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Selective Modification of the Catalytic Subunit of cAMP-Dependent Protein Kinase with Sulfhydryl-Specific Fluorescent Probes†

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ABSTRACT: The catalytic subunit of cAMP-dependent protein kinase contains only two cysteine residues, and the side chains of both Cys 199 and Cys 343 are accessible. Modification of the catalytic subunit by a variety of sulfhydryl-specific reagents leads to the loss of enzymatic activity. The differential reactivity of the two sulfhydryl groups at pH 6.5 has been utilized to selectively modify each cysteine with the following fluorescent probes: 3,6,7-trimethyl-4-(bromomethyl)-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine, and 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole. The most reactive cysteine is Cys 199, and exclusive modification of this residue was achieved with each reagent at pH 6.5. Modification of Cys 343 required reversible blocking of Cys 199 with 5,5'-dithiobis(2-nitrobenzoic acid) followed by reaction of Cys 343 with the fluorescent probe at pH 8.3. Treatment of this modified catalytic subunit with reducing reagent restored catalytic activity by unblocking Cys 199. In contrast, catalytic subunit that was selectively labeled at Cys 199 by the fluorescent probes was catalytically inactive. Even though Cys 199 is presumably close to the interaction site between the regulatory subunit and the catalytic subunit, all of the modified C-subunits retained the capacity to aggregate with the type II regulatory subunit in the absence of cAMP, and the resulting holoenzymes were dissociated in the presence of cAMP. The fluorescent properties of the modified catalytic subunits have been characterized and are consistent with both Cys 199 and Cys 343 being located in relatively hydrophobic environments. In addition, the Stokes radii of the modified catalytic subunits is 26 Å based on Perrin analysis of the polarized fluorescence emission.

During the past 20 years, fluorescence spectroscopy and the covalent attachment of fluorescent probes to macromolecules have been used extensively to investigate biological systems. Covalently bound fluorophores have been used to probe the local environment of the chromophore (McClure & Edelman, 1966; Brand et al., 1971; Lakowicz, 1980; MacGregor & Weber, 1981), to determine the rotational diffusion of the macromolecule to which the fluorophore is attached (Weber, 1952a,b, 1953), and to determine the distance between two fluorophores attached to different sites (Forster, 1948, 1966; Stryer, 1978). This latter application permits estimations of both intramolecular and intermolecular distances and provides a sensitive method for monitoring conformational changes that alter the distance between the two probes. A prerequisite for using fluorescence in structural studies is the ability to label specific sites on the macromolecule uniquely and quantitatively. We describe here a method for the selective modification of either Cys 199 or Cys 343 in the C-subunit of cAMP-dependent protein kinase by several different fluorescent probes.

The catalytic (C) subunit of cAMP-dependent protein kinase is a monomeric protein that contains only two cysteine residues, Cys 199 and Cys 343 (Shoji et al., 1983). These cysteines can be modified by a variety of reagents, including 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB),[1] *N*-ethylmaleimide (NEM), and iodoacetic acid (IAA) (Sugden et al., 1976; Bechtel et al., 1977; Armstrong & Kaiser, 1978; Nelson & Taylor, 1981). Modification of the C-subunit by sulfhydryl-reactive reagents inhibits catalytic activity but not the capacity to bind to the regulatory subunit (First & Taylor, 1988).

Jimenez et al. (1982) investigated the reaction of DTNB with the C-subunit and found that the kinetics of cysteine

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[1] Abbreviations: IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; IANBD, 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole; monobromobimane, 3,6,7-trimethyl-4-(bromomethyl)-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione; DTNB, 5,5'-dithiobis(nitrobenzoic acid); IAA, iodoacetic acid; NEM, *N*-ethylmaleimide; MgATP, magnesium chelate of adenosine 5'-triphosphate.

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modification were biphasic. The reaction rate of each cysteine was found to be dependent on both pH and ionic strength, and the inactivation of the C-subunit coincided with the modification of the rapidly reacting cysteine. This differential reactivity of the cysteine residues has been exploited here to modify either Cys 199 or Cys 343 selectively with a variety of different sulfhydryl-specific, fluorescent probes. Cys 199 and Cys 343 have been selectively modified by either (a) 3,6,7-trimethyl-4-(bromomethyl)-1,5-diazabicyclo[3.3.0]-octa-3,6-diene-2,8-dione (monobromobimane), (b) *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS), or (c) 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD). The specificity of this modification procedure and the fluorescent properties of the modified C-subunits are described as well as the usefulness of such probes for studying structural features of the C-subunit and its interaction with the type II regulatory (R^{II}) subunit.

Experimental Procedures

Materials. Chemicals and reagents were obtained from the following sources: monobromobimane and *N*-ethylmaleimide (NEM) (Calbiochem, La Jolla, CA); IAEDANS and IANBD (Molecular Probes, Junction City, OR); [^{14}C]iodoacetic acid (IAA) (Amersham, Arlington Heights, IL); 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and L-1-(tosylamino)-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin (Sigma Chemicals, St. Louis, MO); synthetic peptide L-R-R-W-S-L-G (Peptide–Oligonucleotide Facility, University of California, San Diego, La Jolla, CA).

Protein Purification. The R^{II}-subunit and C-subunit were purified from porcine heart as described by Nelson and Taylor (1981). Typically, 8 kg of heart yielded 70–90 mg of C-subunit and 30–50 mg of R^{II}-subunit. The C-subunit was stored at −20 °C in 40 mM potassium phosphate, pH 6.5, 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% glycerol (buffer A). The R^{II}-subunit was eluted from the cAMP affinity resin with 25 mM cGMP, concentrated to approximately 1 mg/mL by using an Amicon ultrafiltration apparatus, and stored frozen in 25 mM potassium phosphate (pH 6.5) containing 25 mM cGMP, 2 mM EDTA, and 5 mM 2-mercaptoethanol (buffer B).

