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Binding of Cibacron blue F3GA to proteins containing the dinucleotide fold

(lactate dehydrogenase/phosphoglycerate kinase/spectral titration/NAD domain/transition state analog)

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ABSTRACT **A simple, convenient, and sensitive spectrophotometric procedure is described for quantitative measurement of nucleoside phosphate binding sites constructed by the dinucleotide fold. The procedure involves difference spectral titration of such enzymes with the dye Cibacron blue F3GA in a spectral region remote from the intrinsic absorbance of proteins or natural ligands. The titration curves can be analyzed to determine the affinity of nucleoside phosphate binding sites for both the dye and the natural ligand over a potentially wide range of experimental conditions. The interaction of the dye with two proteins which contain the dinucleotide fold, lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) and phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3), is illustrated.**

We have recently demonstrated (1) that blue dextran covalently attached to Sepharose can be used to prepare an affinity column specific for those proteins whose nucleoside phosphate binding sites are constructed of the supersecondary structure called the dinucleotide fold (2, 3). Since the aromatic ring(s) of nucleoside phosphates such as NAD and ATP are inserted into apolar pockets when bound to these proteins (4–7), we anticipate that binding of the chromophore of blue dextran, Cibacron blue F3GA, at the nucleoside phosphate site should produce a red shift in the absorption spectrum of the dye. Accordingly, we have investigated the spectral consequences of the interaction of Cibacron blue F3GA dye with several proteins known to possess the dinucleotide fold. Particular emphasis has been placed on the interaction of the dye with lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) as a model system, since this protein contains a single dinucleotide fold per subunit and binds a single dinucleotide noncooperatively with a high affinity.

EXPERIMENTAL PROCEDURES

Rabbit muscle M_4 (isozyme 5) and beef heart H_4 (isozyme 1) lactate dehydrogenase were purchased from the Sigma Chemical Co. Yeast phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) and rabbit muscle glyceraldehyde-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD$^+$ oxidoreductase (phosphorylating), EC 1.2.1.12] were purchased from Boehringer Mannheim while subtilisin Novo (EC 3.4.21.4) was obtained from Novo Industri, Japan. A purified sample of Cibacron blue F3GA dye was kindly provided by Dr. H. Bosshard of Ciba-Geigy, Basel, Switzerland. The structural formula of this dye has been given previously (1). All proteins and the dye were found to be homogeneous preparations as judged by polyacrylamide electrophoresis measurements. Protein concentrations were measured spectrophotometrically at 280 nm using extinction coefficients of 162 (8), 210 (9), and 115 (10) mM^{-1} cm^{-1} for M_4 lactate dehydrogenase, H_4 lactate dehydrogenase, and glyceraldehyde-phosphate dehydrogenase, respectively. The concentrations of phosphoglycerate kinase and subtilisin were determined using $E^{1\%}_{280}$ values of 4.9 (11) and 11.7 (12) and molecular weights of 47,000 (11) and 27,500 (12) for the two proteins, respectively. The dye concentration was also measured spectrophotometrically at 610 nm using an extinction coefficient of 13.6 mM^{-1} cm^{-1} determined by dry weight measurements.

Difference spectra and difference spectral titrations were performed at room temperature in cuvettes having 10 mm light paths using a Cary Model 14 and a Gilford spectrophotometer, respectively. Exactly 1 ml of a protein solution and 1 ml of the protein solvent were placed in the sample and reference cuvettes, respectively, and the baseline difference spectrum or difference absorbance was measured. Identical volume increments of a concentrated solution of Cibacron blue F3GA dye were added to both cuvettes and the difference spectrum or absorbance was obtained after each incremental addition. The dissociation constant for the protein:dye binary complex was obtained by fitting the experimental values to the equilibrium expression,

$$K_{dye} = \frac{[Dye][Protein]}{[Complex]} = \frac{(D_T - [PD])(P_T - [PD])}{[PD]} \quad [1]$$

