Probing the Multidomain Structure of the Type I Regulatory Subunit of cAMP-Dependent Protein Kinase Using Mutational Analysis: Role and Environment of Endogenous Tryptophans†

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ABSTRACT: The regulatory R-subunit of cAMP-dependent protein kinase (cAPK) is a thermostable multidomain protein. It contains a dimerization domain at the N-terminus followed by an inhibitor site that binds the catalytic C-subunit and two tandem cAMP-binding domains (A and B). Two of the three tryptophans in the RIα subunit, Trp188 and Trp222, lie in cAMP-binding domain A while Trp260 lies at the junction between domains A and B. The unfolding of wild-type RIα (wt-RI), monitored by intrinsic fluorescence, was described previously [Leon, D. A., Dostmann, W. R. G., and Taylor, S. S. (1991) *Biochemistry 30*, 3035 (*1*)]. To determine the environment of each tryptophan and the role of the adjacent domain in folding and stabilization of domain A, three point mutations, W188Y, W222Y, and W260Y, were introduced. The secondary structure of wt-RI and the point mutants has been studied by far-UV circular dichroism spectropolarimetry (CD). The CD spectra of wt-RI and the three point mutants are practically identical, and the thermal unfolding behavior is very similar. Intrinsic fluorescence and iodide quenching in the presence of increasing urea established that: (a) Trp222 is the most buried, whereas Trp188 is the most exposed to solvent; (b) Trp260 accounts for the quenching of fluorescence when cAMP is bound; and (c) Trp222 contributes most to the intrinsic fluorescence of the wt-RI-subunit, while Trp188 contributes least. For wt-RI, rR(W188Y), and rR(W260Y), removal of cAMP causes a destabilization, while excess cAMP stabilizes these three proteins. In contrast, rR(W222Y) was not stabilized by excess cAMP.

The major receptor for cAMP[1] in eukaryotic cells, cAMP-dependent protein kinase (cAPK), is present in all mammalian tissues, and regulates a wide variety of metabolic processes. In the absence of cAMP, cAPK exists as an inactive tetramer consisting of a dimeric regulatory (R) subunit and two catalytic (C) subunits. Binding of cAMP to the R-subunits causes the tetrameric complex to dissociate into an R-dimer and two free C-subunits. In addition to its role as an inhibitor of the C-subunit, the R-subunit anchors the holoenzyme to specific intracellular sites (*2*), and it prevents the C-subunit from entering the nucleus (*3*).

Although several forms of R-subunit are expressed, all have a conserved and well-defined domain structure (*4*). This domain structure, characterized originally by limited proteolysis, was subsequently confirmed using recombinant techniques (*5*). The N-terminus is a requirement for dimerization (*6*−*10*) and for anchoring or docking at specific subcellular locations (*10*−*13*). A dominant feature of this structure is two tandem cAMP-binding domains at the C-terminus, designated as A and B (*14*, *15*). These two cAMP-binding domains, presumably resulting from gene duplication, show extensive sequence similarity and bind cAMP cooperatively (*16*, *17*).

A model for each cAMP-binding domain was proposed based on the crystal structure of the homologous catabolite gene activator protein (CAP) from *E. coli* (*18*), and subsequently the crystal structure of a deletion mutant was determined (*19*).

The two general classes of R-subunits, type I and type II, differ by autophosphorylation, amino acid sequence, tissue distribution, and antigenicity (*19*−*21*). The RIα subunit (Figure 1) contains three tryptophans (*14*). Two of these, Trp188 and Trp222, are located in cAMP-binding domain A. The third, Trp260, lies at the junction between domains A and B, and is a major site of covalent modification by $8N_3$-cAMP (*22*). Also, the RI-subunit exhibits quenching of fluorescence upon cAMP binding (*23*).

Since the three tryptophan residues are located within or near domain A of the RI-subunit, they may provide useful environment-sensitive probes to monitor its unfolding.

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[1] Abbreviations: cAPK, cAMP-dependent protein kinase; C, catalytic subunit; rR, recombinant regulatory subunit; wt-RI, wild-type RIα regulatory subunit; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine 3′,5′-cyclic monophosphate; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism; Trp, tryptophan.

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EXPERIMENTAL PROCEDURES

Mutagenesis. Mutations were introduced using the oligonucleotide-directed in vitro Mutagenesis System (Amersham) in the M13 vector pUC18 that contained the RI-gene (*24*). Oligonucleotide primers were synthesized on an Applied Biosystems DNA Synthesizer. The resulting mutant clones were identified by DNA sequencing of randomly picked plaques following transformation of *E. coli* JMIO1. DNA sequencing was carried out according to the dideoxy method (*25*) using Sequenase (United States Biochemical). The mutant RI-genes were expressed in *E. coli* 222 according to Saraswat et al. (*26*).

Purification of Proteins. The wild-type (wt-RI) and mutant proteins were purified by anion exchange chromatography (*1*) or by affinity chromatography using cAMP−agarose resin (Diller et al., unpublished results). For the second process, cell homogenates were subjected to two ammonium sulfate precipitation steps. The pellet resulting from a first precipitation with 30% ammonium sulfate was discarded, and the ammonium sulfate concentration in the supernatant was increased to 60%. The pellet resulting from this second precipitation was resuspended, incubated overnight with the cAMP-resin, and eluted with an excess of cAMP at room temperature. Recombinant C-subunit (isoform II) was purified as described by Herberg et al. (*27*). Purified proteins were concentrated using Centricon-10 concentrators and stored in 50% glycerol at −80 °C. Protein purity was confirmed by SDS−PAGE in 12.5% acrylamide gels.

Preparation of "Stripped" RIα. To remove the tightly bound cAMP from the R-subunits, purified proteins were incubated with 8 M urea in 5 mM MOPS, 0.5 mM EDTA, l00 mM KCl, and 5 mM β-mercaptoethanol, pH 7.0 (Buffer A), for 3 h. A Pharmacia NAP-10 column, prepacked with Sephadex G-25 and equilibrated with 8 M urea in Buffer A, was used to separate the protein from the cAMP. After pooling the peak fractions containing RI-subunit, urea was removed by dialysis against Buffer A (*28*). Since the RI-subunit is more labile to proteolysis when cAMP is not bound or when it is not part of a holoenzyme complex (*13*), experiments were performed within 2 days after preparing stripped RI-subunit.

Determination of Extinction Coefficients. The absorbance spectra of all protein samples (~0.25 mg/mL) were measured in Buffer A in a Hewlett-Packard Diode Array spectrophotometer. Absorbance scans were performed from 240 to 300 nm with a 1 cm path length cell. Concentrations were determined from either amino acid analysis and/or the Bradford method (Coomassie Protein Assay Reagent, Pierce Biochemicals). Experimental extinction coefficients (ϵ) were calculated using the Beer−Lambert Law.

Circular Dichroism (CD) Wavelength Measurements and Thermal Unfolding. Proteins (0.05 mg/mL) were in 25 mM potassium phosphate buffer and 2 mM EDTA, pH 6.8. Circular dichroism measurements were performed on an AVIV 202 CD spectropolarimeter using a 0.1 cm path length microcuvette (40 μL capacity). CD spectra were scanned at 25 and 80 °C from 260 to 190 nm, at a 0.2 nm resolution and integration time of 4 s. For thermal unfolding curves, samples were scanned at 20 °C/h from 30 to 80 °C using a 20 s integration time. Each measurement was performed in triplicate. Deviations between scans were negligible. Baseline subtraction and conversion of observed ellipticities (mdeg) to mean residue ellipticities (deg·cm^2·dmol^{-1}) was performed using the AVIV CDS program. Fast Fourier Transform filter smoothing of the curves was performed using the Microcal Origin version 3.5 program.

