Majaama, K., Kuutti-Savolainen, E.-R., Tuderman, L., & Kivirikko, K. I. (1979) *Biochem. J. 178*, 313–322.
Majaama, K., Hanauske-Abel, H. M., & Günzler, V. (1984) *Eur. J. Biochem. 138*, 236–245.
McGee, J. O'D., Langness, U., & Udenfriend, S. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1585–1589.
Myllylä, R., Majaama, K., Günzler, V., Hanauske-Abel, H. M., & Kivirikko, K. I. (1984) *J. Biol. Chem. 259*, 5403–5405.
Nietfeld, J. J., & Kemp, A. (1980) *Biochim. Biophys. Acta 613*, 349–358.
Ofengand, J., Schwartz, I., Chinali, G., Hixson, S. S., & Hixon, S. H. (1977) *Methods Enzymol. 46*, 683–703.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Prockop, D. J., & Kivirikko, K. I. (1969) *J. Biol. Chem. 244*, 4838–4842.
Prockop, D. J., Berg, R. A., Kivirikko, K. I., & Uitto, J. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N., & Reddi, A. H., Eds.) pp 163–273, Plenum Press, New York.
Rao, N. V., & Adams, E. (1978) *J. Biol. Chem. 253*, 6327–6330.
Siegel, B. (1979) *Bioorg. Chem. 8*, 219–226.
Sokal, R. R., & Rohlf, F. J. (1981) *Biometry. The Principles and Practice of Statistics in Biological Research*, 2nd ed., Freeman, San Francisco.
Tuderman, L., Kuutti, E.-R., & Kivirikko, K. I. (1975) *Eur. J. Biochem. 52*, 9–16.
Tuderman, L., Myllylä, R., & Kivirikko, K. I. (1977) *Eur. J. Biochem. 80*, 341–348.
Weeke, B. (1973) in *A Manual of Quantitative Immunoelectrophoresis. Methods and Applications* (Axelsen, N. H., Kroll, J., & Weeke, B., Eds.) pp 37–46, Universitetsforlaget, Oslo, Bergen, and Tromso.

Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase: Fluorescent Affinity Labeling of the Catalytic Subunit from Bovine Skeletal Muscle with *o*-Phthalaldehyde†

Rajinder N. Puri,* Deepak Bhatnagar, and Robert Roskoski, Jr.
Department of Biochemistry, Louisiana State University Medical Center, New Orleans, Louisiana 70119
Received March 27, 1985

ABSTRACT: The catalytic subunit of adenosine cyclic 3′,5′-monophosphate dependent protein kinase from bovine skeletal muscle was rapidly inactivated by *o*-phthalaldehyde at 25 °C (pH 7.3). The reaction followed pseudo-first-order kinetics, and the second-order rate constant was 1.1×10^2 M^{-1} s^{-1}. Absorbance and fluorescence spectroscopic data were consistent with the formation of an isoindole derivative (1 mol/mol of enzyme). The reaction between the catalytic subunit and *o*-phthalaldehyde was not reversed by the addition of reagents containing free primary amino and sulfhydryl functions following inactivation. The reaction, however, could be arrested at any stage during its progress by the addition of an excess of cysteine or less efficiently by homocysteine or glutathione. The catalytic subunit was protected from inactivation by the presence of the substrates magnesium adenosine triphosphate and an acceptor serine peptide substrate. The decrease in fluorescence emission intensity of incubation mixtures containing iodoacetamide- or 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine-modified catalytic subunit and *o*-phthalaldehyde paralleled the loss of phosphotransferase activity. Catalytic subunit denatured with urea failed to react with *o*-phthalaldehyde. Inactivation of the catalytic subunit by *o*-phthalaldehyde is probably due to the concomitant modification of lysine-72 and cysteine-199. The proximal distance between the ε-amino function of the lysine and the sulfhydryl group of the cysteine residues involved in isoindole formation in the native enzyme is estimated to be approximately 3 Å. The molar transition energy of the catalytic subunit–*o*-phthalaldehyde adduct was 121 kJ/mol and compares favorably with a value of 127 kJ/mol for the 1-[(β-hydroxyethyl)thio]-2-(β-hydroxyethyl)isoindole in hexane, indicating that the active site lysine and cysteine residues involved in formation of the isoindole derivative of the catalytic subunit are located in a hydrophobic environment. *o*-Phthalaldehyde probably acts as an active site specific reagent for the catalytic subunit.

A large number of physiological effects of adenosine cyclic 3′,5′-monophosphate (cAMP)[1] in eukaryotic organisms are the result of phosphorylation of specific cellular proteins by cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) (Krebs, 1972; Rubin & Rosen, 1975; Rosen et al., 1977; Cohen, 1978; Krebs & Beavo, 1979), which are widely distributed in nature (Kuo & Greengard, 1969). The inactive holoenzyme consists of two identical regulatory subunits (R) and two catalytic subunits (C) held together by noncovalent interactions (Krebs & Beavo, 1979). The tetrameric inactive enzyme undergoes dissociation in the presence

† This work was supported by U.S. PHS Grant NS-15994 and fellowships (R.N.P. and D.B.) from the American Heart Association—Louisiana.

[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; C subunit, catalytic subunit of type II cAMP-dependent protein kinase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; EA, 1-[(β-hydroxyethyl)thio]-2-(β-hydroxyethyl)isoindole; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/85/0424-6499$01.50/0

of cAMP into a regulatory subunit dimer and two active C subunits according to the scheme (Corbin et al., 1978; Weber et al., 1979)

$$R_2C_2 \text{ (inactive)} + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C \text{ (active)}$$

Early studies indicated that incubation of the C subunit of the type II cAMP-dependent protein kinase with excess DTNB or NBD-Cl resulted in the modification of two cysteine residues with concomitant loss of phosphotransferase activity (Armstrong & Kaiser, 1978; Peters et al., 1977; Hartl & Roskoski, 1982). However, at low ionic strength, chemical modification of only one cysteine residue by DTNB brings about complete loss of the phosphotransferase activity (Jimenez et al., 1982). Nelson & Taylor (1983) demonstrated that of the two cysteines (199 and 343) of the catalytic subunit, only cysteine-199 is essential for catalytic activity.

Treatment of the C subunit with FSBA, an active site directed reagent, leads to an irreversible loss of its phosphotransferase activity with concomitant modification of only the lysine-71 residue (Hixson & Krebs, 1979; Zoller & Taylor, 1979; Zoller et al., 1981). The effects of covalent modification of lysine-71 and cysteine-199 of the C subunit of type II isozyme on nucleotide binding have been characterized by fluorescence polarization titrations with various nucleotides. The results show that chemical modification of these residues inactivates the C subunit by inhibiting nucleotide binding (Bhatnagar et al., 1984).

The complete amino acid sequence of the catalytic subunit (Shoji et al., 1981, 1983; Carr et al., 1982) of the type II isozyme from bovine cardiac muscle has become available. These studies place the active site lysine residue previously ascribed to position 71 (Zoller et al., 1981) at position 72 (Shoji et al., 1983). On the basis of the sequence, the predicted secondary structure is thought to contain three distinct domains (Carr et al., 1982). The overall composition of C subunit is thought to be about 49% α-helix, 20% β-sheet, and 31% remainder (Reed & Kinzel, 1984).

