Fluorescence Energy Transfer between Cysteine 199 and Cysteine 343: Evidence for MgATP-Dependent Conformational Change in the Catalytic Subunit of cAMP-Dependent Protein Kinase†

Eric A. First,‡,§ David A. Johnson,‖ and Susan S. Taylor*,‡

Department of Chemistry, University of California, San Diego, La Jolla, California 92093, and Division of Biomedical Sciences, University of California, Riverside, Riverside, California 92521

Received July 20, 1988; Revised Manuscript Received November 28, 1988

ABSTRACT: The catalytic subunit of cAMP-dependent protein kinase has two cysteine residues, Cys 199 and Cys 343, which are protected against alkylation by MgATP [Nelson, N. C., & Taylor, S. S. (1981) *J. Biol. Chem. 256*, 3743]. While Cys 199 is in close proximity to the active site of the catalytic subunit and is probably directly protected against alkylation by MgATP, the mechanism by which MgATP prevents alkylation of Cys 343 is unclear. To determine whether MgATP directly protects Cys 343 from alkylation by being in close proximity to both Cys 199 and the MgATP binding site, fluorescence resonance energy transfer techniques were used to measure the distance between Cys 199 and Cys 343. Two different donor–acceptor pairs containing 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole at Cys 199 as the acceptor and either 3,6,7-trimethyl-4-(bromomethyl)-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione or *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine at Cys 343 as the donor were prepared following the method described in the preceding paper [First, E. A., & Taylor, S. S. (1989) *Biochemistry* (preceding paper in this issue)]. From the efficiencies of fluorescence resonance energy transfer for each donor–acceptor pair, the distance between Cys 199 and Cys 343 was estimated to be between 31 and 52 Å. Since Cys 199 is close to the MgATP binding site and since MgATP cannot extend beyond a distance of 16 Å, it is unlikely that Cys 343 at a distance of at least 31 Å from Cys 199 is in direct contact with the bound nucleotide. The 31–52-Å distance between the two cysteines suggests instead that the binding of MgATP induces a conformational change in the catalytic subunit, which leads to the protection of Cys 343.

The catalytic (C) subunit of cAMP-dependent protein kinase (EC 2.7.1.37) exists in two aggregation states. The active form of the enzyme is a dissociated monomer (M_r = 40 862). The activity of the enzyme is regulated by interaction with specific regulatory (R) subunits. These R-subunits represent a family of dimeric proteins with each promoter having a molecular mass of approximately 47 000–48 000 daltons [for review see Beebe and Corbin (1986)]. In the absence of cAMP, two C-subunits aggregate with an R-subunit dimer to form an inactive tetrameric holoenzyme (R_2C_2). The frictional coefficient (f/f_0 = 1.18) of free C-subunit is consistent with a spherical globular protein, while the frictional coefficients of both the holoenzyme (f/f_0 = 1.5–1.6) and R-subunit dimer (f/f_0 = 1.4–1.45) suggest significant dimensional asymmetry (Nimmo & Cohen, 1977; Taylor & Stafford, 1978; Carlson et al., 1979).

The activity of the catalytic subunit is known to be sensitive to inhibition by sulfhydryl-specific reagents (Sugden et al., 1976; Armstrong & Kaiser, 1978; Nelson & Taylor, 1981). The C-subunit contains only two cysteine residues, Cys 199 and Cys 343 (Shoji et al., 1983). The most reactive cysteine is Cys 199, and loss of activity by sulfhydryl specific reagents is due to the specific modification of this residue (Nelson & Taylor, 1983; First & Taylor, 1989). Investigation of the binding of *lin*-benzoadenine nucleotides to C-subunit modified with either 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl)[1] or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) suggests that modification of Cys 199 specifically interferes with the portion of the ATP binding site that recognizes the β- and γ-phosphates (Bhatnagar et al., 1984). Affinity labeling with a peptide analogue has indicated that Cys 199 is close to the active site of the enzyme (Bramson et al., 1983). Interaction with the regulatory subunits protects Cys 199 from alkylation by iodoacetic acid (IAA), whereas Cys 343 is still accessible to alkylation (Nelson & Taylor, 1983). The addition of MgATP, however, protects both Cys 199 and Cys 343 against alkylation by IAA (Nelson & Taylor, 1983). Since only one molecule of MgATP binds per C-subunit (Sugden et al., 1976), MgATP could protect Cys 343 from alkylation by two possible mechanisms: either the binding of MgATP to the C-subunit physically prevents access to Cys 343 by IAA or the MgATP binding induces a conformational change in the C-subunit that renders Cys 343 inaccessible to IAA.

If MgATP physically prevents IAA access to both Cys 343 and Cys 199, then the two cysteines should be within 16 Å of each other, MgATP's maximum extended span. We,

† This research was supported in part by USPHS Grant GM19301 to S.S.T. E.A.F. was supported in part by USPHS Training Grant AM07233.

* To whom correspondence should be addressed.

‡ University of California, San Diego.

§ Present address: Department of Chemistry, Imperial College, London SW7 2AY, England.

‖ University of California, Riverside.

[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); FRET, fluorescence resonance energy transfer; IAA, iodoacetic acid; NBD-Cl, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole; MgATP, magnesium chelate of adenosine 5'-triphosphate.

0006-2960/89/0428-3606$01.50/0

therefore, measured the distance between these two cysteines by using fluorescence resonance energy transfer (FRET) techniques. Cys 199 and Cys 343 were specifically modified with donor and acceptor fluorescent probes following the method described previously (First & Taylor, 1989). Two different donor–acceptor pairs were formed by first modifying Cys 199 in the C-subunit with the acceptor, 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole (IANBD) and subsequently modifying Cys 343 with one of two donors, either 3,6,7-trimethyl-4-(bromomethyl)-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione (monobromobimane) or *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS). Also, in addition to examining the isolated C-subunit, type II holoenzyme was formed with modified C-subunits to investigate the effect of R^{II}-subunit on the distance between Cys 199 and Cys 343.

Experimental Procedures

Materials. Chemicals and reagents were obtained from the following sources: monombromobimane (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).

Purification of Proteins. The catalytic and type II regulatory subunits of the cAMP-dependent protein kinase were purified as described previously (Nelson & Taylor, 1983; First & Taylor, 1988). Catalytic subunit was stored in 40 mM potassium phosphate, pH 6.5, 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% glycerol (buffer A). R^{II}-subunit was stored in 25 mM potassium phosphate, pH 6.5, 2 mM EDTA, 5 mM 2-mercaptoethanol, and 25 mM cGMP (buffer B). Formation of holoenzyme from the fluorescently modified C-subunits and unmodified R^{II}-subunit was previously described (First & Taylor, 1989).