Preparation of Proteins for Fluorescence Measurements. Stock solutions of 8 M urea and 16.7 mM cAMP were prepared in Buffer A and/or Buffer B (Buffer A without KCl). The 8 M urea solutions were used within a week of preparation. The 3 M KI, 3 M NaCl, and 10 mM sodium thiosulfate were prepared in Buffer B. All of the solutions were made with reagent grade or higher grade chemicals and filtered prior to use.

Equilibrium Dialysis. cAMP binding was measured by equilibrium dialysis in a 250 μL eight-cell chamber apparatus (Hoefer Scientific). The binding reaction mixture (200 μL) containing RI-subunit dimer (1 μM) and cAMP (4 μM) was placed in one side of the dialysis membrane, and 200 μL of binding buffer (8 μM [^{3}H]cAMP, in 100 mM MOPS, 5 mM β-mercaptoethanol, 1 mM EDTA, and 150 mM KCl) was on the opposite side. The RI-subunit dimer was denatured with 4.5 M urea for 3 h, and then incubated in the reaction chamber at 25 °C for 16 h. Ten microliter aliquots were transferred into scintillation vials and counted in 5 mL of Ecolume (ICN). All measurements were done in duplicate.

Urea Unfolding of the RI-Subunits. Proteins (0.5−1.0 μM) were unfolded in various concentrations of urea (0−8 M) for 3 h at room temperature. Overnight incubation produced no additional changes in the fluorescence emission. Fluorescence measurements were carried out in 1 cm quartz cuvettes at 23 °C using a Hitachi F4010 fluorometer. Samples were excited at 293 nm, and tryptophan emission was monitored from 300 to 450 nm. Fractional unfolding curves were constructed assuming a two-state model, and using the following relationships:

$$F_N + F_U = 1$$

$$F_U = 1 - [(R - R_U)/(R_F - R_U)]$$

where F_U is the fraction of unfolded protein, F_N is the fraction of folded protein, R is the observed ratio of intensity at 353/340 nm at various urea concentrations, and R_F and R_U represent the values of R for the folded and unfolded states, respectively.

Potassium Iodide (KI) Quenching. Fluorescence measurements were carried out on samples containing the following: RI-subunit (0.5 μM), KI (0−0.3 M), and selected urea concentrations (0, 3, and ~7 M). KI was dissolved in Buffer B. Since I_3^- absorbs in the region of tryptophan fluorescence, a small amount of sodium thiosulfate (0.1 mM) was added to the KI solution. Ionic strength ($\mu = 0.3$) was kept constant with NaCl. The fluorescence quenching data were plotted using the Stern−Volmer equation: $F_o/F = 1 + K[X]$, where F_o/F is the fractional decrease in fluorescence due to quencher X and K is the Stern−Volmer quenching constant (*29*). The fraction of tryptophan fluorescence quenched was then estimated by plotting the fluorescence quenching data against the quencher concentration using the modified Stern−Volmer equation:

$$F_o/\Delta F = 1/f_a K[Q] + 1/f_a$$

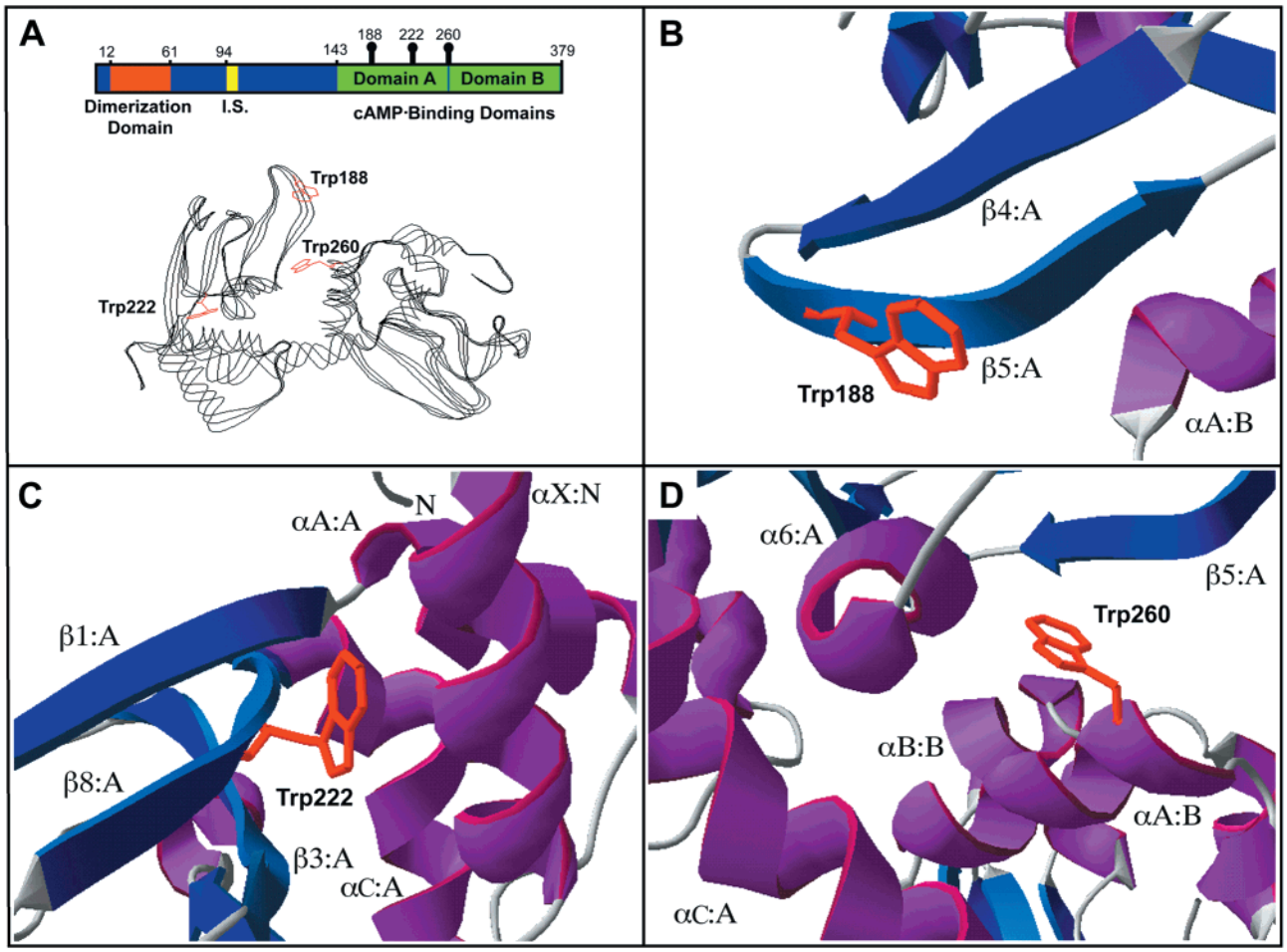

FIGURE 1: Summary of the domain structure and tryptophan mutants of the type I regulatory subunit of the cAMP-dependent protein kinase, based on the crystal structure of the RIα subunit (PDB accession code 1RGS). (A, top) Schematic diagram showing the location of the N-terminal dimerization domain (red), autoinhibitor region (yellow), cAMP-binding domains A and B (green), and mutation sites on residues Trp188, Trp222, and Trp260. (A, bottom) Ribbon diagram showing the relative location of tryptophans 188, 222, and 260. (B) Trp188 is located on a surface loop between β-strands β4:A and β5:A. (C) Trp222 is located on the β8:A β-strand, deeply buried in cAMP-binding domain A. (D) Trp260 is located on the αA:B helix, which is located in the cAMP-binding domain B.

where F_o is the fluorescence in the absence of quencher, ΔF is F_o minus the fluorescence observed in the presence of a quencher, f_a is the fraction of accessible tryptophans, K is the Stern−Volmer constant of quenching, and [Q] is the molar concentration of quencher (*30*).

