Use of Blue Dextran as a Probe for the Nicotinamide Adenine Dinucleotide Domain in Proteins

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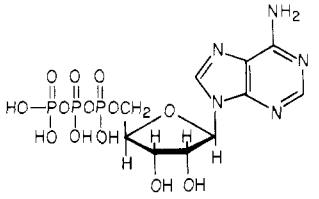
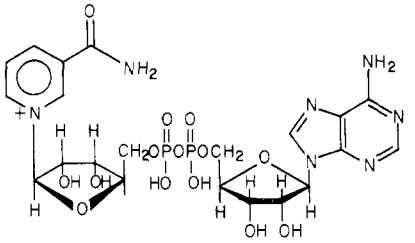
Nucleotides such as adenosine 5′-triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) are reactants common to several hundred different biological reactions, each catalyzed by a different enzyme. The mononucleotide ATP commonly functions as the phosphoryl donor for phosphate transfer reactions catalyzed by kinases specific for a wide variety of phosphate acceptors, while the dinucleotide NAD functions as a reversible hydrogen acceptor in a variety of biological oxidative reactions catalyzed by dehydrogenases.



adenosine triphosphate(ATP)



nicotinamide adenine dinucleotide (NAD^+)

The recent outpouring of enzyme structures described to high resolution by x-ray crystallographers has led to the recognition that enzymes which catalyze reactions involving ATP or NAD frequently possess a common architectural feature called the NAD binding domain.[1] This domain, illustrated schematically in Figure 1, involves a continuous 150-residue segment of a polypeptide chain arranged into a β-sheet core composed of four to six parallel β strands connected by helical intrastrand loops located above and below the β sheet. The amino acid residues surrounding the C terminus of the β sheet form a high affinity binding site for a mono- or dinucleotide and dictate its selectivity. Crystallographic measurements have demonstrated that the NAD domain in certain dehydrogenases,[1] kinases,[1,2] a phosphorylase,[3] and a mutase[4] forms the binding sites for NAD, ATP, adenine ribose 5′-phosphate (AMP), and a sugar phosphate, respectively.

Blue dextran is a high molecular weight (about 2×10^6) water-soluble conjugate of dextran and a blue dye commonly used to measure the void volume of exclusion chromatographic columns. Many investigators have inadvertently observed that blue dextran associates rather tightly with certain proteins when it is added to protein solutions prior to exclusion chromatography in solvents of low ionic strength. Among the earliest reported blue dextran–protein complexes are those involving pyruvate kinase[5] and phosphofructokinase.[6] Such complexation has subsequently been exploited to selectively enrich[7] or deplete[8] heterogeneous protein solutions of a desired component using either mobile[7a-d,8] or immobilized[7e-o] blue dextran.

Recent chromatographic[7i] and spectrophotometric[9] measurements indicate that the blue dye in blue dextran specifically complexes with protein ligand sites constructed by the NAD binding domain. This Account will review the evidence for the specificity of complexation of the blue dye of blue dextran with proteins and illustrate the broad utilization of this specificity for structural predictions and protein manipulations.

Dye Specificity

The blue dye in blue dextran is reactive blue 2, Color Index No. 61-211, commercially known as Cibacron Blue F3GA. Comparison of a space-filling model of reactive blue 2 with that of NAD oriented[1] as it binds to the NAD binding domain of a dehydrogenase reveals distinct structural similarities. As shown in Figure 2,

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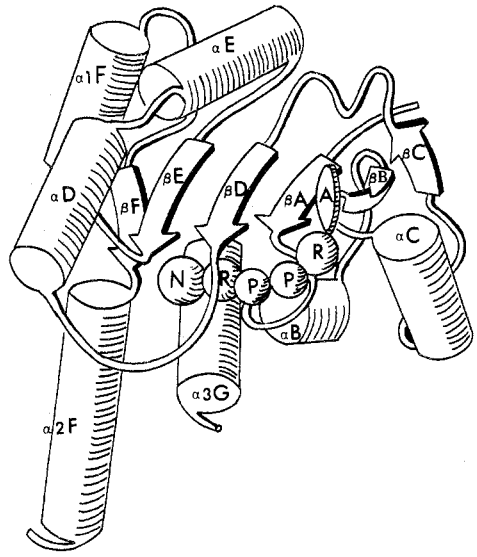
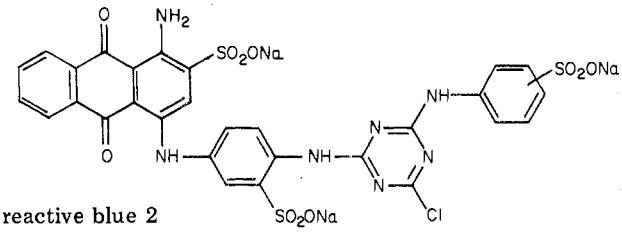