Palczewski et al. (1983) characterized the inhibition of rabbit muscle aldolase using *o*-phthalaldehyde. This compound forms an isoindole adduct by cross-linking ϵ-amino and sulfhydryl functions of the active site lysine and cysteine residues, respectively. Simons et al. (1979) also studied the chemistry of the reaction of *o*-phthalaldehyde with the glucocorticoid receptor. The work cited in the preceding paragraph indicates the presence of cysteine and lysine residues in the active site of the cAMP-dependent protein kinase. Our previous work suggested that these two groups are in the subsite corresponding to the polyphosphate binding region of ATP (Bhatnagar et al., 1984). We hypothesized that *o*-phthalaldehyde might effectively cross-link these residues and thereby inhibit phosphotransferase activity of the C subunit. The present work shows that this reagent is a good inhibitor of the enzyme. A preliminary account of this work has recently appeared (Puri et al., 1985a).

Materials and Methods

The synthetic heptapeptide (Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly) used as substrate in enzyme assays was purchased from Boehringer Mannheim Biochemicals. Carrier-free [γ-^{32}P]ATP was obtained from Amersham. Rabbit skeletal muscle aldolase, *o*-phthalaldehyde, and other biochemicals were obtained from Sigma. The rest of the chemicals used in this work were reagent-grade. Concentrations of the following reagents were determined spectrophotometrically with the given absorption coefficients: ATP, 15.4 mM^{-1} cm^{-1} at 259 nm (Bock et al., 1956); *o*-phthalaldehyde, 1 mM^{-1} cm^{-1} at 337 nm (West, 1976); isoindole ring, 7.66 mM^{-1} cm^{-1} at 337 nm (Simons & Johnson, 1978a). *o*-Phthalaldehyde was prepared freshly in methanol prior to each experiment, and the final concentration of methanol in the reaction mixtures was 1% (v/v). Methanol alone (1%) had no effect on enzyme activity.

Preparation of the Catalytic Subunit of cAMP-Dependent Protein Kinase and Its Activity Measurements. Catalytic subunit of type II cAMP-dependent protein kinase from bovine skeletal muscle was purified to electrophoretic homogeneity as described by Hartl & Roskoski (1982). Phosphotransferase activity of the C subunit was measured as described by Roskoski (1983). Protein concentrations were measured by the method of Lowry et al. (1951) with bovine serum albumin as standard. The enzyme used in these studies had a specific activity of 14 μmol min^{-1} (mg of protein)$^{-1}$, and this value represents the control value (100%) given in the results. Polyacrylamide gel electrophoresis was performed on 10% gels as described by Laemmli (1970). Corrected mobilities of protein bands were calculated according to the procedure of Weber & Osborn (1969). The molar concentration of the C subunit was calculated by assuming a *Mr* of 40 000 (Hartl & Roskoski, 1983).

Spectral Determinations. Fluorescence measurements were made with an SLM 4800 spectrofluorometer. This instrument was interfaced with a Hewlett-Packard Model HP 9825A desk calculator. The spectrofluorometer was also equipped with Hewlett-Packard Model 7225B plotter. The SLM PR-8015 spectrum processor program was used for recording both fluorescence excitation and emission spectra. All operations were performed at 25 °C, with 4-nm excitation and emission slit widths. All spectra were recorded in microcuvettes of 3-mm path length. The absorption spectra were recorded on a Gilford Model 2600 spectrophotometer equipped with a Hewlett-Packard Model 7225B plotter in quartz cuvettes of 1-cm path length.

Results

Inactivation of cAMP-Dependent Protein Kinase Catalytic Subunit by o-Phthalaldehyde. Purified catalytic subunit was inactivated upon incubation with *o*-phthalaldehyde at 25 °C. The time course of inactivation at various concentrations of the reagent is shown in Figure 1. After 1 min of incubation with 0.6 mM *o*-phthalaldehyde, greater than 90% of the original activity of the C subunit was lost. A plot of the natural logarithm of percent residual activity vs. time of incubation with *o*-phthalaldehyde gave a straight line. The slope of this line yields an apparent or observed pseudo-first-order rate constant (k_{obsd}) according to eq 1, where E is the activity at

$$-\ln (E/E_0) = k_{obsd}t \quad (1)$$

any given time and E_0 is the activity at zero time. Pseudo-first-order reaction rates of inactivation of the C subunit were linear with respect to *o*-phthalaldehyde concentration in the range of 0.15–0.6 mM (Figure 1, inset). Linear dependence of the pseudo-first-order rate constants on the concentration indicates that the reaction does not follow saturation kinetics. The second-order rate constant for the inactivation of the C subunit by *o*-phthalaldehyde calculated from the slope of this plot was 1.1×10^2 M^{-1} s^{-1}. An important finding in this study was that sulfhydryl reagents, e.g., cysteine and β-mercaptoethanol, do not cause the reversal of inactivation; these reagents, moreover, were employed to terminate the inactivation reaction.

We found that the loss of phosphotransferase activity corresponded well with the increase in relative fluorescence of the

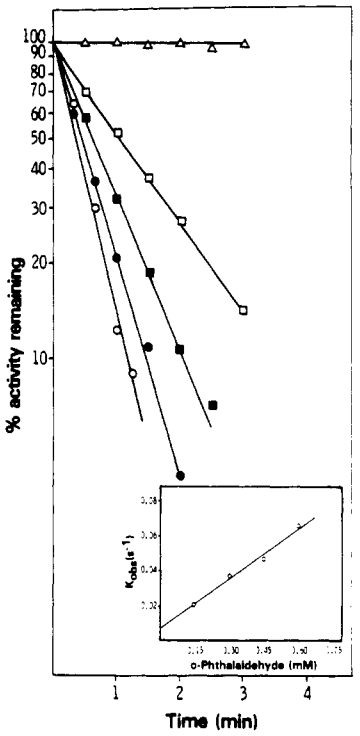


FIGURE 1: Time course of inactivation of catalytic subunit by *o*-phthalaldehyde. The catalytic subunit (1 μg/0.5 mL) was incubated with *o*-phthalaldehyde in 100 mM Hepes–NaOH, pH 7.3, 0.1 mM EDTA, and 1% methanol at 25 °C. At the times specified, the reaction was terminated by transferring a portion of the mixture to a solution of cysteine and β-mercaptoethanol in the same buffer, yielding final concentrations of 20 and 5 mM, respectively. Phosphotransferase activity was then measured as described under Materials and Methods. The data are plotted as the natural logarithm of percent activity remaining vs. time. *o*-Phthalaldehyde concentrations were as follows: (△) 0, (□) 0.15, (■) 0.3, (●) 0.45 and (○) 0.6 mM. The inset shows a plot of the pseudo-first-order rate constants vs. concentration of *o*-phthalaldehyde.

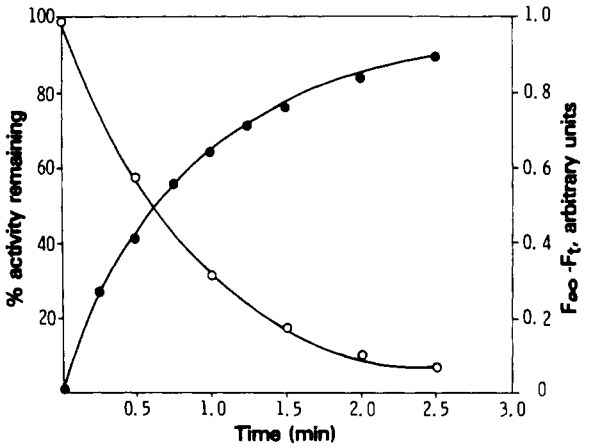


FIGURE 2: Time course of the loss of phosphotransferase activity and increase in fluorescence intensity of the catalytic subunit following treatment with *o*-phthalaldehyde. The enzyme (1 μg/0.5 mL) was treated with 0.3 mM reagent for the specified time, and portions were withdrawn and quenched with cysteine and β-mercaptoethanol. Phosphotransferase activity (○) was measured as described in Figure 1. In a parallel experiment, the catalytic subunit was incubated with 0.3 mM *o*-phthalaldehyde, and the relative fluorescence (●) was measured at the specified times. The data are presented as $F_\infty - F_t$ vs. time. F_∞ and F_t represent the fluorescence end point and relative fluorescence at time t, respectively (Palczewski et al., 1983). The excitation and emission wavelengths were 338 and 405 nm, respectively.

