APPLICATIONS OF BLUE DEXTRAN AND CIBACRON BLUE F3GA IN PURIFICATION AND STRUCTURAL STUDIES OF NUCLEOTIDE-REQUIRING ENZYMES

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SUMMARY: Cibacron Blue F3GA, the chromophore of Blue Dextran, interacted with all eight nucleotide-requiring enzymes that were examined. The conjugated chromophore (Blue Dextran) was more selective, interacting with enzymes including (but perhaps not restricted to) those known to possess the "dinucleotide fold". The discriminating ability of these ligands should prove useful in elucidating the nature of nucleotide binding sites, and facilitate purification of selected nucleotide-requiring enzymes using affinity chromatography methods.
Based on their comparable interactions with Cibacron Blue F3GA and weak interactions with Blue Dextran, it is suggested that neither yeast nor rat brain hexokinase contains the dinucleotide fold as a structural feature.

Several previous investigators (for references, see 1) have observed that various proteins complex with Blue Dextran; the basis for this complexation was examined by Thompson et al. (1) who concluded that it resulted from the binding of Blue Dextran to the "dinucleotide fold" (2,3), an evolutionarily conserved feature of the tertiary structure which serves as a nucleotide binding site on certain proteins. Subsequently, Thompson and Stellwagen (4) showed that the binding of the chromophore of Blue Dextran, Cibacron Blue F3GA, to proteins containing the dinucleotide fold could be followed by difference spectroscopy, and that this method could be used to quantitatively study nucleotide binding to these proteins. The emphasis in the papers of Stellwagen and his colleagues (1,4) has been on the interaction of Blue Dextran or its chromophore, Cibacron Blue F3GA, with proteins containing the dinucleotide fold and it might be inferred that proteins which do not possess this structural feature would not interact with these ligands. However, such is not the case as demonstrated by the present study, which provides additional information facilitating the judicious use of Blue Dextran and Cibacron Blue F3GA in purification and

structural studies of nucleotide-requiring enzymes.

MATERIALS AND METHODS

Rat brain hexokinase was prepared as previously described (5). All other enzymes and biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Blue Dextran-Sepharose was prepared according to Ryan and Vestling (6) except that the cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). The Cibacron Blue F3GA - Sepharose 6B was a special sample furnished by Pharmacia Fine Chemicals. The Cibacron Blue F3GA was a gift of Dr. Robert Roskoski, Department of Biochemistry, University of Iowa.

Hexokinase (5), lactate dehydrogenase (7), malate dehydrogenase (8), and dihydrofolate reductase (9) were assayed by previously published procedures. Glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase were assayed in 0.04 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.5, containing 6.7 mM $MgCl_2$, 10 mM thioglycerol, 0.5 mg/ml NADP, and 1 mM concentrations of the appropriate substrate. All of these assays were monitored by changes in absorbance at 340 nm, and conducted at 25°. Affinity chromatography on Blue Dextran - Sepharose columns was performed under the conditions of Thompson et al. (1).

RESULTS

Kinetic Studies and Affinity Chromatography on Blue Dextran-Sepharose - As expected from the results of Thompson et al. (1), Blue Dextran was a competitive (with respect to NADH) inhibitor of beef heart lactate dehydrogenase (Fig. 1); Thompson et al. (1) showed an analogous effect of Blue Dextran vs. the nucleotide

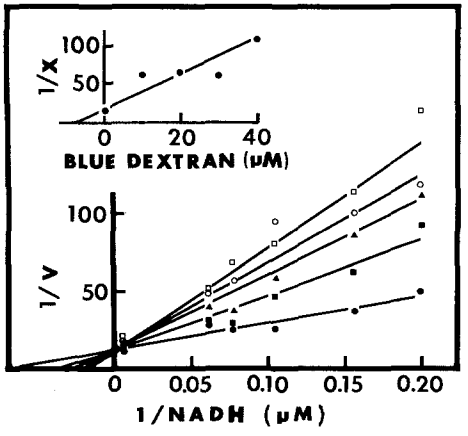

Figure 1. Inhibition of Beef Heart Lactate Dehydrogenase by Blue Dextran. [Pyruvate] was 1 mM in all assays. [Blue Dextran] determined spectrophotometrically (4).