Figure 1. A diagrammatic representation of the NAD binding domain of lactate dehydroganse.[10] The parallel β strands are labeled β_A. . .β_F while the α helices are labeled α_B. . .α_{3G}. The position of the structural elements of NAD, adenine, ribose, phosphate, and nicotinamide, denoted as A, R, P, and N, respectively, are indicated as they bind to the C terminus of the β sheet of the NAD binding domain.



reactive blue 2

the anthraquinone and terminal phenylsulfonate rings of the dye can be oriented as the adenine and nicotinamide rings of NAD, respectively. The two internal rings of the dye mimic the two ribosofuranose rings of NAD, and two of the dye anionic sulfonate groups are positioned similarly to the two anionic phosphates of NAD. The structure of reactive blue 2 is also similar to the structures of ATP and coenzyme A as shown in Figure 2.

The interaction of reactive blue 2 with the M_4 isozyme of lactate dehydrogenase, whose four NAD binding sites are constructed by an NAD binding domain,[10] has been studied[9] in detail. Equilibrium dialysis measurements demonstrate that four dye molecules bind independently to tetrameric lactate dehydrogenase, each with an association constant of 1.4×10^6 M. Double-reciprocal plots of the dependence of the rate of catalysis on the concentration of NADH in the presence and absence of dye indicate that the dye is a competitive inhibitor with NADH. The reciprocal of the competitive inhibition constant, $7.7 \pm 0.6 \times 10^6$ M, compares favorably with the association constant measured by equilibrium dialysis. Thus the single dye-binding site on each subunit must also be the NAD site constructed by the NAD binding domain.

This conclusion is supported by difference spectral measurements of the dye–enzyme complex in the presence of ligands specific for the NAD binding domain. If the anthraquinone and terminal phenylsulfonate rings of the dye are inserted into the apolar pockets in the NAD binding domain designed to bind the adenine and nicotinamide rings of NAD, then complexation of the dye with the enzyme should cause a red shift in the absorption spectrum of the dye. This red shift does occur, producing a difference spectrum having a positive maximum at 660 nm, as shown in the difference spectra illustrated in Figure 3A. Both the occurrence of the isosbestic point at 584 nm (Figure 3A) and the hyperbolic dependence of the difference absorbance at 660 nm on the total dye concentration (Figure 3B) indicate formation of a discrete dye–enzyme complex. An association constant of $2.3 \pm 0.7 \times 10^6$ M calculated from this hyperbolic dependence is in good agreement with the association constant for the dye–enzyme complex calculated from equilibrium dialysis and kinetic measurements.

Addition of increasing concentrations of the ligands NAD or NADH to the dye–enzyme complex causes a progressive decrease in the difference absorbance maximum, as shown in Figure 3C. Most significantly, the ligand which binds most tightly to the NAD binding domain, the reduced form of NAD (NADH), is most effective in reducing the difference absorbance. Association constants for the NADH–enzyme and NAD–enzyme complexes calculated from the values shown in Figure 3C, $1.7 \pm 0.7 \times 10^5$ M and $2.3 \pm 1.4 \times 10^3$ M, respectively, are in good agreement with association constants previously measured[10] for these complexes. The ability of relatively high concentrations (>0.2 M) of the nonspecific ligand NaCl to dissociate the dye–enzyme complex indicates that at least a portion of the interaction between the dye and the NAD binding domain is electrostatic as suggested above.