C subunit following *o*-phthalaldehyde treatment (Figure 2). When the C subunit was incubated with 0.3 mM *o*-phthalaldehyde at 25 °C, there was a loss of 92% of phosphotransferase activity in 2.5 min while there was a 90% increase in relative fluorescence. The two curves intersect at a point that corresponds to the loss of the same amount of phosphotransferase activity as the increase in relative fluorescence at that time. It is, therefore, clear that at a given concentration of *o*-phthalaldehyde the loss of phosphotransferase activity of the C subunit parallels the corresponding increase in relative fluorescence during inactivation by *o*-phthalaldehyde and they both follow the same pseudo-first-order kinetics.

Table I: Effect of Potential Protecting Reagents on the Inactivation of Catalytic Subunit by *o*-Phthalaldehyde[a]

addition to incubation mixture	% activity remaining
none	8
nucleotides (no metal)	
ATP (1 mM)	9
ADP (1 mM)	6
AMP (1 mM)	5
cAMP (50 μM)	7
nucleotides and 10 mM $MgCl_2$	
ATP (1 mM)	46
ADP (1 mM)	32
AMP (1 mM)	15
cAMP (50 μM)	7
metal ions	
Mg^{2+} (10 mM)	6
Mn^{2+} (5 mM)	17
Ca^{2+} (5 mM)	7
Co^{2+} (1 mM)	12
nucleosides	
adenosine (1 mM)	17
peptide substrates and analogues	
Ser-peptide (1 mM)	34
protamine sulfate (1 mg/mL)	7
guanethidine (2 mM)	5
chelators	
EDTA (1 mM)	9
EGTA (1 mM)	12

[a] Protection studies were carried out by incubating solutions containing 50 nM C subunit, 100 mM Hepes–NaOH, pH 7.3, 0.1 mM EDTA, with or without 0.3 mM *o*-phthalaldehyde, 1% methanol, and the reagent at 25 °C for 2 min. The reaction was terminated by the addition of cysteine and β-mercaptoethanol as described in legend to Figure 1. Phosphotransferase activity remaining in the reaction mixtures was subsequently determined.

Effect of Substrates and Other Reagents on the o-Phthalaldehyde Reaction. The *o*-phthalaldehyde reaction was carried out in the presence of various nucleotides, nucleosides, metal ions, and protein and peptide substrates. The data showed that free nucleotides (without the presence of metal ions) failed to provide any protection at a concentration of 1 mM (Table I). In the presence of 10 mM Mg^{2+}, however, the protection provided by adenine nucleotides follows the order ATP > ADP > AMP. Partial protection was provided by free adenosine. Previous results obtained from fluorescence polarization titrations in our laboratory show that the K_d's of ATP, ADP, and AMP for the C subunit follow the same order (Bhatnagar et al., 1983). No significant protection was provided by the metal ions alone or by chelating agents, e.g., EGTA and EDTA. Among the peptide and protein substrates, only the synthetic substrate, Ser-peptide, was found to protect the C subunit from inactivation by *o*-phthalaldehyde. Guanethidine, a guanidinium compound, which is a competitive inhibitor with respect to Ser-peptide (Cook et al., 1982), failed to protect against inactivation. These results show that the substrates MgATP and Ser-peptide protect the C subunit from inactivation by *o*-phthalaldehyde. They also suggest that the residues whose modification results in a loss of phosphotransferase activity may be located in or near the active site.

Characterization of the Product Formed in the Reaction between the Catalytic Subunit and o-Phthalaldehyde. The

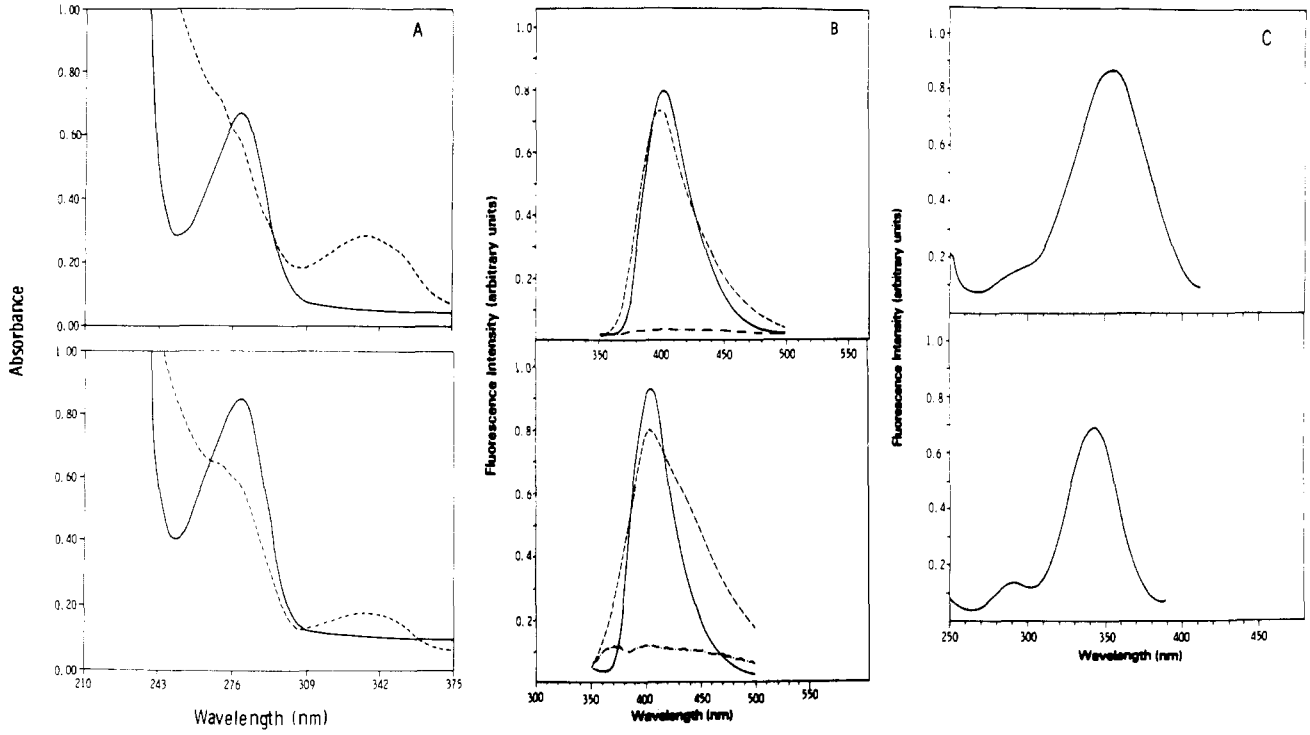


FIGURE 3: Absorption and fluorescence excitation and emission spectra of the catalytic subunit–*o*-phthalaldehyde adduct. C subunit (5 μM) was allowed to react with 10 mM *o*-phthalaldehyde for 3 min at 25 °C in 100 mM Hepes–NaOH, pH 7.3, 0.1 mM EDTA, and 1% methanol. The reaction was terminated by addition of 20 mM cysteine and 5 mM β-mercaptoethanol (final concentrations). The solution was subjected to Sephadex G-25 (0.45 × 18.5 mm) gel filtration in a column equilibrated with the same buffer (minus methanol) with a flow rate of 3.5 mL/min, and the protein-containing fractions were collected. Alternatively, the reaction mixture was dialyzed overnight against three changes of the same buffer. The isoindole derivative of rabbit muscle aldolase (Palczewski et al., 1983) was prepared in an identical fashion. (A) Absorption spectra: (top) 4.7 μM aldolase tetramer (—) and 3.5 μM aldolase–*o*-phthalaldehyde adduct (– –); (bottom) 14 μM C subunit monomer (—) and 8 μM C subunit–aldehyde adduct (– –). (B) Fluorescence emission spectra: (top) 2.2 μM aldolase–*o*-phthalaldehyde adduct; (bottom) 2.2 μM C subunit–*o*-phthalaldehyde adduct. Excitation was at 338 (–) and 295 (– –) nm in each case. (C) Fluorescence excitation spectra: (top) 6.6 μM aldolase adduct; (bottom) 2 μM C subunit adduct. Spectra were obtained as described under Materials and Methods.