where P_T and D_T are the total concentrations of protein subunits and dye present, respectively, and PD is the concentration of complex, which is equal to $\Delta A_{observed}/\Delta\epsilon_{max}$. The ability of selected ligands to displace the dye from the protein was examined by measuring either the difference spectrum or difference absorbance following addition of identical incremental volumes of a concentrated solution of the ligands to both a sample cuvette containing protein and dye and a reference cuvette containing the same concentration of dye only. The dissociation constant for the protein:ligand binary complex was obtained by fitting the experimental values to the expression

$$K_{ligand} = \frac{[Protein][Ligand]}{[Complex]} = \frac{([P])(L_T - [PL])}{[PL]} = \frac{\left(K_{dye}[PD]\right)\left(L_T - P_T + [PD] + \frac{K_{dye}[PD]}{D_T - [PD]}\right)}{\left(D_T - [PD]\right)\left(P_T - [PD] - \frac{K_{dye}[PD]}{D_T - [PD]}\right)} \quad [2]$$

where the concentration of unbound protein, P, equals $K_{dye}[PD]/D_T - [PD]$ from Eq. 1, the concentration of protein:ligand binary complex, PL, equals $P_T - [P] - [PD]$, and L_T is the total concentration of ligand present.

Equilibrium dialysis measurements were obtained using Plexiglas cells having two 1-ml chambers separated by a

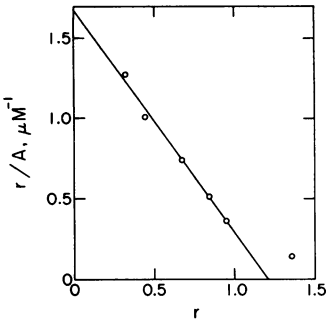

FIG. 1. Equilibrium dialysis measurements of the binding of Cibacron blue F3GA dye by M_4 lactate dehydrogenase. The initial concentration of dye ranged from 3.3 to 38.6 μM, the initial concentration of protein subunits was 14.15 μM, and the solvent was 10 mM Tris·HCl buffer, pH 7.5, containing 0.5 mM mercaptoethanol. All measurements were made after equilibration of the protein with the dye in the dark for 6 days at 4°. The quantity r is the micromoles of dye bound per micromole of protein subunit while A is the concentration of unbound dye in μM. The values shown have been corrected for binding of the dye to the membrane.

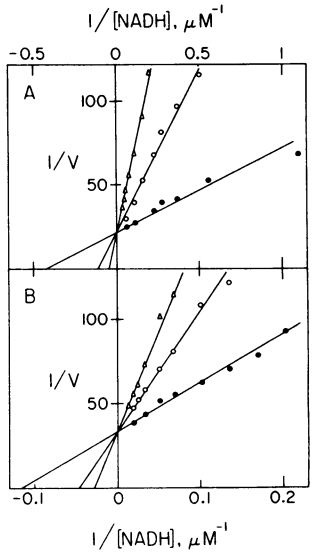

FIG. 2. The inhibition of lactate dehydrogenase catalysis by Cibacron blue F3GA dye. All measurements were made at room temperature using the spectrophotometric assay procedure described in the *Experimental Procedures* section. The initial rates of catalysis, V, have the units ΔA_{340}/min. (A) M_4 lactate dehydrogenase. The concentrations of dye present were: ●, none; ○, 0.27 μM; and △, 0.86 μM. (B) H_4 lactate dehydrogenase. The concentrations of dye present were: ●, none; ○, 2.15 μM; and △, 6.83 μM.

Visking membrane. Cells containing 14.15 μM protein subunit in 0.35 ml of 20 mM Tris·HCl buffer, pH 7.5, containing 1mM mercaptoethanol were equilibrated with 0.35 ml of various concentrations of dye in water for 6 days at 4° with gentle shaking. The cells were covered with aluminum foil during equilibration to prevent any dye-mediated photo-oxidation reactions. Blank cells containing no protein were also equilibrated simultaneously at each dye concentration to measure the binding of the dye to the Visking membrane.