Similar results[9] were obtained in studying the interaction between ATP and the dye with the NAD binding domain of phosphoglycerate kinase which forms the ATP binding site in this enzyme.[2] Catalytic measurements indicate that the dye is a competitive inhibitor for ATP. Addition of the dye to the enzyme produces difference spectra characteristic for insertion of the dye into the adenine pocket to form a discrete dye–enzyme complex, while addition of ATP to the complex indicates that the natural ligand displaces the dye from the NAD binding domain. The association constant for the ATP–enzyme complex falls within the range of values measured using other procedures.[11]

The specific complexation of the dye with the NAD binding domain of a variety of proteins was also investigated using immobilized blue dextran covalently linked through at least one dye moiety of each dextran to Sepharose. Thus the remaining dye molecules attached to each dextran are in principle available for interaction with proteins. Such interaction with the NAD binding domain is feasible since the covalent linkage of dextran to the triazine ring of the dye occurs on the side of the dye opposite to that considered (Figure 2) to contact the domain. As shown in Table I, each of the eight proteins known from crystallographic measurements[1-4] to contain an NAD binding domain specifically binds to blue dextran-Sepharose chromatographic columns. By contrast, proteins known not to contain an NAD binding domain[12] or to contain

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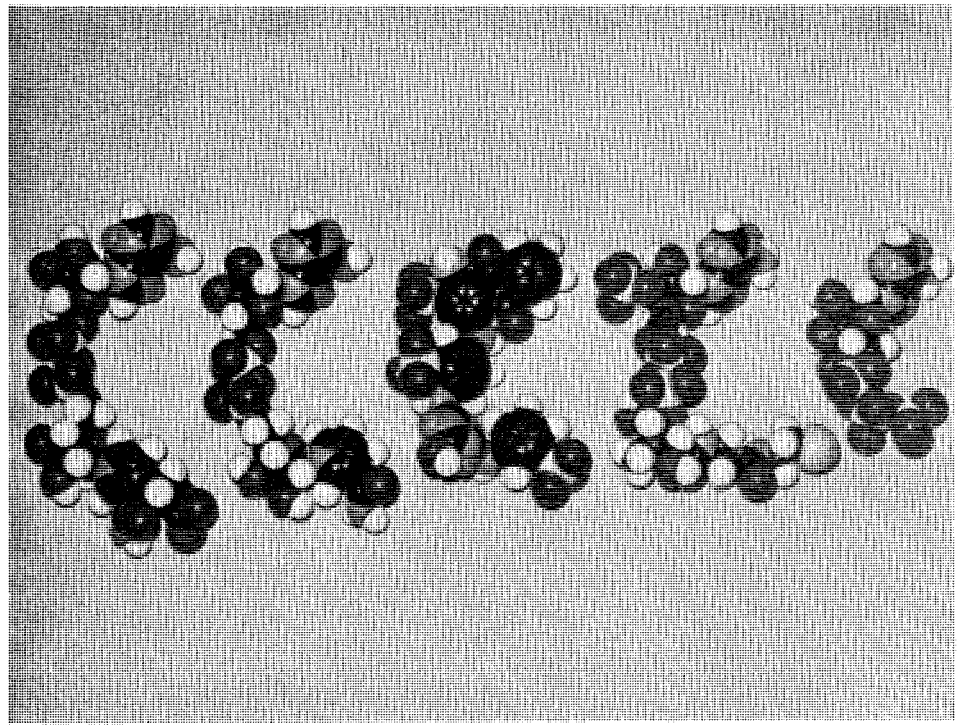
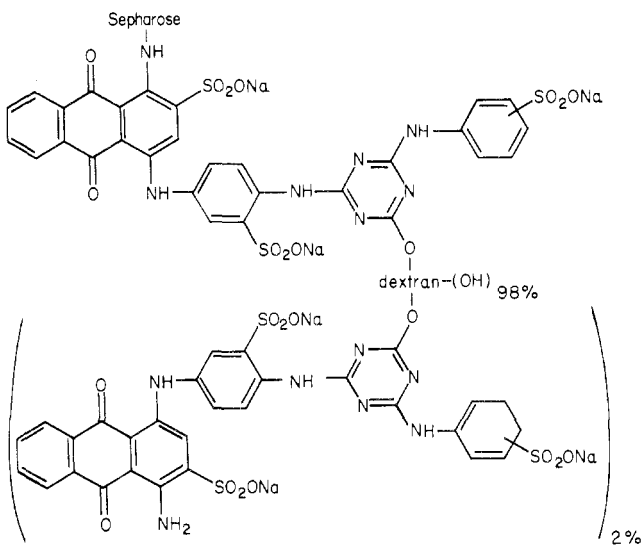


Figure 2. Comparison of a Corey–Pauling–Koltun (CPK) model of reactive blue 2 and various nucleoside phosphates. The models represent, from left to right, the NAD–pyruvate adduct, NAD, reactive blue 2, coenzyme A, and ATP.



domains either without an adenine pocket[13] or having their secondary structural elements connected in a different order[14] do not bind to these columns.