Type II holoenzyme was prepared by combining R^{II}-subunit with a 10% excess of C-subunit followed by overnight dialysis at 4 °C against 10 mM potassium phosphate (pH 6.5) containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% glycerol (buffer C). Excess C-subunit was removed by the addition of CL CM-Sepharose (Pharmacia), which binds the free C-subunit but not holoenzyme ($^1/_2$ mL of resin/mg of excess C-subunit).

Enzyme activity was monitored according to the coupled peptide assay described by Cook et al. (1982), using the synthetic peptide L-R-R-W-S-L-G. In this assay the conversion of ATP to ADP is coupled to the synthesis of pyruvate from phosphoenolpyruvate followed by the reduction of pyruvate to lactate by lactate dehydrogenase. Activity is followed by the decreased absorbance of NADH at 340 nm. The R^{II}-subunit concentration was determined by the method of Bradford (1976), using bovine serum albumin as standard. The concentration of the unmodified C-subunit was determined from its absorbance at 280 nm (Peters et al., 1977). The concentrations of the modified C-subunits were determined by amino acid analysis, by absorbance at 280 nm (corrected for the absorbance of the fluorophore), and by the method of Bradford (1976).

Modification of Cys 199. Prior to labeling Cys 199 with monobromobimane, excess 2-mercaptoethanol was removed by dialysis at 4 °C against a minimum of five changes (1 L each) of 10 mM potassium phosphate, pH 6.5, containing 2 mM EDTA. The final dialysis buffer also contained 10% glycerol. C-subunit (1 mg/mL) was made 1 mM in monobromobimane using a 100 mM stock solution of monobromobimane in methanol. The reaction was incubated at room temperature until less than 5% of the initial catalytic activity remained (3–4 h) and then was quenched with dithiothreitol (DTT) at a final concentration of 20 mM. Excess reagents were removed by dialysis against buffer A at 4 °C. The dialysis buffer (1 L) was changed a minimum of five times over a 36-h period.

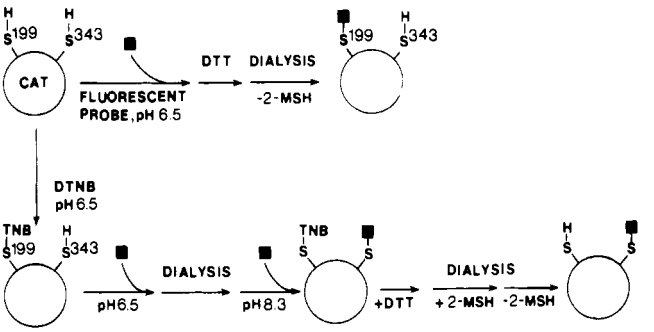

FIGURE 1: Procedure for modification of either Cys 199 or Cys 343 of the C-subunit by sulfhydryl-specific fluorescent probes. Outlined is the procedure for modification of the C-subunit by the fluorescent probes. Modification reactions were all done at room temperature, while dialyses were done at 4 °C. The fluorescent probe is represented by a solid square. 2-MSH is 2-mercaptoethanol.

The procedure for labeling Cys 199 by IAEDANS was the same as above except that the final concentration of IAEDANS was 10 mM. Prior to its addition to the C-subunit, IAEDANS (50 mM) was dissolved in 25 mM HEPES, pH 7.4. The loss of catalytic activity was 95% complete after 6.5 h at room temperature.

The same procedure was used to label Cys 199 with IANBD; the final concentration of IANBD was 500 μM. Prior to its addition to the C-subunit, IANBD was dissolved in acetonitrile at a concentration of 50 mM. Ninety-five percent of the catalytic activity was lost after 6.5 h at room temperature.

Modification of Cys 343. In order to exclusively label Cys 343 with monobromobimane, IAEDANS, or IANBD, Cys 199 was protected first by modification with DTNB, by incubating the C-subunit (25 μM) in 10 mM potassium phosphate, pH 6.5, with DTNB (30 mM) for 4 h at room temperature. Less than 10% of the initial activity remained. The reaction mixture was then made 5 mM in either monobromobimane or IAEDANS or 2.5 mM in IANBD and dialyzed against buffer A at 4 °C overnight to remove excess DTNB. The reaction mixture was then made either 5 mM in monobromobimane, 10 mM in IAEDANS, or 2.5 mM in IANBD after which the pH was raised to 8.3 by the addition of $^1/_{10}$ volume of 1 M Tris (pH 8.5). After incubating for 4 h at room temperature, DTT was added to a final concentration of 50 mM. The addition of DTT not only quenched the reaction between the fluorescent probe and the C-subunit but also released the TNB anion from Cys 199. Excess reagents were removed by dialysis against buffer A at 4 °C. The procedures used for modifying either Cys 199 or Cys 343 are summarized in Figure 1.

Determination of Extent and Specificity of Modification. Since modification of Cys 199 leads to inactivation of the C-subunit, the extent of modification of Cys 199 was determined by simply assaying for catalytic activity. The extent and specificity of modification of the C-subunit at either Cys 199 or Cys 343 with monobromobimane, IAEDANS, or

IANBD were determined by incubating aliquots of labeled C-subunit with a 10-fold excess of [^{14}C]IAA (50 μCi/mmol) at pH 8.3 in the presence of 8 M urea for 5 h at 37 °C. Excess reagents were removed by dialysis against 10 mM KPO_4, pH 6.5, and 2 mM EDTA at 4 °C. Aliquots were then made 100 mM in Tris-HCl, pH 8.3, and incubated at 37 °C overnight with TPCK–trypsin (1/50 w/w). The tryptic peptides were resolved by high-performance liquid chromatography (HPLC) on a Vydac C-18 column, and 1-mL fractions were collected. Elution of the tryptic peptides was achieved by using a binary gradient of 0%–40% solvent B in 80 min, where solvent A was 0.1% TFA in H_2O and solvent B was 0.1% TFA in acetonitrile. The elution of peptides was monitored by using a Hitachi 100-10 flow-through spectrophotometer and a Gilson Spectraglo/filter fluorometer. The [^{14}C]IAA-labeled peptides were located by monitoring radioactivity. Peptides were identified by amino acid analysis and/or peptide sequencing. Amino acid compositions were determined by using an LKB Biochrom Model 4400 automated amino acid analyzer. Samples were hydrolyzed in vacuo in 6 N HCl for 24 h. Sequencing was carried out on an Applied Biosystems Model 470A protein sequencer. Phenylthiohydantoin (PTH) derivatives were identified by HPLC using an IBM Cyano column.