absorbance spectrum of the C subunit modified with *o*-phthalaldehyde exhibits a maximum centered at 337 nm characteristic of an isoindole derivative formation (Simons et al., 1979) and a maximum at 280 nm characteristic of proteins (Figure 3A, bottom panel). The absorbance spectral properties of the catalytic subunit–*o*-phthalaldehyde adduct prepared following gel filtration or dialysis (12–18 h) were experimentally indistinguishable. The absorption spectra of the C subunit– and aldolase–*o*-phthalaldehyde (Figure 3A, top panel) adducts are very similar. Excitation of the C subunit–*o*-phthalaldehyde adduct at either 338 or 295 nm resulted in the appearance of a fluorescence emission maximum at 405 nm (Figure 3B, bottom panel), also characteristic of an isoindole ring (Simons & Johnson, 1978a; Simons et al., 1979). These results compare favorably with those obtained for the aldolase–*o*-phthalaldehyde adduct by Palczewski et al. (1983). The excitation spectrum of the C subunit–*o*-phthalaldehyde adduct (emission wavelength at 405 nm) shows the presence of a major excitation maximum at 346 nm and a shoulder at 288 nm characteristic of an isoindole ring (Figure 3C, bottom panel) (Simons et al., 1979).

Stoichiometry of the Reaction between the Catalytic Subunit and o-Phthalaldehyde. Absorbance measurements at 337 nm following complete inactivation of the C subunit by *o*-phthalaldehyde indicate that 1.03 ± 0.25 mol of isoindole groups (extinction coefficient 7.66 mM^{-1} cm^{-1}) per mole of the C subunit are formed. A linear relationship between increase in absorbance (hence, the increase in isoindole groups formed) with increase in enzyme concentration was observed. These results are in agreement with a value of 1.15 ± 0.16 mol of isoindole groups formed per mole of the C subunit obtained by the relative fluorescence emission intensity measurements at 405 nm with the aldolase adduct (4 mol of isoindole/mol of aldolase; Palczewski et al., 1983) as standard. Furthermore, it was found that the fluorescence emission intensity of the C subunit–*o*-phthalaldehyde adduct also bears a linear relationship to the concentration of the C subunit.

Characterization of the Site of Reaction between Catalytic Subunit and o-Phthalaldehyde. Chemical modification of the C subunit with 6.4 mM iodoacetamide (15 h) at 37 °C abolished 80% of the phosphotransferase activity. When the modified enzyme was allowed to react with *o*-phthalaldehyde and subsequently examined spectrofluorometrically, the fluorescence emission intensity was only one-fourth of that obtained from an identically treated unmodified C subunit with *o*-phthalaldehyde (Figure 4A). Modification of the C subunit with 2 mM FSBA produced a 99% loss of phosphotransferase activity. Fluorescence emission intensity measurement of the adduct between FSBA-modified C subunit and *o*-phthalaldehyde showed that about 95% of the C subunit in the reaction mixture failed to react with *o*-phthalaldehyde (Figure 4B). Addition of iodoacetamide or FSBA to a solution containing C subunit–*o*-phthalaldehyde adduct had no quenching or enhancing effect on the fluorescence emission intensity. When the C subunit was denatured with 6 M urea, there was a complete loss of phosphotransferase activity. The incubation mixture containing the denatured C subunit, moreover, did not react with *o*-phthalaldehyde when examined spectrofluorometrically (Figure 4C). These results combined with those obtained from the stoichiometry of the reaction between the C subunit and *o*-phthalaldehyde (preceding section) lead us to suggest that a cysteine residue at or near the active site

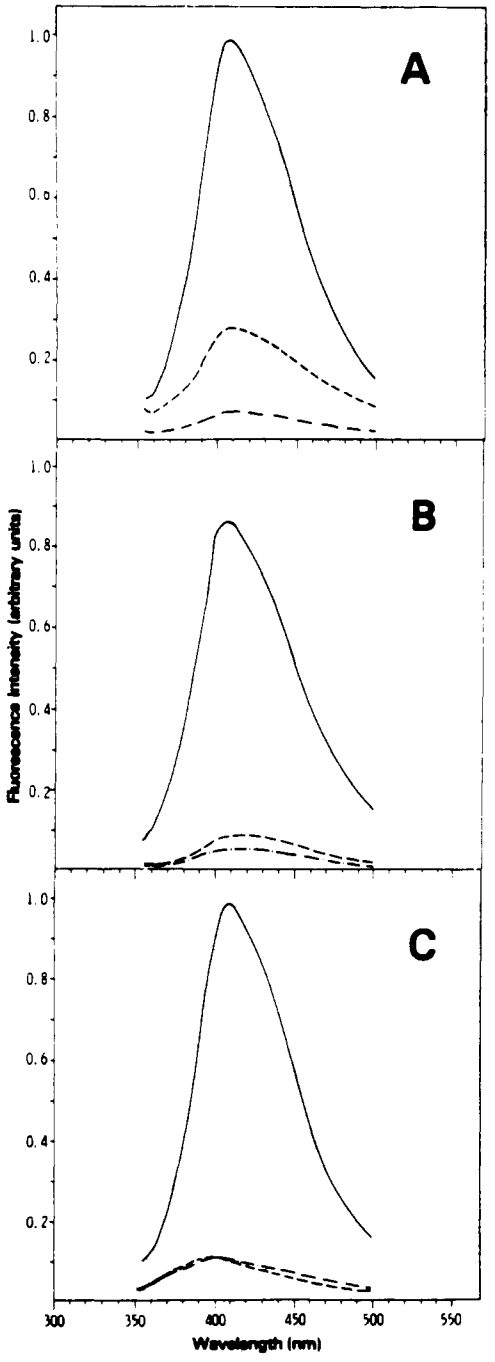


FIGURE 4: Fluorescence emission spectra of chemically modified catalytic subunit. (A) Iodoacetamide treatment. A solution containing 0.6 μM C subunit with or without 6.4 mM iodoacetamide was incubated overnight at 37 °C in 100 mM Hepes–NaOH, pH 7.3. Portions were taken, and activity was measured as described by Roskoski (1983). Portions were also treated with 3 mM *o*-phthalaldehyde (1% methanol final) for 5 min at 25 °C in a 3-mm cuvette, and the emission spectrum was obtained (338-nm excitation). (– –) Iodoacetamide-modified catalytic subunit; (– · –) iodoacetamide-modified C subunit incubated with *o*-phthalaldehyde; (—) untreated catalytic subunit and *o*-phthalaldehyde. (B) FSBA treatment. Reaction mixtures containing 0.6 μM C subunit, with or without 2 mM FSBA, 100 mM Hepes–NaOH, pH 7.3, and 10% (v/v) dimethyl sulfoxide were incubated overnight at 25 °C. Portions were taken for enzyme activity measurements and treatment with 3 mM *o*-phthalaldehyde (5 min at 25 °C in a 3-mm cuvette). (– · –) FSBA-treated C subunit; (– –) FSBA-modified C subunit and *o*-phthalaldehyde; (—) C subunit and *o*-phthalaldehyde. (C) Urea treatment. Solutions of 0.6 μM catalytic subunit with or without 6 M urea were allowed to stand for 30 min at 25 °C in 100 mM Hepes–NaOH, pH 7.3. Solutions were treated as described above. (– · –) urea-treated C subunit; (– –) urea-treated C subunit and *o*-phthalaldehyde; (—) C subunit and *o*-phthalaldehyde.

