Adenosine Cyclic 3',5'-Monophosphate Dependent Protein Kinase: Active Site Directed Inhibition by Cibacron Blue F3GA

Jonathan J. Witt and Robert Roskoski, Jr.*

ABSTRACT: Cibacron Blue F3GA, the polycyclic blue chromophore of Blue Dextran 2000, inhibits the catalytic subunit of bovine brain protein kinase. The rate of inactivation exhibits a hyperbolic dependence on the dye concentration. This suggests that an enzyme–dye complex forms prior to inactivation. Protein and peptide substrates or MgATP protects the enzyme against dye inactivation. Kinetic measurements show that the dissociation constant is \( \sim 100 \mu M \) and the maximal rate of inactivation is 0.13 min\(^{-1}\) at 22 °C. Inactivation is temperature and time dependent. Exhaustive dialysis, gel filtration, or the addition of substrate fails to reactivate the inhibited enzyme. The failure to reverse the inhibition suggests that the dye forms a covalent complex with the enzyme. Denaturation by sodium dodecyl sulfate also fails to dissociate the dye from enzyme. The hyperbolic kinetics, moreover, suggest that the dye functions as an active site directed reagent. The holoenzyme is resistant to Cibacron Blue inactivation. Addition of cAMP converts the enzyme to a form susceptible to inhibition. In agreement with our previous studies, these results also suggest that the regulatory subunit shields, either physically or functionally, the active site of the catalytic subunit.

Adenosine cyclic 3',5'-monophosphate (cAMP)\(^1\) dependent protein kinase consists of two dissimilar subunits (Gill & Garen, 1970; Tao et al., 1970; Kumon et al., 1970; Reichmann et al., 1971). The activation of this enzyme by cAMP is summarized by

\[
R_2C_2 + 2cAMP \rightleftharpoons (R-cAMP)_2 + 2C
\]

in which \( R_2C_2 \) is the holoenzyme and \( R \) and \( C \) are the regulatory and catalytic subunits, respectively (Rosen & Erlichman, 1975; Hofmann et al., 1975). The free \( C \) subunit is active and cAMP independent. Combining \( C \) subunit and the \( (R-cAMP)_2 \) complex results in the release of cAMP and the regeneration of the cAMP-dependent holoenzyme.

We reported that the free \( C \) subunit binds to Blue Dextran-Sepharose affinity resin. The blue chromophore of Blue Dextran, Cibacron Blue F3GA, is a nucleotide analogue (Thompson et al., 1975). These investigators proposed that this nucleotide analogue binds to those proteins which contain the super secondary structure termed the dinucleotide fold. The binding of free \( C \) subunit to Blue Dextran-Sepharose and the failure of the holoenzyme \( (R_2C_2) \) to bind support the notion that the active site is inaccessible in the holoenzyme form.

We present experiments which show that the free chromophore of Blue Dextran, which contains a triazine ring chloride, inhibits the free \( C \) subunit in an apparently irreversible manner and exhibits the characteristics of an active site directed reagent. Furthermore, RI, RII, and the protein kinase inhibitor protein (PKI) also protect against blue dye inactivation. These results support the idea that each of these proteins physically or functionally shields the active site of the enzyme.

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1 Abbreviations used: C, catalytic subunit; RI and RII, regulatory subunits I and II, respectively; cAMP, adenosine cyclic 3',5'-monophosphate; PKI, protein kinase inhibitor protein; Mops, 3-(N-morpholinol)propanesulfonic acid.
C subunit and thereby inhibits it. Some of these experiments have been reported in preliminary form (Witt & Roskoski, 1978).

Methods and Materials

Protein Kinase Preparation and Assay. Bovine brain iso-

The catalytic subunit had a specific activity of 1.0–1.5 μmol/(min mg of protein), using histone as substrate. Some of the variability encountered in specific activity was related to the use of different lots of histone IIa (Sigma Chemical Co.).

With histone as substrate, the assays were performed as previously described (Witt & Roskoski, 1975b). With the synthetic heptapeptide corresponding to the phosphorylatable sequence of pig liver pyruvate kinase isoenzyme IIa (Leu-Arg-Arg-Ala-Ser-Leu-Gly from Peninsula Laboratories, Inc.), the assay mixture contained 200 μM peptide substrate and the activity was measured as previously described (Witt & Roskoski, 1975b) except that 30% acetic acid (v/v) was substituted for water to elute [γ-32P]ATP and metabolites from the phosphocellulose strips (Glass et al., 1978). Values for enzyme activity presented in the tables and figures represent means of triplicate assays from at least three different experiments. The standard errors of the mean are less than 6% of the values given.