Formation of Holoenzyme. Holoenzyme was formed by reassociation of the R^{II}-subunit and the C-subunit as described previously (First & Taylor, 1988) and was monitored by ion-exchange chromotography according to Bubis and Taylor (1985) and by visualization of protein bands following polyacrylamide gel electrophoresis carried out in the presence of sodium dodecyl sulfate (SDS) according to Laemmli (1970).

Fluorescence and Absorbance Measurements. Fluorescence measurements were made with a Perkin-Elmer MPF 66 spectrofluorometer. Absorbance measurements were made with a Perkin-Elmer λ 3B spectrophotometer. Both the spectrofluorometer and the spectrophotometer were interfaced to a Perkin-Elmer 7300 microcomputer. Fluorescence lifetimes were determined by using an EEY pulsed, single-photon-counting nanosecond fluorescence lifetime instrument. To eliminate the effects of anisotropic contributions to the observed decay, a Polaroid HNP1B dichroic film was placed in front of the photomultiplier tube and rotated at an angle of 54° from the vertical position. Fluorescence lifetimes were resolved and assessed as either single- or double-exponential functions, by using a method of moments analysis. Analysis of three exponential functions was accomplished by using the Marquardt analysis method (Marquardt, 1963). The weighted average lifetime was determined by using the equation:

$$\tau_{av} = \sum_{i=1}^{n}\left(\frac{A_i\tau_i}{\sum_{i=1}^{n}A_i}\right) \quad (1)$$

where τ_{av} is the average lifetime, A_i is the exponential coefficient of the decay component, τ_i is the lifetime of the ith decay component, and n is the total number of exponentials used to fit the lifetime decay. The time shift between the lamp pulse and the observed response introduced by the spectral response properties of the detecting photomultiplier tube was corrected with a time shift introduced by the computer. Excitation and emission bands were selected with the appropriate filters: bimane, Oriel 400-nm narrow-band interference excitation filter and Oriel 500-nm broad-band emission filter; AEDANS, Corning 7-60 excitation filter and Oriel 500-nm broad-band emission filter; ANBD, Oriel 450-nm broad-band excitation filter and Corning 3-66 emission filter. For all spectral measurements, samples were suspended in 100 mM HEPES, pH 7.3.

Quantum Yield Determination. The quantum yields (Q) were determined by the ratio procedure of Chen (1965a), using quinine sulfate in 0.1 N H_2SO_4 (Q = 0.70) (Scott et al., 1970) as the reference fluorophore.

Determination of the Fluorescence Polarization. Polarization measurements were determined on a Perkin-Elmer steady-state spectrofluorometer equipped with Melles–Grant dichroic film polarization filters placed in the path of the excitation and emission beams. The temperature was held at 20 °C by a thermostated cuvette holder.

Fluorescence polarization was determined from the equation (Chen, 1965b)

$$P = \frac{I_{vv} - G(I_{vh})}{I_{vv} + G(I_{vh})} \quad (2)$$

where P is the fluorescence polarization, I_{vv} is the fluorescence intensity when both the excitation and emission polarization filters are in the vertical positions, I_{hh} is the intensity when both the excitation and emission polarization filters are in the horizontal positions, I_{vh} is the intensity when the excitation polarization filter is in the vertical position and the emission polarization filter is in the horizontal position, and G is I_{hv}/I_{hh}. I_{hv} is the intensity when the excitation polarization filter is in the horizontal position and the emission polarization filter is in the vertical position. The fluorescence polarization (P) can be related to the the rotational correlation time by the equation (Perrin, 1925, 1926)

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{\tau_f}{\tau_c}\right) \quad (3)$$

where P_0 is the limiting polarization that would be observed at infinite viscosity, τ_f is the fluorescence lifetime, and τ_c is the rotational correlation time. The rotational correlation time of a spherical protein of molecular volume (V_0) is given by the Stokes–Einstein expression (Pesce et al., 1971)

$$\tau_c = \eta V_0/kT \quad (4)$$

where k is the Boltzmann constant, η is the viscosity of the solution, and T is the absolute temperature. The limiting polarization P_0 was determined from extrapolation of a plot of $1/P$ vs T/η to infinite viscosity. The viscosity of the solution was varied by adding aliquots of a 70% (w/v) sucrose solution. The molecular volume of each fluorescently modified C-subunit, assuming a spherical shape, was calculated from the slope of this plot. The Stokes radius R_s of the modified C-subunit was determined from the calculated molecular volume by using the equation $V_0 = (4/3)\pi R_s^3$.

Results

Modification of Cys 199 in the C-Subunit. Incubation of C-subunit at pH 7.4 with monobromobimane, IAEDANS, or IANBD resulted in the rapid loss of catalytic activity. The half-times of inactivation using the conditions described under Experimental Procedures were 30 s, 6 min, and 100 min, respectively. Modification by all three reagents at pH 6.5 also resulted in a similar loss of activity, although the rate of inactivation was substantially decreased at this pH (data not shown). When tryptic digests of the modified C-subunits were submitted to HPLC, a single fluorescent peak eluting at approximately 35% acetonitrile was seen (Figures 2 and 3). The products of modification by either monobromobimane or IAEDANS were followed by monitoring fluorescence. The unmodified cysteines were monitored by alkylation of the

