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Inactivation of Guanosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase from Bovine Lung by *o*-Phthalaldehyde†

Rajinder N. Puri,*,‡ Deepak Bhatnagar,‡ David B. Glass,§ and Robert Roskoski, Jr.‡

Department of Biochemistry, Louisiana State University Medical Center, New Orleans, Louisiana 70119, and Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

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ABSTRACT: Guanosine cyclic 3′,5′-monophosphate (cGMP) dependent protein kinase is inactivated by *o*-phthalaldehyde. The loss of phosphotransferase activity following treatment with *o*-phthalaldehyde was rapid, and the second-order rate constant at 25 °C and pH 7.3 was 35 M^{-1} s^{-1}. The inactivation reaction did not follow saturation kinetics. The cGMP-dependent protein kinase was protected from inactivation by its substrates, MgATP and Ser-peptide. Fluorescence excitation and emission spectroscopic data showed that an isoindole derivative was formed following the reaction between cGMP-dependent protein kinase and *o*-phthalaldehyde. Four moles of isoindole per mole of the cGMP-dependent protein kinase dimer was formed following complete inactivation by *o*-phthalaldehyde. In the absence of cGMP, the protein kinase lost only 50% of its cGMP binding activity while there was almost a complete loss of its phosphotransferase activity. Studies in the presence of 20 μM cGMP, however, showed that about 2 mol of isoindole groups per mole of the protein kinase dimer was formed following complete inactivation by *o*-phthalaldehyde. The second-order rate constant for inactivation of cGMP-dependent protein kinase by *o*-phthalaldehyde in the presence of 20 μM cGMP was 40 M^{-1} s^{-1}. Fluorescence measurements of samples containing inactivated, iodoacetamide-modified, or 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine-modified, cGMP-dependent protein kinase and *o*-phthalaldehyde showed that the intensity of fluorescence in each case was about 50% of that obtained from unmodified, active cGMP-dependent protein kinase and *o*-phthalaldehyde. It is concluded that 2 mol of the adduct is formed by a reaction of *o*-phthalaldehyde within the catalytic domain and an additional 2 mol by its reaction within the regulatory domain. Fluorescence measurements showed that cGMP-dependent protein kinase denatured with urea did not react with *o*-phthalaldehyde, indicating that cysteine and lysine residues participating in isoindole derivative formation are close together in the tertiary structure. The cysteine and lysine residues participating in isoindole derivative formation in the catalytic and regulatory domains are oriented such that the sulfhydryl and ε-amino functions, respectively, are about 3 Å apart. The molar transition energy of the cGMP-dependent protein kinase–*o*-phthalaldehyde adduct was 121 kJ/mol and showed that the regions of catalytic and regulatory domains containing the cysteine and lysine residues involved in isoindole formation are hydrophobic. The overall nature of the reaction between cGMP-dependent protein kinase and *o*-phthalaldehyde was similar to that between the catalytic subunit of adenosine cyclic 3′,5′-monophosphate dependent protein kinase and *o*-phthalaldehyde.

The presence of guanosine cyclic 3′,5′-monophosphate (cGMP)[1] dependent protein kinase in mammalian tissues was first demonstrated by Kuo (1974). The enzyme has been purified from bovine lung (Gill et al., 1976, 1977; Lincoln et al., 1977) and heart muscle (Flockerzi et al., 1978). Several reviews reflecting the progress of our understanding of the structural and functional aspects as well as the biological role of cGMP-dependent protein kinase have appeared (Gill & McCune, 1979; Glass & Krebs, 1980; Flockhart & Corbin, 1982; Lincoln & Corbin, 1983).

The catalytically inactive holoenzyme of cGMP-dependent protein kinase consists of two identical covalently-linked polypeptide chains, each with a molecular weight of about 75000 (Lincoln et al., 1977; Gill et al., 1977). Each subunit contains two cyclic nucleotide binding sites (Mackenzie, 1982; Corbin & Døskeland, 1983) and one phosphotransferase catalytic site

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‡ Louisiana State University Medical Center.
§ Emory University School of Medicine.

[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; C_I or C_{II}, catalytic subunit of type I or type II cAMP-dependent protein kinase, respectively; R_I or R_{II}, regulatory subunit of type I or type II cAMP-dependent protein kinase, respectively; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; histone H2B-(29–35), Arg-Lys-Arg-Ser-Arg-Lys-Glu.