RESULTS

Protein Purification. A ribbon diagram based upon the crystal structure of the RIα regulatory subunit of protein kinase A is shown in Figure 1A. Three mutants in which tryptophan residues 188, 222, and 260 were replaced by tyrosines (Figure 1A cartoon) as well as the wt-RI-subunit and cAMP-stripped R-subunit were prepared as described under Experimental Procedures. Proteins were purified to apparent homogeneity, based on SDS−PAGE. Typical yields were 8−15 mg/L of cell culture.

General Physical Properties of the Tryptophan Mutants. Since tryptophan and tyrosine side chains have similar average buried volumes and hydrophobicities, the amino acid replacement was not expected to significantly disrupt the native folding (*31*, *32*). Tryptophan replacement by tyrosine will, however, affect the absorbance. Therefore, the molar extinction coefficients at 280 nm, ϵ_{280} (M^{-1} cm^{-1}), for the native and mutant proteins were determined (Table 1). The observed 260/280 nm ratios were high because the cAMP-binding sites remain fully saturated when the protein is purified.

Table 1: Absorption Properties of Wild-Type and Mutant RI-Subunits[a]

protein	A^{280}	280 nm/260 nm	theoretical ϵ_{280} (M^{-1} cm^{-1})	observed ϵ_{280} (M^{-1} cm^{-1})
$R_2(cAMP)_4$	1.05	0.9	7.3×10^4	1.00×10^5
stripped RI	0.96	2.1	6.5×10^4	9.2×10^4
W188Y	1.00	1.2	6.6×10^4	4.7×10^4
W222Y	0.76	1.4	6.6×10^4	7.3×10^4
W260Y	0.79	1.2	6.6×10^4	7.6×10^4

[a] The A^{280}, ϵ_{280}, and 280/260 nm determinations were performed in Buffer A. The protein concentration (1.0 mg/mL) was based on amino acid analysis.

Holoenzyme Formation. All three mutant R-subunits formed holoenzyme when dialyzed with C-subunit at a rate that was the same as wild-type RIα (data not shown). The holoenzymes were activated completely after the addition of cAMP. All K_a's for activation by cAMP were less than 1 μM.

Evaluation of Changes in Secondary Structure by Circular Dichroism. To evaluate whether the mutant RI-subunits had similar global secondary structures, the far-UV CD spectrum

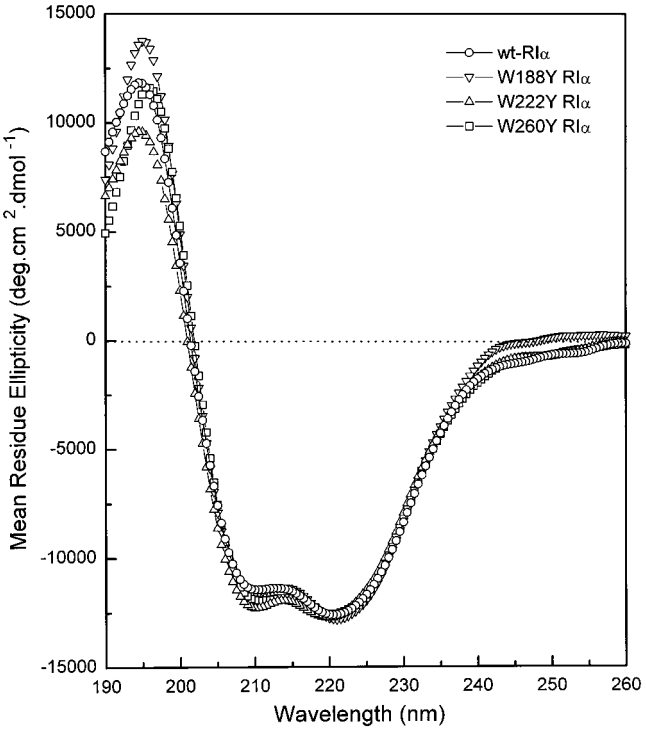

FIGURE 2: Low-temperature circular dichroic spectra of wild-type and tryptophan mutant proteins. The circular dichroic spectrum of each protein is designated as follows: wild-type RIα (○), W188Y (▽), W222Y (△), and W260Y (□). Protein concentration was 0.05 mg/mL. The circular dichroic spectrum was scanned from 260 to 190 nm at 25 °C. Mean residue ellipticity is shown as a function of wavelength.

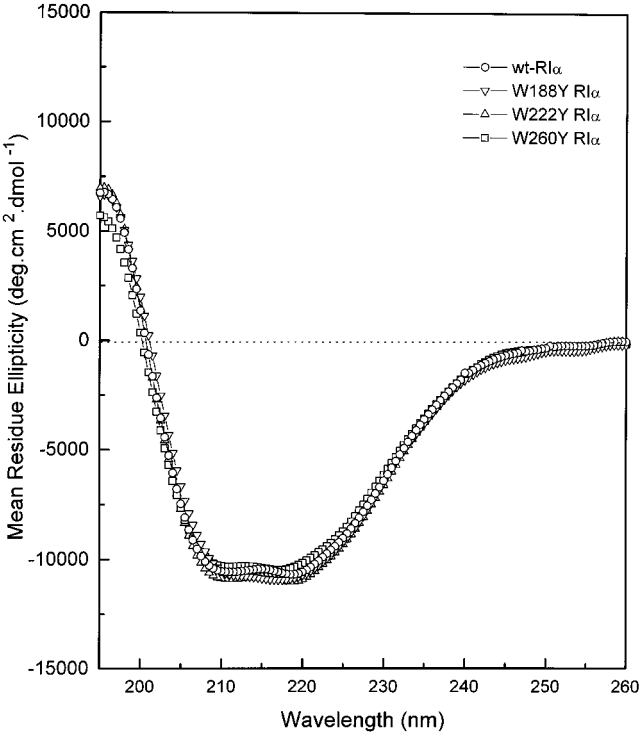

FIGURE 3: High-temperature circular dichroic spectra of wild-type and tryptophan mutant proteins. The circular dichroic spectrum of each protein is designated as follows: wild-type RIα (○), W188Y (▽), W222Y (△), and W260Y (□). Protein concentration was 0.05 mg/mL. The circular dichroic spectrum was scanned from 260 to 190 nm at 80 °C. Mean residue ellipticity is shown as a function of wavelength.

of each protein was measured as indicated. The CD spectra of the wild-type R-subunit and the tryptophan mutants showed two minima at 209 and 222 nm, indicating a high content of α-helix. At 25 °C, the mutants had CD spectra practically identical to that of the wild-type protein (Figure 2); therefore, it was concluded that the global secondary structures of the mutant proteins were not affected in a major way by the point mutations. The spectra of the mutants at high temperature (Figure 3) were also very similar.