Catalytic Subunit Inactivation by Cibacron Blue F3GA.

Purified Cibacron Blue F3GA (a generous gift of Dr. H. Bosshard of Ciba Geigy, Basel, Switzerland) was examined by silica gel thin-layer chromatography and found to migrate as a single component with Rf values at 0.50, 0.62, and 0.58, respectively, using (a) methanol, (b) butanol-acetone-acetic acid–5% NH₄OH–water (7:5:3:3:2 v/v), and (c) tetrahydrofuran–water (48.7:7.3 v/v). Dye concentration was determined spectrophotometrically at 610 nm by using an extinction coefficient of 13600 M⁻¹ cm⁻¹ (Thompson & Stellwagen, 1976). With the blue dye, on the other hand, fails to stabilize dilute C subunit. This concentration of acid-5% NH₄OH–water (7:5:3:3:2 v/v), and (c) tetrahydro-


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coefficient of 13600 M⁻¹ cm⁻¹ (Thompson & Stellwagen, 1976). The C subunit was prepared to apparent homogeneity as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using the procedure of Beavo et al. (1974). The catalytic subunit had a specific activity of 1.0–1.5 μmol/(min mg of protein), using histone as substrate. Some of the variability encountered in specific activity was related to the use of different lots of histone IIa (Sigma Chemical Co.).

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The sources of materials and other methods were previously described (Witt & Roskoski, 1975a,b).

Results

Specific Elution of the Catalytic Subunit from Blue Dextran–Sepharose. Previous studies with the nucleotide analogue, the blue chromophore of Blue Dextran, showed that the bound C subunit is eluted with the substrate MgATP. Guanethidine, at 1 and 5 mM concentrations, also elutes 21 and 76% of the C subunit, respectively, from the nucleotide affinity resin (Table I). Guanethidine dissociates the protein kinase inhibitor from bovine heart C subunit (Demaille et al., 1977) and is proposed to act like the arginyl determinants of protein substrates (Kemp et al., 1976; Demaille et al., 1977). It is not, however, a nucleotide analogue. We propose, therefore,
CAMP-DEPENDENT PROTEIN KINASE

(Continued)

The inactivation of the catalytic subunit by Cibacron Blue F3GA is an active site directed reagent, C subunit was incubated with the blue dye in the presence of substrates and competitive enzyme inhibitors. $K_{app}$ values were measured and graphed according to eq 2. MgATP, however, reduces enzyme inactivation by the blue chromophore (Figure 2). The lines representing inactivation in the presence or absence of MgATP have a common ordinate intercept, thereby indicating that the maximum rate of inactivation ($k_3$) is unchanged. Analogous to competitive inhibition patterns obtained from double-reciprocal plots (Cleland, 1967), this result indicates that the dye and MgATP compete for the same site. From eq 3, the $k_3$ of MgATP is 620 $\mu$M. This differs from the $K_m$ of $\sim 10 \mu$M and provides another example that the dissociation constant is not necessarily equal to the $K_m$ (Jencks, 1969).

Since MgATP and guanethidine elute the C subunit from Blue Dextran-Sepharose synergistically (Table I), we propose that its blue chromophore occupies portions of both the nucleotide and protein substrate domains of the active site. Supporting this proposal, the synthetic heptapeptide substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly) and guanethidine (a protein substrate competitive inhibitor) also retard the rate of inactivation at a given concentration of blue dye. By use of the procedure outlined in Figure 2, the maximum rate of inactivation ($k_3$), determined from the ordinate intercept, is unchanged. From eq 3, the $k_3$ for the synthetic peptide and guanethidine is 0.3 and 1.6 $\mu$M, respectively. Benzamidine, a feeble competitive inhibitor of C subunit with respect to protein substrate, has no effect on the inactivation process with concentrations to 20 $\mu$M. Nucleotide and protein substrate protection at a given dye concentration support the notion that the blue dye is an active site directed reagent.