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(Lincoln et al., 1977). Binding of cGMP to the inactive holoenzyme results in the formation of an active enzyme complex according to the following scheme, where E denotes an enzyme monomer (Takai et al., 1976; Gill et al., 1977; Lincoln et al., 1977; Corbin & Døskeland, 1983):

$$E_2 \text{ (less active)} + 4\text{cGMP} \rightleftharpoons E_2(\text{cGMP})_4 \text{ (more active)}$$

This is in marked contrast to the mechanism of activation of the cAMP-dependent protein kinase by cAMP whereby the binding of cAMP to the inactive tetrameric enzyme results in dissociation of the holoenzyme into two catalytic subunits and a dimeric regulatory subunit–$(cAMP)_4$ complex (Corbin et al., 1978). The two enzymes, however, bear similarities in size and shape (Erlichman et al., 1973; Lincoln et al., 1977; Lincoln & Corbin, 1977), in amino acid compositions (Takio et al., 1983, 1984a,b; Shoji et al., 1983), in substrate specificities (Lincoln & Corbin, 1977; Edlund et al., 1977), and in their ability to undergo autophosphorylation (Erlichman et al., 1974; DeJong & Rosen, 1977; Foster et al., 1981). Fluorescence polarization titration and steady-state kinetic studies (Bhatnagar et al., 1985) have also emphasized similarities in nucleotide binding and metal ion requirements between the catalytic subunit of type II cAMP-dependent protein kinase and cGMP-dependent protein kinase. Amino acid sequences of the type II cAMP-dependent (Takio et al., 1983) and cGMP-dependent (Takio et al., 1984b) protein kinases have also confirmed the earlier view held by other investigators (Lincoln & Corbin, 1977, 1978; Gill, 1977) that they are highly homologous enzymes.

FSBA, an affinity reagent, inactivates the catalytic subunit of cGMP-dependent protein kinase by modifying one lysine residue at position 389 located in the catalytic domain (Hixson & Krebs, 1981; Hashimoto et al., 1982). Although the presence of a cysteine residue at or near the active site of the catalytic domain of the type II cAMP-dependent protein kinase has been established (Nelson & Taylor, 1983), the presence of a cysteine residue in the catalytic domain of the cGMP-dependent protein kinase has not been demonstrated by chemical-modification studies. The work described in this paper shows that *o*-phthalaldehyde inhibits phosphotransferase activity of the cGMP-dependent protein kinase by simultaneously modifying cysteine and lysine residues in its catalytic domain. In addition, *o*-phthalaldehyde also reacts with another pair of cysteine and lysine residues in the regulatory domain of cGMP-dependent protein kinase.

Materials and Methods

Purification of cGMP-Dependent Protein Kinase. The cGMP-dependent protein kinase from bovine lung, as purified by the method of Glass & Krebs (1979), had a specific activity of 5.5 units/mg of protein (1 unit is defined as the amount of enzyme that will catalyze the transfer of 1 μmol of ^{32}P to histone H2B at 30 °C, pH 7.0) with 0.5 mg/mL histone H2B as substrate. It compares well with specific activity values reported previously (Flockerzi et al., 1978; Glass et al., 1978). The −cGMP/+cGMP activity ratio of this preparation of enzyme with 25 μM histone H2B-(29–35) (Glass & Krebs, 1982) as substrate was 0.09, indicating an 11-fold stimulation of activity by cGMP. The specific activity of cGMP-dependent protein kinase with Ser-peptide as substrate was 500 nmol min^{-1} mg^{-1}, and this compares favorably with 580 nmol min^{-1} mg^{-1} as described by Corbin & Døskeland (1983). The enzyme examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was pure except for the presence of a trace amounts of a lower molecular weight degradation product probably resulting from proteolytic digestion (Gill et al., 1981) of the enzyme during purification or storage.

cGMP-Dependent Protein Kinase Activity Measurements. Phosphotransferase activity of the enzyme in this work was measured with Ser-peptide as the substrate. The assay mixture contained 50 mM Mops–NaOH, pH 7.0, 10 mM $MgCl_2$, 3 mM DTT, 100 μM [γ-^{32}P]ATP (30 dpm/pmol), 1.5 mM Ser-peptide, and 2 μM cGMP. Typical concentrations of the enzyme in the assay mixture were 10–50 nM. The radiolabeled product was resolved from substrate by phosphocellulose paper adsorption (Roskoski, 1983).

The cGMP binding activity was measured by a method similar to that employed by Sugden & Corbin (1976) for the binding of cAMP to the regulatory subunit of the cAMP-dependent protein kinase. cGMP-dependent protein kinase, 1 μg, was incubated at 30 °C for 60 min in 0.11 mL of a solution containing 50 mM potassium phosphate, pH 6.8, 1 mM EDTA, 0.5 mg/mL histone H2A, 2 M sodium chloride, and 1 μM [8-^3H]cGMP (3000 dpm/pmol). The reaction was terminated by the addition of an ice-cold solution, 2.6 mL, containing 10 mM potassium phosphate (pH 6.8) and 1 mM EDTA (buffer A). The reaction mixture was filtered through 0.45-μm Millipore filters. The reaction tube was rinsed with an additional 2.6 mL of buffer A over the Millipore filter, which was further washed 3 times with 2.6-mL portions of ice-cold buffer A. The filters were dried at 105 °C, and radioactivity was measured by liquid scintillation spectrometry.

A M_r of 150 000 (Foster et al., 1981) was used to calculate molar concentrations of the cGMP-dependent protein kinase. The enyzme concentration was determined either by the method of Lowry et al. (1951) with bovine serum albumin as standard or by absorbance with an extinction coefficient of $E_{280}^{0.1\%} = 1.05$ (Foster et al., 1981). Polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate was performed by the method of Laemmli (1970) on 10% gels, and corrected mobilities were calculated as described by Weber & Osborn (1969).

