Cibacron Blue 3G-A (CB3G-A) was investigated as a ternary complex analogue of lactate dehydrogenase and phosphoglycerate kinase as had been suggested earlier (Stellwagen, E. (1977) Accts. Chem. Res. 10, 92-98). CB3G-A and Procion Brilliant Blue (PBB, II), a structural isomer of the Cibacron dye without the sulfonated benzoyl moiety, were attached covalently to the columns and then eluted by substrates in various combinations. CB3G-A and PBB interact similarly with the two enzymes in spite of the structural differences between the dyes indicating that the specific structure of CB3G-A is not a ternary complex analogue. Inhibition studies of phosphoglycerate kinase by CB3G-A suggest that 2 molecules of dye bind per monomer and are consistent with multiple substrate binding sites. It is suggested that the kinetic mechanism of phosphoglycerate kinase is best described as steady state random.

Chromatography resins with the anthraquinone reactive dye, Cibacron Blue 3G-A (CB3G-A, I) attached have become very popular tools in enzyme purification. The mode of action of these resins in the purification process is still not clear. It has been suggested that they have hydrophobic (1-3) or a combination of hydrophobic and electrostatic interactions (4, 5). It has also been suggested that they are nucleotide-specific (6) or are "dinucleotide fold"-specific (5, 7). This latter process is an affinity proposed to be due to a particular folding pattern exhibited by several proteins binding dinucleotides. This folding pattern is supported as an evolutionary nucleotide binding domain that consists of six parallel β-pleated sheets flanked by four α helices (8). The active sites of these proteins have been found predominantly at the COOH terminus of the β-pleated sheet (see references in 8 and 9).

Many proteins exhibit a polypeptide-folding pattern similar to the dinucleotide fold (see references in 5 and 9). Those studied have an affinity for CB3G-A and CB3G-A chromatography resins can be used as affinity columns, the proteins eluted by low concentrations of their substrates or effectors (5, 7). It is questionable whether the proteins affinity is due to this tertiary protein structure (3, 4).

An example of a protein with a dinucleotide fold domain is lactate dehydrogenase (8). CB3G-A acts as a competitive inhibitor of NADH and has been shown to bind stoichiometrically and noncooperatively to each active subunit of lactate dehydrogenase by equilibrium dialysis and spectrophotometric titration (10). The enzyme cannot be eluted from CB3G-A resins by 15 mM NAD or 10 mM pyruvate, but can be eluted by a solution containing 1 mM NAD and 1 mM pyruvate (11). In addition, Corey-Pauling-Koltun space filling models of CB3G-A and the NAD-pyruvate adduct, known to form a complex with lactate dehydrogenase (12, 13) are remarkably similar. This has led to the suggestion that CB3G-A is a ternary complex analogue spanning the NADH and pyruvate binding sites of the enzyme (5, 10). Unfortunately lactate dehydrogenase has an ordered addition kinetic mechanism (12) making formation of this type of complex difficult to determine kinetically.

Multisubstrate or geometric analogues should bind very tightly to an enzyme if they mimic the transition state (14). CB3G-A does bind very tightly to many enzymes and if it is a multisubstrate analogue it should block both substrate sites simultaneously. This effect can be determined most easily in a random mechanism (15). Phosphofructokinase has a random mechanism (16) and binds tightly to a CB3G-A chromatography resin (17, 18). However, its protein structure is unknown and chromatography work indicates that the nucleotide substrate, ATP, is effective at displacing the enzyme but that fructose 6-phosphate has little effect on its elution (18). In addition, the enzyme has multiple effector sites. Yeast phosphoglycerate kinase binds to CB3G-A both on a resin (7, 9) and free in solution (10). It has a folding structure similar to a dinucleotide fold with a somewhat different chain homology (19) and is eluted from the dye columns by either of its substrates separately with an additive effect of its two ligands in its elution from Blue Dextran Sepharose (5). The available kinetic and binding data have been interpreted as being consistent with a rapid equilibrium random mechanism (20-22). All of these factors make phosphoglycerate kinase an effective model to study whether CB3G-A is a possible ternary complex analogue as predicted earlier (5, 10).

