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HAZARDS IN THE USE OF CIBACRON BLUE F3GA IN STUDIES OF PROTEINS

Bruce H. Weber[1], Kenneth Willeford[1], John G. Moe[2], and Dennis Piszkiewicz[3]

[1]Department of Chemistry and Institute of Molecular Biology, California State University, Fullerton, California 92634

[2]Department of Biological Chemistry, University of California, Irvine, California 92717

[3]Department of Chemistry, Duquesne University, Pittsburgh, Pennsylvania 15219

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SUMMARY: Cibacron Blue F3GA from several commercial sources is shown to be heterogeneous. This crude dye inactivates both phosphoglycerate kinase and isoleucyl-tRNA synthetase. Purification of Cibacron Blue F3GA to homogeneity results in a dramatic decrease in inactivation of these enzymes. The inactivation is shown to be due to covalent modification of phosphoglycerate kinase and probably isoleucyl-tRNA synthetase by a minor component present in crude Cibacron Blue F3GA.

INTRODUCTION

Blue dextran and its derivatives have achieved wide use as probes of proteins that normally bind nucleotides (1). Many of these proteins catalyze reactions involving ATP and NAD, and possess a common feature of their tertiary structure known as the NAD binding domain (2, 3). Blue dextran and its parent dye Cibacron Blue F3GA have been covalently bound to insoluble matrices and used as affinity chromatographic media for the purification of a great number of proteins (1,2,3). The parent dye Cibacron Blue F3GA has been used in kinetic (4,5,6) equilibrium binding (3), and spectroscopic studies (3,5,6) as a probe of the structure and function of many of these proteins.

Our laboratories have been using commercially available preparations of Cibacron Blue F3GA in experiments with yeast phophoglycerate kinase and E.coli isoleucyl-tRNA synthetase. We have found these commercial preparations to be heterogeneous in composition. We now report the resolution of components of commercial Cibacron Blue F3GA, some of their effects on the two enzymes under study, and the implications of these studies.

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MATERIALS AND METHODS

Phosphoglycerate kinase was prepared from yeast by the method of Brake and Weber (7) or obtained as the crystalline suspension from Boehringer-Mannheim Corporation, as were the glyceraldehyde 3-phosphate dehydrogenase, ATP, 3-phosphoglycerate, and NADH. Its activity was assayed as described previously (8).

Homogeneous isoleucyl-tRNA synthetase was prepared by the method of Eldred and Schimmel (9). L- [^{3}H] isoleucine was from INC, E. coli B tRNA was from Schwarz-Mann, and ATP was purchased from Calbiochem. Synthetase activity was assayed by the esterfication of L- [^{3}H] isoleucine to tRNA in 0.05 M Tris-HCl at 30° C according to the method of Muench and Berg (10).

Samples of Cibacron Blue F3GA were obtained from Polysciences, Inc., Pierce Chemical Company and Ciba-Geigy (gift of Dr. A. Yoshida). Ultrapure guanidine-HCl was purchased from Mann Research. Activated silica gel thin-layer chromatography plates were from Eastman. Silica gel was from J.T. Baker Chemical Company. All other chemicals were of the highest quality available.

Reactive dyes were analyzed by thin-layer chromatography on activated silica gel developed by tetrahydrofuran:water (48:7, v/v). Cibacron Blue F3GA was purified by preparative column chromatography on silica gel. In a typical experiment, 600 mg of the crude dye was dissolved in water and mixed with 3 g of silica gel. This mixture was evaporated to dryness at 40° C in a heated vacuum dessicator. The dried mixture was suspended in ethyl acetate: tetrahydrofuran:water (48:48:4, v/v/v), applied to a column (2.0 x 44 cm) of silica gel equilibrated with the same solvent, and then washed with one liter of this solvent. The eluting solvent was then changed to ethyl acetate: tetrahydrofuran:water (20:48:7, v/v/v). Two liters of this mixture were passed through the column while 20 ml fractions were collected. The fractions were assayed by thin-layer chromatography as described above. The fractions containing the major blue component (R_f = 0.57) were pooled. The organic solvents were removed by rotary evaporation and the aqueous dye solution was lyophilized. This procedure resulted in the isolating of 340 mg of pure Cibacron Blue F3GA.

Unfortunately this polysulfonated derivative of a chlorotriazine did not lend itself to infrared, proton magnetic resonance, or mass spectral analysis. Therefore, another apporach to its characterization was taken. The purified dye was coupled to dextran by the method of Böhme et al (11). The blue product and authentic Blue Dextran 2000 were hydrolyzed in 6 N HCl at 30° C for 60 hrs. Aliquots were subjected to thin-layer chromatography under the conditions described above. The mobilities of both hydrolysis products, presumably the hydroxyl derivative of Cibacron Blue F3GA, were identical (R_f = 0.28).