The materials used in this work were the same as those described in the preceding paper (Puri et al., 1985). In addition, [8-^3H]cGMP (6 Ci/mmol) was obtained from ICN, and cGMP was supplied by Sigma.

Results

Inactivation of cGMP-Dependent Protein Kinase by o-Phthalaldehyde. The time course of inactivation of cGMP-dependent protein kinase by *o*-phthalaldehyde is shown in Figure 1. When the natural logarithm of percent activity remaining was plotted against time, linear pseudo-first-order plots were obtained in the concentration range of 0.2–0.8 mM for *o*-phthalaldehyde. At the lowest concentration of *o*-phthalaldehyde, 0.2 mM, more than 90% of its phosphotransferase activity was lost in 5 min whereas the same loss of activity occurred in about 1.5 min when the concentration of *o*-phthalaldehyde was 0.8 mM.

The pseudo-first-order reaction rates, K_{obsd}, were calculated as described previously (Puri et al., 1985). When these were plotted against *o*-phthalaldehyde concentration, a linear relationship was obtained. The slope of the plot yields a second-order rate constant, K, equal to 35 M^{-1} s^{-1} (Figure 1, inset). The absence of hyperbolic dependence of the pseudo-first-order reaction rates on *o*-phthalaldehyde concentration suggests that the reaction with cGMP-dependent protein kinase does not follow saturation kinetics. The inactivation of cGMP-dependent protein kinase by *o*-phthalaldehyde in the presenceof 20 μM cGMP follows the same pattern as in the absence of cGMP. The second-order rate constant in the

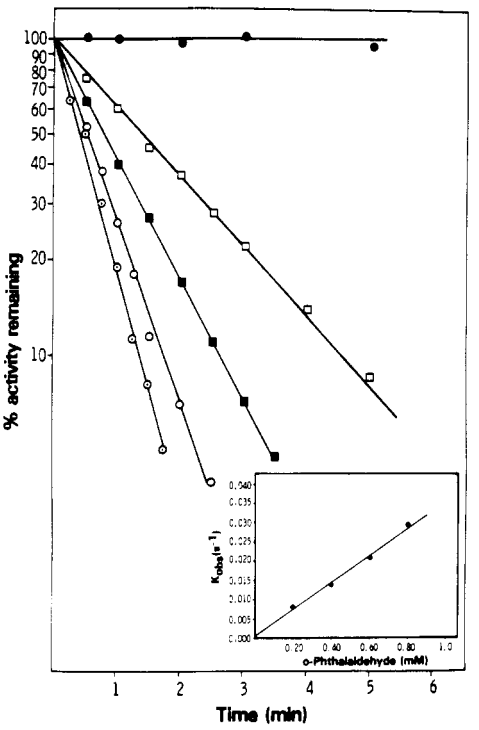

FIGURE 1: Time course of inactivation of cGMP-dependent protein kinase by *o*-phthalaldehyde. Each incubation mixture contained 0.14 μM cGMP-dependent protein kinase, 100 mM Hepes–NaOH, pH 7.3, 0.1 mM EDTA, 1% methanol, and *o*-phthalaldehyde at 0 (●), 0.2 (□), 0.4 (■), 0.6 (○), and 0.8 mM (⊙). The incubations were carried out at 25 °C. Aliquots of the incubation mixtures were transferred into vials containing a solution of cysteine and β-mercaptoethanol. The final concentrations of the latter in the reaction mixtures were 20 and 5 mM, respectively. The mixtures were assayed for phosphotransferase activity as described under Materials and Methods. The inset shows the plot of K_{obsd} vs. *o*-phthalaldehyde concentration.

presence of cGMP was similar (40 M^{-1} s^{-1}; data not shown).

Since regulatory and catalytic domains of the cGMP-dependent protein kinase are located on the same polypeptide chain (Gill et al., 1976; Lincoln et al., 1977; Lincoln & Corbin, 1983), it was of interest to examine the cGMP binding activity. When cGMP-dependent protein kinase was incubated with 0.4 mM *o*-phthalaldehyde at 25 °C, there was a loss of about 60% phosphotransferase activity (Figure 1) and 32% loss of the cGMP binding activity at 1 min (Figure 2). At 3 min, *o*-phthalaldehyde brought about a 95% loss of the phosphotransferase activity but only an approximately 50% loss of cGMP binding activity, which remained constant thereafter.

Effect of Potential Protecting Agents on the Inactivation of cGMP-Dependent Protein Kinase. The effect of various reagents on the inactivation of cGMP-dependent protein kinase phosphotransferase activity by *o*-phthalaldehyde is summarized in Table I. MgATP completely protected the enzyme, and MgADP, Ser-peptide, and histone H2B partially protected the enzyme from inactivation. The nucleotides in the absence of Mg^{2+}, guanethidine, protamine sulfate, and chelators were ineffective. cGMP (50 μM or 1 mM) in the presence or absence of Mg^{2+} was completely ineffective in preventing the enzyme from losing phosphotransferase activity.