During the course of these studies, the rate of inactivation declined over a period of several days. Dye decomposition in aqueous solution failed to explain this observation since freshly prepared dye solutions gave $K_{app}$ values similar to solutions prepared 2 months previously. On the other hand, if $K_{app}$ values obtained from fresh and aged (3 weeks) enzyme are graphed according to eq 1, the corresponding lines share a common intercept but exhibit a different slope. These results show that the maximum rate of inactivation ($k_3$) is unchanged; enzyme aging, however, is associated with a decreased affinity of the dye for the enzyme. The $k_3$ for fresh and aged (3 weeks) enzyme was 100 and 210 $\mu$M, respectively. Some older enzyme preparations (6 weeks), moreover, were resistant to dye inactivation. However, they retained their original specific activity.

Preincubation of aged (3 weeks) C subunit with MgCl$_2$ (30 min, 4 °C), however, restores sensitivity to dye inactivation. The MgCl$_2$ effect might be related to a direct effect on the enzyme protein or perhaps to the formation of a Mg-dye complex analogous to MgATP. A fivefold excess of EDTA was added to the MgCl$_2$-treated enzyme prior to addition of the dye to distinguish between these possibilities. The $K_{app}$ for the MgCl$_2$-treated enzyme (minus EDTA) was equivalent to that following EDTA treatment; both, however, were greater than that for the MgCl$_2$-untreated enzyme (Table II). These

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**Figure 2:** MgATP protects C subunit against inactivation by Cibacron Blue F3GA. The C subunit (5 $\mu$g) was incubated with the indicated concentrations of blue dye without addition ( ), with 310 $\mu$M ATP alone ( ), or with 310 $\mu$M ATP and 5 $\mu$M MgCl$_2$. The rate of inactivation was measured as described under Methods and Materials, and the values for $K_{app}$ were graphed according to eq 1. Blue subunit activity, however, is not restored by addition of large concentrations of MgATP or protein substrate during the assay. These experiments demonstrate that there is a difference in the mechanism by which this chromophore interacts with the protein kinase C subunit when compared with other enzymes.

Specific elution of C subunit from Blue Dextran-Sepharose affinity resins by MgATP and guanethidine is consistent with the hypothesis that the chromophore binds at the active site of the enzyme. The inactivation of the C subunit by the free chromophore may result, therefore, from complex formation and reaction of the blue dye at the active site. Active site directed enzyme inactivation has kinetic characteristics which are distinguishable from simple bimolecular reactions. Following the presentation of Kitz & Wilson (1962), the reaction between an enzyme with an active site directed agent (similar to that with substrate) can be formulated by

$$E + I \rightarrow \frac{k_1}{k_3} E-I \rightarrow E-I \rightarrow EI$$

where $E$ and $I$ are free enzyme and inhibitor, $E-I$ is an enzyme–inhibitor Michaelis complex, $EI$ is the inhibited enzyme, and $k_3$ is the rate-limiting step. A steady-state treatment of the process yields the equation (Kitz & Wilson, 1962)

$$\frac{1}{K_{app}} = \frac{1}{k_3} + \frac{k_1}{k_3} \frac{1}{[I]}$$

where $K_{app}$ is the observed rate of inactivation for a given concentration of inhibitor, $k_3$ is the maximum rate of inactivation, and $k_1$ is the dissociation constant of inhibitor for the enzyme ($k_3/k_1$).

The inactivation of the catalytic subunit by Cibacron Blue F3GA at pH 7.0, 22 °C, follows pseudo-first-order kinetics. $K_{app}$ values for varying inhibitor concentrations were measured and plotted in double-reciprocal form (eq 2). A straight line and positive ordinate intercept result (Figure 2), thereby indicating saturating kinetics (and not simple second-order kinetics) for the inactivation process. The maximum rate of inactivation ($k_3$) is 0.13 min$^{-1}$, and the $k_3$ is 100 $\mu$M, as determined from the ordinate and abscissa intercepts, respectively. These results are consistent with the contention that inactivation of C by Cibacron Blue F3GA occurs after the formation of a reversible E-I complex.

Substrates and competitive inhibitors characteristically reduce the rate of enzyme inactivation by active site directed reagents. The rate equation is an extension of eq 2, which may be written as (Kitz & Wilson, 1962)

$$\frac{1}{K_{app}} = \frac{1}{k_3} + \frac{k_1}{k_3} \left[\frac{1}{[I]} + \frac{[A]/[k_3]}{/[I]}\right]$$