Even in the absence of detectable differences in secondary structure, thermal denaturation is a sensitive method that permits the detection of changes in the cAMP-binding properties of the R-subunits (unpublished observations). Therefore, we resorted to this technique to assess possible changes in the cAMP-binding properties of the tryptophan mutants.

Estimation of Thermal Stability by CD. The thermal stability of the point mutants was assessed from CD thermal unfolding profiles recorded at 222 nm. The wt-RI-subunit showed a remarkable thermal stability: even at 80 °C it stayed in solution and its secondary structure was still partly α-helical (Figure 3).

Figure 4 shows the thermostability of stripped wt-RI in the presence or absence of cAMP compared to that of native wt-RI and the tryptophan mutants. Removal of cAMP (stripping) from the dimeric wt-RI resulted in a biphasic profile, corresponding to precipitation of the protein, with a T_m of 45 °C. Addition of cAMP to the stripped wt-RI-subunit produced a shift of the T_m from 45 to 62 °C. Although the loss of thermal stability produced by stripping could be partially corrected by the addition of cAMP, the protein did not remain in solution at high temperature. Additional experiments under conditions which perturbed the binding of cAMP but had no effect on the secondary structure of the protein as evaluated by CD, like lowering the pH to 4.0, showed the same loss of thermal stability (results not shown).

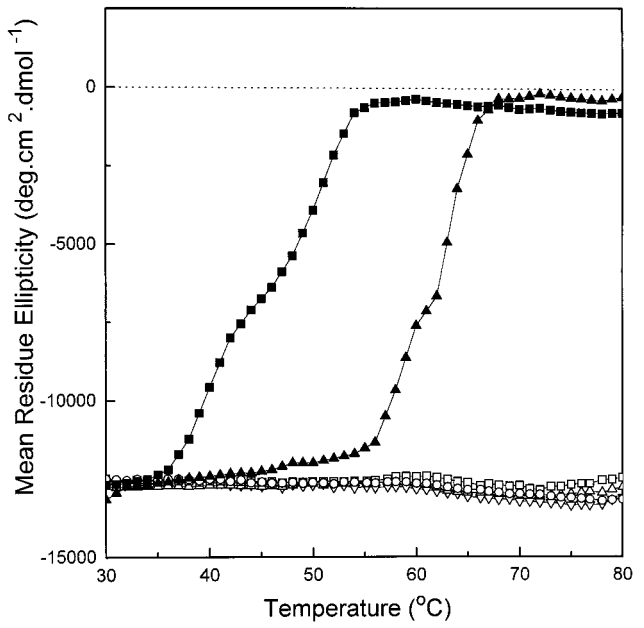

FIGURE 4: Thermal denaturation profiles of wild-type, stripped, and tryptophan mutant proteins. Wild-type RIα (○), W188Y (▽), W222Y (△), W260Y (□), and stripped wild-type RIα without (■) and with cAMP (▲). Samples (0.05 mg/mL) were scanned at 20 °C/h from 30 to 80 °C using a 20 s integration time. Ellipticity was monitored at 222 nm.

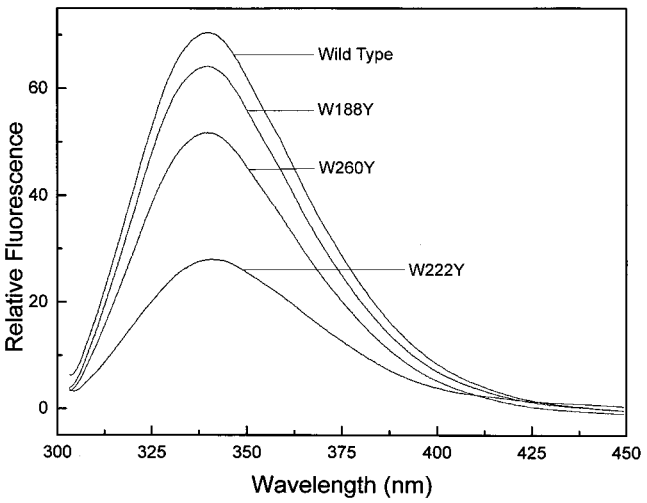

FIGURE 5: Comparison of the fluorescence emission spectra of wild-type and tryptophan mutants of the RI-subunit. Samples (0.5 μM) containing wild-type RIα, and the W188Y, W260Y, and W222Y mutants were excited at 293 nm at 23 °C. The buffer contained 5 mM MOPS, pH 7.0, 0.5 mM EDTA, 100 mM KCl, and 5 mM β-mercaptoethanol. Each protein (0.5 μM R-dimer) was saturated with cAMP.

The thermal denaturation scans at pH 4 showed a transition with a T_m of 62 °C for RI at low pH (not shown), which was in agreement with the T_m of stripped RI after the addition of cAMP.

On the other hand, the wt-R-subunit and the tryptophan mutants stayed in solution, supporting the notion that the introduction of the tryptophan point mutations is not significantly altering the thermostability of the R-subunit.

Comparison of the Fluorescence Emission Spectra. The fluorescence emission from a protein depends on both the number of tryptophans and the environment of each tryptophan. Normally, each tryptophan does not contribute equally to the total protein fluorescence. The three tryptophan mutants allowed us to determine the contribution that each tryptophan makes to the overall fluorescence of the native RI-subunit. All showed a maximum fluorescence emission at 340 ± 2 nm, and each tryptophan to tyrosine mutant had less fluorescence than the wild-type RI-subunit (Figure 5). The contribution from each tryptophan in the native protein was as follows: Trp188, 10%; Trp222, 65%; and Trp260, 25%.

Effect of cAMP on Intrinsic Fluorescence of the Native R-Subunit. To determine how cAMP binding affects the fluorescence of each mutant, the wild-type and mutant proteins were stripped of endogenous cAMP as described previously (*28*). This was necessary because the R-subunits, when isolated, remain fully saturated with cAMP. Using 0.25 μM stripped R-subunit, maximum fluorescence quenching was obtained with 1 μM cAMP. This indicates that all four cAMP-binding sites in the R-dimer were functional. The K_d's were also less than 1 μM.

Approximately 25−30% of the fluorescence of the stripped wild-type RI-subunit was quenched by cAMP (Figure 6). The stripped tryptophan mutants W188Y and W222Y both exhibited quenching similar to the native protein, but only 5% quenching was observed for the W260Y mutant. Therefore, it was concluded that cAMP specifically quenched the fluorescence associated with Trp260. In the presence of cAMP, the λ_{max} of wild-type RI, W188Y, and W222Y, but not W260Y, exhibited a small shift to the blue, indicating that the environments of Trp260 became more hydrophobic.