Nature of the Product Formed in the Reaction between cGMP-Dependent Protein Kinase and o-Phthalaldehyde. Fluorescence excitation and emission spectroscopy were used to characterize the cGMP-dependent protein kinase–*o*-phthalaldehyde adduct. The emission spectra showed a maximum at 405 nm following excitation at 295 or 338 nm. The excitation spectrum exhibited a maximum at 345 nm and

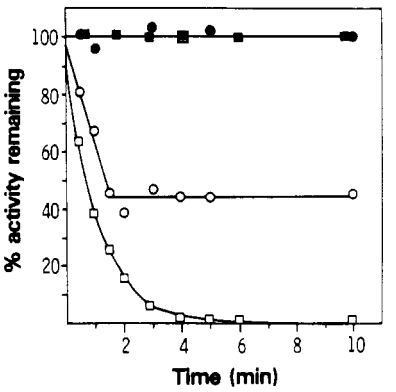

FIGURE 2: Time course of the inhibition of cGMP binding activity by *o*-phthalaldehyde. A solution containing 0.13 μM cGMP-dependent protein kinase, 100 mM Hepes–NaOH, pH 7.3, 0.1 mM EDTA, 1% methanol, and 0.4 mM *o*-phthalaldehyde was incubated at 25 °C. Aliquots of the incubation mixture were withdrawn at the time intervals indicated and transferred to a solution containing cysteine and β-mercaptoethanol as described in Figure 1. Portions were subsequently assayed for cGMP binding activity (○) and phosphotransferase activity (□). Appropriate controls for cGMP binding activity (●) and phosphotransferase activity (■) were included in the experiment.

Table I: Effect of Potential Protecting Reagents on the Inactivation of cGMP-Dependent Protein Kinase by *o*-Phthalaldehyde[a]

addition to incubation mixture	% activity remaining
none	14
nucleotides	
ATP (1 mM)	19
ADP (1 mM)	11
AMP (1 mM)	13
cGMP (1 mM)	17
cGMP (50 μM)	10
nucleotides and 10 mM Mg^{2+}	
ATP (1 mM)	100
ADP (1 mM)	36
AMP (1 mM)	22
cGMP (1 mM)	15
cGMP (50 μM)	12
metal ions	
Mg^{2+} (10 mM)	17
Mn^{2+} (5 mM)	7
Ca^{2+} (5 mM)	17
Co^{2+} (1 mM)	7
nucleosides	
adenosine (1 mM)	16
chelators	
EDTA (1 mM)	14
EGTA (1 mM)	15
proteins	
Ser-peptide (1 mM)	42
histone H2B (2 mg/mL)	31
guanethidine (2 mM)	14
protamine sulfate (1 mg/mL)	14

[a] Solutions containing 6.7 μM cGMP-dependent protein kinase, 100 mM Hepes–NaOH, pH 7.3, 0.1 mM EDTA, 1% methanol, and 0.2 mM *o*-phthalaldehyde were incubated for 3 min at 25 °C in the presence of the specified concentration of the various reagents. The reactions were terminated as described in Figure 1. Appropriate controls devoid of *o*-phthalaldehyde were included in each case. Residual phosphotransferase activities in the reaction mixtures were subsequently measured as described under Materials and Methods.

a shoulder at 290 nm when an emission wavelength of 405 nm was used (Figure 3). These spectral characteristics are consistent with the formation of an isoindole derivative in the reaction between cGMP-dependent protein kinase and *o*-phthalaldehyde (Simons et al., 1979; Palczewski et al., 1983; Puri et al., 1985).

Stoichiometry of the cGMP-Dependent Protein Kinase–o-Phthalaldehyde Adduct. The stoichiometry of the cGMP-dependent protein kinase–*o*-phthalaldehyde adduct was ex-