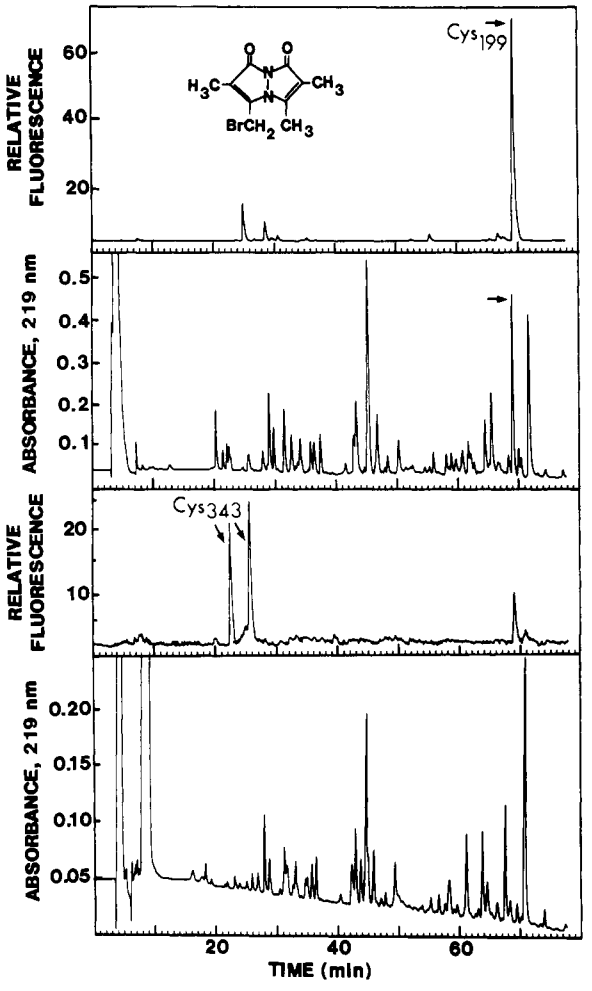

FIGURE 2: Extent and specificity of modification of Cys 199 and Cys 343 by monobromobimane. The extent and specificity of the modification of each cysteine residue by monobromobimane was determined by modifying the fluorescently labeled protein with [^{14}C]IAA, digesting with trypsin, and separating the digest on HPLC. The top two panels show the elution pattern of the fluorescent peptides (top) and of all the tryptic peptides based on absorbance at 219 for C-subunit modified at Cys 199 by monobromobimane. The lower two panels show the elution of the fluorescent peptides and of the total digest for C-subunit modified at Cys 343 by monobromobimane. The radioactivity profiles of peptides that contained unmodified sulfhydryl groups and, thus, were alkylated by [^{14}C]IAA are not shown. The structure of monobromobimane is shown as an inset on the top panel.

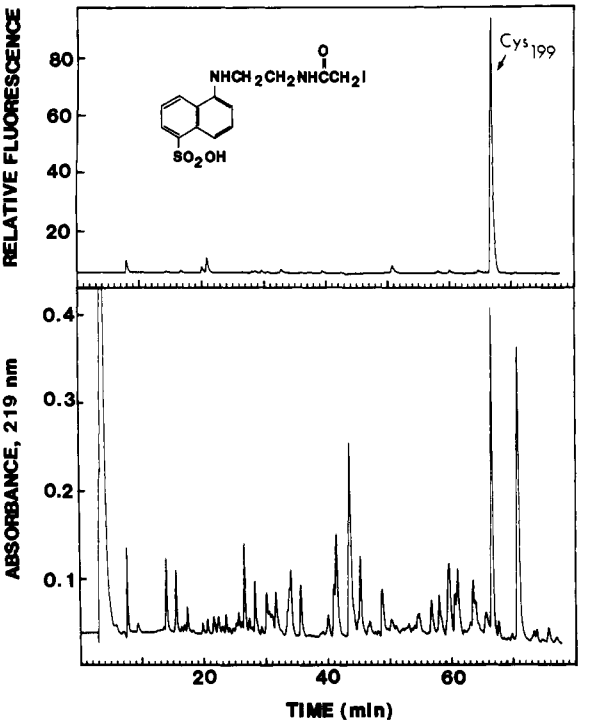

FIGURE 3: Modification of Cys 199 by IAEDANS. The elution of the tryptic peptides from the HPLC column was monitored by fluorescence and by absorbance at 219 nm. The single major fluorescent peak eluting at 67 min corresponds to the tryptic peptide containing Cys 199. The structure of IAEDANS is shown in the inset of the upper panel.

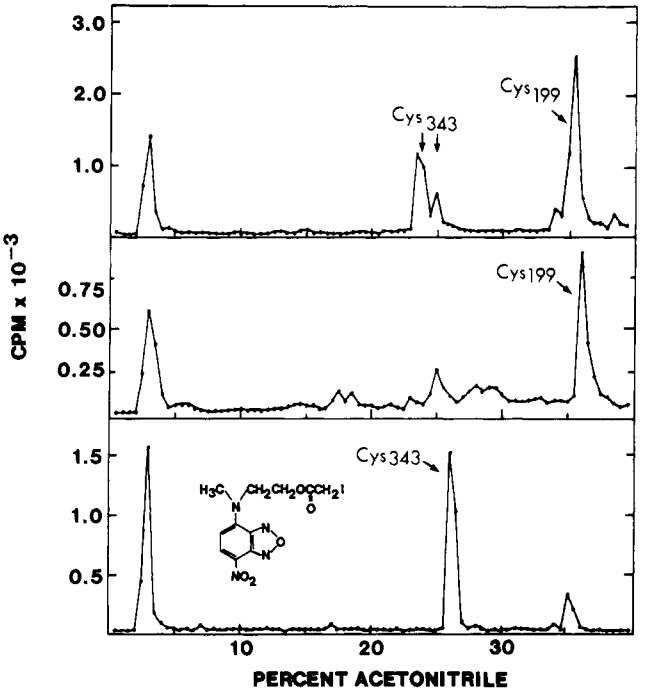

FIGURE 4: Modification of Cys 343 and Cys 199 by IANBD. The modification of cysteine residues was followed by identifying the unreacted cysteines by alkylation with [^{14}C]IAA. The upper panel shows the radioactive profile that is obtained when native C-subunit is alkylated with [^{14}C]IAA. The elution positions of the tryptic peptides containing Cys 199 and Cys 343 are indicated. The middle and lower panels show the alkylation of C-subunit that had been modified at Cys 343 and Cys 199, respectively, with IANBD. The structure of IANBD is shown as an inset in the lower panel.