Phosphoglycerate kinase (0.1 mg/ml) in 0.090 M Tris-nitrate buffer at pH 8.50 was incubated with reactive dyes at 37° C. After sixty minutes of incubation an aliquot was removed for thin-layer chromatography, and the remaining reaction mixt was passed through a Sephadex G-25 column (0.9 x 10 cm) equilibrated with 0.10 M Tris-nitrate pH 8.5 buffer. Fractions were assayed by the method of Lowry (12); those containing protein were pooled and assayed for enzyme activity.

Isoleucyl-tRNA synthetase (0.1 mg/ml) in 5 mM sodium phosphate buffer at pH 7.00 containing 10 mM β-mercaptoethanol was incubated with reactive dyes at 30° C, After 30 minutes aliquots were withdrawn and assayed for activity as described above.

Sedimentation coefficients of native and modified phosphoglycerate kinase were determined in a Beckman Model E analytical ultracentrifuge at 60,000 rpm

TABLE 1

Effects of Various Dyes on the Catalytic Activities of Phosphoglycerate Kinase and Isoleucyl-tRNA Synthetase

Addition		Phosphoglycerate Kinase % Activity[a]		Isoleucyl-tRNA Synthetase % Activity[a]
None		100		100
Blue Dextran 2000 (100 μM)[b]		—		80
Crude Cibacron Blue F3GA[c]	(0.5 μM)	93	(0.4 μM)	60
	(5.0 μM)	62	(0.6 μM)	48
	(50 μM)	6	(0.8 μM)	35
	(100 μM)	0	(1.0 μM)	21
Purified Cibacron Blue F3GA	(1000 μM)	97	(2.0 μM)	91
Purified component[d] with R_f = 0.77 from crude Cibacron Blue F3GA	(0.05 mg/ml)	60		—
	(0.5 mg/ml)	0		—

[a]Activities indicated are the percent of initial activity measured after 60 minutes incubation for phosphoglycerate kinase followed by gel filtration, and after 30 minutes incubation for isoleucyl-tRNA synthetase.

[b]Calculated concentration of dye assuming the chromophore has the same extinction coefficient (E_{610} = 13.6 mM^{-1} cm^{-1}) as Cibacron Blue F3GA.

[c]These concentrations are approximate since it was assumed that there was a single component.

[d]The molecular weight of this component is unknown.

employing double-sector cells. Both schlieren optics and absorption optics, at 280 and 625 nm, were employed.

RESULTS

In our initial studies we noticed that incubation of phosphoglycerate kinase or isoleucyl-tRNA synthetase with commercial samples of Cibacron Blue F3GA resulted in inactivation and incorporation of blue dye into the protein. These observations led to our systematic studies of the effects on these enzymes of the crude Cibacron Blue F3GA and its major component which was isolated by silica gel chromatography.

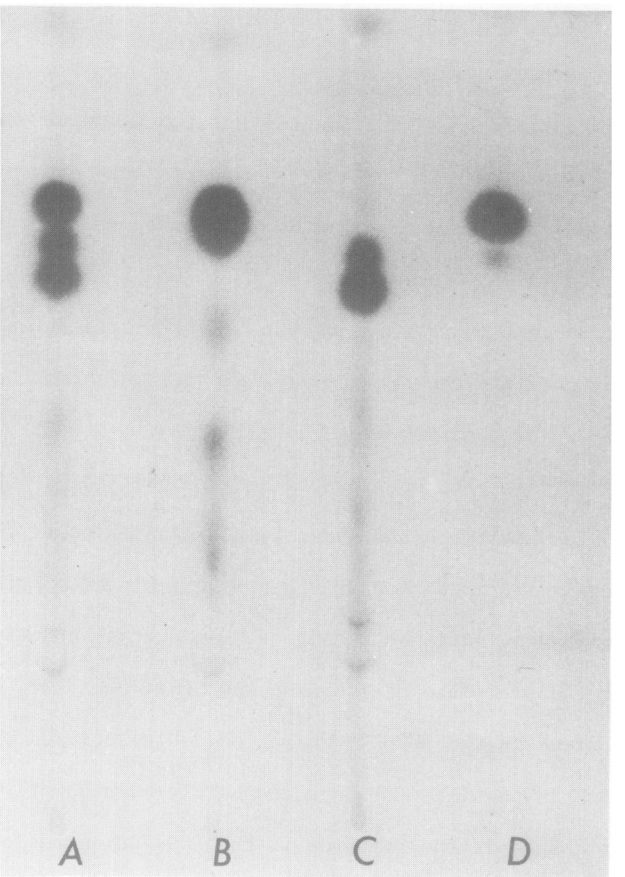

Figure 1. Thin-layer chromatography of dyes on silica gel (see text for details). A through C are crude Cibacron Blue F3GA from Pierce Chemical, Polysciences, Inc., and Ciba-Geigy, respectively. D is purified Pierce Cibacron Blue F3GA.