Each of the eight proteins containing an NAD binding domain is most readily eluted from blue dextran-Sepharose columns by solvents containing the nucleoside or sugar phosphate ligand which binds most tightly to the NAD binding domain of the protein being

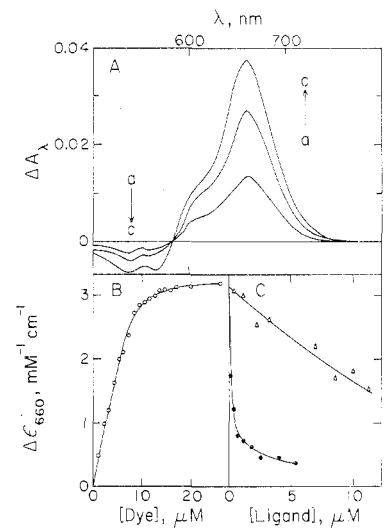


Figure 3. Spectral measurements of reactive blue 2/M_4-lactate dehydrogenase mixtures. (A) Difference spectra of the dye in the presence of increasing concentrations of the enzyme. The sample cuvette initially contained 12 μM enzyme subunits while both the sample and reference cuvettes contained (a) 2.15 μM, (b) 4.28 μM, and (c) 7.47 μM dye. (B) Difference extinction at 660 nm of the dye, expressed in enzyme subunit concentration units, as a function of the total dye concentration present. The sample cuvette initially contained 12 μM enzyme subunits while the indicated total concentrations of dye were added to both the sample and reference cuvettes. (C) The effect of increasing concentrations of the ligands NAD (△) or NADH (●) on the difference extinction of a dye:enzyme mixture measured at 660 nm. The sample cuvette initially contained 11.6 μM enzyme subunits and 26.1 μM dye only. The indicated concentrations of NAD or NADH were added to both cuvettes. The solvent for all these spectral measurements was 10 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM 2-mercaptoethanol.

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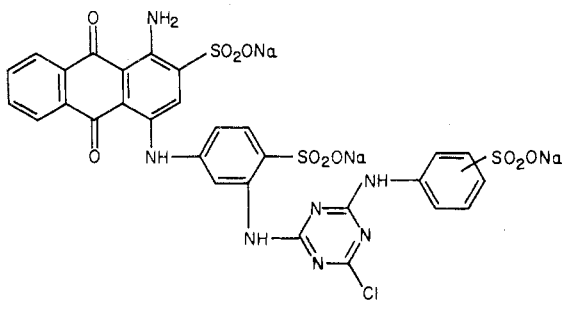
Table I
Interaction of Proteins with Blue Dextran-Sepharose

Specific interaction	Nonspecific interaction	No interaction
Proteins of known tertiary structure		
Lactate dehydrogenase[7f,i]	Ribonuclease[7f,i]	Hemoglobin[7f]
Malate dehydrogenase[7i]	Chymotrypsinogen[7f]	Thermolysin
Alcohol dehydrogenase[7f,i]	Cytochrome *c*[7f,i]	Superoxide dismutase[18b]
Glyceraldehyde-phosphate dehydrogenase[7i]		Micrococcal nuclease[7i]
Adenylate kinase[7i]		Subtilisin[7i]
Phosphoglycerate kinase[7i]		Apoflavodoxin[7i]
Phosphoglucomutase[7i]		Hexokinase[17]
Phosphorylase *a*[17]		Triosephosphate isomerase[17]
Proteins of unknown tertiary structure		
Pyruvate kinase[7f,i]	Serum albumin[7f,8]	Ovalbumin[7f]
Phosphofructokinase[7e,i]	Enolase[17]	Thyroglobulin[7f]
Protein kinase[7k,m]	Interferon[7o]	Aspartate transcarbamylase
Adenylate cyclase[7p]	Clotting factor X[7n]	Dihydrofolate reductase[7i]
DNA polymerase I[7i]		Phosphoglucomutase[17]
Phosphogluconate dehydrogenase[7f,i]		Phosphoglucoisomerase[17]
Nitrate reductase[7j]		
Aldolase[17]		
Fructose bisphosphatase[7i]		
Choline acetyltransferase[7l]		
Isoleucyl-tRNA synthetase[7q]		
cAMP phosphodiesterase[18a]		
Methylmalonyl-CoA isomerase[18c]		
Citrate synthetase[18d]		