Enzymic activity of M_4 lactate dehydrogenase was measured spectrophotometrically by observing the loss of absorbance at 340 nm. The assay solutions contained 10 mM Tris·HCl buffer, pH 7.5, 0.5 mM mercaptoethanol, 0.7 mM sodium pyruvate, the indicated concentrations of NADH and dye, and about 0.04 nM enzyme. All measurements were made in cuvettes having path lengths of 50 mm. Catalysis was initiated by addition of pyruvate. Assay solutions for the H_4 isozyme were identical except that the concentration of pyruvate was 0.33 mM, the concentration of enzyme was 0.1 nM, and the cuvettes had path lengths of 10 mm. Michaelis and inhibition constants were calculated by a computer fit of the experimental values to the equation for competitive inhibition using a weighted least squares program.

RESULTS

M_4 lactate dehydrogenase

Equilibrium dialysis measurements of the binding of Cibacron blue F3GA dye by M_4 lactate dehydrogenase are shown in Fig. 1 in the form of a Scatchard plot. These results indicate that each subunit of lactate dehydrogenase binds one dye molecule and that the dissociation constant for the protein subunit:dye binary complex is 0.71 μM.

Kinetic measurements of the initial rates of the reaction catalyzed by M_4 lactate dehydrogenase in the presence and absence of Cibacron blue F3GA dye are shown in Fig. 2A as double reciprocal plots. Analysis of these results indicates that the dye is a competitive inhibitor for the substrate NADH, having a K_i of 0.13 ± 0.01 μM. The results obtained from equilibrium dialysis and from enzymic activity measurements together indicate that a single dye molecule binds to each protein subunit at its NADH binding site as predicted from previous affinity chromatographic measurements using blue dextran-Sepharose (1).

Solutions of Cibacron blue F3GA dye in 10 mM Tris·HCl buffer, pH 7.5, exhibit a rather broad spectral absorption maximum centered at 610 nm. In the presence of M_4 lactate dehydrogenase the absorption spectrum of the dye undergoes a red shift, producing difference spectra having positive maxima at 660 nm and an isobestic point at 584 nm, as shown in Fig. 3A. The increase in the extinction at 660 nm exhibits a hyperbolic dependence on the concentration of added dye, as shown in Fig. 4A, indicating the formation of a protein:dye complex. Since the affinity of the protein for dye is very strong, only the experimental $\Delta\epsilon$ values collected near the limiting value of 3.2 mM^{-1} cm^{-1}, where an appreciable amount of unbound dye is present, can be used to estimate a dissociation constant. Assuming that the difference spectral measurements also reflect the binding of a single dye per protein subunit, a dissociation constant of 0.49 ± 0.16 μM was calculated using Eq. 1.

The difference spectrum produced by addition of M_4 lactate dehydrogenase to solutions of the dye is quite similar to the difference spectrum resulting from the presence of 50% ethylene glycol in the dye solvent as shown in Fig. 3B. A solvent containing 50% ethylene glycol would be expected to be 40% as effective as a protein fabric in perturbing the absorbance spectrum of a chromophore (13). The favorable comparison of the two difference spectra in Fig. 3 suggests that the dye occupies the hydrophobic pockets designed to bind the aromatic rings of NAD or NADH. If this interpretation is correct, then addition of the natural ligands NAD or NADH to the M_4 lactate dehydrogenase:dye complex should displace the dye, resulting in the loss of the observed difference spectrum. As shown in Fig. 4B, addition of increasing concentrations of either of these ligands results in a progressive decrease in the difference absorbance measured at 660 nm. The average dissociation constants for both protein:ligand binary complexes (calculated using Eq. 2) are listed in