where $A$ is a substrate or competitive inhibitor and $k_1$ is its dissociation constant. For substantiation of the contention that Cibacron Blue F3GA is an active site directed reagent, C subunit was incubated with the blue dye in the presence of substrates and competitive enzyme inhibitors. $K_{app}$ values were measured and graphed according to eq 2. ATP alone fails to alter the rate of dye inactivation (Figure 2). MgATP, however, reduces enzyme inactivation by the blue chromophore (Figure 2). The lines representing inactivation in the presence or absence of MgATP have a common ordinate intercept, thereby indicating that the maximum rate of inactivation ($k_3$) is unchanged. Analogous to competitive inhibition patterns obtained from double-reciprocal plots (Cleland, 1967), this result indicates that the dye and MgATP compete for the same site. From eq 3, the $k_3$ for MgATP is 620 $\mu$M. This differs from the $K_m$ of $\sim 10 \mu$M and provides another example that the dissociation constant is not necessarily equal to the $K_m$ (Jencks, 1969).
results are consistent with the notion that MgCl₂ has a direct effect on the aged enzyme protein.

**General Characteristics of Cibacron Blue F3GA Inactivation of the Catalytic Subunit.** In addition to time dependence, blue dye inactivation is also temperature dependent. The rate of inactivation at 0 °C is ~3% that at 30 °C. The rate of inactivation was insensitive to pH (6.0–8.0). Since many dyes inhibit enzymes by photooxidation, experiments were performed under various degrees of illumination to test for blue dye mediated photoinactivation. In these experiments, C subunit was incubated for 7 min at 22 °C with 50 μM Cibacron Blue F3GA. Incubations were performed in the dark, under moderate (room fluorescent lighting) or high (375-W bulb, 23-cm distance) intensity lighting. Since no differences in activation were observed, enzyme inactivation is unrelated to photooxidation.

Several attempts were made to reverse the blue dye inactivation. For example, low concentrations of substrate nucleotides or high NaCl concentrations (200 mM) displace Cibacron Blue F3GA from lactate dehydrogenase and phosphoglycerate kinase as measured spectrophotometrically (Thompson & Stellwagen, 1976). In the present study, if 5 mM MgATP or guanethidine is added alone or in combination to an enzyme solution containing the blue dye, then enzyme inactivation is terminated. This treatment, however, fails to restore lost enzyme activity. Unlike lactate dehydrogenase or phosphoglycerate kinase, the addition of NaCl (to 500 mM) also fails to reactivate the enzyme. Enzyme inactivation is also not reversed by Sephadex G-25 gel filtration or by exhaustive dialysis. Since Thompson et al. (1976) demonstrated that Blue Dextran–Sepharose quantitatively displaces NAD⁺ (kd = 10⁻⁶ M) from glyceraldehyde-3-phosphate dehydrogenase (which can be recovered by salt elution), a similar approach was attempted. If Cibacron Blue F3GA binds noncovalently to the active site of the C subunit, then the Blue Dextran–Sepharose resin may displace it and reverse the inhibition. After treatment of C subunit (6 μg) with Cibacron Blue F3GA, inhibited catalytic subunit was applied to a small Blue Dextran–Sepharose column and treated with low ionic strength and then high ionic strength buffer to elute specifically adsorbed enzyme, as described in Table I. No activity was detected in any of the elution fractions, indicating that the blue dye was not displaced by the affinity resin. Protein analysis (Bradford, 1976) also shows that the dye-inactivated enzyme fails to bind to the affinity resin.

Chemical attachment of the dye to the enzyme would most likely involve displacement of the chloride group of the triazine ring by a nucleophilic side-chain residue. Blue Dextran–Sepharose, in which sugar alcohols replace the dye chloride, interacts with C in a reversible manner (Table I; Witt & Roskoski, 1975a). For a further test of this hypothesis, the methoxy derivative of the blue dye was synthesized. This compound is a reversible inhibitor of C subunit activity (Table III).

Because of the inability to reactivate the enzyme under nondenaturing conditions, attempts were made to dissociate the blue dye by denaturation. After incubation of 50 μM blue dye for 30 min at 22 °C as specified under Methods and Materials, after Sephadex G-50 medium gel filtration (4 × 1 cm) using 220 mM sodium phosphate (pH 7.0) as eluant, fractions (0.4 mL) were collected and taken for protein and enzyme activity determinations. The content of blue dye was determined spectrophotometrically at 610 nm by using an extinction coefficient of 15 600 M⁻¹ cm⁻¹ (Thompson & Stellwagen, 1976). These experiments represent the mean plus or minus the standard error of the mean of triplicate determinations.

