Unfolding of the Regulatory Subunit of cAMP-Dependent Protein Kinase I†

Darryl A. Leon, Wolfgang R. G. Dostmann, and Susan S. Taylor*
Department of Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0654
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ABSTRACT: The unfolding of the recombinant regulatory subunit of cAMP-dependent protein kinase I was followed by monitoring the intrinsic protein fluorescence. Unfolding proceeds in at least two stages. First, the quenching of fluorescence due to cAMP binding is abolished at relatively low levels of urea (<2 M) and is observed as an increase in intensity at 340 nm. The high-affinity binding of cAMP is retained in 3 M urea even though the quenching is lost. The second stage of unfolding, presumably representing unfolding of the polypeptide chain, is seen as a shift in λ_{max} from 340 to 353 nm. The midpoint concentration, C_m, for this process is 5.0 M. Cyclic AMP binding activity is lost at a half-maximal urea concentration of 3.5 M and precedes the shift in λ_{max}. Unfolding of the protein in the presence of urea was fully reversible; furthermore, the presence of excess levels of cAMP stabilized the regulatory subunit. A free energy value ($\Delta G_D^{H_2O}$) of 7.1 ± 0.2 kcal/mol was calculated for the native form of the protein when denaturation was induced with either urea or guanidine hydrochloride. Iodide quenching of tryptophan fluorescence was used to elucidate the number of tryptophan residues accessible during various stages of the unfolding process. In the native cAMP-bound form of the regulatory subunit, only one of the three tryptophans in the regulatory subunit is quenched by iodide while more than two tryptophans can be quenched with iodide in the presence of 3 M urea.

The major receptor for adenosine cyclic 3′,5′-phosphate (cAMP)[1] inside the cell is cAMP-dependent protein kinase (cAPK). 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 regulatory subunits causes the tetrameric complex to dissociate into an R dimer and two free catalytic subunits. The active catalytic subunits are then capable of phosphorylating other proteins containing the general consensus sequence Arg-Arg-X-Ser/Thr [for reviews, see S. J. Beebe and S. Taylor (Beebe & Corbin, 1986; Taylor et al., 1990)].

Although several forms of regulatory subunits are expressed in eukaryotic cells, each has a conserved and well-defined domain structure. This domain structure was originally characterized by limited proteolysis and has subsequently been confirmed by using recombinant techniques to construct a variety of deletion mutants (Ringheim et al., 1988). A dominant feature of the domain structure is two tandem cAMP-binding domains at the C-terminus designated as sites A and B (Titani et al., 1981; Takio et al., 1982). These two cAMP-binding domains show extensive sequence similarity and clearly result from gene duplication. Binding of cAMP to these two domains shows positive cooperativity (Døskeland & Øgreid, 1984; Robinson-Steiner & Corbin, 1983). A model for each cAMP-binding domain has been proposed based on the crystal structure of the homologous catabolite gene activator protein (CAP) in *Escherichia coli* (Weber et al., 1987).

There are two general classes of R subunits, designated as type I and type II, which differ both in physical properties such as amino acid sequence, autophosphorylation, and antigenicity and also in tissue distribution (Rosen et al., 1975; Hofmann et al., 1975). The type I regulatory subunit contains three tryptophans (Takio et al., 1982). Two of these, Trp-188 and Trp-222, are located in cAMP-binding domain A. The third, Trp-260, lies at the junction between domain A and domain B and is a major site of covalent modification by $8N_3$-cAMP (Bubis & Taylor, 1987). The type I regulatory subunit exhibits quenching of fluorescence as a consequence of cAMP binding. In contrast, the type II regulatory subunit containing a single tryptophan, Trp-226, shows no quenching of fluorescence (LaPorte et al., 1980). The intrinsic fluorescence of these three tryptophan residues can be used as a monitor to examine the conformational and thermodynamic changes in the regulatory subunit of cAMP-dependent protein kinase I. Changes in the intrinsic protein fluorescence of the recombinant type I bovine regulatory subunit were followed by using both urea and guanidine hydrochloride (GdnHCl) as denaturants. In addition to following the changes in intrinsic fluorescence, the solvent accessibility of the tryptophan residues at various stages of unfolding was monitored by iodide quenching.

Experimental Procedures

Materials. Reagents were purchased as follows: MOPS, guanidine hydrochloride (grade I), MES, benzamidine hydrochloride, leupeptin, BSA, TLCK, TPCK, and PMSF, Sigma Chemical Co.; EDTA, EGTA, KCl, 2-mercaptoethanol, $MgCl_2$, sodium chloride, and ammonium sulfate, Fisher Scientific; potassium iodide (high grade) and sodium thiosulfate (high grade), Aldrich Chemical Co.; ultrapure urea and [^{3}H]cAMP, ICN; cAMP and ultrapure sodium dodecyl sulfate, United States Biochemical; bacto-tryptone and yeast extract, Difco Laboratories; ampicillin, Mannheim Boehringer; and HAWP filters, Millipore.