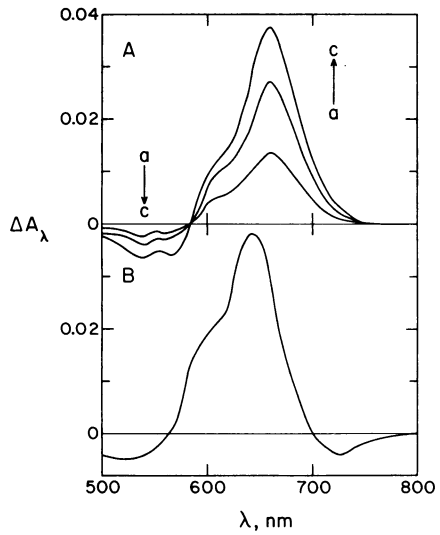

FIG. 3. Difference spectra of Cibacron F3GA dye in the presence of lactate dehydrogenase or ethylene glycol. (A) Protein difference spectra. The sample cuvette contained 12 μM M_4 lactate dehydrogenase subunits while both the sample and reference cuvettes contained (a) 2.15 μM dye, (b) 4.28 μM dye, and (c) 7.47 μM dye. The solvent was 10 mM Tris·HCl buffer, pH 7.5, containing 0.5 mM mercaptoethanol. (B) Ethylene glycol difference spectrum. The sample cuvette contained 50% (vol/vol) ethylene glycol while both sample and reference cuvettes contained 21.5 μM dye. The solvent was water. Both cuvettes in (B) had a path length of 8.75 mm.

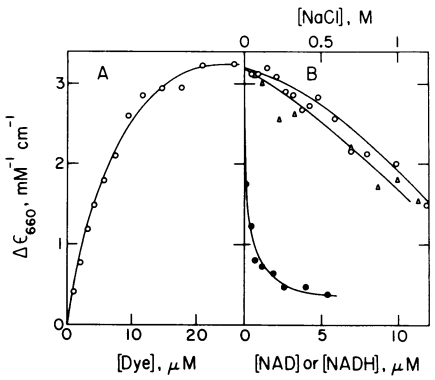

FIG. 4. Difference spectral titrations of M_4 lactate dehydrogenase solutions with Cibacron blue F3GA dye. (A) The difference extinction coefficient at 660 nm as a function of the total dye present. The initial protein subunit concentration was 12.0 μM. (B) The effect of increasing concentrations of NAD (○), NADH (●), and NaCl (△) on the difference extinction coefficient. All solutions initially contained 11.6 μM protein subunits and 26.1 μM dye. The solvent for all measurements was 10 mM Tris·HCl buffer, pH 7.5, containing 0.5 mM mercaptoethanol.

Table 1. These values together with K_{NADH} for the H_4 enzyme:NADH binary complex described below are within the range of values measured previously by fluorometric titrations (8, 14, 15).

Since NaCl also displaces M_4 lactate dehydrogenase from blue dextran-Sepharose affinity columns (1), addition of NaCl to the protein:dye complex should cause the loss of the difference spectrum. The relatively high concentrations of NaCl required to cause the loss of the difference extinction measured at 660 nm, shown in Fig. 4B, are commensurate with the concentrations of NaCl required to displace the protein from the affinity columns.

H_4 lactate dehydrogenase

The H_4 isozyme of lactate dehydrogenase appears to bind less tightly to a blue dextran-Sepharose affinity column than does the M_4 isozyme, particularly at pH values above pH 7.5 (C. S. Vestling, personal communication). Addition of the H_4 isozyme to a solution of the dye at pH 7.5 produces a difference spectrum similar to that shown in Fig. 3A but having a positive maximum at 665 nm. Analysis of the difference spectral titration of the H_4 isozyme measured at 665 nm indicates that the H_4 isozyme:dye complex has a dissociation constant of 2.8 ± 1.2 μM, about six times larger than that of the M_4 isozyme:dye complex. Addition of NADH to the H_4 isozyme:dye complex causes the disappearance of the difference spectrum from which a dissociation constant for the H_4 isozyme:NADH complex of 5.9 ± 3.2 μM was calculated. Kinetic measurements of H_4 isozyme catalysis indicate that the dye functions as a competitive inhibitor for NADH as shown in Fig. 2B. The inhibition constant for the H_4 isozyme:dye complex is 2.1 ± 0.2 μM.