examined. In all cases, the ionic strength of ligand solutions required to rapidly elute each protein is at least an order of magnitude less than that of NaCl solutions which effect a similar rapid elution of protein. These results suggest that complexation of the dye with the NAD binding domain involves apolar, electrostatic, and perhaps hydrogen-bond interactions similar to those involving complexation of the specific natural ligand.

As shown in Table I, three basic proteins known[15] not to contain an NAD binding domain, ribonuclease, chymotrypsinogen, and cytochrome *c*, are also retained by the anionic blue dextran-Sepharose columns. However, each of these proteins is eluted by a concentration of NaCl equivalent in ionic strength to the concentration of specific ligand. Thus, specific complexation of the immobilized dye with proteins containing the NAD binding domain can be distinguished from ion-exchange chromatography of the anionic dye with cationic proteins by comparing the effectiveness of solutions of specific and nonspecific ligands of comparable ionic strength in eluting bound proteins.

Immobilized dye-Sepharose columns have also been prepared using reactive blue 2 and its isomer, reactive blue 5 (1). Phosphofructokinase has been reported to



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bind specifically to reactive blue 2-dextran-Sepharose columns but not to reactive blue 5-dextran-Sepharose columns.[16] Since ATP both[16] elutes the enzyme from immobilized reactive blue 2 columns and competes with mobile reactive blue 2 for the allosteric binding site, it is very likely that the allosteric site on phosphofructokinase is constructed by an NAD binding domain. The failure of reactive blue 5 to bind to this site suggests that the interaction between reactive blue 2 and an NAD binding domain requires a precise orientation of apolar and ionic groups and is not the result of a rather nonspecific apolar and/or ionic interaction of a portion of the dye with enzyme surfaces.

Structural Predictions

Since blue dextran-Sepharose functions as an affinity column for ligand sites constructed by the NAD binding domain, it should be possible to predict the occurrence of this domain in the structures of proteins which have not yet been subjected to crystallographic analyses. A protein containing an NAD binding domain would be expected to bind to the column and be eluted by a solvent containing the ligand specific for the protein having an ionic strength at least an order of magnitude less than the NaCl solvents which produce a comparable elution of the protein. Using these criteria, rabbit muscle phosphorylase *a* was predicted to contain the NAD binding domain while yeast hexokinase was predicted not to contain the NAD binding domain.[17] Subsequent crystallographic measurements[3,14] have substantiated both of these predictions.

It should be relatively simple to predict the presence of the NAD binding domain in a wide range of proteins. All that is required in principle is a small blue dextran-Sepharose chromatographic column, a crude homogenate containing the protein of interest and a specific assay procedure for the biological function of the protein. As shown in Table I, a variety of enzymes

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which bind ATP, such as pyruvate kinase, protein kinase, adenylate cyclase, and DNA polymerase I, appear to contain the NAD binding domain, while aspartate transcarbamylase does not. Similarly, some enzymes which bind NAD and/or NADP, such as nitrate reductase and phosphogluconate dehydrogenase, appear to contain the NAD binding domain, while dihydrofolate reductase does not. Several enzymes specific for coenzyme A or acyl coenzyme A, including citrate synthetase, choline acetyltransferase, and methylmalonyl-CoA isomerase, bind specifically to blue dextran-Sepharose columns, suggesting that the coenzyme A sites can also be constructed by the NAD binding domain. The binding of fructose bisphosphatase to blue dextran-Sepharose columns and its specific elution with the effector 5′-AMP suggest that allosteric as well as substrate sites may also be constructed by the NAD binding domain. Finally, the specific interaction of aldolase with blue dextran-Sepharose suggests that enzymes other than phosphoglucomutase may have sugar phosphate binding sites constructed by an NAD binding domain.

These results suggest that a variety of proteins having a wide range of biological functions appear to contain the NAD binding domain. This chromatographic procedure can be used to systematically search for proteins containing the NAD binding domain in order to construct evolutionary relationships. It would be of particular interest to survey the enzymes involved in nucleic acid, protein, and fatty acid synthesis for the presence of the NAD binding domain.