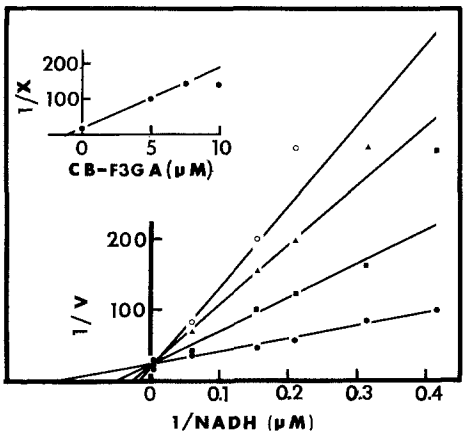

Figure 2. Inhibition of Beef Heart Lactate Dehydrogenase by Cibacron Blue F3GA. Conditions as in Fig. 1.

substrates of rabbit muscle lactate dehydrogenase and phosphoglycerate kinase. Similar competitive inhibition by the free chromophore, Cibacron Blue F3GA, can also be demonstrated (Fig. 2, and reference 4). We have extended these kinetic studies to several other enzymes with the results summarized in Table I.

Rat brain hexokinase was competitively inhibited by Cibacron Blue F3GA with K_i of 30 μM. There was very slight (∿ 10% inhibition) by 110 μM Blue Dextran at 0.4 mM ATP (∿ K_m). This made detailed kinetic studies impractical, but assuming that the inhibition was competitive with ATP, one can estimate that the K_i > 400 μM, clearly indicating a weak interaction of the enzyme with the conjugated ligand. Furthermore, adsorption to Blue Dextran-Sepharose columns was, in contrast to the other biospecifically adsorbed enzymes, relatively weak. Using comparable amounts of protein, virtually 100% of the other biospecifically adsorbed enzymes but only 55-60% of the rat brain hexokinase was adsorbed; about 70% of the adsorbed hexokinase was eluted with 10 mM ATP.

In contrast to all of the other enzymes, inhibition of dihydrofolate reductase by Cibacron Blue F3GA was, under these assay conditions, noncompetitive with respect to the nucleotide substrate, NADPH (Fig. 3), but competitive with respect to dihydrofolate (Fig. 4). These results suggest that

Table I. Comparison of Cibacron Blue F3GA and Blue Dextran as Inhibitors of Various Enzymes; Correlation with Biospecific Adsorption to Blue Dextran Sepharose Columns and Presence of Dinucleotide Fold

Enzyme	K_i (μM)[a] Cibacron Blue F3GA	K_i (μM)[a] Blue Dextran[b]	Biospecifically[c] Adsorbed to Blue Dextran-Sepharose	Dinucleotide Fold Present[d]
Beef Heart Lactate Dehydrogenase (NADH)	1	8	Yes[e]	Yes
Rabbit Muscle Lactate Dehydrogenase (NADH)	0.13[f]	-	Yes[e]	Yes
Yeast Glucose-6-P Dehydrogenase (NADP)	1	10	Yes	Unknown
Yeast 6-Phosphogluconate Dehydrogenase (NADP)	0.4	5	Yes[e]	Unknown
Pig Heart Malic Dehydrogenase (NADH)	0.3	0.2	Yes[e]	Yes
Rat Brain Hexokinase (ATP)	30	> 400 μM	Weakly	Unknown
Yeast Hexokinase (ATP)	10	No Inhibition[g]	No[e]	No
Bovine Liver Dihydrofolate Reductase	35 (vs. NADPH) 5 (vs. Dihydrofolate)	No Inhibition[g]	No[e]	Unknown

[a] For all enzymes except dihydrofolate reductase, inhibition was competitive with the nucleotide substrate (indicated in parentheses after the enzyme). K_i values were obtained by replotting the reciprocal of the intercept on the 1/s axis vs. [inhibitor] (see inset to Figs. 1 and 2). For the case of dihydrofolate reductase, see the text and Figs. 3 and 4. All lines were determined by least squares analysis of the data.