Formation of Donor and Acceptor Pairs. Preparation and characterization of various donor and acceptor forms of the C-subunit modified at a single site have been described previously (First & Taylor, 1989). In addition, two donor–acceptor pairs were prepared. The first donor–acceptor pair was C-subunit modified with IANBD at Cys 199 and with monobromobimane at Cys 343 and is referred to as B343–N199. It was prepared by taking 2 mL of C-subunit modified at Cys 199 by IANBD (N199, 20 μM in buffer A) and bringing it to 100 mM Tris, pH 8.3. Following addition of monobromobimane (100 mM in methanol) to a final concentration of 10 mM, the solution was incubated for 4 h at room temperature, and then the reaction was quenched by the addition of 50 μL of 1 M dithiothreitol. Excess reagents were removed by repeated dialysis against 1 L of 10 mM potassium phosphate, pH 6.5, containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% glycerol, at 4 °C. The second donor–acceptor pair, D343–N199, was C-subunit modified with IANBD at Cys 199 and with IAEDANS at Cys 343 and was prepared by the same procedure as B343–N199, except that reaction mixture contained 10 mM IAEDANS instead of monobromobimane, and the IAEDANS (100 mM) was initially dissolved in 1 M Tris, pH 8.3. The procedures that were used to modify the C-subunit are summarized schematically in Figure 1.

Determination of the Extent of Modification and Integrity of the Donor–Acceptor Pairs. The extent of cysteine modification was determined by reacting aliquots of the labeled proteins with [^{14}C]IAA (10 mM, 50 μCi/mL) as described previously (First et al., 1989). After incubating overnight with trypsin (1/50 w/w) at 37 °C, the resulting tryptic digests were separated by reverse-phase high-performance liquid chromatography (HPLC) on a Vydac C-18 column. 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% (w/v) trifluoroacetic acid in H_2O and solvent B was 0.1% (w/v) trifluoroacetic acid in acetonitrile. The functional activity of each donor–acceptor pair was assessed by its capacity to form intact holoenzyme when combined with the R^{II}-subunit. Cation-exchange chromatography was used to assess the integrity of holoenzyme formed between the R^{II}-subunit and the donor–acceptor pairs. This method is based on the capacity of CL CM-Sepharose CL-6B (Pharmacia) cation-exchange resin to bind free C-subunit, but not holoenzyme, and has previously been described in detail (First & Taylor, 1988).

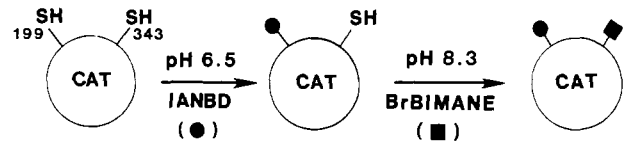

FIGURE 1: Procedure for formation of the B343–N199 donor–acceptor pair. C-subunit was treated with dithiothreitol following its reaction with IANBD and again following its reaction with monobromobimane (BrBIMANE). Excess IANBD and dithiothreitol were removed by dialysis prior to the reaction of IANBD-modified C-subunit with monobromobimane.

Determination of Sample Concentrations. Sample concentration was determined by amino acid analysis, by absorbance at 280 nm (corrected for the absorbance of the fluorophore), and by the method of Bradford (1976) using bovine serum albumin as a standard. The total nanomoles of protein were determined from amino acid analysis using the average of the amino acids Asp, Glu, Ala, and Leu divided by the number of each residue per mole. The concentration of each sample was calculated from the average of five separate hydrolyses. Amino acid analysis was carried out on an LKB Biochrom Model 4400 automated amino acid analyser in vacuo in 6 M HCl at 110 °C for 24 h.

Fluorescence and Absorbance Measurements. Fluorescence measurements were made with a Perkin-Elmer MPF 66 spectrofluorometer. Absorbance measurements were made on a Perkin-Elmer λ 3B spectrophotometer. Both the spectrofluorometer and spectrophotometer were interfaced to a Perkin-Elmer 7300 microcomputer. Fluorescence lifetimes were determined by using a EEY pulsed, single-photon-counting nanosecond fluorescence lifetime instrument. To eliminate the effects of anisotropic contributions to the observed decay, a Polaroid HNP'B dichroic film was placed in front of the photomultiplier tube, and rotated at an angle of 54° from the vertical position (Spencer & Weber, 1970). Fluorescence decay rates were resolved and assessed as either single- or double-exponential functions by using a method of moments analysis. Analyses of three-exponential functions were accomplished by using the Marquardt analysis method (Marquardt, 1963). Excitation and emission bands were selected with the appropriate filters: momobromobimane, Oriel 400-nm narrow-band 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.

The overlap integrals between the donor and acceptor were calculated from the equation (Fairclough & Cantor, 1978)

$$J = \frac{\int I_D(\lambda)\epsilon_A(\lambda)\lambda^4\, d\lambda}{\int I_D(\lambda)\, d\lambda} \quad (1)$$

The overlap integral corresponds to the probability of resonance between the excited-state donor dipole and the ground-state acceptor dipole and is evaluated as the integrated mutual area of overlap between the donor emission spectrum $[I_D(\lambda)]$ and the acceptor absorption spectrum $[\epsilon_A(\lambda)]$. Wavelength λ is in centimeters. The efficiency of FRET was determined by three different methods: steady-state fluorescence donor quenching, steady-state acceptor sensitization, and fluorescence donor lifetime measurements. The apparent efficiency of FRET, E_D, measured as the extent of quenching of the donor quantum yield or lifetime (Fairclough & Cantor, 1978), was calculated from

$$E_D = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (2)$$

where I_{DA} and I_D are the fluorescence intensities in the presence and absence of acceptor, respectively. The respective lifetimes are denoted by τ_{DA} and τ_D. When multiple lifetimes are observed, the apparent transfer efficiencies in the absence of "static" components can be calculated from

$$E_D = 1 - \frac{\sum_{i=1}^{n} a_i\tau_i}{\sum_{i=1}^{n} a_i^0\tau_i^0} \quad (3)$$

The terms in the numerator and denominator denote the total fluorescence intensity for the donor in the presence and absence of acceptor, respectively, and are calculated from the normalized experimental amplitudes (a_i) and lifetimes τ_i. The efficiency of FRET was also estimated from steady-state acceptor sensitization, E_A, by using the equation (Fairclough & Cantor, 1978)

$$E_A = \frac{I_{DA}(\lambda_D,\lambda) - I_D(\lambda_D,\lambda) - I_A(\lambda_D,\lambda)}{[A_D(\lambda_D)/A_A(\lambda_D)]I_A(\lambda_D,\lambda) - I_D(\lambda_D,\lambda)} \quad (4)$$

where $I_{DA}(\lambda_D,\lambda)$ is the emission spectrum of the donor–acceptor pair in the region of acceptor emission of the sample, $I_D(\lambda_D,\lambda)$ is the emission spectrum of the donor which has been separated from the acceptor, $I_A(\lambda_D,\lambda)$ is the emission spectrum of the acceptor which has been separated from the donor, and $A_D(\lambda)/A_A(\lambda)$ is the ratio of the absorbances of the donor and acceptor at the excitation wavelength. The sample temperatures were maintained at 20 °C by using a thermostatted cuvette holder. All spectroscopic analyses were performed with samples suspended in 100 mM HEPES, pH 7.3.