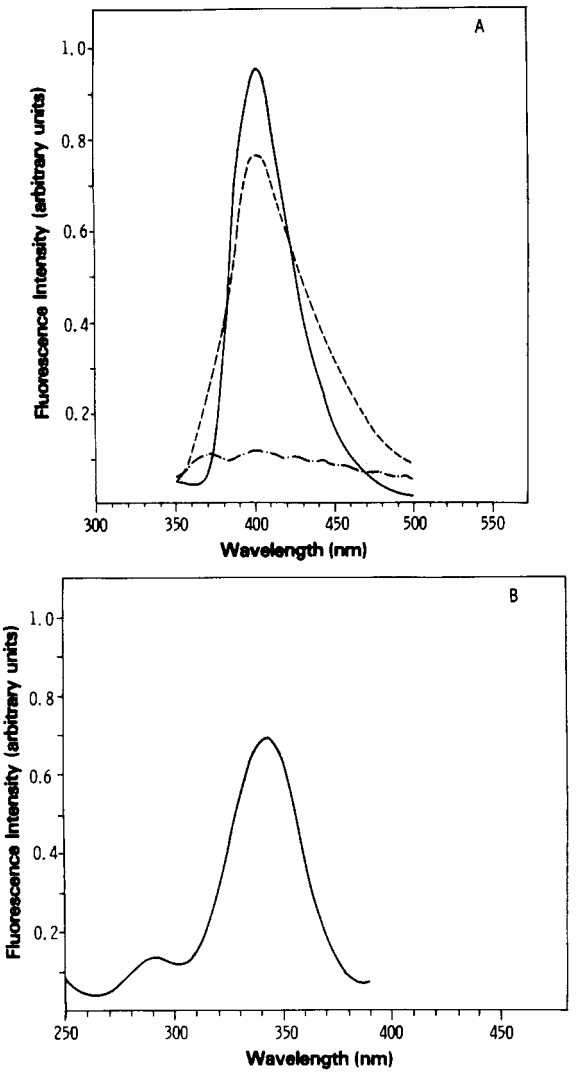

FIGURE 3: Characterization of the product of reaction between cGMP-dependent protein kinase and *o*-phthalaldehyde. cGMP-dependent protein kinase, 2.2 μM, and *o*-phthalaldehyde, 8 mM, were allowed to react in a solution containing 100 mM Hepes–NaOH, pH 7.3, 0.1 mM EDTA, and 1% methanol at 25 °C for 3 min, and the fluorescence emission spectra were recorded (panel A) with excitation wavelengths of 295 (—) and 338 nm (--) as described previously (Puri et al., 1985). An identically constituted reaction mixture was used for recording the fluorescence excitation spectrum (panel B) with an emission wavelength of 405 nm.

amined by absolute and relative methods (Palczewski et al., 1983; Puri et al., 1985). Absorption spectroscopy data (absorption maximum at 337 nm) and fluorescence emission intensity data (emission maximum at 405 nm) with aldolase as the standard show that 3.5 ± 0.12 and 4.06 ± 0.27 mol of isoindole groups are formed per mole of cGMP-dependent protein kinase, respectively, following complete inactivation by *o*-phthalaldehyde. In the presence of 20 μM cGMP, only 2.42 ± 0.07 mol of isoindole/mol of cGMP-dependent protein kinase is formed following complete inactivation as determined by fluorescence or absorbance measurements.

Characterization of Sites of Isoindole Derivative Formation in the Reaction between cGMP-Dependent Protein Kinase following Complete Reaction with o-Phthalaldehyde. When cGMP-dependent protein kinase was treated with iodoacetamide, 90% of the phosphotransferase activity was lost (Table II). When the incubation mixture containing iodoacetamide-modified cGMP-dependent protein kinase was subsequently treated with *o*-phthalaldehyde, the intensity of the fluorescence emission resulting from isoindole formation was half that

Table II: Phosphotransferase Activity of cGMP-Dependent Protein Kinase following Modification by Iodoacetamide or FSBA and Denaturation by Urea[a]

reagent	concn	% activity remaining
none		100
iodoacetamide	6.4 mM	10
FSBA	4.0 mM	1
urea	6.0 M	0

[a] The experimental details of modification reactions are provided in Figure 5.

obtained from unmodified protein kinase and *o*-phthalaldehyde (Figure 4A). FSBA modification of the cGMP-dependent protein kinase abolished 99% of its phosphotransferase activity. Subsequent reaction of the FSBA-modified cGMP-dependent protein kinase with *o*-phthalaldehyde showed that the intensity of fluorescence was also half of that obtained from unmodified protein kinase and *o*-phthalaldehyde (Figure 4B). Although chemical modification of cGMP-dependent protein kinase by iodoacetamide or FSBA abolishes its phosphotransferase activity, the modified enzyme still retains its ability to react with *o*-phthalaldehyde, but with a 50% reduced stoichiometry. Iodoacetamide and FSBA themselves did not affect the fluorescence emission properties of the cGMP-dependent protein kinase–*o*-phthalaldehyde adduct (data not shown). Treatment of cGMP-dependent protein kinase with 6 M urea causes complete and rapid loss of phosphotransferase activity of the enzyme. The cGMP-dependent protein kinase denatured by urea was incapable of isoindole derivative formation in a subsequent reaction with *o*-phthalaldehyde as shown by the fluorescence spectrum (Figure 4C).