[b] Concentration of Blue Dextran is expressed in terms of its chromophore, using an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ at 610 nm (4).

[c] We use the term "biospecific", suggested by O'Carra et al. (10), to imply that adsorbed enzyme can be eluted by low (∿ 1-10 mM) concentrations of its nucleotide substrate. This excludes adsorption phenomena presumably due to nonspecific hydrophobic or electrostatic interaction.

[d] See Thompson et al. (1) for pertinent references.

[e] Confirms the results of Thompson et al. (1).

[f] Data of Thompson and Stellwagen (4).

[g] No inhibition at concentrations < 110 μM and limiting concentrations (∿ K_m) of the indicated substrate.

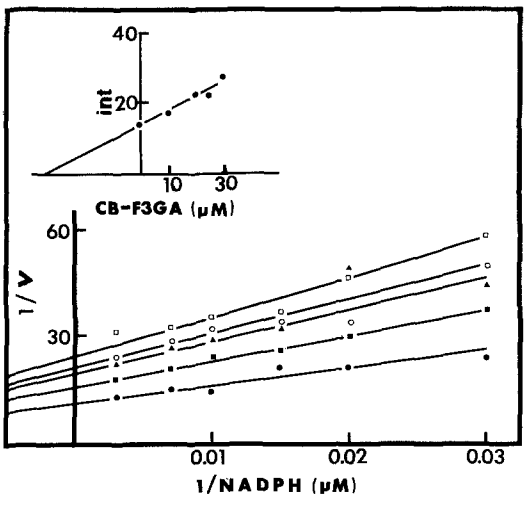

Figure 3. Noncompetitive Inhibition (vs. NADPH) of Dihydrofolate Reductase by Cibacron Blue F3GA. [Dihydrofolate] was 0.067 mM in all assays. The K_i was determined by replotting the intercepts on the 1/v axis vs. [Cibacron Blue F3GA] (inset).

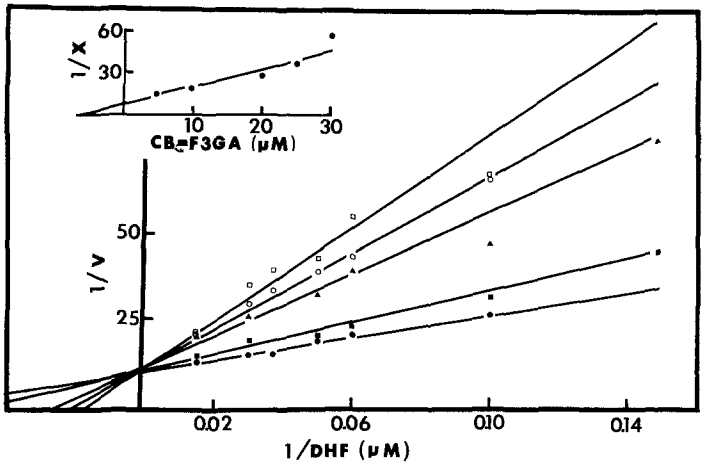

Figure 4. Competitive Inhibition (vs. Dihydrofolate) of Dihydrofolate Reductase by Cibacron Blue F3GA. [NADPH] was 0.34 mM in all assays.

Cibacron Blue F3GA can bind to both the NADPH and dihydrofolate sites.

Difference Spectra - Thompson and Stellwagen (4) observed that binding of Cibacron Blue F3GA to lactate dehydrogenase and phosphoglycerate kinase caused a red shift in the absorption spectrum of the chromophore; difference spectra

with both enzymes showed a maximum at 660-680 nm. We have obtained similar difference spectra resulting from binding of Cibacron Blue F3GA to beef heart and rabbit muscle lactate dehydrogenase and to yeast hexokinase, and from binding of Blue Dextran to beef heart lactate dehydrogenase; no difference spectrum resulted from mixing Blue Dextran with yeast hexokinase, an observation in keeping with the absence of demonstrable interaction between yeast hexokinase and Blue Dextran in kinetic and affinity chromatography experiments.