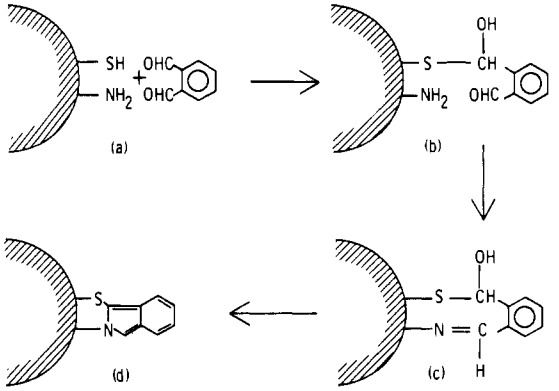
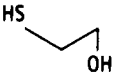
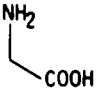
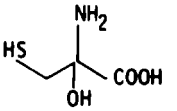
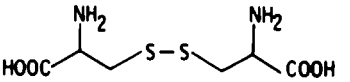
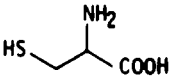
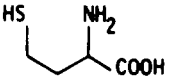
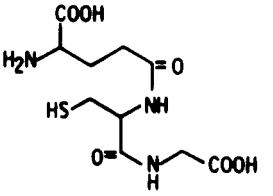


FIGURE 5: Schematic representation of the reaction between the C subunit and *o*-phthalaldehyde.

(Armstrong & Kaiser, 1978; Jimenez et al., 1982; Nelson & Taylor, 1983) and a lysine residue close to or within the same site (Hixson & Krebs, 1979; Zoller & Taylor, 1979; Zoller et al., 1981) react with *o*-phthalaldehyde to form an isoindole derivative. Inactivation of C subunit following treatment with *o*-phthalaldehyde is a direct consequence of isoindole derivative formation. It is also reasonable to conclude from these studies that treatment of the C subunit with urea abolishes the favorable orientation of the cysteine and lysine residues for reaction with *o*-phthalaldehyde.

Proximity of the Cysteine and Lysine Residues Participating in Isoindole Derivative Formation in Reaction of the Catalytic Subunit with o-Phthalaldehyde. The essential steps of reaction between the C subunit and *o*-phthalaldehyde are schematically illustrated in Figure 5 (Simons & Johnson, 1978a). Cysteine effectively terminates the reaction between the C subunit and *o*-phthalaldehyde at any stage of its progression (Table II). It is, therefore, reasonable to assume that the proximity of SH function of the cysteine residue and ϵ-NH_2 function of the lysine residue in the C subunit participating in reaction with *o*-phthalaldehyde is comparable to that of SH and NH_2 functions in cysteine. It is concluded that the distance between the SH and ϵ-NH_2 functions of the participating cysteine and lysine residues in the C subunit and the average distance between SH and NH_2 functional groups in one of the rotomers of cysteine are comparable to that between the two CHO functions in *o*-phthalaldehyde. Several reagents that contain SH and NH_2 functions in varying distances were examined for their ability to terminate the reaction between the C subunit and *o*-phthalaldehyde. When the C subunit was treated with 0.3 mM *o*-phthalaldehyde at 25 °C and the reaction terminated after 0.5 min with a mixture of 20 mM cysteine and 5 mM β-mercaptoethanol (used to prevent C subunit cysteines from autooxidation), phosphotransferase activity remaining in the incubation mixture was 60% of that in an incubation mixture identically treated in the absence of *o*-phthalaldehyde. Glycine or β-mercaptoethanol was unable to stop the reaction between the C subunit and *o*-phthalaldehyde. A mixture of glycine and β-mercaptoethanol, however, was nearly as effective as cysteine. Cysteine alone or a mixture of β-mercaptoethanol and glycine when treated with *o*-phthalaldehyde was indeed found to exhibit fluorescence emission maximum at 445 nm (Figure 6) characteristic of an isoindole derivative formation in an aqueous environment as discussed later. It was found that cystine (no free SH group) was completely ineffective in terminating the reaction. Homocysteine and glutathione, in which the distance between SH and NH_2 functions is somewhat different from that in cysteine, were moderately effective in terminating the reaction. All

Table II: Comparison of the Ability of Various Reagents Containing Sulfhydryl and Amino Functions To Terminate the Reaction between Catalytic Subunit and *o*-Phthalaldehyde[a]

compd	concn (mM)	structure	% activity remaining
β-mercaptoethanol	5		0
glycine	20		0
β-mercaptoethanol and glycine	5 and 20		50
cystine	20		0
cysteine	20		63
homocysteine	20		40
glutathione	20		43

[a] Each incubation mixture contained 50 nM C subunit, 100 mM Hepes–NaOH, pH 7.3, 0.1 mM EDTA, 0.3 mM *o*-phthalaldehyde, and 1% methanol. The reaction was allowed to proceed at 25 °C for 0.5 min at which time the reagent indicated in the table was added. In each case, a control without *o*-phthalaldehyde was set up under identical conditions. Residual phosphotransferase activity in the incubation mixtures was subsequently determined.

these reagents, moreover, were ineffective in reversing the reaction (isoindole derivative formation) between the C subunit and *o*-phthalaldehyde.

Environment of the Cysteine and Lysine Residues Participating in the Reaction between the C Subunit and o-Phthalaldehyde. In order to learn something about the microenvironment of the cysteine and lysine residues participating in the formation of an isoindole derivative in the reaction between the C subunit and *o*-phthalaldehyde, molar transition energies (E_T) (Dimroth et al., 1963) of EA [1-[(β-hydroxyethyl)thio]-2-(β-hydroxyethyl)isoindole] in various solvents (Palczewski et al., 1983) were compared with those of the isoindole derivatives formed from the reaction between *o*-phthalaldehyde and cysteine, glycine and β-mercaptoethanol, aldolase, and catalytic subunit. The molar transition energies can be calculated from the fluorescence emission maximum of an isoindole with the linear free energy relationships described by the equations (Dimroth et al., 1963; Palczewski, 1983):

$$E_T = 2.985\lambda_{em} - 1087.28 \quad (2)$$

$$E_T = 3.521\lambda_{em} - 1294.31 \quad (3)$$

where λ_{em} corresponds to the wavelength of the fluorescence emission maximum of an isoindole derivative. Equation 2 has been used to calculate molar transition energies of an isoindole derivative in solvents of different polarities (Palczewski et al., 1983) and was employed to calculate the values of E_T for reactants (except methanol) described in Table III. Equation 3 has been used to calculate E_T for an isoindole derivative, only in a series of similar solvents, e.g., alcohols and methanol (Palczewski et al., 1983). A value of 121 kJ/mol for the molar