The effects of crude and purified Cibacron Blue F3GA on the activities of phosphoglycerate kinase and isoleucyl-tRNA synthetase are summarized in Table 1. The crude dye was found to inactivate both enzymes; however, the purified dye had no significant effect on the activities of either enzyme.

Samples of Cibacron Blue F3GA obtained from three sources were examined by thin-layer chromatography (Figure 1). Each of these samples were resolved into a major blue component (R_f = 0.57) and at least eight other colored components. The major component was purified in quantity by column chromatography on silica

gel, and upon reaction with dextran yielded a product indistinguishable from that derived from authentic Blue Dextran.

When unpurified Cibacron Blue F3GA (m$\underline{M}$) was reacted with one mg of phosphoglycerate kinase followed by thin-layer chromatography, no change was apparent in the major components, but several minor spots disappeared relative to the control. The most obvious change was the loss of the fast-moving component (R_f = 0.77) of the crude Cibacron Blue F3GA. This fast component was purified employing silica gel column chromatography and was shown capable of inactivation (see Table 1).

To determine whether phosphoglycerate kinase modified by crude Cibacron Blue F3GA represented tightly bound dye molecule(s) as opposed to covalent modification, several experimental approaches were employed. Attempts to reverse binding of dye by addition of ATP or Mg-ATP up to concentrations of 100 m$\underline{M}$, following the procedure of Thompson and Stellwagen (3), produced no change in the spectrum in the 500-700 nm region. Blue-colored phosphoglycerate kinase was made 8 $\underline{M}$ in gaunidine-HCl and then dialyzed exhaustively against 8 $\underline{M}$ guanidine-HCl; no change in the spectrum in the 500-700 nm region was observed. Dye-reacted phosphoglycerate kinase was subjected to SDS elctrophoresis (13) and the resultant gels scanned at 200 nm and 625 nm; the absorbances coincided and correspond to the position of control phosphoglycerate kinase. In sedimentation velocity experiments, enzyme modified by crude Cibacron Blue F3GA showed that both the 625 and 280 nm absorbances had $S_{20,w}$ = 3.7 s; the unmodified enzyme showed $S_{20,w}$ = 3.4 s. Taken together, these data indicated that some component of Cibacron Blue F3GA covalently modified phosphoglycerate kinase.

The product of the reaction of isoleucyl-tRNA synthetase with Cibacron Blue F3GA was also examined to determine if covalent bond formation had taken place. The blue dye could not be separated from the protein by column chromatography on Sephadex G-25 (1.5 x 23 cm) in the presence of 6 $\underline{M}$ urea or 6 $\underline{M}$ guanidine-HCl.

After storage for several months the purified Cibacron Blue F3GA when examined by thin-layer chromatography showed several components, some of which corresponded to those observed for the crude dyes.

DISCUSSION

Our results demonstrate that there are contaminants in commercial Cibacron Blue F3GA that can covalently modify and inactivate phosphoglycerate kinase and isoleucyl-tRNA synthetase. Recently, others have also reported inactivation or covalent modification of proteins by preparations of Cibacron Blue F3GA of unspecified purity. Apps and Gleed (5) have reported that pigeon liver NAD kinase when exposed to Cibacron Blue F3GA acquired a blue color which could not be removed by dialysis, gel filtration, charcoal adsorption or electrophoresis on SDS gels. Witt and Roskowski (14) have reported that Cibacron Blue F3GA inactivates the catalytic subunit of cAMP dependent protein kinase probably as the result of covalent bond formation.

Since preparations of Cibacron Blue F3GA covalently modify proteins, caution must be taken in the use of the dye in kinetic studies. The non-competitive inhibition of RNA polymerase by it (6) might also be explained by covalent modification and consequent inactivation. Therefore, all studies, either physical or chemical, employing Cibacron Blue F3GA should ascertain two facts, the purity of the reagent and whether or not covalent modification is occurring. Furthermore, since the purified dye is unstable, it should be used freshly purified.

The fact that Cibacron Blue F3GA, or contaminants found in commercial preparations of it, may react with proteins should not be surprising as compounds such as it have been used extensively for dying proteins (15-20). Furthermore, since reactive chlorotriazine dyes, such as Cibacron Blue F3GA are reactive with proteins, they may be useful reagents for protein modification, especially if they show an affinity for the NAD-binding domain.

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