Discussion

cGMP-dependent protein kinase rapidly lost phosphotransferase activity following chemical modification by *o*-phthalaldehyde. Although the rate of reaction between cGMP-dependent protein kinase and *o*-phthalaldehyde was somewhat slower (K = 35 M^{-1} s^{-1}) than that between the catalytic subunit of type II cAMP-dependent protein kinase (Puri et al., 1985) or aldolase (Palczewski et al., 1983), they were similar in that none followed saturation kinetics in the concentration range of the inhibitor investigated. The fluorescence excitation and emission spectra of the cGMP-dependent protein kinase–*o*-phthalaldehyde adduct demonstrated the formation of an isoindole derivative (Simons & Johnson, 1978; Simons et al., 1979; Puri et al., 1985). Polyacrylamide gel electrophoresis of the reaction product in the absence of sodium dodecyl sulfate showed no higher molecular weight bands other than the one corresponding to cGMP-dependent protein kinase (data not shown). These findings indicate that the reaction between cGMP-dependent protein kinase and *o*-phthalaldehyde was intramolecular and not intermolecular in nature. As in the case of the catalytic subunit of cAMP-dependent protein kinase (Puri et al., 1985), treatment of cGMP-dependent protein kinase with 6 M urea destroys the proximal integrity of the cysteine and lysine residues present in the native enzyme for reaction with *o*-phthalaldehyde (Figure 4C). We, therefore, conclude that the loss of phosphotransferase activity of the cGMP-dependent protein kinase following reaction with *o*-phthalaldehyde is a consequence of simultaneous modification of cysteine and lysine residues at or near the active site of the catalytic domain. During isoindole formation, these residues must be oriented such that their SH and ϵ-NH_2 functions are about 3 Å apart (Puri et al., 1985). These results are further corraborated by the findings that among several reagents examined for their

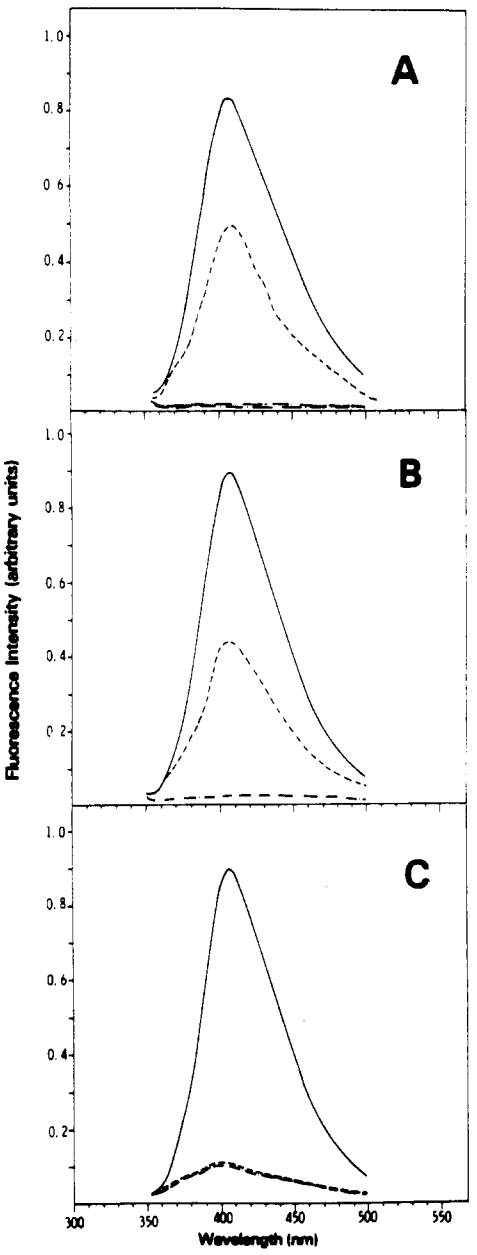

FIGURE 4: Fluorescence emission spectra of incubation mixtures containing either the iodoacetamide-modified, the FSBA-modified, or the urea-denatured cGMP-dependent protein kinase and *o*-phthalaldehyde. A solution containing 0.58 μM cGMP-dependent protein kinase, 100 mM Hepes–NaOH, pH 7.3, and 0.1 mM EDTA was incubated with either 6.4 mM iodoacetamide, 4 mM FSBA, or 6 M urea. Incubation with iodoacetamide was carried out overnight at 37 °C. The reaction with FSBA was carried out overnight in the presence of 5% dimethyl sulfoxide at 25 °C while the denaturation of enzyme with urea was carried out for 0.5 h at 25 °C. Appropriate controls were incubated simultaneously. Small portions of the incubation mixtures were withdrawn and assayed for phosphotransferase activity. Aliquots containing 0.43 μM protein were withdrawn from control and reaction mixtures, containing modified or denatured cGMP-dependent protein kinase, and treated with 3 mM *o*-phthalaldehyde for 3 min at 25 °C. These were subsequently examined by fluorescence emission spectroscopy as previously described (Puri et al., 1985). (Panel A) cGMP-dependent protein kinase (– –), iodoacetamide-modified cGMP-dependent protein kinase (– · –), and iodacetamide-modified cGMP-dependent protein kinase and *o*-phthalaldehyde (- - -); (panel B) FSBA-modified cGMP-dependent protein kinase (– –) and FSBA-modified cGMP-dependent protein kinase and *o*-phthalaldehyde (- - -); (panel C) urea and *o*-phthalaldehyde (- - -) and urea-denatured cGMP-dependent protein kinase and *o*-phthalaldehyde (– · –). All three panels include control enzyme treated under conditions identical with the modification reaction, i.e., cGMP-dependent protein kinase and *o*-phthalaldehyde (—).