Other proteins

Crystallographic studies have shown that the dinucleoside phosphate binding site of glyceraldehyde-phosphate dehydrogenase (6) and the mononucleoside phosphate binding site of phosphoglycerate kinase (16, 17) are constructed by dinucleotide folds, while the aromatic specificity site of subtilisin (2, 3) is constructed by a remnant of the fold. Previous measurements (1) have demonstrated that the dehydrogenase and the kinase are retained by blue dextran-Sepharose affinity chromatographic columns while subtilisin is not. Addition of a 4-fold molar excess of dye to solutions of either the kinase or dehydrogenase but not subtilisin produced significant visible difference spectra having positive maxima at 680 nm. Thus formation of a difference spectrum having a

Table 1. Dissociation constants for protein:dye and protein:ligand complexes

Protein	Measurement	K_{dye}, μM	Ligand	K_{ligand}, μM
M_4 Lactate dehydrogenase	Spectral titration	0.49 ± 0.16	NADH	7.0 ± 2.8
			NAD	660 ± 390
	Enzyme catalysis	0.13 ± 0.01	NADH	3.4 ± 0.3
	Equilibrium dialysis	0.71		
H_4 Lactate dehydrogenase	Spectral titration	2.8 ± 1.2	NADH	5.9 ± 3.2
	Enzyme catalysis	2.1 ± 0.2	NADH	8.9 ± 0.9
Phosphoglycerate kinase	Spectral titration	6.9 ± 2.0	ATP	420 ± 110
			3-PGA	470 ± 170

All measurements were made in 10 mM Tris·HCl buffer, pH 7.5, containing 0.5 mM mercaptoethanol at room temperature except for the dialysis measurements which were done at 4°. The following abbreviation is used: 3-PGA, 3-phosphoglycerate.

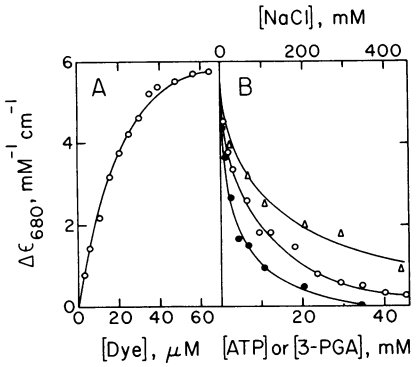

FIG. 5. Difference spectral titrations of phosphoglycerate kinase solutions with Cibacron blue F3GA dye. (A) The difference extinction coefficient at 680 nm as a function of the total dye present. The initial protein concentration was 12.05 μM. (B) The effects of increasing concentrations of ATP (●), 3-phosphoglycerate (3-PGA) (○), and NaCl (△) on the difference extinction coefficient are shown. All solutions initially contained 10.95 μM protein and 39.12 μM dye. The solvent for all measurements was 10 mM Tris·HCl buffer, pH 7.5, containing 0.5 mM mercaptoethanol.

positive maximum in the range 660–680 nm appears characteristic for complexation of the dye with proteins containing the dinucleotide fold. Variations in the position of the positive maxima may reflect subtle differences in the orientation of the dye when bound to the protein.

We have also investigated the interaction of the dye with phosphoglycerate kinase in some detail, since the dinucleotide fold in this monomeric protein forms the binding site for a mononucleoside phosphate as contrasted with the dinucleoside phosphate binding site in lactate dehydrogenase. The spectral titration of phosphoglycerate kinase with dye is shown in Fig. 5A. A dissociation constant of 6.9 ± 2.0 μM was calculated for the enzyme:dye binary complex from these titration data using Eq. 1. Addition of increasing concentrations of either ATP or 3-phosphoglycerate to the enzyme:dye binary complex results in a progressive decrease in the difference extinction measured at 680 nm as shown in Fig. 5B. Though not shown, ADP was equally as effective as ATP in decreasing the difference extinction. The average dissociation constants for both enzyme:ligand binary complexes calculated form the data shown in Fig. 5B using Equation 2 are listed in Table 1. These values are in excellent agreement with both the K_m values (11) and K_d values (18) reported for the phosphoglycerate kinase:ATP and phosphoglycerate kinase:3-phosphoglycerate complexes. Addition of increasing concentrations of NaCl to the phosphoglycerate kinase:dye complex also results in a progressive decrease in the difference extinction. As shown in Fig. 5B, about an order of magnitude higher concentration of NaCl is required to produce the same loss of $\Delta\epsilon_{680}$ produced by ATP. At least 200 mM NaCl is required to rapidly elute phosphoglycerate kinase from a blue dextran-Sepharose affinity column, in agreement with the special titration data shown in Fig. 5B.