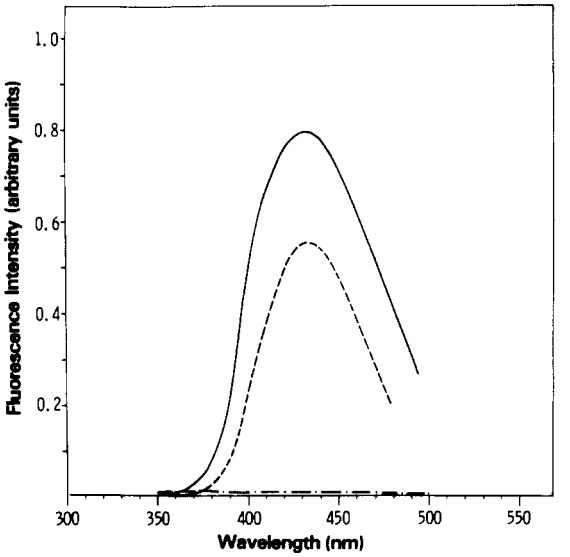


FIGURE 6: Fluorescence emission spectra of cysteine and a mixture of glycine and β-mercaptoethanol following treatment with *o*-phthalaldehyde. A solution of 5 mM cysteine, 100 mM Hepes–NaOH, pH 7.3, 0.1 mM EDTA, and 1% methanol was incubated with 10 mM *o*-phthalaldehyde (—) at 25 °C for 3 min. A separate incubation mixture containing 5 mM β-mercaptoethanol and glycine was treated with *o*-phthalaldehyde (– –). (–·–) Mixture of the buffer and *o*-phthalaldehyde as a blank.

transition energy of the isoindole derivative between the C subunit and *o*-phthalaldehyde is identical with that obtained for aldolase. An examination of Table III also shows that the value of the molar transition energy for the adduct between the C subunit and *o*-phthalaldehyde is close to that of synthetic

Table III: Molar Transition Energies[a] of Isoindole Derivatives Formed in the Reaction of the Following with *o*-Phthalaldehyde

reactant	solvent	molar transition energy (kJ/mol)
EA[b]	water	267
EA	methanol	233
EA	acetonitrile	194
EA	dioxane	155
EA	diethyl ether	138
EA	hexane	127
cysteine	Hepes, pH 7.3	205
β-mercaptoethanol + glycine	Hepes, pH 7.3	205
aldolase	Hepes, pH 7.3	121
catalytic subunit	Hepes, pH 7.3	121

[a] Equation 2 was used to calculate molar transition energy in every case except methanol, for which eq 3 was used (see Results). [b] EA, 1-[(β-hydroxyethyl)thio]-2-(β-hydroxyethyl)isoindole (Palczewski et al., 1983).

isoindole, EA, in hexane. The data therefore suggest that the microenvironment of the active site of the C subunit containing the cysteine and lysine residues participating in the reaction with *o*-phthalaldehyde is fairly hydrophobic. A similar conclusion has been made earlier by Bhatnagar et al. (1983) with fluorescence polarization studies.

Discussion

The evidence presented in this work shows that the C subunit of type II cAMP-dependent protein kinase is inactivated by *o*-phthalaldehyde. The inactivation is a consequence of the formation of an isoindole derivative as ascertained from its absorbance and fluorescence properties (Simons & Johnson, 1978a; Simons et al., 1979; Palczewski et al., 1983). The reaction follows second-order kinetics with a rate constant of 1.1×10^2 M^{-1} s^{-1} and compares favorably with a value of 1.1×10^3 M^{-1} s^{-1} for a similar reaction between aldolase and *o*-phthalaldehyde (Palczewski et al., 1983). The difference between the second-order rate constants for the two enzymes may be attributed to the higher pH of the reaction medium (50 mM borate buffer, pH 8.4) in which the reaction with aldolase was investigated (Palczewski et al., 1983). Our preliminary experiments showed that the reaction between the C subunit and *o*-phthalaldehyde at pH 8.3 proceeded faster than at pH 7.3. To decrease the rates so that a reasonable number of time points could be accurately taken, we chose pH 7.3 to study the reaction between the C subunit and *o*-phthalaldehyde.

The second-order rate constant for the inactivation of the C subunit by *o*-phthalaldehyde ($K = 1.1 \times 10^2$ M^{-1} s^{-1}) was twice that for modification of cysteine in the active site of C subunit by DTNB (50 M^{-1} s^{-1}; Jimenez et al., 1982). NBD-Cl, 0.5 mM, inactivated the C subunit in 30 min at pH 7.0, 30 °C (Hartl & Roskoski, 1982), with a 95% loss of phosphotransferase activity, and the overall reaction followed hyperbolic kinetics. FSBA, 1 mM, inactivated the C subunit in 40 min at pH 7.5, 30 °C (Hixson & Krebs, 1979), with almost complete loss of phosphotransferase activity, and the overall reaction also followed hyperbolic kinetics. Modification of the C subunit with 0.6 mM *o*-phthalaldehyde at pH 7.3, 25 °C, brought about almost complete loss of phosphotransferase activity in about 1 min (Figure 1), and the overall reaction rate showed a linear dependence on the *o*-phthalaldehyde concentration. Differences between the modification of either SH group (DTNB, NBD-Cl) or NH_2 group (FSBA), on the one hand, and the SH group and NH_2 group simultaneously by *o*-phthalaldehyde, on the other, may be explained as follows: (1) the intermediate b (Figure 5) formed from the nucleophilic attack of a cysteine sulfhydryl on the CHO function of *o*-phthalaldehyde is in a favorable conformation for the nucleophilic attack by the nitrogen of the Schiff base c (Figure 6) (formed between an NH_2 function and the second CHO function of *o*-phthalaldehyde) to form the isoindole derivative d (Figure 6); (2) the conversion of the intermediate c to the isoindole derivative d (Figure 6) is thermodynamically favorable since such a process converts the benzenoid ring of *o*-phthalaldehyde, a six overlapping π-electron system to an isoindole ring system, a ten overlapping π-electron system. Thus, the driving force for and enhanced rate of reaction between the C subunit and *o*-phthalaldehyde are aided by (1) intramolecular anchimeric assistance (Bruice & Benkovic, 1966) and possibly orbital steering (Dafforn & Koshland, 1973) and (2) enhanced thermodynamic stability of the isoindole ring in the C subunit–*o*-phthalaldehyde adduct compared to benzene ring in *o*-phthalaldehyde (LeNoble, 1974). Evidence presented in this work (Table II) shows that the reaction between the C subunit and *o*-phthalaldehyde is irreversible and the isoindole derivative formed is stable under the experimental conditions over a period of at least 24 h. Both the irreversibility of the reaction and stability of the product of the reaction between the C subunit and *o*-phthalaldehyde are explained on the basis of the ease of formation of the ten overlapping π-electron (ring) system from a six overlapping π-electron (ring) system and not vice versa under the described experimental conditions. From a consideration of bond angles and bond distances of the two aldehydic functions in *o*-phthalaldehyde (March, 1977), we conclude that SH and NH_2 functions (and not the residues containing these functions) participating in isoindole formation in the reaction are about 3 Å apart. This is the likely explanation for the observation that homocysteine and glutathione, in which the SH and NH_2 functions are not able to assume the same spatial orientation with respect to each other (3-Å distance) as in cysteine, are not as efficient reaction terminators (Table II). Cystine with no free SH function completely lacks the ability to terminate reaction between the C subunit and *o*-phthalaldehyde.

Fluorescence emission spectroscopy constitutes a very sensitive technique to detect and quantitate isoindole derivatives (Weidkamm et al., 1973; Simons & Johnson, 1978b). Very efficient electronic energy transfer from tryptophan (an indole derivative) to the C subunit–*o*-phthalaldehyde adduct (an isoindole derivative) may in part be due to the fact that indole and isoindole rings are isoelectronic and contain similar ten overlapping π-electron ring systems. Under identical conditions, the emission spectra (Figure 3B) and excitation spectra (Figure 3C) of aldolase–*o*-phthalaldehyde and the C subunit–*o*-phthalaldehyde adducts are very similar. Thus, spectroscopic results obtained with the C subunit–*o*-phthalaldehyde adduct are consistent with the formation of an isoindole derivative.