Calculation of the Forster Critical Distance and the Actual Distance between the Donor and Acceptor. The Forster critical distance R_0 is the distance at which the efficiency of FRET is 0.5 and can be calculated by using the equation

$$R_0 = (9.765 \times 10^3)(\kappa^2 J Q_D n^{-4})^{1/6} \quad (5)$$

where n is the refractive index of the medium between the donor and acceptor, Q_D is the donor quantum yield, J is the overlap integral, and κ^2 is the orientation factor and accounts for the relative orientation of the donor emission and acceptor absorption transition dipoles. For proteins, n has been taken to be 1.4. While it is usually not possible to directly measure κ^2, upper and lower bounds were obtained by measurement of the limiting anisotropies of the donor and acceptor. When both axial depolarization factors are positive, the upper and lower limits of the orientation factor can be calculated from the equations (Dale & Eisinger, 1975):

$$\kappa_{max}^2 = \tfrac{2}{3}[1 + \langle d_D^x\rangle + \langle d_A^x\rangle + 3\langle d_D^x\rangle\langle d_A^x\rangle] \quad (6)$$

$$\kappa_{min}^2 = \tfrac{2}{3}[1 - (\langle d_D^x\rangle + \langle d_A^x\rangle)/2] \quad (7)$$

where the terms $\langle d_D^x\rangle$ and $\langle d_A^x\rangle$, respectively, denote the axial depolarization factors for donor and acceptor. These axial depolarization factors were estimated from the individual emission anisotropies of each labeled C-subunit at various viscosities (η). From plots of reciprocal anisotropy versus temperature divided by viscosity (T/η), the intrinsic anisotropy of each labeled C-subunit was obtained from the interpolation to infinite viscosity. The square root of each intrinsic anisotropy divided by 0.4 yielded estimates for the axial depolarization factors. By substitution of κ_{min}^2 and κ_{max}^2 into eq 5, minimum (R_0^{min}) and maximum (R_0^{max}) values of R_0 were obtained.

The maximum and minimum distances between the donors and acceptors (R) were determined from the efficiencies of FRET by using the equations (Forster, 1965)

$$R_{max} = R_0^{max}(1/E - 1)^{1/6} \quad (8)$$

$$R_{min} = R_0^{min}(1/E - 1)^{1/6} \quad (9)$$

Results

Extent and Specificity of Donor–Acceptor Pair Formation. When each of the two C-subunits modified with both donor and acceptor probes was denatured, treated with [^{14}C]IAA, digested with TPCK–trypsin, and separated on HPLC, no radioactivity could be detected in any of the tryptic peptides, indicating that both Cys 199 and Cys 343 had been modified completely by the fluorescent labels. The HPLC profile for one of these pairs, B343–N199, is shown in Figure 2. Since the ANBD label does not undergo fluorescence emission at low pH, only the peptides modified by monobromobimane are detected when fluorescence is directly monitored (middle panel). The fluorescent peaks eluting from the HPLC at approximately 20% acetonitrile were identified as peptides that contained Cys 343 which had been modified by monobromobimane. Both the inactivation of the C-subunit by IANBD (not shown) and the lack of incorporation of [^{14}C]-IAA into B343–N199 (bottom panel) indicated that Cys 199 was modified by IANBD. This is indirectly confirmed by the absence of a significant fluorescent peak eluting a 37% acetonitrile, which would correspond to the Cys 199 peptide being modified by monobromobimane (middle panel). The absorbance spectrum of B343–N199 also is consistent with the formation of C-subunit that was modified at one cysteine residue by IANBD and at the other cysteine residue by monobromobimane (Figure 3). The HPLC profiles for the other donor–acceptor pair, D343–N199, were very similar to those obtained from the B343–N199 donor–acceptor pair, confirming that in this pair Cys 343 was modified by the donor, IAEDANS, and Cys 199 was modified by the acceptor, IANBD.

Integrity of Modified C-Subunits. The capacity of CM-Sepharose CL-6B cation-exchange resin to retain the free C-subunit but not the holoenzyme was utilized to determine whether the modified C-subunits retain their native conformation and are still functionally capable of forming holoenzyme. Although the modifications to the C-subunit may alter the kinetics of C-subunit interaction with the R^{II}-subunit and the rate of dissociation of the holoenzyme by cAMP, all of the modified C-subunits employed in this study were still capable of forming intact holoenzyme. As can be seen in Figure 4, CM-Sepharose CL-6B resin did not bind either donor–acceptor pair when it is complexed with the R^{II}-subunit. In contrast, in the presence of cAMP, the free C-subunit

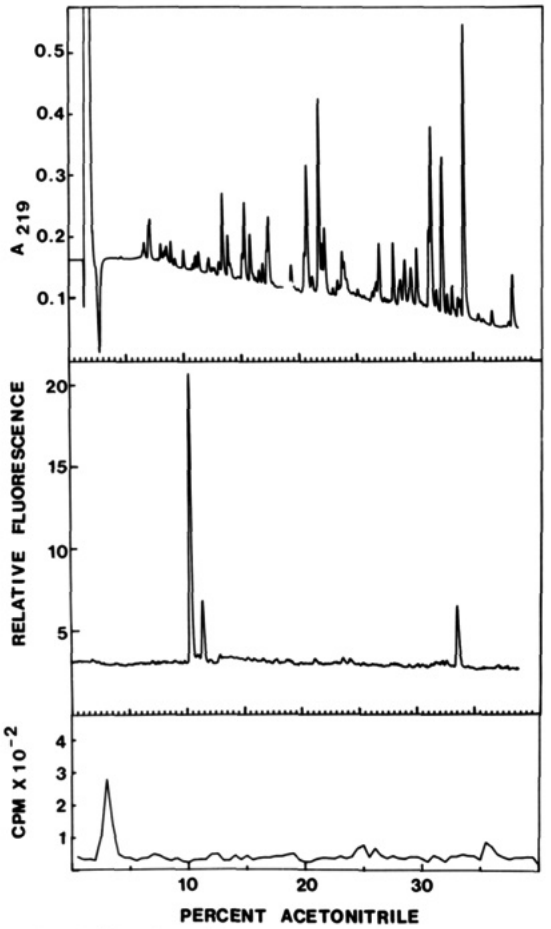

FIGURE 2: Modification of C-subunit at Cys 199 with IANBD and at Cys 343 with monobromobimane. The HPLC separation of a tryptic digest of B343–N199 C-subunit that had been incubated with [^{14}C]IAA prior to digestion is shown. The eluate from the HPLC column was monitored as follows: the upper panel shows the elution profile at A_{219}, the middle panel shows the elution profile of all fluorescent tryptic peptides, and the lower panel shows the elution profile for [^{14}C]IAA incorporation.