denatured protein with [^{14}C]iodoacetic acid (IAA), using conditions that quantitatively label free cysteines. Under the low-pH conditions (pH 2.1) employed to separate the tryptic peptides, the ANBD label does not undergo fluorescence emission. Consequently, only [^{14}C]IAA incorporation was used to identify the modified cysteine residues for the C-subunit modified with IANBD (Figure 4). For C-subunit modified at pH 6.5 by monobromobimane and IAEDANS, amino acid analysis and peptide sequencing identified the first 10 residues of the fluorescent peptide as T-W-T-L-C-G-N-P-Q-Y. In both cases, this fluorescent peak eluted just before the corresponding elution position of the peptide alkylated with IAA, which coelutes with another major absorbing peptide at approximately 37% acetonitrile (Figures 2 and 3). The single site covalently modified by the fluorescent probes was thus identified as Cys 199. In addition, [^{14}C]IAA was found in all cases to be incorporated almost exclusively into Cys 343 of the modified C-subunit, indirectly confirming that the fluorescent labels were attached to Cys 199.

Modification of Cys 343. The selective modification of Cys 343 by the same three fluorescent labels was achieved by first labeling Cys 199 reversibly with DTNB and then reacting the C-subunit with each of the fluorescent labels at pH 8.3 to modify Cys 343. In each case, treatment of these fluorescently modified C-subunits with 50 mM DDT restored better than

Table I: Spectral Properties of the Fluorescently Modified Catalytic Subunits[a]

sample	absorbance maxima (nm)	extinction coefficients (M^{-1} cm^{-1})	emission maxima (nm)	quantum yields
C-subunit modified at Cys 199 by monobromobimane	394 (±5)	4.8 (±0.2) × 10^3	480 (±2)	0.15 (±0.02)
C-subunit modified at Cys 343 by monobromobimane	387 (±3)	5.5 (±0.3) × 10^3	481 (±2)	0.15 (±0.02)
cysteine modified by monobromobimane	392 (±2)	4.7 (±0.9) × 10^3	484 (±2)	0.45 (±0.05)
C-subunit modified at Cys 199 by IAEDANS	336 (±2)	6.8 (±0.2) × 10^3	498 (±2)	0.20 (±0.03)
C-subunit modified at Cys 343 by IAEDANS	334 (±2)	6.1 (±0.3) × 10^3	497 (±2)	0.15 (±0.02)
cysteine modified by IAEDANS	338 (±2)	5.0 (±0.1) × 10^3	508 (±1)	0.34 (±0.04)
C-subunit modified at Cys 199 by IANBD	350 (±2)	4.6 (±0.2) × 10^3	547 (±2)	0.02 (±0.01)
	493 (±2)	1.3 (±0.3) × 10^4		
C-subunit modified at Cys 343 by IANBD			545 (±3)	0.02 (±0.01)
cysteine modified by IANBD	356 (±2)	6.4 (±0.2) × 10^3	562 (±5)	0.01 (±0.01)
	500 (±2)	2.5 (±0.3) × 10^4		

[a] All measurements were done on at least three different samples, and the mean value was determined. The range of values obtained is indicated in parentheses.

Table II: Fluorescence Lifetimes of the Modified Catalytic Subunits[a]

sample	analysis of fluorescence decay curves						
	A_1	τ_1	A_2	τ_2	A_3	τ_3	τ_{av}
C-subunit modified at Cys 199 by monobromobimane	0.21 (±0.05)	4 (±1)	0.65 (±0.05)	12.2 (±0.6)	0.15 (±0.06)	21 (±1)	11.9 (±0.5)
C-subunit modified at Cys 343 by monobromobimane	0.40 (±0.09)	3.0 (±0.5)	0.46 (±0.08)	8.7 (±0.8)	0.16 (±0.05)	17 (±1)	7.8 (±0.5)
cysteine modified by monobromobimane	1.0	7.5 (±0.5)					7.5 (±0.5)
C-subunit modified at Cys 199 by IAEDANS	0.4 (±0.2)	6 (±3)	0.4 (±0.1)	12 (±3)	0.12 (±0.05)	20 (±3)	10 (±3)
C-subunit modified at Cys 343 by IAEDANS	0.5 (±0.1)	4.8 (±0.5)	0.42 (±0.08)	12 (±3)	0.06 (±0.09)	21 (±3)	10 (±1)
cysteine modified by IAEDANS	0.3 (±0.1)	7 (±1)	0.7 (±0.2)	11 (±1)			9.8 (±0.5)
C-subunit modified at Cys 199 by IANBD	0.86 (±0.08)	1.1 (±0.4)	0.11 (±0.05)	5 (±2)	0.04 (±0.02)	9.6 (±0.6)	1.8 (±0.3)
C-subunit modified at Cys 343 by IANBD	0.7 (±0.2)	1.0 (±0.3)	0.2 (±0.1)	4 (±1)	0.07 (±0.02)	9.2 (±0.8)	2.1 (±0.3)
cysteine modified by IANBD	1.0	2.5 (±0.3)					2.5 (±0.3)

[a] The lifetimes indicated are the mean values determined from three different samples. The range of values obtained is indicated in parentheses.

90% of the catalytic activity. Modification of the labeled C-subunits by [^{14}C]IAA, followed by tryptic digestion and separation of the tryptic peptides on HPLC, confirmed that Cys 343 was preferentially modified by the fluorescent reagents under the conditions described. The patterns of fluorescence and [^{14}C]IAA incorporation indicate that the labeling by either monobromobimane or IAEDANS was complete and selective (Figures 2 and 3). It was particularly important to correlate the incorporation of [^{14}C]IAA into C-subunit labeled at Cys 343 by IANBD with fluorescence, because incorporation of the fluorescent label was variable. The incorporation of [^{14}C]IAA ensured that the prior reaction had gone to completion (Figure 4).