Two approaches were taken to determine whether CB3G-A is a ternary complex analogue for phosphoglycerate kinase and also lactate dehydrogenase. The first approach was a
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MATERIALS AND METHODS

NAD, NADH, ATP, 3-phosphoglycerate, dithiothreitol, yeast phosphoglycerate kinase, rabbit muscle lactate dehydrogenase, rabbit muscle glyceraldehyde phosphate dehydrogenase, and rabbit muscle glyceraldehyde phosphate dehydrogenase/triosephosphate isomerase were obtained from Sigma. Pyruvate was from the J. T. Baker Co., Cibacron Blue 3G-A (CB3G-A, C.I. reactive blue 2, C.I. constitution Number 61211) was a gift from Ciba-Geigy and Procion Brilliant Blue MR (PBB, C.I. reactive blue 4, C.I. constitution Number 61205) was supplied by ICN K & K Laboratories, Inc. Blue Sepharose CL-4B (CB3G-A Sepharose CL-6B) and Sepharose CL-4B were purchased from Pharmacia. Bio-Gel P-2 was obtained from Bio-Rad Laboratories as were the polypropylene disposable columns (0.7 x 4.0 cm) used.

All materials were used as supplied except CB3G-A which was purified as previously described (3). Nucleotide concentrations were obtained by absorbance measurements and 3-phosphoglycerate concentrations were determined enzymatically (23). PBB-Sepharose CL-6B was synthesized as described earlier (4). All enzymes used in column chromatography were dialyzed extensively at 4°C against 10 mM Tris, 0.5 mM EDTA, and 1 mM dithiothreitol, pH 7.5 (standard buffer). A 100- to 200-μg sample of protein was applied to a 1-ml column of PBB or CB3G-A Sepharose CL-6B in a plastic column (0.7 x 4 cm) equilibrated in the standard buffer. Each column was then successively washed with 5 column volumes of standard buffer and then 5 column volumes of increasing concentrations of one of the substrates of the enzyme in standard buffer. The columns were then extensively washed with 2 M KCl, re-equilibrated with standard buffer, reloaded with protein, and the elution procedure repeated with the other substrate of the enzyme. The columns were stripped with 2 M KCl again, equilibrated with standard buffer, reloaded, and the elution procedure repeated with both substrates in the standard buffer. All fractions were collected and assayed for lactate dehydrogenase (24) and phosphoglycerate kinase.

RESULTS AND DISCUSSION

It has been suggested that CB3G-A is a transition analogue for both substrates with lactate dehydrogenase (5, 7) and possibly 3-phosphoglycerate kinase (5, 10). Two approaches were used to test this theory. First, the elution by substrates of both enzymes from dye-substituted columns was evaluated and a kinetic study was then made of the inhibition of 3-phosphoglycerate kinase by CB3G-A.

The elution characteristics of lactate dehydrogenase from CB3G-A and PBB-Sepharose CL-6B are remarkably similar as shown in Fig. 1 (C and D). Lactate dehydrogenase is not eluted from either resin by concentrations of pyruvate under 20 mM. These results are similar to those obtained with Blue Dextran Sepharose (11). It is however, eluted at lower concentrations of NAD and pyruvate from both resins when the substrates are used in combination. The elution characteristics of phosphoglycerate kinase on CB3G-A and PBB-Sepharose CL-6B are also quite similar and follow closely the results obtained previously with Blue Dextran Sepharose (5) (Fig. 1, A and B). Either 3-phosphoglycerate or ATP can elute the enzyme from the resins, but a combination of 3-phosphoglycerate and ATP elutes the enzyme at lower concentrations.