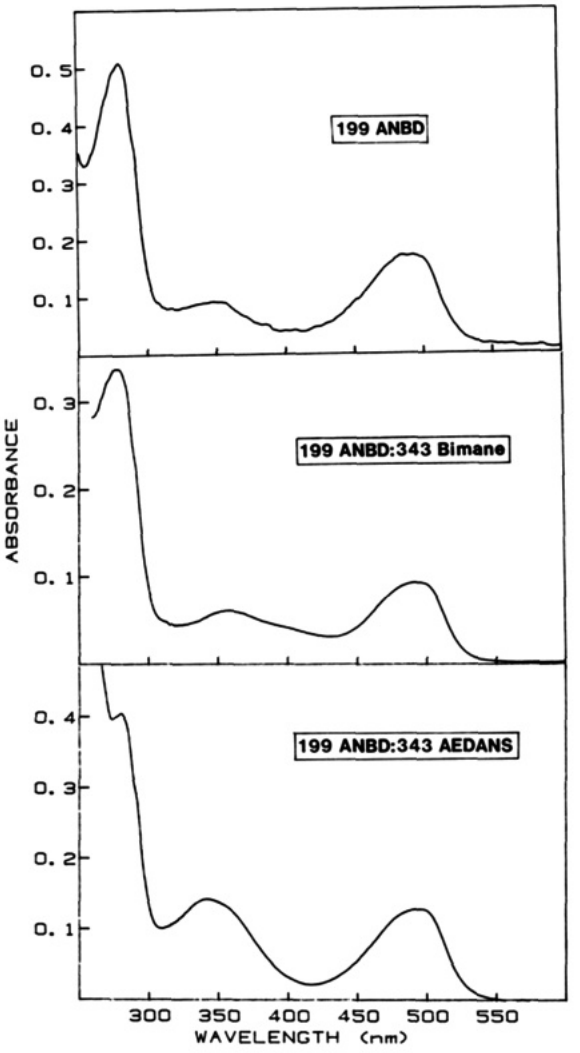

FIGURE 3: Absorbance spectra of modified catalytic subunits: N199 (top panel, 13 μM); B343–N199 (middle panel, 7.5 μM); and D343–N199 (bottom panel, 10 μM). All samples were 10 μM suspended in potassium phosphate and 2 mM EDTA, pH 6.5.

(either B343–N199 or D343–N199) was bound to the resin (lanes 2 and 4).

Calculation of R_0's. The overlaps between the emission spectrum of the donor, either C-subunit modified at Cys 343 by monobromobimane, B343, or C-subunit modified at Cys 343 by IAEDANS, D343, and the absorption spectrum of the acceptor, C-subunit modified at Cys 199 by IANBD, N199, are shown in Figure 5. The respective overlap integrals were calculated to be 3.9 ($\pm$0.5) $\times$ 10^{-14} cm^3 M^{-1} for B343–N199 and 3.3 ($\pm$0.5) $\times$ 10^{-14} cm^3 M^{-1} for D343–N199. The quantum yields for C-subunit modified at Cys 343 by either monobromobimane or IAEDANS were previously determined to be 0.15 ($\pm$0.02) for both modified C-subunits (First & Taylor, 1989). If the labels rotated isotropically and rapidly relative to their fluorescent lifetimes, then κ^2 would take a value of 0.67, and the resulting Forster critical distances calculated from these measurements are 28 Å for the B343–N199 pair and 27 Å for the D343–N199 pair. Using estimates of the minimum and maximum κ^2 values based on axial depolarization factors yields minimum and maximum Forster critical distances of 26 and 40 Å for the B343–N199 pair and 26 and 35 Å for the D343–N199 pair (Table I).

Determination of Efficiency of Fluorescence Resonance Energy Transfer. The steady-state emission spectrum created by numerically adding the donor and acceptor spectra is compared to the emission spectrum obtained for the B343–N199 donor–acceptor pair in Figure 6 (top). Analysis of the donor steady-state quenching (450–480 nm) yielded an effi-

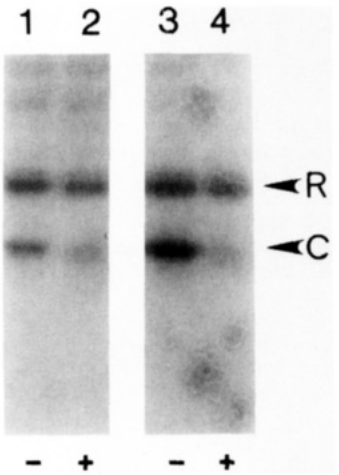

FIGURE 4: Formation of holoenzyme by C-subunits containing the donor–acceptor pairs. Cation-exchange chromatography on CM-Sepharose CL-6B was employed to determine whether or not the donor–acceptor pairs were capable of forming holoenzyme that could be dissociated by cAMP. The holoenzyme used in lanes 2 and 4 contained cAMP. Lanes 1 and 2 contained holoenzyme formed from the B343–N199 C-subunits, and lanes 3 and 4 contained holoenzyme formed from the D343–N199 C-subunits.

