flow rate was 0.1 ml/min. Protein was determined by the Folin–Lowry method (Lowry *et al.*, 1951), after precipitation with trichloroacetic acid, with bovine serum albumin as standard.

Results and Discussion

Reciprocal plots (Hanes, 1932) with NAD⁺ or MgATP²⁻ as the varied substrate suggested that Cibacron Blue was competitive with NAD⁺ and non-competitive with MgATP²⁻ (Fig. 1). However, determination of the individual parameters of the rate equation

$$v_0 = V_{\max} \left(1 + \frac{K_a}{[\text{MgATP}^{2-}]} + \frac{K_b}{[\text{NAD}^+]} + \frac{K_{ab}}{[\text{MgATP}^{2-}][\text{NAD}^+]} \right)^{-1}$$

revealed that this pattern was due to an increase in all of the parameters except $V_{\max}$ (Table 1). From this it can be deduced that the dye binds strongly to the NAD⁺ site in the enzyme–MgATP²⁻ complex ($K_1 = 7 \mu\text{M}$), less strongly to the MgATP²⁻ site in enzyme–NAD⁺ ($K_1 = 14 \mu\text{M}$), and only weakly to these sites in the free enzyme. Although the apparent K_m for NAD⁺ is independent of the concentration of MgATP²⁻, and vice versa (Apps, 1968), different strengths of binding to free enzyme and to binary complexes have been observed with other nucleotides (Apps & Marsh, 1972), and may indicate the occurrence of conformation changes on substrate binding.

The interaction between dye and protein was investigated by studying the perturbation of the absorption spectrum of the dye (Thompson & Stellwagen, 1976). Difference spectra obtained after successive additions of dye to enzyme in the presence of high concentrations of NAD⁺ or MgATP²⁻ showed no isosbestic point, suggesting that more than one type of complex was formed in each case; determination of dissociation constants was not possible under these circumstances. In the absence of other ligands absorbance differences at 660 nm approximately fitted a binding curve with $K_1 = 21 \mu\text{M}$, assuming one binding site per NAD kinase subunit of mol.wt. 34000 (Apps, 1975).

Fig. 1. Inhibition of NAD kinase by Cibacron Blue F3GA

(a) $[\text{NAD}^+]$ varied, $[\text{MgATP}^{2-}] = 2.1 \text{ mM}$. Cibacron Blue concentrations: $\circ$, 0; $\bullet$, 18.7; Δ , 37.3; $\blacktriangle$, 56.0 μM . (b) $[\text{MgATP}^{2-}]$ varied, $[\text{NAD}^+] = 0.50 \text{ mM}$. Cibacron Blue concentrations: $\circ$, 0; $\bullet$, 17.9; Δ , 35.8; $\blacktriangle$, 53.8 μM . Rate measurements were performed in 0.1 M-triethanolamine/HCl buffer, pH 7.4, at 30°C , with a free Mg^{2+} concentration of 10 mM. The units of v_0 are $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

Table 1. Kinetic parameters of the rate equation for the NAD kinase reaction

Rates were determined in 0.1 M-triethanolamine/HCl buffer, pH 7.4, at 30°C .

	No inhibitor	+14.3 μM -Cibacron Blue F3GA
K_a (mM)	2.30	4.65
K_b (mM)	0.58	1.75
K_{ab} (mM ²)	1.12	2.00
V_{max} ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$)	0.92	0.90

The spectra of ternary enzyme-substrate-dye complexes relative to the free dye were characterized by a positive maximum at 670 nm, and a trough at 575 nm (Fig. 2); in the absence of substrates, the maximum was at 660 nm, and the much smaller trough at 565 nm.

Chromatography of NAD kinase on various immobilized forms of Cibacron Blue showed that the enzyme did not bind to Blue-Dextran-Sepharose, nor to Cibacron Blue attached through the carboxyl group of a six-carbon spacer, but was almost completely retained by Sepharose with directly coupled Cibacron Blue. It was not eluted by KCl (1 M), NAD^+ (5 mM), NADH or NADPH (2 mM), MgATP^{2-}

Fig. 2. Difference spectra produced when Cibacron Blue F3GA binds to NAD kinase

The sample cuvette contained 47.4 μM -Cibacron Blue and 1.48 mg of NAD kinase/ml in 0.1 M-potassium phosphate buffer, pH 7.4; the reference cuvette contained 47.4 μM -Cibacron Blue. Additional components in both cuvettes were: (a) none; (b) 6.3 mM- NAD^+ ; (c) 12.5 mM- MgATP^{2-} . (d) is the instrument baseline.

or ADP^{3-} (10 mM), nor by NADH and MgATP^{2-} together; but could be eluted by 0.5 mM-Cibacron Blue, with up to 70% recovery. The specific activity

of the eluate was 0.4 unit/mg, if the column was loaded with enzyme partially purified through two steps (Apps, 1968), and washed with ADP and NADH before elution. This preparation contained several protein components, and the specific activity is lower than that achieved by the previously described procedure (Apps, 1975); this is presumably because many proteins are capable of binding to immobilized Cibacron Blue. Complete removal of the eluting dye from the enzyme could not be achieved by dialysis, gel filtration or charcoal adsorption, and binding of dye to NAD kinase subunits was apparent even during polyacrylamide-gel electrophoresis in presence of sodium dodecyl sulphate, and this suggests tighter binding than the $21 \mu\text{M}$ value for K_i might indicate.

The results of kinetic experiments suggest that Cibacron Blue F3GA is able to compete with both NAD^+ and MgATP^{2-} , but that the dye binds best to binary enzyme-substrate complexes. Binding of the dye to the free enzyme is confirmed by spectral titrations, but this may occur at sites other than substrate-binding regions, not therefore necessarily being inhibitory or revealed by kinetic studies. Chromatography shows that the free enzyme binds to the immobilized dye, and is not released by substrates, again suggesting binding that does not compete with nucleotides. The usefulness of this technique as a purification step is decreased by the failure to remove the dye subsequently, but NAD kinase so prepared is readily immobilized in

'activated' nylon tubes (P. V. Sundaram & D. K. Apps, unpublished work). The complexity of the interaction of Cibacron Blue F3GA with NAD kinase limits its usefulness in investigating the properties of this enzyme.

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