Formation of Holoenzyme from Modified C-Subunits. In order to establish whether the modified C-subunits are capable of forming holoenzyme, they were dialyzed with R^{II}-subunit and then passed through a column of CL CM-Sepharose cation-exchange resin to remove any free C-subunit. In each case, the eluate contained equivalent amounts of both the R^{II}-subunit and the C-subunit. Recovery of the R^{II}-subunit was nearly quantitative; only the excess C-subunit was retained on the CL CM-Sepharose resin (Figure 5). When the eluate was incubated with cAMP (100 μM) and rechromatographed on CL CM-Sepharose resin, only the R^{II}-subunit remained unbound. Holoenzyme that was formed with unmodified C-subunit was indistinguishable from holoenzyme formed with the modified C-subunits. Since C-subunit modified exclusively at Cys 343 was still active, holoenzymes formed from these modified C-subunits were assayed directly for catalytic activity in the presence and absence of cAMP to assess the integrity of the holoenzyme that formed. All of these modified C-subunits formed holoenzyme that was dissociated in the presence of cAMP (data not shown).

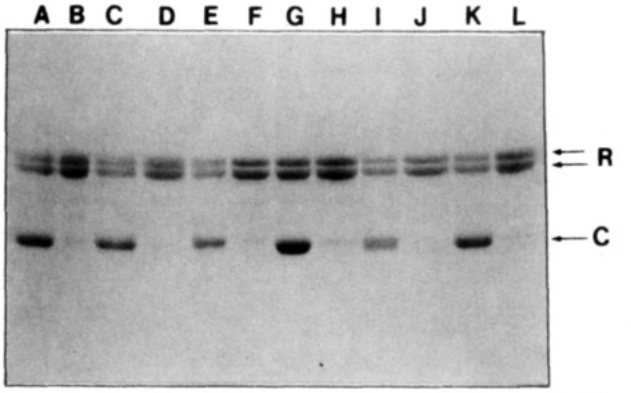

FIGURE 5: Formation of holoenzyme with modified C-subunits. The ability of the fluorescently labeled C-subunits to form type II holoenzyme was determined by chromatography on CL CM-Sepharose. Holoenzyme that has been incubated in the presence (B, D, F, H, J, L) or absence (A, C, E, G, I, K) of 100 μM cAMP is shown. The C-subunits used to form the holoenzymes present in the different lanes were as follows: lanes A and B, C-subunit modified at Cys 199 with monobromobimane; lanes C and D, C-subunit modified at Cys 343 with monobromobimane; lanes E and F, C-subunit modified at Cys 199 with IAEDANS; lanes G and H, C-subunit modified at Cys 343 with IAEDANS; lanes I and J, C-subunit modified at Cys 199 with IANBD; lanes K and L, C-subunit modified at Cys 199 with IANBD.

Spectral Properties of Modified C-Subunits. The spectral properties of the modified C-subunits are summarized in Table I and in Figure 6. In each case the modified C-subunits are compared to free Cys modified with the same probe.

Fluorescence Lifetime Analysis. The fluorescent lifetimes for each modified C-subunit are summarized in Table II. Attempts were made to fit the fluorescence decay rates to one, two, and three exponential functions. In most cases a three-exponential fit of the fluorescence decays was required to

Table III: Limiting Polarizations, Rotational Relaxation Times, and Stokes Radii for the Modified Catalytic Subunits[a]

sample	P_0	τ_c (ns)	R_s (Å)
C-subunit modified at Cys 199 by monobromobimane	0.31 (±0.02)	19 (±2)	26 (±0.3)
C-subunit modified at Cys 343 by monobromobimane	0.29 (±0.02)	16 (±2)	25 (±0.3)
cysteine modified by monobromobimane	0.025 (±0.006)	8 (±2)	13 (±0.4)
C-subunit modified at Cys 199 by IAEDANS	0.20 (±0.01)	17 (±2)	30 (±0.3)
C-subunit modified at Cys 343 by IAEDANS	0.18 (±0.01)	14 (±2)	24 (±0.3)
cysteine modified by IAEDANS	0.03 (±0.005)	4.0 (±0.5)	16 (±0.3)
C-subunit modified at Cys 199 by IANBD	0.38 (±0.03)	13 (±2)	23 (±0.3)
C-subunit modified at Cys 343 by IANBD	0.42 (±0.01)	14 (±2)	23 (±0.3)
cysteine modified by IANBD	0.21 (±0.08)	1.7 (±0.5)	11 (±0.4)

[a] Values obtained are the mean measurements determined from three different samples. The range of values obtained is indicated in parentheses.