Table I: Energy-Transfer Parameters, Efficiency of Energy Transfer, and the Calculated Distances between Cys 199 and Cys 343[a]

donor–acceptor pair	$J \times 10^{14}$ ($cm^3\ M^{-1}$)	$\langle d_D^x \rangle$	$\langle d_A^x \rangle$	Q_D	efficiency of energy transfer	R_0^{min} (Å)	R_0^{max} (Å)	$R_0^{2/3}$ (Å)
C-subunit modified at Cys 343 with bimane and at Cys 199 with IANBD (B343–N199)	3.9 (±0.5)	0.73	0.85	0.15 (±0.02)		24	40	31
					0.20[b]	30	50.4	39
					0.40[c]	26	42.8	33
					0.17[d]	31.3	52.1	40.4
					$X = 0.26$	28.6	47.6	36.9
C-subunit modified at Cys 343 with AEDANS and at Cys 199 with IANBD (D343–N199)	3.3 (±0.5)	0.57	0.85	0.15 (±0.02)		25	38	30
					0.20[b]	31.5	47.9	37.8
					0.10[c]	36.1	54.8	43.3
					$X = 0.15$	33.4	50.7	40.1

[a] The overlap integral J was calculated by using eq 1. $\langle d_D^x \rangle$ and $\langle d_A^x \rangle$ were calculated by taking the square root of the donor and acceptor anisotropies [from First and Taylor (1989)] divided by 0.4, respectively. The donor quantum yields are from First and Taylor (1989). The Forster critical distances were calculated by using a κ^2 factor of 0.67 (R_0), as well as the minimum value for the κ^2 factor (R_0^{min}) and the maximum value for the κ^2 factor (R_0^{max}) calculated by using eq 7 and 6, repsectively. The standard deviations are shown in parentheses. Each of the values for the efficiency of energy transfer represents the mean of three separate samples. The transfer efficiency using lifetime analysis for the D343–N199 is not included because the donor lifetime was distorted by significant contributions from the acceptor emission. The distances between the B343–N199 donor–acceptor pair were calculated on the basis of the efficiency of energy transfer derived from both steady-state quenching of donor fluorescence and steady-state sensitization of acceptor fluorescence. In addition, the distance was calculated by using a value of 0.17 transfer efficiency, which is based on the reduction of donor lifetime. The distances based on an average ($\bar{X}$) of all three methods are also included. The distances between the D343–N199 donor–acceptor pair were calculated by using a transfer efficiency of 0.15, which is the average of the steady-state donor quenching and acceptor sensitization efficiencies. The $R^{2/3}$ distances represent the calculated distance assuming a κ^2 equal to $^2/_3$. The R^{max} and R^{min} distances assume calculated estimates of κ_{max}^2 and κ_{min}^2 based on eq 6 and 7, respectively. [b] Energy transfer determined by steady-state quenching (E_D). [c] Energy transfer determined by steady-state sensitization (E_A). [d] Energy transfer determined by donor lifetime analysis.

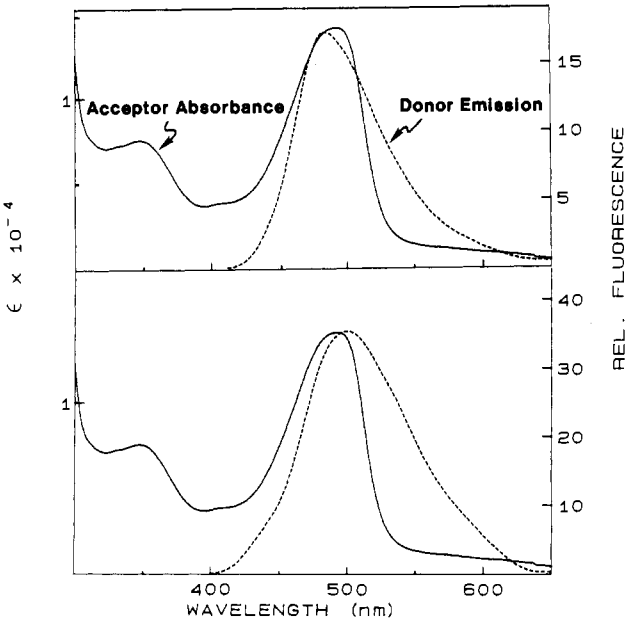

FIGURE 5: Spectral overlap between the emission spectrum of donor and absorbance spectrum of acceptor. Top panel: Spectral overlap for B343–N199. The donor, B343, corrected emission spectrum produced (ex: 390 nm) is indicated by a dashed line. The acceptor, N199, absorbance spectrum is indicated by a solid line. The concentrations of B343 and N199 were 0.6 and 10 μM, respectively. Bottom panel: Spectral overlap for D343–N199. The donor, D343, corrected emission spectrum (ex: 340 nm) is indicated by a dashed line. The acceptor, N199, absorbance spectrum is indicated by a solid line. The concentrations of D343 and N199 were 1 and 10 μM, respectively.

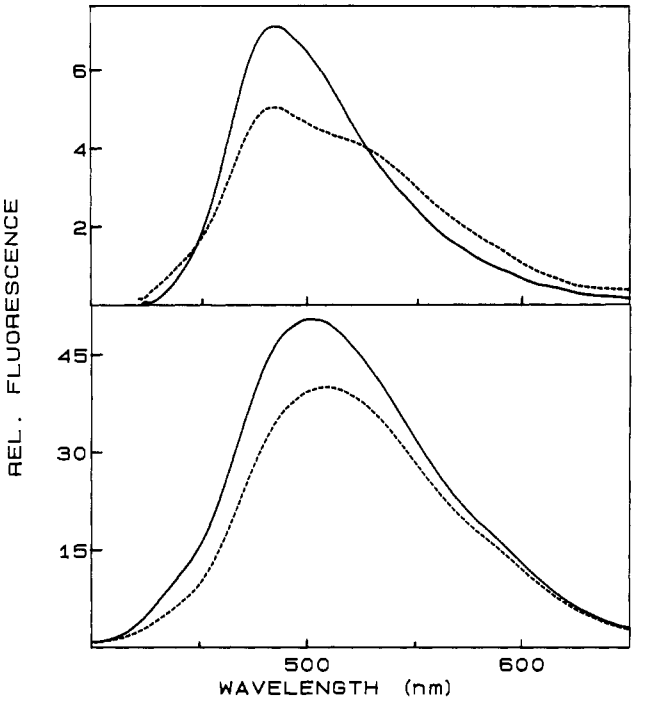

FIGURE 6: Comparison of the emission spectrum for the donor–acceptor pair with the emission spectrum of the isolated donor and acceptor. Top panel: B343–N199. The corrected emission spectrum of the donor–acceptor pair in the B343–N199 C-subunit (dashed line) is compared to the spectrum generated from the addition of the emission spectrum of the isolated donor, B343, and the isolated acceptor, N199 (solid line). All samples were excited at 390 nm and were at a concentration of 0.5 μM. Bottom panel: D343–N199. The corrected emission spectrum of the D343–N199 C-subunit (dashed line) is compared to the spectrum generated from the addition of the emission spectrum of the isolated donor, D343, and the isolated acceptor, N199 (solid line). All species were excited at 340 nm and were at a concentration of 3 μM.

ciency of FRET of 0.2 between the bimane and ANBD labels (Figure 6 and Table I). A value of 0.4 was obtained when acceptor sensitization (530–560 nm) was used to determine the efficiency of FRET. Analysis of donor quenching and acceptor sensitization in D343–N199 gave efficiencies of FRET of 0.2 and 0.10, respectively (Table I). Determination of the efficiency of FRET from the quenching of the donor fluorescence lifetime is shown in Figure 7. In the absence of the ANBD acceptor species, C-subunit modified by monobromobimane at Cys 343 has an weighted average lifetime of 8.6 ns. When ANBD was also attached to the monobromobimane-modified C-subunit, the weighted average fluorescent lifetime was 7.1 ns, indicating that the efficiency of transfer between bimane and ANBD, by lifetime analysis, was 0.17. The efficiency of FRET for D343–N199 determined from the donor lifetime was not reliable because the donor lifetime was distorted by significant contribution from the acceptor emission, and therefore was not included.