It has been previously shown that a cysteine (Peters et al., 1977; Armstrong & Kaiser, 1978; Nelson & Taylor, 1983) and a lysine residue (Hixson & Krebs, 1979; Zoller & Taylor, 1979; Zoller et al., 1981) at or near the active site correspond to positions 199 and 72, respectively, in the revised primary sequence of the C subunit (Shoji et al., 1983). Treatment of the C subunit with iodoacetamide causes a 80% loss of phosphotransferase activity (due to modification of only one cysteine residue at position 199; Nelson & Taylor, 1983) and a corresponding decrease in the intensity of fluorescence emission maximum resulting from subsequent reaction with *o*-phthalaldehyde. Treatment of the C subunit with FSBA

(due to modification of a single lysine residue at position 72; Hixon & Krebs, 1979; Zoller et al., 1981) brings about 99% loss of phosphotransferase activity and a 95% decrease in fluorescence emission intensity compared to a control in a subsequent reaction with *o*-phthalaldehyde. Data presented in Table I showed that the substrates, ATP and to a lesser extent Ser-peptide, protect the C subunit from modification by *o*-phthalaldehyde. These observations, coupled with the fact there is 1 mol of isoindole derivative formed per mole of the C subunit, suggest that the cysteine-199 and lysine-72 at or near the active site of the C subunit are more likely to be involved in isoindole formation. There is another set of cysteine (343) and lysine residues (342, 345) in proximity to each other in the amino acid sequence of the C subunit (Shoji et al., 1983), but data presented in this work do not favor the involvement of these cysteine and lysine residues in isoindole formation in reaction with *o*-phthalaldehyde. These results are further corroborated by the finding that the C subunit denatured with urea does not react with *o*-phthalaldehyde when examined spectrofluorometrically. Thus, the results obtained from chemical modification studies with *o*-phthalaldehyde indirectly favor the presence of a cysteine residue and lysine residue at or near the active site of the C subunit. The formation of the isoindole adduct does not depend on the adventitious proximity of cysteine and lysine residues in the primary structure but rather on the specific proximity in the tertiary structure. The formation of an isoindole derivative involving lysine-72 and cysteine-343 is unlikely since it has been demonstrated by Nelson & Taylor (1983) that cysteine-343 is located farther away from the active site of the enzyme, and therefore, the SH group of cysteine 343 must be must more than 3 Å away from the ϵ-NH_2 group of lysine-72 in the active site. Polyacrylamide gel electrophoresis of the C subunit–*o*-phthalaldehyde adduct showed that there was no intersubunit cross-linking as evidenced by the absence of any bands corresponding to higher molecular weight aggregates.

Spectrofluorometric examination of the reaction mixtures containing synthetic peptides (NH_2 function donors), for example, des-Arg-Bradykinin (Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), *N*-acetylangiotensin I, (N^{α}-Ac-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), and Bz-Gly-His-Leu, and *o*-phthalaldehyde in the presence or absence of β-mercaptoethanol (SH function donor) did not show the formation of isoindole derivative (data not presented). However, a reaction mixture containing Ser-peptide (with a free NH_2 function at the N-terminus) and β-mercaptoethanol shows the formation of an isoindole derivative. The results of these investigations suggest that only the ϵ-NH_2 function provided by a lysine residue (and not the secondary amino or primary amino functions of histidine or arginine, respectively) participates in an isoindole derivative formation in the reaction between the C subunit and *o*-phthalaldehyde.

On the basis of primary sequence of the C subunit, Carr et al. (1982) predicted that it is organized into three domains as follows: residues 1–110 constitute domain I; residues 111–232 constitute domain II; the rest are part of the third domain. Since the ϵ-NH_2 function of lysine-72 and the SH function of cysteine-199 residues of the C subunit are involved in reaction with *o*-phthalaldehyde and their spatial disposition must be about 3 Å with respect to each other in order to form an isoindole derivative, it may be suggested that there is interaction between domains I and II in the tertiary structure of the protein.

The fluorescence emission maxima of aldolase– and the C subunit–*o*-phthalaldehyde adducts in an aqueous buffer are located at 405 nm (Figure 3B), whereas the cysteine–*o*-phthalaldehyde adduct and the one derived from a mixture of glycine and β-mercaptoethanol exhibit the maxima at about 450 nm in the same solvent (Figure 6). The data presented in Table III show that molar transition energy of cysteine–*o*-phthalaldehyde or that of a mixture of glycine and β-mercaptoethanol–*o*-phthalaldehyde adduct in an aqueous buffer is similar to that of a synthetic isoindole, EA, in a fairly polar solvent, e.g., methanol. However, the molar transition energy values of the C subunit–*o*-phthalaldehyde adduct and of the aldolase–*o*-phthalaldehyde adduct are close to the value obtained for EA in hexane. These observations lead to the suggestion that either the cysteine and lysine residues participating in the isoindole derivative formation between the C subunit and *o*-phthalaldehyde are located in a very hydrophobic environment or the environment may be nonrelaxing during the fluorescence lifetime of isoindole (Palczewski et al., 1983). The former seems a more plausable reason in the light of evidence gathered in our laboratory which shows that the region containing the ATP binding site in the C subunit is hydrophobic (Bhatnagar et al., 1983; Fry et al., 1985) and that reagents like DTNB, NBD-Cl, and FSBA that modify the cysteine-199 and lysine-72 residues, respectively, inactivate the C subunit by inhibiting ATP binding. This effect involves the region on the C subunit complimentary to the β- and γ-phosphates of the ATP molecule (Bhatnagar et al., 1984). In their reaction coordinate scheme involving MgATP, Ser-peptide, and the C subunit, Granot et al. (1980) have shown that the OH function of serine in Ser-peptide is 5.3 ± 0.7 Å away from the γ-phosphate of ATP. Therefore, it is reasonable to suggest that Ser-peptide also binds in part to a region in a hydrophobic environment of the C subunit that contains the lysine-72 and cysteine-199 residues. Indeed, Ser-peptide provides partial protection to the C subunit from inactivation by *o*-phthalaldehyde (Table I).

The role of lysine in the active site of pyruvate kinase (Hollenberg et al., 1971), creatine kinase (James & Cohn, 1974), histone kinase (Kochetkov et al., 1977), and other enzymes (Easterbrook-Smith et al., 1976; Dallochio et al., 1976) has been investigated, and it may be concluded that the lysine residue serves as a cationic locus that binds the polyphosphate chain of the nucleotide substrate to the anchoring site of the enzyme. Evidence presented in this work and previous work in our laboratory (Bhatnagar et al., 1983) favors this view. However, the role of the cysteine residue is unclear (Nelson & Taylor, 1983). It has been argued earlier in this work that the SH function of cysteine-199 and the ϵ-amino function of lysine-72 in the active site of the C subunit are only 3 Å apart and this region is involved in the ATP binding (Bhatnagar et al., 1984). It is possible that the SH group of cysteine interacts with the ϵ-NH_2 group of the lysine residue and thus serves to maintain the conformation of the active site. In any case, more work is needed to substantiate this notion. The possibility that the sulfhydryl group participates in the formation of a phosphoenzyme intermediate in the catalytic cycle of the C subunit has been discounted by Granot et al. (1980); furthermore, there is no precedent for phosphoenzyme intermediates involving SH function in phosphotransferase reactions involving ATP (Walsh, 1979).

Carr et al. (1982) pointed out that distant homologies in the amino-terminal region where the ATP binding site is located in nucleotide binding domains exist in several proteins. These include the γ-subunit of phosphorylase *b* kinase (Reimann et al., 1984), cGMP-dependent protein kinase (Hashimoto et al., 1982; Takio et al., 1984), the catabolite

gene activator protein termed CAP (Weber et al., 1982), and two tyrosine–specific viral kinases, pp^{60src} of Rous sarcoma virus (Barker & Dayhoff, 1982) and $pp^{90gag-yes}$ of avian sarcoma virus Y73 (Kitamura et al., 1982). It is therefore, anticipated that *o*-phthalaldehyde may react with the active site of these proteins in a similar manner. Indeed, this has been found to be the case with cGMP-dependent protein kinase (Puri et al., 1985b).