DISCUSSION

Based on the above results as well as the work from Stellwagen's laboratory (1,4), the following generalizations appear in order:

1) all nucleotide-requiring enzymes interact with Cibacron Blue F3GA presumably due to structural similarity between the dye and nucleotides (1).

2) enzymes possessing the dinucleotide fold show strong interactions with Cibacron Blue F3GA and its conjugate, Blue Dextran. Kinetically determined K_i values with the free chromophore are $\sim$ 0.1 - 1 μM, and the K_i values with Blue Dextran are usually about 10 x higher. These enzymes also show relatively strong biospecific adsorption to Blue Dextran-Sepharose columns. If yeast hexokinase can be taken as typical, enzymes which do not possess the dinucleotide fold interact relatively weakly with the free dye (K_i > 10 μM) and very weakly or not at all with the conjugated form, Blue Dextran.

3) interaction with Cibacron Blue F3GA or Blue Dextran can be detected kinetically, spectrally, or by affinity chromatography; we suggest that kinetic measurements offer the most convenient method for rapidly obtaining quantitative measures of the interaction.

Cibacron Blue F3GA may be a universal ligand for nucleotide, including cyclic nucleotide (11), (and dihydrofolate and/or folate?) binding sites. However, when the chromophore is coupled to the dextran matrix, the binding becomes more selective. It seems probable that this selectivity may be due to steric constraints resulting from the conjugation of the chromophore to the

dextran matrix; presumably the dinucleotide fold is sufficiently "open" to accommodate the chromophore surrounded by its bulky dextran matrix whereas some other types of nucleotide binding sites are more restrictive in their accessibility. However, there may still be nucleotide binding sites other than the dinucleotide fold that are sufficiently "open" to permit binding of Blue Dextran and thus it seems somewhat tenuous to interpret binding of Blue Dextran as a priori evidence for the existence of the dinucleotide fold (cf., 12). Conversely, since every enzyme known to have the dinucleotide fold has also been found to interact strongly with Blue Dextran, the absence of marked interactions with Blue Dextran might reasonably be taken as an indication that the dinucleotide fold is not present in the enzyme.

As noted above, it might be inferred from the papers of Stellwagen and his colleagues (1,4) that interaction with either Blue Dextran or the chromophore Cibacron Blue F3GA would depend upon the existence of the dinucleotide fold. Consequently, investigators finding a lack of interaction with the more readily available Blue Dextran might consider it unlikely that interaction would be attained with Cibacron Blue F3GA and prematurely discount some potentially useful applications of Cibacron Blue F3GA. For example, the failure to achieve biospecific adsorption of a dehydrogenase or kinase to a Blue Dextran Sepharose column cannot be taken as an indication that such biospecific adsorption would also fail with a Cibacron Blue F3GA-Sepharose column and Easterday and Easterday (13) have demonstrated the usefulness of such columns in purification of various nucleotide-requiring enzymes. Although rat brain hexokinase is only weakly adsorbed to Blue Dextran-Sepharose, we have readily achieved complete adsorption and extensive purification with high recoveries using Cibacron Blue F3GA-Sepharose ("Blue Sepharose" (13)).

Departing from the more "applied" aspects of this study, we would like to comment on the results obtained with the hexokinases. Rat brain hexokinase consists of a single polypeptide chain of molecular weight 98,000 (5) while the yeast enzyme is a dimer of subunits having a molecular weight of 50,000 (14).

Despite other notable differences between the yeast and mammalian hexokinases, some authors (14,15) have suggested an evolutionary relationship between these enzymes, with the 98,000 molecular weight mammalian hexokinase being a result of duplication and fusion of the gene for the 50,000 molecular weight subunit of the yeast enzyme. While we do not believe that the available evidence provides a particularly strong basis for the speculated evolutionary relationship, it should be noted that the present results are at least consistent with it, i.e., neither the yeast nor the brain hexokinase strongly interact with Blue Dextran, suggesting that their nucleotide binding sites are similar in the negative sense that neither consists of the dinucleotide fold.

ACKNOWLEDGEMENTS

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