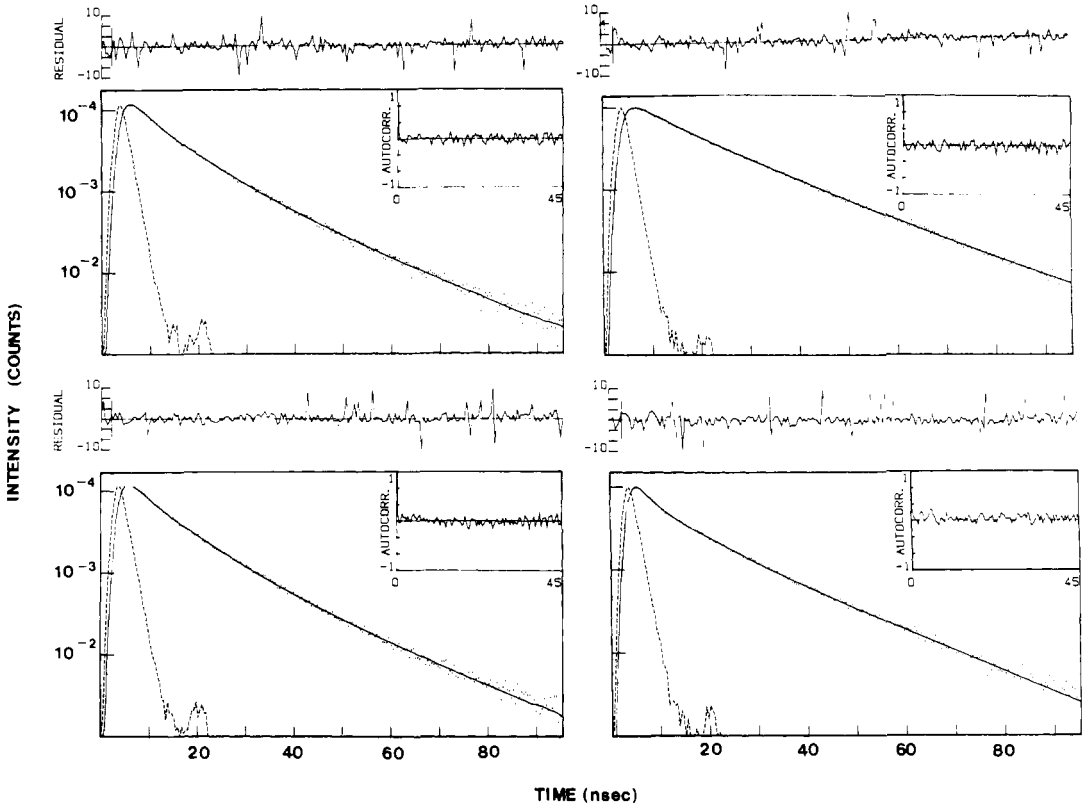

FIGURE 7: Comparison of the donor fluorescence decays for the isolated donor and the donor–acceptor pair. The fluorescence decays for the B343–N199 donor–acceptor pair (lower left), B343 donor (upper left), D343–N199 donor–acceptor pair (lower right), and D343 donor (upper right) are shown. Fluorescence decays and lamp pulses are indicated by solid and dashed lines, respectively. The concentration of all C-subunits was 5 μM. All samples were suspended in 100 mM HEPES, pH 7.3.

The distance between Cys 199 and Cys 343 was calculated for each donor–acceptor pair as described under Experimental Procedures (Table I). For the B343–N199 donor–acceptor pair, the distance calculated from the efficiency of FRET determined from the decrease in the donor lifetime is probably the most reliable, since this value is independent of concentration differences between the donor and the donor–acceptor pair, and there is little contribution from the acceptor emission. The distance obtained by using the efficiency of energy transfer calculated from the donor lifetime (0.17) and assuming a κ^2 value of 0.67 was 40 Å, while the minimum and maximum distances were 31 and 52 Å, respectively. The steady-state donor quenching and steady-state acceptor sensitization for the D343–N199 donor–acceptor pair are in reasonable agreement with each other. The distance between the AEDANS and ANBD labels using the average efficiency of FRET determined by steady-state donor quenching and acceptor sensitization (0.15), and assuming a κ^2 value of 0.67, gave a distance of 40 Å, while the minimum and maximum distances were 33 and 51 Å, respectively.

Effect of the R^{II}-Subunit on the Distance between Cys 199 and Cys 343. In order to determine the distance between Cys 199 and Cys 343 in holoenzyme, the B343–N199 donor–acceptor pair was combined with unmodified R^{II}-subunit to form holoenzyme. The emission spectrum of this holoenzyme was obtained in the presence and absence of cAMP. As can be seen in Figure 8, addition of cAMP slightly alters the emission spectrum of the modified holoenzyme. The actual magnitude of this change is difficult to accurately assess from sensitization of the acceptor emission, since the quantum yield of ANBD attached to Cys 199 of the C-subunit increases by approximately 2-fold when the C-subunit is in the holoenzyme (First & Taylor, 1989), while the quantum yield of the bimane label attached to Cys 343 of the C-subunit in the holoenzyme increases only slightly (5% ± 3%). Consequently, the steady-state donor emission probably more accurately reflects the actual change in the efficiency of FRET between the bimane and ANBD labels when B343–N199 is in holoenzyme compared to when it is free in solution. Assuming the dissociation of the holoenzyme results in a 5% decrease in the quantum yield of the bimane label, the quenching of steady-state donor quenching observed in Figure 8 is 0.11 (±0.03). This corresponds to a change of approximately 4 Å between the bimane and ANBD labels, assuming a κ^2 of 0.67.