The similar affinity and elution profiles of lactate dehydrogenase and phosphoglycerate kinase with both CB3G-A and PBB-Sepharose CL-6B indicate that the binding is not inherently dependent on the overall structure of CB3G-A. Thus, CB3G-A is not a necessarily specific multisubstrate or ternary complex analogue for either enzyme, as has been previously suggested (5, 10). The sulfonated benzoyl moiety of CB3G-A, which is missing from its structural isomer, PBB, does not act as pyruvate in an analogous structure to the NAD pyruvate adduct for lactate dehydrogenase as Stellwagen has suggested from model studies (5, 7, 10). Also, it is not likely that it mimics 3-phosphoglycerate with phosphoglycerate kinase. The data suggest that lactate dehydrogenase is bound to the dye resins at or near its NAD site. The pyruvate site may be blocked by the size of the dyes. A combination of pyruvate and NAD may also be more effective in eluting lactate dehydrogenase due to the tighter binding of the NAD-pyruvate adduct (13, 25) or possibly the NAD-pyruvate ternary complex may form. A similar line of reasoning would explain the binding and elution patterns of phosphoglycerate kinase, with CB3G-A binding at or near its ATP site. If the dye molecules function as specific geometric but not necessarily ternary complex analogues of the substrates they should still exhibit unique inhibitory behavior toward enzymes with a random binding mechanism. The available kinetic data suggest that phosphoglycerate kinase is random although the studies are certainly not clear (20-22). It would have been useful to compare the inhibition of both CB3G-A and PBB, but PBB is a dichloro-triazine dye and reacts with the protein at a significant rate precluding its use for inhibition studies.

To study the inhibition of phosphoglycerate kinase by CB3G-A required several controls to assure the inhibition observed was not an artifact of the assay. The primary concern was to insure the accuracy of the coupled assay used to measure phosphoglycerate kinase activity. The most common assay procedure of coupling glyceraldehyde phosphate dehydrogenase to the 1,3-diphosphoglycerate production of phosphoglycerate kinase was used. The problems with various assays for phosphoglycerate kinase have recently been extensively studied by Scopes (21). It was found with using this assay that CB3G-A inhibited glyceraldehyde phosphate dehydrogenase. To circumvent the problem, first the activity of glyceraldehyde phosphate dehydrogenase was determined at the highest concentration of CB3G-A (18.33 μM) used for phosphoglycerate kinase inhibition studies. This assay was...
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not under ideal conditions and should be considered the minimal activity for glyceraldehyde phosphate dehydrogenase under the coupled assay conditions. An activity of 0.028 I.U. is obtained for the amount of coupling enzyme used in the phosphoglycerate kinase assay (6.7 µg/ml). The method of Storer and Cornish-Bowden (26) was used to calculate the amount of coupling enzyme needed to insure an accurate assay for the primary enzyme. An activity of 0.0091 I.U. of glyceraldehyde phosphate dehydrogenase is needed to insure a lag time of 30 s or less at the highest activity (0.0011 I.U.) of phosphoglycerate kinase in the presence of 18.33 µM CB3G-A. With this level of coupling enzyme all assays were linear for 5 min and no lag phase was observable. The highest absolute absorbance at 340 nm in the inhibition assays was 1.2 A, therefore there should be little stray light error. Moreover, increasing concentrations of phosphoglycerate kinase gave a linear response in the presence of 18.33 µM CB3G-A.

Since a difference spectra was observed upon addition of CB3G-A to glyceraldehyde phosphate dehydrogenase (10), the effective free dye concentration could be reduced effectively by addition of excess coupling enzyme causing an apparent reduction in the inhibition. To determine maximum levels of coupling enzyme that could be added without reducing the effective free CB3G-A concentration, a series of assays was done in the presence of 18.33 µM CB3G-A. An addition of 3.35 µg of glyceraldehyde phosphate dehydrogenase gives a lag time in the standard assay of greater than 90 s and the assay never truly reaches steady state. An addition of 4.7 µg gives a lag time of 10 s in the assay and is linear for 5 min. Up to 20 µg of coupling enzyme may be added without phosphoglycerate kinase activity being affected by its addition. At higher levels of coupling enzyme the inhibition can be almost totally relieved. Therefore, in the inhibition kinetics done, 6.7 µg/ml of glyceraldehyde phosphate dehydrogenase was used for the assays. This level did not affect the free dye concentration significantly at any concentration.