Denaturation with Urea. The changes in intrinsic protein fluorescence of the various R-subunits using urea as a denaturant are shown in Figure 7. The unfolding process can be divided into two components. First, between 0 and 3 M urea, there was an increase in the fluorescence intensity at 340 nm with no major shift in wavelength. Second, between 3 and 8 M urea, there was a shift in the λ_{max} from 340 to 353 nm, corresponding to the global unfolding of the protein and a loss of cAMP.

As shown previously (*1*), the fluorescence intensity of the unfolded wt-RI was greater than the intensity of the native form. This kind of behavior is opposite to what is observed in most proteins (*33*). For the wt-RI, there was a 35% increase in fluorescence as the urea concentration increased to 3 M. As shown previously, this did not correspond to a release of bound cAMP since cAMP binds with the same high affinity in the presence of 3 M urea (*1*). The W188Y and W260Y mutants showed a 25% and a 12% increase in fluorescence, respectively. In contrast, for the W222Y mutant, there was a 3% decrease in fluorescence when the urea concentration increased to 3 M.

The mutation of residue Trp188 did not significantly alter the increase in fluorescence observed in the wt-RI-subunit, which implied that residues Trp222 and Trp260 were mostly responsible for that increase. The effect of mutations on residues Trp260 and Trp222 indicated that the increase in fluorescence intensity at 3 M urea was mainly due to changes in the environment surrounding Trp222 (Figure 7). There were minor but consistent changes in λ_{max} during this first phase of the unfolding process. There were small shifts to blue (1.5 and 1 nm) for the W188Y and W260Y mutants, and a shift to red (5 nm) for the W222Y (indicated by arrows in Figure 7) when the urea concentration was increased. Since each mutant is in fact monitoring the environment surrounding the two remaining tryptophan residues, the observed shifts in λ_{max} ($\Delta\lambda_{max} = \lambda_{max}^{3M} - \lambda_{max}^{0M}$) are the sum of the shifts corresponding to both residues. This can be systematized in the following equation system:

$$\Delta\lambda_{max}^{W188Y} = \Delta\lambda_{max}^{W222} + \Delta\lambda_{max}^{W260} = -1.5 \text{ nm}$$

$$\Delta\lambda_{max}^{W222Y} = \Delta\lambda_{max}^{W188} + \Delta\lambda_{max}^{W260} = +5 \text{ nm}$$

$$\Delta\lambda_{max}^{W260Y} = \Delta\lambda_{max}^{W188} + \Delta\lambda_{max}^{W222} = -1.5 \text{ nm}$$

which performing Gaussian elimination gives $\Delta\lambda_{188} = +2.75$ nm, $\Delta\lambda_{222} = -3.75$ nm, and $\Delta\lambda_{260} = +2.25$ nm. Thus, the environments surrounding Trp188 and Trp260 become more polar whereas the surroundings of W222 become more hydrophobic, therefore explaining the increase in the emission of Trp222 and the unusual behavior of the RI regulatory subunit at low urea concentrations.

During the second phase of the unfolding process (at urea concentrations over 3 M), the W188Y and W260Y mutants showed a decrease in fluorescence intensity with respect to control conditions when they are unfolded, while W222Y showed the opposite behavior. Unfolding of the W222Y was accompanied by an increase in fluorescence intensity, which implied that the unusual behavior of RI at higher urea

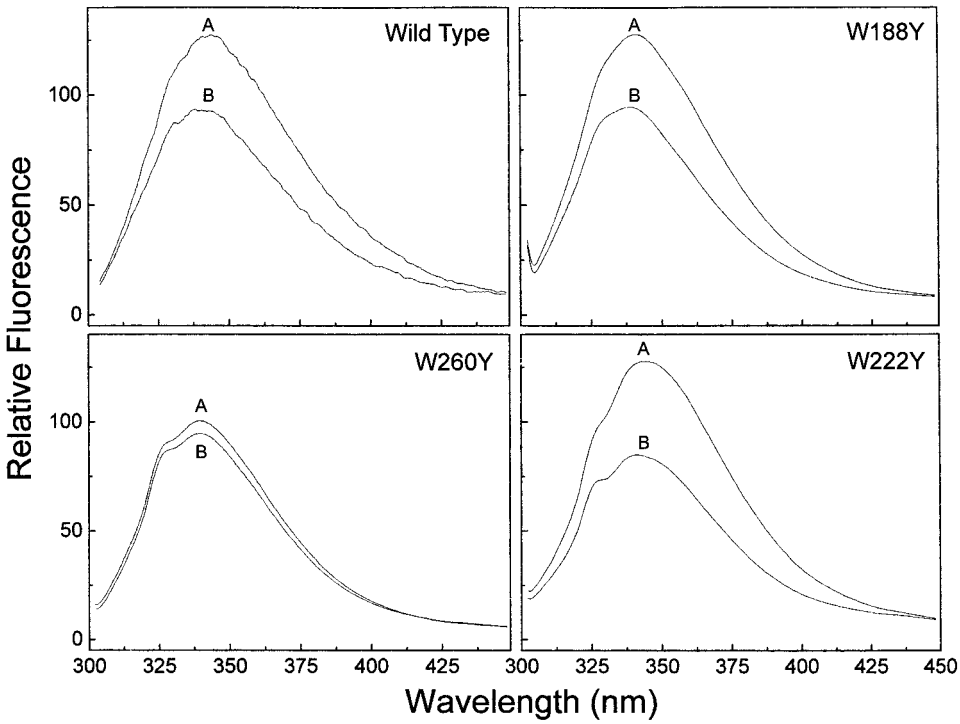

FIGURE 6: Fluorescence spectra of stripped RI-subunit in the absence and presence of cAMP. Each designated RI-subunit (0.5 μM R-dimer) was denatured, stripped of cAMP, and renatured as described under Experimental Procedures. The fluorescence emission was then measured in the absence (A) and presence (B) of 150 mM cAMP in Buffer B, pH 7.0.