Such evolutionary relationships can be extended if the amino acid sequences of proteins which specifically bind to blue dextran-Sepharose are known. The sequences of such proteins can be analyzed[19] for continuous regions likely to form the alternating pattern of β strands and α helices characteristic of an NAD binding domain. The sequence of such regions can then be genetically compared with the sequence forming the NAD binding domain in a model protein such as lactate dehydrogenase using minimum base change per amino acid codon criteria.[1] Such analysis of the sequence of aldolase predicts[20] that residues 146–300 can form the secondary structural elements characteristic of the NAD binding domain and are genetically related to the sequence of lactate dehydrogenase which forms its NAD binding domain.

Exposure of Sites and Resolution of Complexes

A novel use of blue dextran-Sepharose chromatography has been employed in the resolution of cAMP-dependent protein kinase.[7k] This protein consists of a catalytic subunit specific for the phosphate donor ATP and a regulatory subunit which masks the ATP site on the catalytic subunit. Addition of 3′,5′-cyclic AMP (cAMP) to the enzyzme results in the dissociation of the regulatory and catalytic subunits, thereby exposing the ATP binding site on the catalytic subunit. The holoenzyme consisting of both kinds of subunits does not bind to blue dextran-Sepharose columns. In the presence of cAMP, the dissociated catalytic subunit can then be eluted from the column using solvents containing either 1 mM ATP or 0.2 M KCl.

These results indicate that blue dextran-Sepharose chromatography can be used to determine the exposure of ligand sites constructed by an NAD binding domain provided the affinity of the ligand site for a potential masking agent is greater than the affinity of the site for the dye. Preliminary measurements with ribosomes and platelets suggest that these assemblies contain exposed sites constructed by NAD binding domains. The results obtained with protein kinase also demonstrate that blue dextran-Sepharose chromatography can be used to fractionate protein complexes and subcellular assemblies provided one or more of the protein substituents contains an exposed ligand site constructed by an NAD binding domain and that conditions can be found for dissociation of the substituents while retaining their functional integrity.

Ligand Exchange or Removal

A variety of proteins such as glyceraldehyde-phosphate dehydrogenase[21] and phosphofructokinase[22] have such a strong affinity for their ligands that they normally occur as protein–ligand complexes. In order to examine aspects of the structure and function of such proteins, it is often desirable either to prepare the apoenzyme and/or to replace the ligand with an analogue. Tightly bound nucleoside phosphate ligands are usually removed by repeated addition of activated charcoal to protein solutions, a procedure noted for low recovery and possible damage to the apoprotein.[23] By contrast, recent measurements[24] indicate that ligands tightly bound to the NAD binding domain of proteins can be easily removed using blue dextran-Sepharose chromatography resulting in the quantitative recovery of both the bound ligand and the apoprotein. Application of a solution of glyceraldehyde-phosphate dehydrogenase to a small blue dextran-Sepharose column equilibrated with 10 mM Tris-HCl buffer, pH 7.5, results in the retention of the protein and elution of all the formerly protein-bound NAD. The apoenzyme is then eluted by the Tris buffer containing 1 M NaCl, resulting in recovery of at least 95% of the initial enzymic activity.

The success of this procedure depends on the relative affinity of the NAD binding domain of the apoprotein for the immobilized dye and for the bound ligand and on the much greater concentration of immobilized dye relative to bound ligand. Since sites specific for a variety of nucleotide ligands appear to be constructed by NAD binding domains, this procedure should be useful both for preparation of apoproteins from holoproteins and for measuring the molar ratio of ligand and apoprotein in the holoprotein. Alternatively, the natural ligand can be directly replaced with an analogue by elution of the column-bound apoenzyme with solutions containing the desired analogue. Complexation of apoproteins with spin-labeled, ^{13}C-enriched or fluorescent ligand analogues should be achieved in

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excellent yields by these procedures.

Protein Purification

Mobile blue dextran was first used in protein purification as a final step in the purification of pyruvate kinase.[7b,c] Blue dextran was added to a heterogeneous pyruvate kinase solution and the mixture applied to a Sephadex G-200 exclusion column equilibrated and eluted with a solvent of *low* ionic strength. The large proteins and the pyruvate kinase–blue dextran complex were eluted in the void volume in advance of the smaller proteins. Reapplication of the void volume eluate to a second Sephadex G-200 exclusion column equilibrated and eluted with a solvent of *high* ionic strength resulted in the dissociation of the blue dextran–pyruvate kinase complex and the separation of the pyruvate kinase from the large proteins which continued to be totally excluded.