Table III: Peptide Segments Containing Aligned Cysteine Residue in the Catalytic Domain of Protein Kinases

protein	residue nos.	amino acid sequence	ref
C subunit (cAMP-dependent protein kinase)	198–203	L-*C*-G-T-P-E	Shoji et al. (1983)
cGMP-dependent protein kinase	517–522	F-*C*-G-T-P-E	Takio et al. (1984b)

potential protective effect (Table I), the substrates, e.g., MgATP and Ser-peptide, were found to be most effective in protecting the cGMP-dependent protein kinase from inactivation by *o*-phthalaldehyde.

Chemical modification of the cGMP-dependent protein kinase with FSBA, an affinity reagent (Colman, 1983), was accompanied by 99% loss of its phosphotransferase activity (Table II), and subsequent treatment of the modified protein kinase with *o*-phthalaldehyde produces 50% loss of fluorescence emission intensity (Figure 4B). These results show the presence of a lysine residue per protein kinase monomer at or near the ATP binding site in the catalytic domain (Hixson & Krebs, 1981). The presence of a lysine residue at position 389 (Takio et al., 1984b) at or near active site of the catalytic domain of cGMP-dependent protein kinase has been demonstrated by Hashimoto et al. (1982). It is interesting to note that segments of peptides from γ-subunit of phosphorylase kinase (Reimann et al., 1984) and tyrosine kinases pp^{60src} (Barker & Dayhoff, 1982) and $pp^{90gag\text{-}yes}$ (Kitamura et al., 1982) also contain lysine residues corresponding to lysine residues 72 and 389 in the catalytic domain of cAMP-dependent protein kinase and cGMP-dependent protein kinase, respectively: sequence homologies around the critical lysines are noticeable (Reimann et al., 1984). Chemical modification of cGMP-dependent protein kinase by iodoacetamide brings about 90% loss of its phosphotransferase activity (Table II). Subsequent reaction with *o*-phthalaldehyde produces only a 50% loss of the fluorescence emission intensity (Figure 4A). These results are consistent with the presence of a cysteine residue per protein kinase monomer at or near the active site of the catalytic domain of cGMP-dependent protein kinase. A comparison (Table III) of the peptide segment (198–203) of the catalytic subunit of cAMP-dependent protein kinase (Shoji et al., 1983) with a peptide segment (517–522) of cGMP-dependent protein kinase (Takio et al., 1984b) shows that cysteine-199 in the former corresponds to cysteine-518 in the latter and the amino acid sequences around the cysteine are very similar. These observations are in accord with the suggestion made by Takio et al. (1984b) that the catalytic domain of cGMP-dependent protein kinase is located between the residues 474 and 599 in their arbitrary scheme of the functional domains of the kinase. It is important to point out that involvement of lysine-389 and cysteine-518 in the isoindole formation in the catalytic domain of the cGMP-dependent protein kinase was not independently demonstrated and this hypothesis is only one possibility.

Our present data show that about 4 mol of isoindole groups/mol of cGMP-dependent protein kinase dimer is formed in the reaction with *o*-phthalaldehyde. In the presence of 20 μM cGMP, there is only 2.42 ± 0.07 mol of isoindole groups formed per mole of protein kinase dimer, and there is complete loss of phosphotransferase activity. Since there are two essential lysines per mole of the cGMP-dependent protein kinase that react with FSBA (Hixson & Krebs, 1981), there must be two additional pairs of reactive lysine and cysteine

Table IV: Peptide Segments Containing Aligned Cysteine and Lysine Residues Postulated To Participate in Isoindole Formation in the Cyclic Nucleotide Binding Domain of Protein Kinases

protein	residue nos.	amino acid sequence	ref
Aligned Cysteine Residues			
cGMP-dependent protein kinase	310–317	V-T-*C*-L-V-I-D-R	Takio et al. (1984b)
R_I	343–350	L-K-*C*-V-K-L-D-R	Titani et al. (1984)
R_{II}	353–360	V-K-*C*-L-V-M-D-V	Takio et al. (1984a)
Aligned Lysine Residues			
cGMP-dependent protein kinase	205–211	L-I-*K*-H-T-E-Y	Takio et al. (1984b)
R_I	238–244	L-R-*K*-R-K-M-Y	Titani et al. (1984)
R_{II}	242–248	A-K-*K*-R-K-M-F	Takio et al. (1984a)