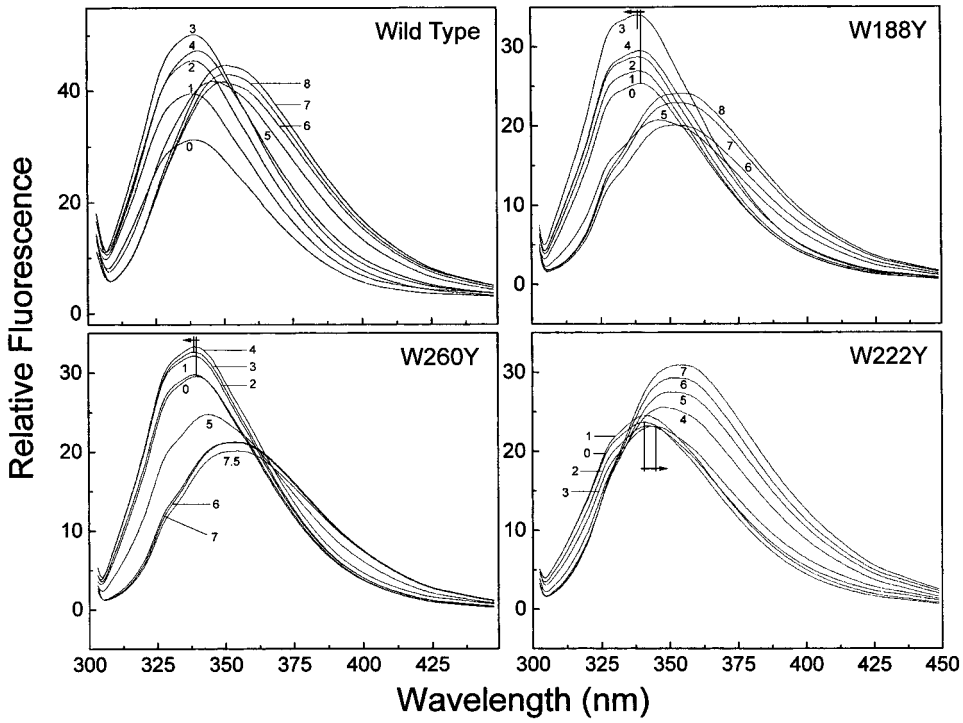

FIGURE 7: Tryptophan fluorescence spectra of the wild-type and mutant RI-subunits at various urea concentrations. The urea concentrations are indicated on each spectrum. The protein samples (0.5 μM) were excited at 293 nm at 23 °C in Buffer B, pH 7.0. Arrows in the W188Y, W222Y, and W260Y panels indicate the direction of the shift in λ_{max} that accompanies the increase in urea concentration from 0 to 3 M.

concentrations was due to either Trp188 or Trp260. The Trp188 mutant showed practically no difference in fluorescence intensity between the folded and unfolded forms, which is consistent with the peripheral location of the residue according to the crystal structure. On the other hand, the unfolded Trp260 mutant showed a marked decrease in fluorescence with respect to the folded form, which suggests that this residue is responsible for the unusual spectroscopic properties of the wt-RI protein at high urea concentrations.

The purified wt-RI dimeric subunit contains four cAMP-binding sites that remain fully saturated despite exhaustive dialysis, and 8 M urea is required to fully strip cAMP from the proteins (*27*). The tryptophan mutations did not decrease the binding of cAMP to any of these purified mutant proteins.

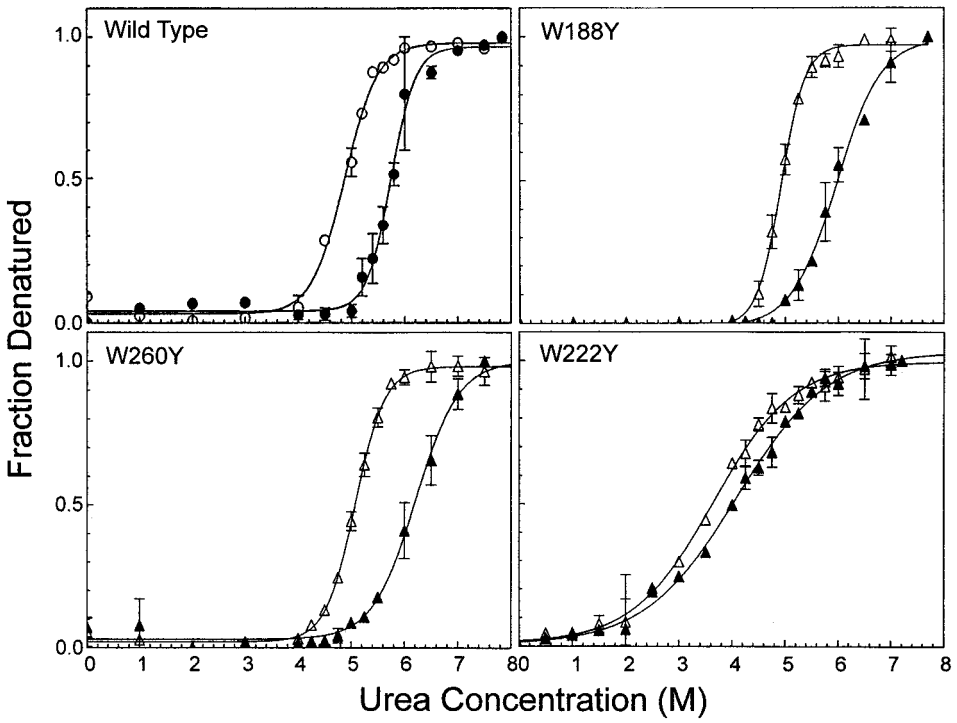

FIGURE 8: Unfolding curves for the RI-subunits as a function of urea concentration. The data were taken from the fluorescence spectra in Figure 7. The R-subunit dimer proteins (0.5 μM) were denatured in the absence of excess cAMP (hollow symbols) or in the presence of 150 mM excess cAMP (filled symbols).

Although CD results strongly suggested that there was no change in the cAMP-binding properties of the tryptophan mutants, this point was confirmed by adding excess cAMP to the mutant R-subunits in the absence of urea (results not shown). If both cAMP-binding sites were not fully saturated, a quenching of fluorescence would be expected, as previously shown with the stripped proteins. Conversely, rR(W188Y) and rR(W222Y R) subunits showed no additional decrease in fluorescence in the absence of urea when a 150-fold excess of cAMP was added. The W260Y mutant could not be monitored in this way because of the reduced quenching observed (maximum quenching was only 5% of the original fluorescence signal). Thus, the observed increase in fluorescence at low levels of urea was indeed distinct from the increase in fluorescence that occurred when cAMP was stripped from the R-subunit.

The second phase of the unfolding process correlated with the shift in λ_{max} of the fluorescence spectra as the urea concentration increased to 8 M. This global unfolding was monitored by measuring the ratio of intensities at 353/340 nm, which would correspond to the fraction of unfolded protein at a certain concentration of urea. The C_m (midpoint concentration at which 50% of the protein is unfolded), a gauge of the intrinsic stability of the protein, was calculated from curves that plotted the protein unfolded fraction vs the concentration of urea (Figure 8). C_m's were between 4.9 and 5.0 M, except rR(W222Y), which was 3.6 M (Table 2).

The unfolding curve for rR(W222Y) showed less cooperativity than those curves corresponding to the other two tryptophan mutants and the wt-RI-subunit. The fact that the unfolding curve for W222Y was less cooperative indicated that the unfolding of the areas surrounding Trp188 and Trp260 was not cooperative. Removal of Trp222 in the W222Y mutant removed the probe monitoring the region that unfolds with more cooperativity, therefore yielding a poorly cooperative unfolding curve, which reflects the behavior of the regions monitored by Trp188 and Trp260. Removal of the Trp188 or Trp260 residues in the W188Y and W260Y mutants did not affect the high cooperativity of the unfolding observed in the wild-type protein, thus confirming the importance of the residues surrounding Trp222 to explain the cooperativity of unfolding of domain A.

Table 2: Midpoint Concentrations (C_m) of Unfolding of Wild-Type and Mutant Forms of the RI-Subunit of cAMP-Dependent Protein Kinase[a]

protein	C_m(M)	C_m (M) + 150 μM cAMP
RI-subunit	5.0 (±0.2)	5.8 (±0.2)
RI-subunit stripped	3.8 (±0.1)	5.8 (±0.2)
rR(Wl88Y)	4.9 (±0.2)	5.9 (±0.2)
rR(W222Y)	3.6 (±0.1)	4.0 (±0.1)
rR(W260Y)	5.0 (±0.1)	6.0 (±0.2)

[a] The midpoint concentrations (C_m) were all calculated from data in Figure 8. The unfolding was carried out using purified proteins where the cAMP binding sites were saturated (−cAMP) and also in the presence of 150 μM excess cAMP (+cAMP). Protein concentration was 0.5 μM R-dimer.