The salient features of the reaction between the C subunit and *o*-phthalaldehyde are summarized as follows: (a) the reaction does not follow saturation kinetics but exhibits rather fast second-order kinetics, $K = 1.1 \times 10^2\ M^{-1}\ s^{-1}$; (b) the reaction is irreversible; (c) the inactivation of the C subunit is a direct consequence of an isoindole derivative formation between the sulfhydryl function of a cysteine and the ϵ-NH_2 function of a lysine probably located at or near the active site; (d) the functional groups participating in isoindole derivative formation are about 3 Å apart as are located in a hydrophobic environment of active site of the C subunit.

ACKNOWLEDGMENTS

We thank Gail Daniels for typing the manuscript and Dr. David B. Glass for reviewing the manuscript.

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.

REFERENCES

Armstrong, R. N., & Kaiser, E. T. (1978) *Biochemistry 17*, 2840.

Barker, W. C., & Dayhoff, M. O. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2836.

Bhatnagar, D., Roskoski, R., Jr., Rosendahl, M. S., & Leonard, N. J. (1983) *Biochemistry 22*, 6310.

Bhatnagar, D., Hartl. F. T., Roskoski, R., Jr., Lessor, R. A., & Leonard, N. J. (1984) *Biochemistry 23*, 4350.

Bock, P. M., Ling, N. S., Morell, S. A., & Lipton, S. N. (1956) *Arch. Biochem. Biophys. 62*, 253.

Bruice, T. C., & Benkovic, S. J. (1966) in *Biorganic Mechanisms*, Vol. 1, p 178, W. A. Benjamin, New York.

Carr, S. A., Biemann, K., Shoji, S., Parmelee, D. C., & Titani, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6128.

Cohen, P. (1978) *Curr. Top. Cell. Regul. 14*, 118.

Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5794.

Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M., & McCarthy, D. (1978) *J. Biol. Chem. 250*, 218.

Dafforn, A., & Koshland, D. E., Jr. (1973) *Biochem. Biophys. Res. Commun. 52*, 780.

Dallochio, F., Negrini, R., Signorini, M., & Rippa, M. (1976) *Biochim. Biophys. Acta 429*, 629.

Dimroth, K., Reichardt, C., Siepmann, T., & Bohlmann, F. (1963) *Justus Liebigs Ann. Chem. 661*, 1.

Easterbrook-Smith, S. B., Wallace, J. C., & Keech, D. B. (1976) *Eur. J. Biochem. 62*, 125.

Fry, D. C., Kuby, S. A., & Mildvan, A. S. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 672.

Granot, J., Mildvan, A. S., Bramson, H. N., & Kaiser, E. T. (1980) *Biochemistry 19*, 3537.

Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5175.

Hartl, F. T., & Roskoski, R., Jr. (1983) *J. Biol. Chem. 258*, 3950.

Hashimoto, E., Takio, K., & Krebs, E. G. (1982) *J. Biol. Chem. 257*, 727.

Hixson, C. S., & Krebs, E. G. (1979) *J. Biol. Chem. 254*, 7509.

Hollenberg, P. F., Flashner, M., & Coon, M. J. (1971) *J. Biol. Chem. 146*, 946.

James, T. L., & Cohn, M. (1974) *J. Biol. Chem. 249*, 2599.

Jimenez, J. S., Kupfer, A., Gani, V., & Shaltiel, S. (1982) *Biochemistry 21*, 1623.

Kitamura, N., Kitamura, A., Toyoshima, K., Hirayama, Y., & Yoshida, M. (1982) *Nature (London) 297*, 205.

Kochetkov, S. N., Bulargina, T. V., Sashcenko, L. P., & Severin, E. S. (1977) *Eur. J. Biochem. 81*, 111.

Krebs, E. G. (1972) *Curr. Top. Cell. Regul. 5*, 99.

Krebs, E. G., & Beavo, J. A. (1979) *Annu. Rev. Biochem. 48*, 923.

Kuo, J. F., & Greengard, P. (1969) *J. Biol. Chem. 244*, 3417.

Laemmli, U. (1970) *Nature (London) 227*, 680.

LeNoble, W. J. (1974) in *Highlights of Organic Chemistry*, Vol. 3, p 261, Marcel Dekker, New York.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

March, J. (1977) in *Advanced Organic Chemistry*, p 22, McGraw-Hill, New York.

Nelson, N. C., & Taylor, S. S. (1983) *J. Biol. Chem. 258*, 10981.

Palczewski, K., Hargrave, P. A., & Kochman, M. (1983) *Eur. J. Biochem. 139*, 429.

Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691.

Puri, R. N., Bhatnagar, D., & Roskoski, R., Jr. (1985a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 702.

Puri, R. N., Bhatnagar, D., Glass, D. B., & Roskoski, R., Jr. (1985b) *Biochemistry* (following paper in this issue).

Reed, J., & Kinzel, V. (1984) *Biochemistry 23*, 1357.

Reimann, E. M., Titani, K., Ericsson, L. H., Wade, R. D., Fischer, E. H., & Walsh, K. A. (1984) *Biochemistry 32*, 4185.

Rosen, O. M., Rangel-Aldo, R., & Erlichmann, J. (1977) *Curr. Top. Cell. Regul. 12*, 39.

Roskoski, R., Jr. (1983) *Methods Enzymol. 99*, 3.

Rubin, C. S., & Rosen, O. M. (1975) *Annu. Rev. Biochem. 44*, 831.

Shoji, S., Parmelee, D., Wade, R. D., Kumar, S., Ericsson, L. H., Walsh, K. A., Neurath, H., Long, G. L., Demaille, J. Q., Fischer, E. H., & Titani, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 848.

Shoji, S., Ericsson, L. H., Walsh, K. A., Fisher, E. H., & Titani, K. (1983) *Biochemistry 22*, 3702.

Simons, S. S., Jr., & Johnson, D. F. (1978a) *J. Org. Chem. 43*, 2886.

Simons, S. S., Jr., & Johnson, D. F. (1978b) *Anal. Biochem. 90*, 705.

Simons, S. S., Jr., Thompson, E. B., & Johnson, D. F. (1979) *Biochemistry 18*, 4915.

Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A., & Titani, K. (1984) *Biochemistry 23*, 4200.

Walsh, C. (1979) in *Enzymatic Reaction Mechanisms*, p 179, W. H. Freeman, San Francisco.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4400.

Weber, I. T., Takio, K., Titani, K., & Steitz, T. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7679.
Weber, W., Vogel, C., & Hilz, H. (1979) *FEBS Lett. 99*, 62.
Weidkamm, E., Wallach, D. F. H., & Flükiger, R. (1973) *Anal. Biochem. 54*, 102.
West, R. C., Ed. (1976) *Handbook of Chemistry and Physics*, p 150, CRC Press, Cleveland, OH.
Zoller, M. J., & Taylor, S. S. (1979) *J. Biol. Chem. 254*, 8363.
Zoller, M. J., Nelson, N. C., & Taylor, S. S. (1981) *J. Biol. Chem. 256*, 10837.

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

Received March 27, 1985

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

† This work was supported by U.S. Public Health Service Grants NS-15994 (R.R.) and GM-28144 (D.B.G.) and fellowships (R.N.P. and D.B.) from the American Heart Association—Louisiana.
‡ 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.

0006-2960/85/0424-6508$01.50/0