residues per enzyme dimer. While there is complete loss of the phosphotransferase activity of cGMP-dependent protein kinase in reaction with *o*-phthalaldehyde, there is only 50% loss of cGMP binding activity (Figure 2). Even though there are four cGMP binding sites per mole of cGMP-dependent protein kinase dimer (Corbin & Døskeland, 1983), our studies indicate that only 2 mol of isoindole groups is formed from cysteine and lysine residues in the cGMP binding domains. The reason may be 2-fold: (a) modification of two cGMP binding sites per mole of the protein kinase dimer may cause a conformational change in the protein kinase that renders the other cysteine and lysine residues incapable of isoindole formation, or (b) the cysteine and lysine residues within the unreactive cGMP binding site lack the necessary proximity to one another for reaction with *o*-phthalaldehyde. Other possibilities may also exist. It is, therefore, reasonable to suggest that loss of cGMP binding activity of the protein kinase is due to isoindole derivative formation between cysteine and lysine residues at or near the cGMP binding site(s) of the protein kinase monomer. This is consistent with the fact that there is only a 50% loss of cGMP binding activity during reaction with *o*-phthalaldehyde. These results are further substantiated by the finding that chemical modification of cGMP-dependent protein kinase by iodoacetamide or FSBA abolishes practically all its phosphotransferase activity but the iodoacetamide- or FSBA-modified enzyme is still capable of reacting with *o*-phthalaldehyde as evidenced by the fluorescence emission spectrum of the adduct (Figure 4A,B). The fluorescence intensity was only half (2 mol of isoindole/mol of enzyme) that of a control incubation mixture containing unmodified cGMP-dependent protein kinase (4 mol of isoindole/mol of enzyme). The failure of iodoacetamide to alter isoindole formation in the regulatory domain indicates that the cysteine in this domain is less reactive than that in the catalytic domain. Similar observations have been made with cysteine residues 326, 355, and 370 in the regulatory domain of the type II cAMP-dependent protein kinase (Nelson & Taylor, 1983). In the presence of cAMP, these cysteines are protected from alkylation by iodoacetamide, and in the absence of cAMP, they undergo alkylation only with much higher concentration (70 mM) of iodoacetamide at 37 °C (Nelson & Taylor, 1983).

On the basis of the previously determined amino acid sequences of the R_I (Titani et al., 1984) and R_{II} (Takio et al., 1984a) subunits of the cAMP-dependent protein kinase and that of the cGMP-dependent protein kinase (Takio et al., 1984b), it is possible to examine which putative cysteine and lysine residues within the cGMP-binding domain of the cGMP-dependent enzyme might be involved in the isoindole derivative formation. This domain of the protein kinase is arbitrarily divided into two peptide segments (Takio et al., 1984b): segment B, residues 102–219, and segment C, residues 220–340. The cAMP-binding domains of the two R subunits of the cAMP-dependent enzyme are similarly divided into two peptide segments designated as B_1 (residues 134–252) and C_1 (residues 253–379) for R_I (Titani et al., 1984) and B_2 (residues 135–256) and C_2 (residues 257–400) for R_{II} (Takio et al., 1984a). A closer examination of these domains reveals that there is a cysteine residue corresponding to Cys-312 of the cGMP-dependent enzyme (Table IV) that is properly aligned in the peptide segments C, C_1, and C_2 of the regulatory domains of the two protein kinases and that the amino acid sequences around this cysteine are also homologous in the two enzymes. Cysteine-312 of the cGMP-dependent protein kinases behaves more like cysteine-355 of R_{II} (Nelson & Taylor, 1983) toward cyclic nucleotide protection and alkylation with iodoacetamide. A lysine residue (corresponding to Lys-207 of the cGMP-dependent enzyme) also exhibits a high degree of alignment within the same peptide segments of the two enzymes (Table IV). This suggests that the two cGMP binding domains in the native cGMP-dependent protein kinase interact. Whether or not the Cys-312 and Lys-207 in the cGMP binding domain of the cGMP-dependent protein kinase are involved in the isoindole formation in reaction with *o*-phthalaldehyde is a hypothesis that should be experimentally verified by independent chemical modification studies.

The molar transition energy of the cGMP-dependent protein kinase–*o*-phthalaldehyde adduct, as calculated from its fluorescence emission maximum (Palczewski et al., 1983; Puri et al., 1985), was 121 kJ/mol. This value is similar to the one obtained for the aldolase or the cAMP-dependent protein kinase catalytic subunit–*o*-phthalaldehyde adducts (Puri et al., 1985). These findings suggest that cysteine and lysine residues participating in isoindole derivative formation in reaction between cGMP-dependent protein kinase and *o*-phthalaldehyde are located in a hydrophobic environment similar to those in aldolase (Palczewski et al., 1983) and the catalytic subunit of type II cAMP-dependent protein kinase (Puri et al., 1985). On the basis of the similarity of results obtained during our work concerning chemical modification of the catalytic subunit of cAMP-dependent protein kinase (Puri et al., 1985) and the catalytic domain of the cGMP-dependent protein kinase (this work), we also suggest that there exists considerable similarity in the tertiary structure of the catalytic domains of the two protein kinases (Weber et al., 1982). It is, therefore, reasonable to predict that chemical modification of R_I and R_{II} by *o*-phthalaldehyde might inhibit cAMP binding to these regulatory subunits. Furthermore, it has been found that *o*-phthalaldehyde also inhibits phosphotransferases unrelated to protein kinases, e.g., yeast hexokinase (Puri et al., 1984).

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Registry No. MgATP, 1476-84-2; Ser peptide, 65189-71-1; L-Lys, 56-87-1; L-Cys, 52-90-4; protein kinase, 9026-43-1; *o*-phthalaldehyde, 643-79-8.

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