To establish the correlation between unfolding and cAMP release, it was necessary to develop a method for measuring cAMP binding under conditions that closely matched those used for unfolding. It was previously established using a filtration assay and 20 nM protein that cAMP did not bind to wt-RI at urea concentrations above 4 M (*1*). The filtration assay is thus inadequate for samples with high levels of cAMP, or high protein concentrations. Therefore, equilibrium dialysis was used to measure cAMP binding and also to mimic the exact protein concentrations used for unfolding. Equilibrium dialysis was carried out using 0.5 μM R-subunit in the presence and absence of 4.5 M urea. The cAMP-

Table 3: Iodide Quenching of Tryptophan Fluorescence of the RI-Subunits[a]

protein	urea concentrations		
	0 M	3 M	7 M
RI-subunit	0.3	0.8	1.0
rR(Wl88Y)	0.1	0.3	1.0
rR(W222Y)	0.3	0.7	0.8
rR(W260Y)	0.3	0.6	1.0

[a] Iodide quenching in the presence of 0, 3, or 7 M urea was expressed as the fraction of tryptophan fluorescence susceptible to iodide quenching, as calculated from the modified Stern−Volmer plot.

binding sites remained fully saturated in the absence of urea whereas 80% of the cAMP-binding sites were saturated in 4.5 M urea, which indicated a correlation between unfolding and cAMP release.

Stabilization of Folded Structure by cAMP. The C_m's calculated for the purified proteins reflected full saturation with cAMP. Therefore, the effect of different cAMP concentrations (or no cAMP bound, in the case of the stripped wt-R-subunit) on the stability of R mutants was explored. When the unfolding of stripped wt-RI was determined, the C_m shifted significantly from 5.0 to 3.8 (not shown). Excess cAMP, on the other hand, was shown to further stabilize the wt-RI-subunit, causing the C_m to shift to 5.8 M (Figure 8).

The effect of excess cAMP on each of the tryptophan mutants was subsequently determined. The unfolding curves were plotted for each protein in the presence of 150 μM cAMP, that is, a >10-fold excess over the bound cAMP (2 μM) (Figure 8). wt-RI, rR(W188Y), and rR(W260Y) all showed a comparable increase in stability when a high concentration of cAMP was used. The single exception was rR(W222Y), which showed a marginal shift in C_m from 3.6 to 4.0 M urea.

Next, to confirm the stabilizing effect of cAMP in the presence of denaturant, wt-RI was incubated with 4.6 M urea, a concentration where approximately half of the protein was unfolded, and increasingly higher concentrations of cAMP were added. The midpoint concentration of cAMP required to shift the λ_{max} from 353 nm back to 340 nm was 25 μM (not shown).

Iodide Quenching. Solvent accessibility of the tryptophan residues at various stages of unfolding was monitored by iodide quenching. The changes in fluorescence as a function of iodide concentration were monitored at 340 nm in the presence of 0 or 3 M urea, and at 353 nm in the presence of 7 M urea. The addition of increasing concentrations of iodide induced a 1−3 nm blue shift. In the absence of denaturant, 30% of the fluorescence of the wild-type RIα subunit was quenched by iodide (Table 3). In 3 M urea, 80% was quenched, while in 7 M urea all of the fluorescence was quenched by iodide.

Each mutant was then characterized. The mutant showing the most protection was rR(W188Y), since in the absence of urea only 10% of the fluorescence was quenched, implying that both of the remaining tryptophans were mostly buried. Even in 3 M urea only 30% of the fluorescence of the remaining two tryptophans was quenched. For rR(W222Y), 30% of the fluorescence was quenched in the absence of urea. At 3 M urea, 70% of the fluorescence of its two tryptophans was quenched. Finally, at 7 M urea, 80% of the fluorescence was quenched by iodide. For rR(W260Y), 30% of the fluorescence was quenched in the absence of urea and 60% was quenched in 3 M urea. Not until the protein fully denatured in 7 M was all of the fluorescence quenched by the presence of 300 mM iodide.

DISCUSSION

Both physical and genetic approaches can help to unravel the factors involved in organizing a polypeptide chain into a functional stable structure. Certainly, the structural integrity of a full-length protein becomes more complex when that protein is composed of multiple well-defined domains, some of which can potentially be stabilized by extrinsic ligands. Such is the case for the R-subunits of cAPK. To evaluate the unfolding process, as well as the structural integrity of individual domains, the unfolding of mutant forms of RI-subunit, lacking selective tryptophans, was characterized. These mutants also allowed for the characterization of each tryptophan in terms of its solvent accessibility, its proximity to cAMP, and its role in stabilizing the RI-subunit. They also provide a basis for determining how cAMP binding contributes to the overall conformational stability of the RI-subunit.

The use of probes to study biological systems relies on the assumption that the modifications introduced in the system are not significantly perturbing it, and that the observations can be extrapolated to the native system. Our CD observations indicate that mutation of the three tryptophans does not alter the secondary structure of the R-subunits in a detectable way, and that the mutations are not producing any significant alteration in the protein thermal stability. Minor differences in the thermal unfolding profiles can be attributed to the differences in CD of the tryptophan groups in the far-UV region. Therefore, it is reasonable to conclude that the introduction of tryptophan mutations in RI did not alter in a noticeable way either its secondary structure or its thermal stability.

CD experiments and fluorescence experiments also substantiated the fact that there were no major differences between the cAMP-binding properties of the mutant proteins and the wild-type R-subunit. Since the modified proteins are virtually identical to the wt-RI-subunit in any aspect that we studied, we must conclude that the use of tryptophan mutations is a valid method to monitor the urea-induced unfolding of the cAMP-binding domains of RI while allowing the extrapolation of our observations to the native protein.

For the native RI-subunit, the fluorescence emission maximum at 340 nm upon excitation at 293 nm was due to emission from three tryptophans: Trp188, Trp260, and Trp222. When the RI-subunit was stripped of cAMP, the fluorescence increased (*1, 23*). When the mutant RI-subunits were stripped of bound cAMP, each gave a similar emission maximum near 340 nm. The addition of cAMP quenched the fluorescence of W188Y and W222Y by 25−30%, similar to the wild-type RI-subunit. However, in the W260Y mutant, the fluorescence emission was only slightly quenched upon cAMP addition. The fluorescence quenching induced by cAMP binding can, therefore, be attributed specifically to Trp260. This is consistent with affinity labeling results, which showed that Trp260 is covalently modified by $8N_3$-cAMP

bound to site A (*22*). Also, the structural information from a deletion mutant of the RI-subunit (Δ1−91 rRIα) indicates that the indole side chain of Trp260 is in close contact with the adenine ring of the cAMP bound to domain A (*19*).

The relative contribution of each tryptophan residue to the emission of the RI-subunit was consistent with the degree of exposure to the solvent deduced from the crystal structure (*18*). Contact of the indole nucleus with polar solvents is known to quench the emission of tryptophan, and according to the crystal structure, Trp188 would be the most exposed residue, followed by Trp260, and the most shielded residue would be Trp222 (*19*).