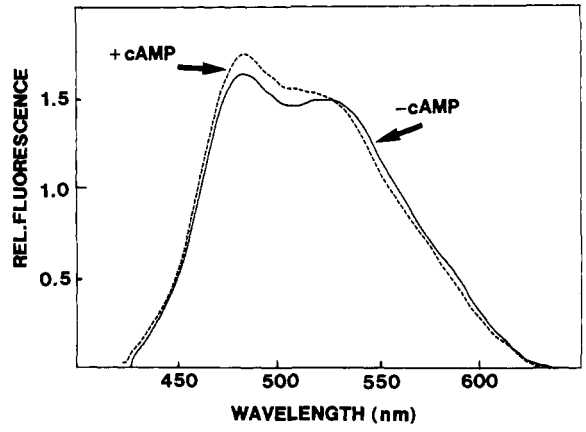

FIGURE 8: Effect of the R^{II}-subunit on the efficiency of energy transfer between the donor and acceptor. Holoenzyme (0.5 μM) formed from B343–N199 C-subunit and unmodified R^{II}-subunit was excited at 390 nm in the presence (dashed line) or absence (solid line) of 0.1 mM cAMP.

Discussion

As previously discussed, the location of Cys 199 in the active site of the C-subunit has been well characterized. In contrast,

relatively little is known about the location of Cys 343 with respect to the active site of the enzyme. The capacity of MgATP to protect this residue from alkylation (Sugden et al., 1976; Armstrong & Kaiser, 1978; Nelson & Taylor, 1981) suggests either that Cys 343 is also near the ATP binding site or that the addition of MgATP induces a conformational change in the C-subunit which prevents this residue from reacting with alkylating reagents. To address this question, we have taken advantage of the different rates of reactivity of these two sulfhydryl groups in the apoenzyme. Specifically, each sulfhydryl group has been selectively labeled with a different fluorescent probe. The probes were chosen such that they could serve as donor–acceptor pairs and thus allow the distance between the two cysteines to be measured by using fluorescence energy transfer techniques (Stryer, 1978).

Two donor–acceptor pairs are described here. For both of these donor–acceptor pairs, Cys 199 is initially modified with the acceptor IANBD. When Cys 343 was subsequently modified with IAEDANS as the donor, the distance between these two cysteine residues was calculated to be between 33 and 51 Å. Alternatively, when Cys 343 was modified with monobromobimane as the donor, the distance between Cys 199 and Cys 343 was calculated to be between 31 and 52 Å. Similar distance measurements were obtained when the acceptor, IANBD, was attached to Cys 343 and either of the two donors was attached to Cys 199 (data not included). These distances are consistent with the prediction of Jimemez et al. (1982), based on the effect of ionic strength on the reactivity of the two cysteines, that the two cysteines are not in close proximity.

The catalytic subunit of cAMP-dependent protein kinase is susceptible to inhibition by several sulfhydryl-specific reagents. Several studies have specifically identified Cys 199 as the residue that is close to the active site of the C-subunit, and inhibition of the catalytic activity appears to be due exclusively to the modification of this cysteine (Jimenez et al., 1982; Nelson & Taylor, 1983; First & Taylor, 1989). Other evidence supports the conclusion that Cys 199 is located in close proximity to the γ-phosphate of ATP (Bhatnagar et al., 1984). Previous studies showed that MgATP protected both Cys 199 and Cys 343 from modification by sulfhydryl-specific reagents (Armstrong & Kaiser, 1978; Nelson & Taylor, 1981). Extended ATP has a maximum length of 16 Å (Kraut, 1963; Stryer, 1981), and the NMR data of Granot et al. (1979) predict either a bidentate α,γ coordination or a tridentate α,β,γ coordination for the bound MgATP. Our results that Cys 199 and Cys 343 are at least 31 Å apart indicate that an ATP-induced conformational change in the C-subunit is responsible for the protection of these cysteines. That this conformational change involves a large portion of the C-subunit is suggested by the distance between the two cysteines (31–52 Å).

Circular dichroism studies indicated that the C-subunit undergoes a substrate-induced conformational change. Reed et al. (1985) showed that the CD spectrum of the C-subunit changes dramatically when the apoprotein is compared to a complex containing both MgATP and a synthetic peptide. The MgATP protection of two residues that are greater than 31 Å apart would be consistent with an extended conformational change of the magnitude seen by circular dichroism. In addition, this predicted conformational change is consistent with the kinetic studies of Whitehouse et al. (1984), who demonstrated that the C-subunit shows a preferred ordered binding pathway, whereby the binding of MgATP induces a change in the protein that enhances the subsequent binding of peptide substrate.

Addition of the R^{II}-subunit was found to slightly alter the efficiency of FRET between fluorescently modified Cys 199 and Cys 343 of the C-subunit. Three possibilities exist for this change in the efficiency of FRET. It is possible that the C-subunit undergoes a conformational change on binding to the R^{II}-subunit which brings Cys 199 and Cys 343 approximately 4 Å closer to each other. Alternatively, the presence of the R^{II}-subunit may simply position the two probes such that they are physically closer together, while leaving the distance between Cys 199 and Cys 343 relatively unchanged. Finally, the R^{II}-subunit may force one or both of the probes to reorient, making FRET from the bimane probe to the ANBD probe more favorable. The last two possibilities seem plausible with respect to the ANBD label attached to Cys 199 since this residue is known to be near the R^{II}-subunit in holoenzyme (Rosen & Ehrlichmann, 1975; First & Taylor, 1989). Regardless of the reason for the spectral shifts resulting from holoenzyme formation, the capacity of the R^{II}-subunit to form holoenzyme with the modified C-subunits supports the assumption that the modified C-subunits retained their functional integrity.

By use of two donor–acceptor pairs the uncertainty that is associated with assuming an orientation factor of 0.67 is reduced. In addition, although an orientation factor of 0.67 was assumed for the FRET calculations, minimum and maximum distances also were calculated by using Forster critical distances determined from the minimum and maximum possible orientation factors. Even when the minimum value for κ^2 is used, the distance between Cys 199 and Cys 343 is greater than 31 Å, lending support to the hypothesis that Cys 343 is not at the MgATP binding site in the C-subunit. This is consistent with the observation that modification of Cys 343 fails to inactivate the C-subunit (Nelson & Taylor, 1983; First & Taylor, 1989).

ACKNOWLEDGMENTS

We are grateful to Brenden McEvilly for technical assistance in the purification of proteins and to Dr. Thomas Vedvick for amino acid analysis. We thank Dr. George Fortas for his helpful discussions.

Registry No. Cys, 52-90-4; MgATP, 1476-84-2; protein kinase, 9026-43-1.

REFERENCES

Armstrong, R. N., & Kaiser, E. T. (1978) *Biochemistry 17*, 2840–2845.

Beebe, S. J., & Corbin, J. D. (1986) in *The Enzymes: Control by Phosphorylation* (Boyer, P. D., & Krebs, E. G., Eds.) Part A, Vol. XVII, Academic Press, Inc., New York.