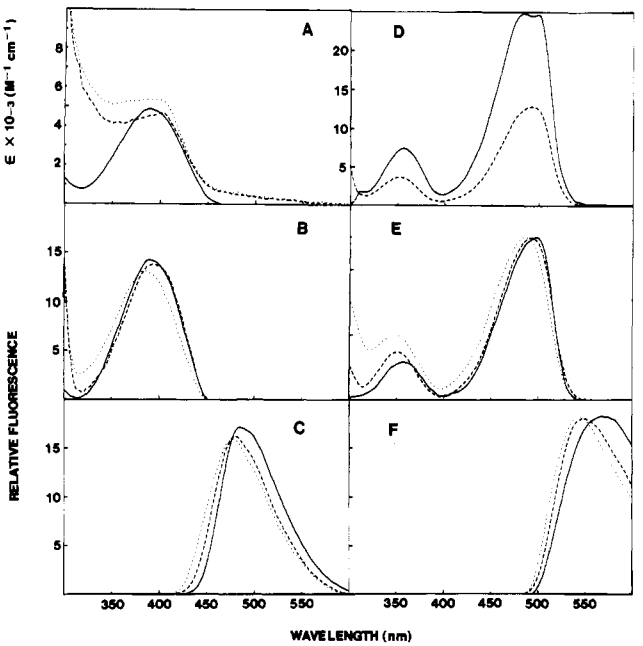

FIGURE 6: Absorbance, excitation, and emission spectra of C-subunits and cysteine modified by monobromobimane and by IANBD. The absorbance spectra (panel A), steady-state fluorescence excitation spectra (panel B), and steady-state fluorescence emission spectra (panel C) are shown for C-subunit modified at Cys 199 (dashed lines), C-subunit modified at Cys 343 (dotted lines), and cysteine modified by monobromobimane (solid lines). The absorbance spectra (panel D), steady-state fluorescence excitation spectra (panel E), and steady-state fluorescence emission spectra (panel F) are shown for C-subunit modified at Cys 199 (dashed lines), C-subunit modified at Cys 343 (dotted lines), and cysteine modified by IANBD (solid lines). The fluorescence excitation and emission spectra were plotted such that the wavelengths of maximum excitation and maximum emission could be readily compared and do not represent the relative intensities of the fluorescence spectra of the samples. The concentration of the C-subunits modified by either monobromobimane or IANBD was 10 μM, and the concentration of the cysteine modified by either monobromobimane or IANBD was 250 μM in the samples used to measure the absorbance spectra. All samples were 100 mM in HEPES (pH 7.4).

generate random residuals. The fluorescence lifetimes for C-subunit modified at Cys 199 by monobromobimane, IAEDANS, and IANBD are shown in Figure 7. In contrast to the C-subunits, the lifetimes of free cysteine modified by the fluorescent probes could usually be fit with a single-exponential decay (Table II).

Perrin Analysis of Fluorescence Polarization. Estimates of the mobility of the fluorescent probes attached to the C-subunit were made by measuring steady-state polarization of fluorescence as a function of solvent viscosity. Results of this analysis are shown in Figure 8. Extrapolation of the plot of reciprocal polarization versus temperature/viscosity to infinite viscosity gives $1/P_0$, where P_0 is the limiting polarization. Substitution of $1/P_0$ into eq 3 and 4 yields the rotational

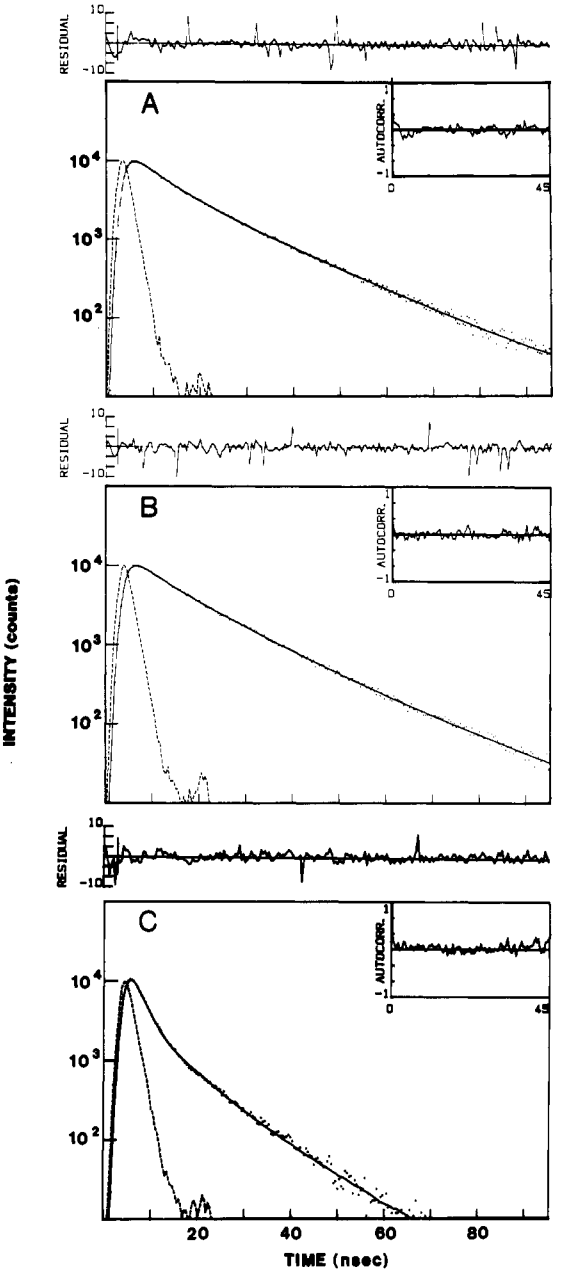

FIGURE 7: Analysis of the fluorescence lifetimes of C-subunits modified at Cys 199 by monobromobimane, IAEDANS, and IANBD. Panels A, B, and C show the fluorescence lifetimes of C-subunit modified at Cys 199 by monobromobimane, IAEDANS, and IANBD, respectively. The lamp pulse is indicated by the dashed curves. All modified C-subunits were 100 mM in HEPES (pH 7.4) and had an approximate concentration of 5 μM.

correlation time. Table III summarizes the limiting polarization values, correlation times, and Stokes radii of the modified C-subunits.