It should also be emphasized that the removal of cAMP did not significantly change the hydrophobic environment of Trp188 and Trp222, while there was a decrease in the hydrophobicity of the environment surrounding Trp260. This was consistent with measurements of the Stokes radius of the stripped and nonstripped RI-subunit. The Stokes radius is reduced when cAMP is removed, indicating that the structure is more compact (*34*). Moreover, that effect correlated with domain A, since no difference in Stokes radius was observed in a mutant unable to bind cAMP in domain B (*34*). According to the crystal structure (*19*), Trp260 begins the A-helix of cAMP-binding domain B (Figure 1D) and is stacked against the adenine ring of cAMP bound to cAMP-binding domain A. Our observations suggest that the effect of the removal of cAMP from domain A is an increased exposure of Trp260 to the solvent.

The unfolding process was divided into two steps, with the initial step correlating with an increase in fluorescence at 340 nm. This initial step must reflect either a conformational change in cAMP-binding site A or a change in the orientation of the two domains relative to one another. It did not, however, correlate with a release of cAMP nor did it correlate with the unfolding, which resulted in a wavelength shift reflecting exposure of the tryptophans to solvent.

The mutants described here have demonstrated unambiguously that the increase in fluorescence emission that results from urea was distinct from the increase that was seen when cAMP was removed from the protein in the absence of urea. The latter was due to Trp260 as discussed above, whereas the increase in fluorescence that resulted from low concentrations of urea is due primarily to Trp222. The W222Y mutant, in contrast to wild-type RI-subunit and the W188Y and W260Y mutants, showed very little increase in fluorescence at 340 nm in 3 M urea, even though cAMP was bound to the protein. Even when excess cAMP was present, there was no increase in fluorescence for the W222Y mutant in the presence of low urea concentrations. From these results, it was concluded that the increase in fluorescence that was observed in wild-type RI-subunit at low urea concentrations was due to changes in the environment surrounding Trp222.

In addition to causing a quenching of tryptophan fluorescence, the addition of cAMP to the stripped RI-subunit also increased the stability of the protein. Several of the mutant proteins also showed that the addition of excess cAMP (150 μM) at urea concentrations between 3.5 and 5.5 M was sufficient to shift the λ_{max} back to 340 nm, thus stabilizing the protein further. This concentration of cAMP, however, was not capable of reversing the fluorescence increase at 3 M urea. Excess cAMP would shift the equilibrium between free and bound cAMP toward the bound form, therefore stabilizing the protein, but it would be incapable of reversing the conformational change produced by 3 M urea. This observation also supports the notion that the first phase of the unfolding process is not linked to changes in the cAMP-binding properties of the R-subunit.

At urea concentrations above 6 M, the fluorescence emission wavelengths were no longer shifted by the addition of 150 μM cAMP. No change was observed for the unfolding curves of the W222Y mutant, indicating that excess cAMP did not stabilize this protein in the presence of urea. In contrast, the unfolding curves for the RI-subunit and W188Y mutant were shifted to higher concentrations of urea by adding 150 μM cAMP. Equilibrium dialysis experiments performed in the presence of 4.5 M urea showed that 0.5 μM RI-subunit dimer could still bind nearly 2 μM cAMP. This result differs from the [^{3}H]cAMP-binding assay performed by Leon et al. (*1*), which showed that binding of cAMP was abolished beyond urea concentrations of 4 M. For that filtration-based assay, the protein concentration was 20 nM. It should be emphasized again that the filtration-based radiolabel assay could not be used successfully under present conditions (high levels of cAMP and high protein concentrations). Since the fluorescence experiments were performed at RI-subunit concentrations of 0.5 μM, identical to the equilibrium dialysis, it was assumed that the RI still bound most of its cAMP in the presence of 4.5 M urea, and that the complex was simply stabilized by mass action due to the high concentration of RI-subunit and cAMP. The complex was further stabilized in the presence of excess cAMP.

Iodide quenching of tryptophan residues provided a valuable insight into which tryptophans were accessible to solvent upon unfolding. At 0 M urea, 30% of the fluorescence was quenched in the wild-type, Trp222, and Trp260 mutants. In contrast, a quenching of only 10% was observed when Trp188 was mutated, indicating that this residue was the most accessible to the solvent and accounted for most of the quenching under these conditions. At 3 M urea, 80% of the fluorescence of the wild-type subunit was quenched. As in the previous case, mutation of the Trp188 residue proved that this residue was the most susceptible to quenching, followed by Trp260 and Trp222. At 7 M urea, all three tryptophans were mostly exposed to solvent. In the case of the W222Y mutant, 20% of fluorescence was still not quenched, probably reflecting some cAMP still bound and interacting with Trp260 or the existence of some folded core even under those conditions.

The fluorescence studies described here have established several features of the RI-subunit structure and have helped to define the unfolding process. In the folded state of the RI-subunit, the fluorescence from Trp260 is quenched by bound cAMP, Trp188 is partially exposed, and Trp222 is fully buried. At intermediate concentrations of urea, Trp188 becomes fully exposed, and the remaining tryptophans become more exposed to solvent. Eventually, at urea concentrations beyond 4 M, the cAMP molecules begin to dissociate from the binding sites, at which point the buried Trp222 and Trp260 become exposed to solvent.

The crystal structure of a 1−91 deletion mutant of the bovine RIα subunit (*19*) is quite consistent with the fluorescence results described here. Although the dimerization domain is absent in that structure, it reveals the detailed

features of each cAMP-binding site and provides a molecular basis to explain the cooperative binding of cAMP and the activation of the holoenzyme. Even though the protein is monomeric, it maintains the autoinhibitor site as well as the two cAMP-binding sites, and also conserves most of properties typical of the wild-type subunit, like cAMP-mediated activation and holoenzyme formation.

Each cAMP-binding domain consists of three major α-helices and eight β-strands (β1−β8). The eight β-strands form a flattened β-barrel, consisting of two antiparallel β-sheets, each with four strands, connected in a jelly roll topology. According to the crystal structure of Δ1−91 RIα, Trp188 and Trp222 are both an integral part of domain A. Based on our results, Trp222 is deeply buried and inaccessible to solvent, while Trp188, at least in the absence of the C-subunit, is quite exposed. The crystal structure reveals that Trp188 is located on an exposed loop in domain A, between β-strands 4 and 5, which is in agreement with our observations. The Trp222 residue is located also in domain A, but in β-strand 8 and deeply buried in cAMP-binding domain A. Whereas the first two tryptophans are located in domain A, Trp260 is located in α-helix A in domain B, although its aromatic side chain stacks with the adenine ring of the cAMP in domain A. That location, which is consistent with affinity labeling (*22*), is now also confirmed by our fluorescence data.

In conclusion, we have confirmed that some important structural features of the monomeric form of RIα used for crystallization are consistent with observations obtained from the dimeric wild-type R-subunit using fluorescence techniques. Also, our observations present valuable insights into the changes undergone by the protein during the urea-induced unfolding process as well as their correlation with the release of cAMP. These findings, together with the mutation of other residues critical for cAMP binding and for the communication between different domains, will provide the basis for a better understanding of the interactions between the R monomers, between them and the catalytic subunit, and explain the unusual fluorescent and thermal stability characteristics of the cAMP-dependent kinase regulatory subunits.

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