DISCUSSION

The principal finding of this report is the development of a simple, rapid and sensitive spectrophotometric procedure utilizing a spectral region remote from the intrinsic absorbance of proteins or natural ligands for measurement of the integrity of either mono- or dinucleotide phosphate binding sites constructed by the dinucleotide fold. The integrity of a fold can be expressed as its affinity for either the dye Cibacron blue F3GA or its natural ligands. The procedure should be amenable in principle to quantitative measurement of the integrity of the binding site in response to changes of pH, temperature, the presence of biochemicals or denaturants, and chemical modification of the protein over a wide range of conditions which often preclude the application of established alternative procedures.

Secondarily, it should be noted that the interactions of the model proteins examined here, lactate dehydrogenase, phosphoglycerate kinase, and subtilisin, with the free dye parallel the interactions of these proteins with the immobilized dye attached through dextran to Sepharose (1). For example, both spectral titration and affinity chromatographic measurements indicate that NADH is more effective than NAD, which is more effective than NaCl, in displacing the dye from lactate dehydrogense:dye binary complex. Similarly, the smaller value for the dissociation constant of the M_4 as opposed to the H_4 lactate dehydrogenase:dye complex parallels the stronger retention of the M_4 isozyme by affinity columns. These favorable comparisons suggest that the previous perception of blue dextran-Sepharose affinity chromatography is correct.

Finally, the nature of protein:dye binary complexes merits comment. As noted in Table 1, the dye binds more tightly to the model proteins than the natural nucleotide ligands specific for the individual proteins. This is somewhat surprising, since the dye contains an anionic group on its terminal six-membered ring which corresponds to the nicotinamide ring of NAD (1). The presence of this anionic group would be expected to decrease rather than enhance the affinity of lactate dehydrogenase for the dye relative to NAD or NADH. However, the covalent adduct NAD-pyruvate, which has an anionic group on its nicotinamide ring, also has a greater affinity for the enzyme than does either NAD or NADH (19). The covalent adduct:enzyme binary complex is known to be isomorphous (4) with ternary complexes such as enzyme:NADH:oxamate considered to represent transition state analogs (20). The similarity of the structure and affinity of dye and NAD-pyruvate adduct suggests that the enzyme:dye binary complex may also be a transition state analog with the dye occupying at least portions of both substrate binding sites. Similar complexation of the dye across the ATP/ADP and 3-phosphoglycerate binding sites of phosphoglycerate kinase, while an interesting possibility, remains only conjecture with the available data. While displacement of the dye from this enzyme:dye binary complex by either substrate would seemingly support such a binding geometry, it should be noted that ADP and 3-phosphoglycerate are mutual competitive inhibitors (21). Accordingly, either substrate would displace the dye from simple binding to the ATP/ADP binding site on the enzyme. However, if crystallographic studies indicate that the dye does bridge the binding sites for two-substrate enzymes containing the dinucleotide fold, the dye could be very useful for further studies of active site geometry and function.

We are grateful to Drs. Susan S. Taylor and Gerald Rawley for independently drawing our attention to the structural comparison of the dye with the NAD-pyruvate adduct. This investigation was supported by U.S. Public Health Service Research Grant GM-13215 and Training Grant T01-GM-550, both from the Institute of General Medicine Sciences. S.T.T. is a U.S. Public Health Service predoctoral trainee.

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