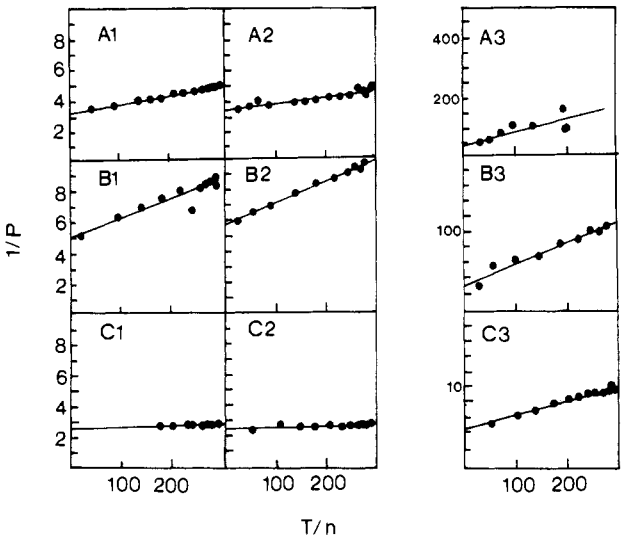

FIGURE 8: Perrin analysis of C-subunit modified at Cys 199 or Cys 343 by either monobromobimane, IAEDANS, or IANBD and cysteine modified by either monobromobimane, IAEDANS, and IANBD. The Perrin plots for the following samples are shown: C-subunit modified at Cys 199 by monobromobimane (panel A1), C-subunit modified at Cys 343 by monobromobimane (panel A2), cysteine modified by monobromobimane (panel A3), C-subunit modified at Cys 199 by IAEDANS (panel B1), C-subunit modified at Cys 343 by IAEDANS (panel B2), cysteine modified by IAEDANS (panel B3), C-subunit modified at Cys 199 by IANBD (panel C1), C-subunit modified at Cys 343 by IANBD (panel C2), and cysteine modified by IANBD (panel C3). The concentration of each sample was approximately 5 μM.

Discussion

The C-subunit of cAMP-dependent protein kinase contains two cysteine residues, Cys 199 and Cys 343 (Shoji et al., 1983). Jimenez and Shaltiel (1982) observed that the rate of the reaction between DTNB and the C-subunit was biphasic, with the biphasic properties being most pronounced at pH 6.5 when the salt concentration was less than 50 mM. Modification of the fast reacting thiol correlated with loss of catalytic activity. We have found that the modification of the C-subunit by monobromobimane, IAEDANS, and IANBD at pH 6.5 and an ionic strength below 50 mM result in the selective modification of Cys 199, thus identifying it unambiguously as the fast reacting thiol. Since modification of this residue correlates with the loss of catalytic activity, Cys 199 most likely is at the active site. This is consistent with the finding of Bramson et al. (1983) that a thiol-reactive peptide substrate analogue both inactivated and selectively modified Cys 199 of the C-subunit. It is also consistent with the observations of Nelson and Taylor (1983), who found that alkylation of the type II holoenzyme of cAMP-dependent protein kinase by iodoacetic acid selectively modified Cys 343 in the C-subunit. This modified C-subunit retained its catalytic activity, indicating Cys 343 was not the cysteine residue associated with the active site of the C-subunit.

The differential reactivity of the cysteine residues in the C-subunit has been used to selectively modify the slow reacting thiol with monobromobimane, IANBD, and IAEDANS. This was achieved by first reacting the C-subunit with DTNB to protect Cys 199, modifying the C-subunit with one of the fluorescent probes, and, finally, treating this modified C-subunit with dithiothreitol to remove the TNB anion from Cys 199. The resulting C-subunit was selectively modified at Cys 343 and retained most of its catalytic activity, confirming that Cys 199 alone is responsible for the loss of activity in the C-subunit following alkylation by thiol-reactive reagents.

The procedures used to selectively modify either Cys 199 or Cys 343 do not appear to have altered the physical properties of the C-subunits drastically. The calculated Stokes radii for all of the modified C-subunits was 26 Å. This is in good agreement with the values obtained previously from both ultracentrifugation and gel filtration (Bechtel et al., 1977; Peters et al., 1977; Zoller et al., 1979), suggesting that the overall structure of the C-subunit is not substantially perturbed by the presence of the fluorescent probes. In addition, the modified C-subunits were capable of reassociating with unmodified R^{II}-subunit to form holoenzyme. This holoenzyme can be dissociated into the free R^{II}-dimer and modified C-subunit by the addition of cAMP. These dissociated modified C-subunits are bound to CL CM-Sepharose cation-exchange resin in a manner that is analogous to the unmodified C-subunit. Finally, in the case of C-subunit that was modified at Cys 343 by the fluorescent probes, the modified C-subunit retained nearly 100% of its catalytic activity, indicating that the active site in these modified C-subunits remains functionally intact.

Several properties of the fluorescently modified C-subunits suggest that both Cys 199 and Cys 343 are located in relatively hydrophobic regions of the C-subunit. First, the emission spectra of the modified C-subunits are blue-shifted relative to the emission spectra of the free label (Table I and Figure 6). This was especially apparent for the C-subunits that were modified by IAEDANS and IANBD. Both of these probes are highly sensitive to the polarity of their environment (Hudson & Weber, 1973; Haugland, 1975). The observed blue shifts in the emission spectra of the modified C-subunit are approximately 50% as large as the blue shifts in the emission spectra that were observed for IAEDANS and IANBD when these probes were covalently bound to residues in the hydrophobic pockets of uridine diphosphoglucose dehydrogenase and myosin, respectively (Franzen et al., 1980; Haugland, 1975). In addition, the fluorescence lifetimes for each of the modified C-subunits were longer than for the corresponding free labels. This is consistent with the conclusion, suggested by the blue shifts in the steady-state emission spectra of the modified C-subunits, that both Cys 199 and Cys 343 are located in hydrophobic environments. In the case of the probes that are introduced at Cys 199, it is possible that these fluorescent groups may occupy the adenine binding site.

In nearly all cases, the exponential fit for the fluorescence lifetimes of the modified C-subunits was substantially improved when a three-exponential fit was employed (Table II). Several factors may contribute to this, such as multiple rotamers of the fluorophore or different conformational states of the protein. The purified C-subunit contains at least two isolectric variants (Peters et al., 1977; Walsh et al., 1982), and this may contribute to some heterogeneity.

In summary, C-subunit has been modified at either Cys 199 or Cys 343 by three different fluorophores: monobromobimane, IAEDANS, and IANBD. These selectively modified C-subunits can now be used to measure the proximity of each cysteine to other regions of the molecule by using fluorescence resonance energy transfer techniques as described in the following paper in this issue (First et al., 1989). These C-subunits also can be used to measure distances in the holoenzyme complex.

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