Immobilization of the blue dextran by covalent attachment to an inert support together with recognition of the specificity of the dye for the NAD binding domain has greatly expanded the utility of blue dextran–protein complexation for protein purification. Both the blue dextran and the dye itself have been covalently attached to Sephadex, Sepharose, and polyacrylamide gels and to cellulose.[7e,f,h,16] Comparison of the binding and elution properties of pyruvate kinase, creatine kinase, and phosphofructokinase with such columns demonstrated that covalent attachment of blue dextran to Sepharose 4B or 6B is most versatile for chromatographic fractionation of proteins.[7f,16] Application of the supernatant from a homogenate of *Escherichia coli* to blue dextran-Sepharose and elution with an ATP gradient result in about a 1500-fold purification of phosphofructokinase in good yield, giving an enzyme solution having the specific activity and gel electrophoretic pattern characteristic of the purified enzyme.[7i,22] A similar one-step purification to homogeneity using blue dextran-Sepharose chromatography has been achieved for nitrate reductase from chlorella or spinach using an NADH gradient to selectively elute the enzyme.[7i] A variety of enzymes of markedly enhanced enrichment can be recovered from a single homogenate by elution with successive concentrations of gradients of different ligands.[7f]

Preliminary measurements with purified lactate dehydrogenase indicate that the interaction of this enzyme with blue dextran-Sepharose chromatographic columns is not perturbed by the presence of nonionic detergents. This result suggested that blue dextran-Sepharose chromatography could be useful for the purification of membrane bound proteins containing the NAD binding domain which are solubilized by nonionic detergents. The chromatographic profile for elution of Lubrol-solubilized adenylate cyclase from bovine brain is illustrated in Figure 4. This chromatographic procedure results in over a 100-fold enhancement of adenylate cyclase activity in good yield.[7p] The purification of NADH-cytochrome b_5 reductase solubilized from rabbit liver microsomes with Triton X-100 can also be achieved using blue dextran-Sepharose chromatography.[18b]

Blue dextran-Sepharose chromatography of proteins offers several advantages over affinity chromatography using immobilized ligand columns. Blue dextran-Sepharose is relatively inexpensive and easily made

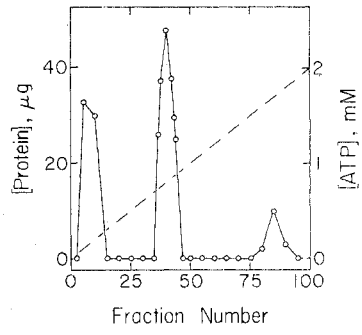

Figure 4. Blue dextran-Sepharose chromatography of bovine brain adenylate cyclase. Frozen bovine brain cortex was homogenized and the insoluble material extracted with 2% Lubrol PX. The Lubrol extract was applied to a 9-mL column of blue dextran-Sepharose equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 5 mM NaF and 1 mM EDTA. The column was washed with the equilibration solvent and then eluted with a linear ATP gradient contained in 100 mL of the equilibration solvent as shown. All of the adenylate cyclase activity bound to the column eluted with the central peak in the elution profile, giving a 150-fold enzyme purification to near homogeneity.

from materials commonly found in research laboratories. The blue chromophore is not biodegradable and thus is stable to the presence of catabolic enzymes in crude cellular extracts such as phosphatases which metabolize nucleoside phosphate affinity columns. Most importantly, a single column can be utilized to selectively enhance the relative concentration of a wide range of proteins, as illustrated in Table I. This selectivity is achieved by the choice and concentration of the mobile ligand used to elute the column containing the bound protein(s) of interest and is based on the relative affinity of proteins for the immobilized dye and for the mobile ligand and the relative concentrations of the immobilized dye and the mobile ligand.