Bhatnagar, D., Hartle, F. T., Roskoski, R., Jr., Lessor, R. A., & Leonard, N. J. (1984) *Biochemistry 23*, 4350–4357.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bramson, H. N., Thomas, N., Matsueda, R., Nelson, N. C., Taylor, S. S., & Kaiser, E. T. (1983) *J. Biol. Chem. 257*, 10575–10581.

Carlson, G. M., Bechtel, P. J., & Graves, D. J. (1979) *Adv. Enzymol. 50*, 41.

Dale, R. E., & Eisinger, J. (1975) in *Biochemical Fluorescence Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol. 1, Dekker, New York.

Fairclough, R. H., & Cantor, C. R. (1978) *Methods Enzymol. 48*, 347–379.

First, E. A., & Taylor, S. S. (1988) *J. Biol. Chem. 263*, 5170–5175.

First, E. A., & Taylor, S. S. (1989) *Biochemistry* (preceding paper in this issue).

Forster, Th. (1965) in *Modern Quantum Chemistry* (Sinanoglu, O., Ed.) Part 3, Academic Press, New York.
Granot, J., Kondo, H., Armstrong, R. N., Mildvan, A. S., & Kaiser, E. T. (1979) *Biochemistry 18*, 2337.
Jimenez, J. S., Kupfer, A., Gani, V., & Shaltiel, S. (1982) *Biochemistry 21*, 1623–1630.
Kraut, J., & Jenson, L. H. (1963) *Acta. Crystallogr. 16*, 79–88.
Kupfer, A., Gani, V., Jiminez, J. S., & Shaltiel, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3073–3077.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 2*, 431–441.
Nelson, N. C., & Taylor, S. S. (1981) *J. Biol. Chem. 256*, 3743–3750.
Nelson, N., & Taylor, S. S. (1983) *J. Biol. Chem. 258*, 10981–10987.
Nimmo, H. G., & Cohen, P. (1977) *Adv. Cyclic Nucleotide Res. 8*, 146.
Reed, J., Kinzel, V., Kemp, B. E., Cheung, H.-C., & Walsh, D. A. (1985) *Biochemistry 24*, 2967–2973.
Rosen, O. M., & Erlichman, J. (1975) *J. Biol. Chem. 250*, 7788–7794.
Shoji, S., Ericsson, L. H., Walsh, K. A., Fischer, E. H., & Titani, K. (1983) *Biochemistry 22*, 3702–3709.
Spencer, R. D., & Weber, G. (1970) *J. Chem. Phys. 52*, 1654–1663.
Stryler, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.
Stryer, L. (1981) in *Biochemistry*, W. H. Freeman and Co., San Francisco.
Sugden, P. H., Holladay, L. A., Reimann, E. M., & Corbin, J. D., (1976) *Biochem. J. 159*, 409–422.
Taylor, S. S., & Stafford, P. H. (1978) *J. Biol. Chem. 253*, 2284–2287.
Whitehouse, S., Feramisco, J. R., Casnellic, J. E., Krebs, E. G., & Walsh, D. A. (1984) *J. Biol. Chem. 258*, 3693–3701.

Inactivation of Bakers' Yeast Glucose-6-phosphate Dehydrogenase by Aluminum†

Sung-Woo Cho and J. G. Joshi*
Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996-0840
Received October 4, 1988; Revised Manuscript Received January 13, 1989

ABSTRACT: Preincubation of yeast glucose-6-phosphate dehydrogenase (G6PD) with Al(III) produced an inactive enzyme containing 1 mol of Al(III)/mol of enzyme subunit. None of the enzyme-bound Al(III) was dissociated by dialysis against 10 mM Tris-HCl, pH 7.0, containing 0.2 mM EDTA at 4 °C for 24 h. Citrate, $NADP^+$, EDTA, or NaF protected the enzyme against the Al(III) inactivation. The Al(III)-inactivated enzyme, however, was completely reactivated only by citrate and NaF. The dissociation constant for the enzyme–aluminum complex was calculated to be 4×10^{-6} M with NaF, a known reversible chelator for aluminum. Modification of histidine and lysine residues of the enzyme with diethyl pyrocarbonate and acetylsalicylic acid, respectively, inactivated the enzyme. However, the modified enzyme still bound 1 mol of Al(III)/mol of enzyme subunit. Circular dichroism studies showed that the binding of Al(III) to the enzyme induced a decrease in α-helix and β-sheet and an increase in random coil. Therefore, it is suggested that inactivation of G6PD by Al(III) is due to the conformational change induced by Al(III) binding.

Environmental stress such as acid rain has aroused interest in aluminum toxicity (Godbold & Huttermann, 1988). Although a unified hypothesis for aluminum toxicity remains to be established, Al(III) affects diverse cellular functions. For example, it inhibits (i) synaptosomal uptake systems with some selectivity toward the uptake of choline, glutamate, and γ-aminobutyric acid (Lai et al., 1980, 1982; Wong et al., 1981), (ii) in vitro assembly of tubulin into microtubules (Macdonald et al., 1987), and (iii) mitosis in murine cells in tissue culture (Jones et al., 1986). Al(III) affects the brain microsomal protein synthesis in immature rats (Magour & Maser, 1981) and the activity of the regulatory component of adenylate cyclase by fluoride (Northup et al., 1983). Al(III) binds to iron storage proteins such as transferrin (Cochran et al., 1984) and ferritin (Fleming & Joshi, 1987) and induces structural and functional changes in calmodulin (Siegel & Haug, 1983). Al(III) inhibits several enzymes such as acetylcholinesterase (Marquis & Lerric, 1982), Na^+,K^+-ATPase (Lai et al., 1980), ferroxidase (Huber & Frieden, 1970), dihydropteridine reductase (Altmann et al., 1987), and hexokinase from brain and yeast (Womack & Colowick, 1979).

We report here that Al(III) also inactivates yeast glucose-6-phosphate dehydrogenase, the first enzyme in the pentose phosphate pathway. Inactivation of G6PD[1] by specific reagents has shown that lysine and histidine residues are involved in the catalytic activity (Kuby & Roy, 1976; Jeffery et al., 1985; Domashke et al., 1969). We used a similar approach to determine the role of these amino acid residues in the binding of Al(III). The enzyme from yeast was chosen

†Supported by research grants to J.G.J. from the Council for Tobacco Research and from the Robert and Monica Cole Neuroscience Foundation.
* Author to whom correspondence should be addressed.

[1] Abbreviations: DEPC, diethyl pyrocarbonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; ASA, acetylsalicylic acid; CD, circular dichroism.

0006-2960/89/0428-3613$01.50/0