The inhibition by CB3G-A toward MgATP$^2-$ was competitive as is shown in Fig. 2. However, the slope replot (Fig. 3) was nonlinear. Similar results were obtained for CB3G-A inhibition relative to 3-phosphoglycerate (Figs. 4 and 5). The data of Figs. 2 and 4 are consistent with the extensive study by Scopes (21) where the initial rate reciprocal plots were shown to be slightly nonlinear. The lines drawn are weighted to the higher concentrations of the varied substrate. The slopes were fitted to a parabola using a least squares fitting program and the lines shown in Figs. 3 and 5 were from the computer fit.

These results are most consistent with a mechanism as shown in Scheme I:

\[
\begin{align*}
E & \rightleftharpoons \text{E-ATP} \\
E-\text{ATP} & \rightleftharpoons E-3-P-G \rightleftharpoons E + ADP + 1,3-DPG \\
K_1 & \approx E-3-P-G \\
E_1 & \rightleftharpoons K_2 \\
\end{align*}
\]

\text{Scheme I}

where 1,3 DPG = 1,3-diphosphoglycerate; 3-P-G = 3-phosphoglycerate.
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FIG. 2. Plot of reciprocal of initial reaction velocity with respect to the reciprocal of the concentration of MgATP$^{2-}$ in the absence and presence of CB3G-A. 3-P-Glycerate was held constant at 0.769 mM. CB3G-A concentrations were 0 (C), 7.73 µM (O), 10.3 µM (C), 13.8 µM (O), and 18.3 µM (A). Other experimental details are described under “Materials and Methods.”

The binding of CB3G-A (I) is competitive with both substrates. The formation of the EI₂ complex results in the parabolic slope replots. The apparent dissociation constants, $K_I$ and $K_{II}$, were calculated to be 11 µM and 4 µM based on the data of Fig. 3, and 4 µM and 8 µM from the data of Fig. 5. It cannot be determined whether the 2 dye molecules bind in an ordered or random fashion but it is clear that no enzyme-substrate dye complexes form since no intercept effect is observed in the primary plots and the difference spectra of CB3G-A-phosphoglycerate kinase can be abolished by addition of either substrate (10). The rate equation describing Scheme I is

$$v = \frac{E_0}{1 + \frac{I}{K_I} + \frac{(I)^2}{K_{II}} + \frac{(I)}{K_{III}}}$$

(1)

The binding of 1 molecule has to block both sites effectively or else both dyes bind in a concerted fashion to fit Equation 1. Thus, the inhibition data are consistent with a random mechanism for phosphoglycerate kinase. Whether CB3G-A is a geometric ternary complex analog is still not clear since the dye may simply bind in the general area of the active site. The 2nd dye molecule does not even have to bind near the active site since formation of the EI₂ complex does not compete directly with substrate binding. The spectral titrations done previously also indicate multiple binding sites for CB3G-A (10).

Until the recent detailed study by Scopes (21), the nonlinear nature of the initial rate reciprocal plots had been ignored or interpreted as substrate activation (21, 22, 27). Multiple substrate sites were suggested as causing the activation (27). Various studies on the number of binding sites give values of either 1 (28-31) or 2 molecules (28, 32) of substrate bound per monomer. The binding of 2 dye molecules per active site would appear to be consistent with multiple sites but should be interpreted with care as the location of the 2nd dye molecule cannot be ascertained from the kinetic behavior.