Investigation of Catalytic Sites

As noted above, reactive blue 2 appears to specifically bind to the NAD binding domain in each subunit of lactate dehydrogenase. The association constant for the enzyme subunit–dye complex[9] is about an order of magnitude greater than the association constant for the subunit–NADH complex and three orders of magnitude greater than the value for the subunit–NAD complex.[10] The greater affinity of the dye is surprising since the dye contains an anionic sulfonate group on its terminal phenyl ring while nicotinamide contains no anionic group. The association constant of each enzyme subunit for the covalent NAD–pyruvate adduct, which has an anionic group on the nicotinamide moiety (Figure 1), is also greater[25] than that for NADH or NAD. Crystallographic measurements[10] of the enzyme–NAD–pyruvate complex indicate that the structure of this binary complex is isomorphous with the enzyme–NAD–oxalate ternary complex and is distinct from the enzyme–NAD binary complex. These comparisons suggest that the dye may span the coenzyme and substrate binding sites and that the enzyme–dye binary complex may be considered as a ternary complex analogue. While lactate dehydrogenase cannot be eluted from blue dextran-Sepharose columns by solvents containing either 15 mM NAD or 10 mM pyruvate, the enzyme can be eluted by the same solvents

(25) L. J. Arnold, Jr., and N. O. Kaplan, *J. Biol. Chem.*, 249, 652 (1974).

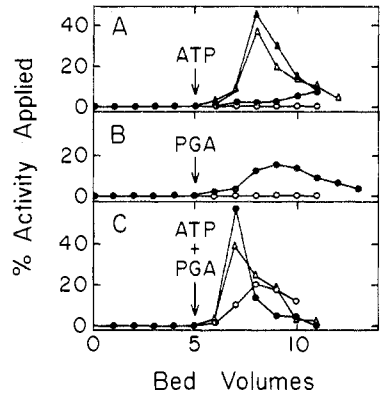

Figure 5. Blue dextran-Sepharose chromatography of yeast phosphoglycerate kinase. Fifty micrograms of the purified enzyme was added to a series of columns each containing 1 mL of blue dextran-Sepharose equilibrated with 10 mM Tris-HCl buffer, 0.5 mM 2-mercaptoethanol, pH 7.5. Each column was washed with five bed volumes of the equilibration solvent, as shown, and then with at least five bed volumes of the equilibration solvent containing the indicated ligand concentrations. (A) concentration of ATP present in the eluting solvents: (○) 0.2 mM, (●) 0.4 mM, (△) 0.7 mM, and (▲) 1.0 mM. (B) Concentration of 3-phosphoglycerate (PGA) present in the elution solvents: (○) 0.1–1.0 mM and (●) 2 mM. (C) Concentration of both ATP and PGA present in the elution solvents: (○) 0.1 mM, (△) 0.2 mM, and (●) 0.4 mM.

containing only 1 mM NAD *and* 1 mM pyruvate.[26] Therefore the coenzyme and substrate together are much more effective in displacing the enzyme from the dye than either individually, as would be expected if the dye occupied the binding sites for both the coenzyme and the substrate. Similar observations pertain to kinase–dye binary complexes. The affinity of phosphoglycerate kinase for the dye is about two orders of magnitude greater[9] than for either substrate ATP or 3-phosphoglycerate.[11] As shown in Figure 5, the enzyme is eluted from blue dextran-Sepharose columns much more easily using a combination of both substrates than either substrate individually. These results also suggest that the enzyme–dye binary complex can be considered as a ternary complex analogue of certain enzymes. The stability, specificity, and chromophoric properties of the dye should be particularly useful in probing the geometry of catalytic sites and the conformational changes resulting from the complexation of the dye at such sites.

(26) B. Nadel-Girard and C. L. Markert, "Isozymes", Vol. 2, C. L. Markert, Ed., Academic Press, New York, N.Y., 1975, p 45.

Concluding Remarks

The experimental results obtained using both mobile and immobilized reactive blue 2 are consistent with the view that the dye has a strong affinity for protein ligand binding sites constructed by an NAD binding domain. This affinity can be utilized to predict the presence of the domain in individual proteins contained in heterogeneous mixture, to determine the exposure of the domain in protein complexes and subcellular assemblies, to greatly simplify the purification of proteins containing the domain, to remove or exchange ligands bound to the domain, and to locate ligand binding sites adjacent to the domain. The growing awareness of the wide distribution of the NAD binding domain among proteins important to DNA and protein biosynthesis, energy production, and endocrine regulation affords great opportunity to utilize blue dextran for protein purification and structural analysis.

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