### Table II: MgCl₂ Treatment Enhancement of Catalytic Subunit Inactivation by Cibacron Blue F3GA³

<table>
<thead>
<tr>
<th>pretreatment</th>
<th>EDTA (mM)</th>
<th>Kapp (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0</td>
<td>27.4</td>
</tr>
<tr>
<td>none</td>
<td>10</td>
<td>29.3</td>
</tr>
<tr>
<td>MgCl₂ (10 mM)</td>
<td>0</td>
<td>15.8</td>
</tr>
<tr>
<td>MgCl₂ (10 mM)</td>
<td>10</td>
<td>18.4</td>
</tr>
</tbody>
</table>

³ Aged C subunit (3 weeks, 30 μg of protein in 200 μL) was incubated for 30 min at 4 °C in the presence or absence of MgCl₂. Portions (20 μL) were then incubated with 50 μM blue dye in 200 mM potassium phosphate (pH 7.0) at 22 °C with EDTA as specified in a final volume of 100 μL. Enzyme activity and Kapp values were determined as described under Methods and Materials.

### Table III: Methoxy Cibacron Blue F3GA Reversible Inhibition of C Subunit Activity⁴

<table>
<thead>
<tr>
<th>treatment</th>
<th>initial activity [μmol/(min mg of protein)]</th>
<th>gel filtration activity [μmol/(min mg of protein)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>1.35</td>
<td>1.27</td>
</tr>
<tr>
<td>100 μM Cibacron Blue F3GA</td>
<td>0.27</td>
<td>0.30</td>
</tr>
<tr>
<td>100 μM methoxy blue dye</td>
<td>0.46</td>
<td>1.30</td>
</tr>
</tbody>
</table>

⁴ Catalytic subunit (18 μg/300 μL) was treated with the specified compound as described under Methods and Materials. After 30 min, samples (5 μL) were assayed for enzyme activity in the presence of 100 μM Cibacron Blue F3GA or its derivative. The remainder was applied to Sephadex G-50 columns (1 × 15 cm) equilibrated with 0.1 M potassium phosphate (pH 6.8). The protein-containing fractions were combined, and enzyme activity was measured as previously described (Witt & Roskoski, 1975b). Protein was measured by the procedure of Bradford (1976). For replacement of the triazine chloride, the methoxy derivative of Cibacron Blue F3GA was prepared according to methodologies outlined by Dudley et al. (1951) for the synthesis of 2,4-bis-(phenylamino)-6-methoxytriazine. The reaction was initiated by the addition of Cibacron Blue F3GA (125 mg, 1.5 × 10⁻⁴ mol) to 50 mL of methanol-dissolved sodium hydroxide (7 mg, 1.7 × 10⁻² mol) at 50°C. After the mixture was refluxed for 2.5 h, the methanol was evaporated. The product was purified by dissolving it in 5 mL of water and then adding 1 g of NaCl. After the mixture was stirred for 2 h at room temperature, the resulting precipitate was collected by suction filtration and washed with 20% NaCl (10 mL). The air-dried precipitate was dissolved in 1 mL of water prior to use.

### Table IV: Stoichiometry of Cibacron Blue F3GA Binding to Catalytic Subunit⁵

<table>
<thead>
<tr>
<th>treatment</th>
<th>dye bound (mol/mol)</th>
<th>act. [μmol/(min mg)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cibacron Blue F3GA</td>
<td>1.21 ± 0.11</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Cibacron Blue F3GA, ATP (5 mM), MgCl₂ (10 mM)</td>
<td>0.12 ± 0.04</td>
<td>1.31 ± 0.11</td>
</tr>
<tr>
<td>no addition</td>
<td>0.00</td>
<td>1.42 ± 0.12</td>
</tr>
</tbody>
</table>

⁵ Catalytic subunit (150 μg/250 μL) was treated with 50 μM blue dye for 30 min at 22 °C as specified under Methods and Materials. After Sephadex G-50 medium gel filtration (4 × 1 cm) using 220 mM sodium phosphate (pH 7.0) as eluant, fractions (0.4 mL) were collected and taken for protein and enzyme activity determinations. The content of blue dye was determined spectrophotometrically at 610 nm by using an extinction coefficient of 15 600 M⁻¹ cm⁻¹ (Thompson & Stellwagen, 1976). These experiments represent the mean plus or minus the standard error of the mean of triplicate determinations.
Table V: Effect of Cibacron Blue F3GA on CAMP-Dependent Protein Kinase Holoenzymes I and II