The product inhibition studies have not been interpreted with acknowledgment of the basic nonlinear nature of the reciprocal leading to suggestions that the type of inhibition (competitive or noncompetitive) depended on the substrate or metal concentration (22). The complications of changing magnesium concentrations with the difficulty in interpretation of the data necessitates further studies before the product inhibition patterns can be analyzed as being due to multiple binding sites on the monomer. Binding of the longer product (i.e., 1,3-diphosphoglycerate) of a reaction to either substrate

FIG. 3. Replot of the slopes of Fig. 2 with respect to the concentration of CB3G-A. Slope is in minutes.

FIG. 4. Plot of reciprocal of initial reaction velocity with respect to the reciprocal of the concentration of 3-P-glycerate in the absence and presence of CB3G-A. MgATP$^{2-}$ was held constant at 0.500 mM. CB3G-A concentrations were 0 (C), 7.73 µM (O), 10.3 µM (C), 13.8 µM (O), and 18.3 µM (A). Other experimental details are described under “Materials and Methods.”

FIG. 5. Replot of the slopes of Fig. 4 with respect to the concentration of CB3G-A. Slope is in minutes.
site is not unusual and is not necessarily related to regulatory sites.

It should be noted that the slope replots of the product inhibition data described previously (22) are linear indicating that inhibition by any product is caused by binding of only 1 molecule, not two as was found with CB3G-A. In a study of sulfate effects, Scopes has found that low concentrations (below 20 μM) activate phosphoglycerate kinase (21). Above this concentration sulfate was competitive with both substrates and it appears to have multiple binding sites relative to 3-phosphoglycerate. The dye may bind at the same inhibitory sites as sulfate but with a stronger affinity allowing multiple interactions to be observed with both substrates.

The nonlinear behavior of the reciprocal plots has been suggested by Scopes (21) as being due to an "activatory site" near the active site. He concluded that the mechanism was not steady state but rapid equilibrium. Results obtained previously with hexokinase (33), phosphofructokinase (34) and other enzymes (35) suggest that kinases more often have steady state than rapid equilibrium mechanisms with product release contributing to the limitations on the activity. Phosphoglycerate kinase also, most likely, has a steady state random mechanism and future work should treat the mechanism as such due to changes in product inhibition patterns and the possible regulatory potential of such mechanisms. The present study on dye binding would be consistent with either a rapid equilibrium or steady state mechanism. It must be pointed out that only a random mechanism could give rise to the type of inhibition observed.

Scopes (36) has very recently confirmed two MgATP sites per phosphoglycerate kinase monomer by gel filtration techniques, one site considerably weaker than the other. Included in this paper was a personal communication from H. C. Watson showing only 1 molecule of CB3G-A bound per enzyme monomer at the sole adenine nucleotide site in the crystalline enzyme. This does not preclude alternate dye sites on the enzyme in solution. Scopes also presents a mechanism by which MgATP, 3-phosphoglycerate, or sulfate accelerate a two-step dissociation of 1,3-diphosphoglycerate. The dissociation of 1,3-diphosphoglycerate is proposed as the rate-limiting step in the enzyme's reaction as it has a dissociation rate of no more than 50 s⁻¹ and the enzyme's catalytic rate is 900 s⁻¹. If this is the case, then the enzyme must have a steady state mechanism.

Rao et al. (37) have also recently studied 3¹P NMR of bound reactants and products with phosphoglycerate kinase. Addition of sulfate causes a change in the active site by altering the environment of bound 3-phosphoglycerate and the equilibrium constant for enzyme-bound reactants and products is altered in favor of ATP formation. These findings show there is indeed a change in the active site conformation upon addition of sulfate, but tend to cast doubts on a two-step dissociation of 1,3-diphosphoglycerate as its 3¹P spectra is not perturbed by sulfate addition. The mechanism of substrate activation of the enzyme could very well be explained by a steady state mechanism. Future studies on this enzyme should include isotope exchange or other isotopic experiments to evaluate rate-limiting steps in the reaction.

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