<table>
<thead>
<tr>
<th>isoenzyme</th>
<th>Control</th>
<th>Cibacron Blue F3GA (50 μM)</th>
<th>CAMP (10 μM)</th>
<th>Cibacron Blue F3GA (50 μM), cAMP (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>125</td>
<td>120</td>
<td>123</td>
<td>88</td>
</tr>
<tr>
<td>II</td>
<td>110</td>
<td>94</td>
<td>107</td>
<td>69</td>
</tr>
</tbody>
</table>

a Bovine muscle protein kinases I and II were prepared by using the methodology of Corbin et al. (1975). In a final volume of 100 μL (200 mM potassium phosphate, pH 7.0), fractions containing protein kinases I and II were incubated for 20 min at 22 °C with 50 μM Cibacron Blue F3GA and cAMP, as specified. Enzyme activity (10 μL) was measured as previously described (Witt & Roskoski, 1975a).

Discussion

Cibacron Blue F3GA, the chromophore of Blue Dextran, inhibits the C subunit of bovine brain and skeletal muscle CAMP-dependent protein kinase in a time- and temperature-dependent fashion. The hyperbolic dependence of inhibition on dye concentration is characteristic of an active site directed process. The protection against inhibition afforded by MgATP, a synthetic heptapeptide substrate, and guanethidine (a substrate analogue) argues that they and Cibacron Blue F3GA interact with the same enzyme domain, namely, the active site.

That Cibacron Blue F3GA interacts with the active site of the C subunit is expected since the dye is a structural analogue of ATP. The apparently irreversible nature of inhibition, however, was unanticipated since its inhibition of other enzymes is reversible (Thompson & Stellwagen, 1976). Weber et al. (1979), on the other hand, reported that commercial sources of Cibacron Blue F3GA inactivate Escherichia coli isoleucine-tRNA synthetase and yeast phosphoglycerate kinase by covalent modification. They find, however, that covalent modification and inactivation are associated with contaminants in the blue dye which can be resolved by thin-layer chromatography. In contrast to the heterogeneity observed in commercial sources of the dye, which we confirmed, the dye obtained from Ciba Geigy was homogeneous. The same sample of dye, moreover, reversibly inhibits phosphoglycerate kinase (Thompson & Stellwagen, 1976); this is consistent with the result of Weber et al. (1979), who showed that the dye purified by silica gel chromatography does not irreversibly inhibit this enzyme; they find, however, that a contaminant in the commercial dye irreversibly inhibits the enzyme. The dye used in the present experiments lacks the contaminant based on thin-layer chromatographic analysis and on its failure to irreversibly inhibit phosphoglycerate kinase. Inactivation of the catalytic subunit, therefore, is associated with Cibacron Blue F3GA per se and not with a contaminant.

The k_d of blue dye for lactate dehydrogenase and C subunit is 0.49 and 100 μM, respectively (Thompson & Stellwagen, 1976, this study). The K_M for NADH and ATP are 7 and 10 μM, respectively (Thompson & Stellwagen, 1976, this study). Thompson & Stellwagen (1976) suggest that the blue dye's affinity for the dehydrogenase may be enhanced because it resembles the NAD–pyruvate adduct and may mimic the transition state. In the case of protein kinase, a portion of the dye may span a portion of the peptide substrate domain of the active site but does not closely resemble the peptide substrate.

Several previous observations indicate that protein kinase undergoes changes during storage. For example, aged enzyme becomes less dependent on CAMP for expression of activity (Brostrom et al., 1970) and is less capable of releasing CAMP upon regeneration of holoenzyme (Sugden & Corbin, 1976). In the present study, MgCl_2 restores the sensitivity of aged enzyme to dye inactivation. This seems to involve a direct effect on the C subunit per se and not interaction of MgCl_2 with the blue dye. It may be that the C subunit, like E. coli glutamine synthetase (Kingdon et al., 1968; Denton &
Ginsburg, 1969), has different conformations which are altered by divalent cations.

The nucleotide and peptide substrates protect the C subunit from blue dye inactivation. The finding that protection occurs over a wide range of substrate and competitive inhibitor concentrations supports the idea that the blue dye interacts with the active site. It seems possible, but less likely, that ligands with such different affinities for the active site would protect against inactivation at a remote site. The ability of the regulatory subunit to protect against inactivation may be due to interaction with the active site of the catalytic subunit. Alternatively, functional shielding of the active site from the dye might involve a conformational change mediated by allosteric interaction of the regulatory with the catalytic subunit. Additional studies are required to test these and other possibilities for the mechanism of inhibition of phosphotransferase activity by the regulatory subunits.

References