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* To whom correspondence should be addressed.

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; SDS, sodium dodecyl sulfate; TLCK, 1-chloro-3-(tosylamino)-7-amino-L-2-heptanone; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; MES, 2-(*N*-morpholino)ethanesulfonic acid; GdnHCl, guanidine hydrochloride; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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Purification of the Recombinant Regulatory Subunit. Luria Bertani (LB) broth (1 L) containing ampicillin (100 μg/mL) was inoculated with a frozen glycerol stock of *E. coli* cells (25 μL) containing a plasmid, pLST2, encoding for the mouse recombinant type I regulatory subunit (Saraswat et al., 1986) which includes a β-galactosidase leader sequence on its N-terminus. The cells were grown for at least 36 h at 37 °C with shaking. All subsequent procedures were carried out at 4 °C. After centrifugation for 25 min at 5000 rpm and resuspension of the pellet in lysis buffer (25 mM MES, 5 mM Na_2EDTA, 5 mM EGTA, 10 mM 2-mercaptoethanol, 10 mM benzamidine hydrochloride, 1 μM leupeptin, 28 μM TLCK, 28 μM TPCK, and 86 μM PMSF, pH 6.4), the cells were ruptured in a French pressure cell. After centrifugation at 12000 rpm for 20 min, the supernatant was collected and precipitated with ammonium sulfate (45%). The resulting precipitate was centrifuged at 10000 rpm for 10 min and resuspended in lysis buffer. The pooled solutions were dialyzed twice for 1 h with buffer A (5 mM 2-mercaptoethanol, 2 mM EDTA, and 10 mM MES, pH 6.5, conductivity 0.8 μS). The dialyzed solution was centrifuged for 10 min at 10000 rpm. The conductivity of the supernatant was adjusted to 0.8 μS with cold H_2O before being loaded onto a DE-52 ion-exchange column (100 mL) equilibrated with buffer A. After the column was washed with 500 mL of buffer, the protein was eluted with a (500 mL) linear NaCl gradient (0–250 mM).

Measurement of cAMP Binding. Cyclic AMP binding as a function of urea concentration was measured by the ammonium sulfate precipitation assay described previously by Døskeland and Øgreid (1988). Briefly, the regulatory subunit (10 nM) was denatured in urea (0–8 M) and incubated at room temperature for 3 h. [^{3}H]cAMP (200 nM) was added, and the incubation was continued at 30 °C for 30 min, a time shown previously to be sufficient to completely exchange with endogenous bound cAMP. After 50-μL aliquots were placed on ice for 30 min, 95% ammonium sulfate (3 mL) was added to each sample. The precipitated samples were collected on Millipore HAWP (0.45 μm) filters and rinsed twice with (3 mL) 65% ammonium sulfate. The filters were then placed in vials containing 2% SDS and scintillation fluid, and counted in a Beckman liquid scintillation counter. All experiments were performed at least in duplicate with experimental variations indicated in each respective figure.

Chemical Preparations for Fluorescence Measurements. Stock solutions of 8 M urea, 6 M guanidine hydrochloride (GdnHCl), and 16.7 mM cAMP were prepared in buffer B, pH 7.0 (5 mM MOPS, 0.5 mM EDTA, 100 mM KCl, and 5 mM 2-mercaptoethanol), and/or buffer C (buffer B 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 C. All of the solutions were made with reagent-grade or higher grade chemicals and were run through a 0.22-μm pore filter before being used.

Denaturation and Renaturation. The regulatory subunit (0.5–1.0 μM) was denatured in various concentrations of denaturant (0–8 M urea or 0–3 M GdnHCl), and the solutions were incubated for 3 h at room temperature, a time that was determined to be sufficient to reach equilibrium. Renaturation was performed by first denaturing the protein in 7 M urea for 3 h at room temperature. Aliquots of this mixture then were diluted in solutions containing 0–7.0 M urea to give a final protein concentration of 0.25 μM and incubated overnight at room temperature. The lowest dilution obtained was 0.35 M urea which essentially produced no spectral difference from the native regulatory subunit. All fluorescence measurements were made in a 1-cm quartz cuvette at 23 °C. Fluorescence measurements were made on an SLM Aminco SPF-500 spectrofluorometer interfaced with a PC/AT computer. The protein samples were excited at 293 nm, and the tryptophan emission was monitored at 347 nm. The resulting emission spectra was then followed from 300 to 450 nm.

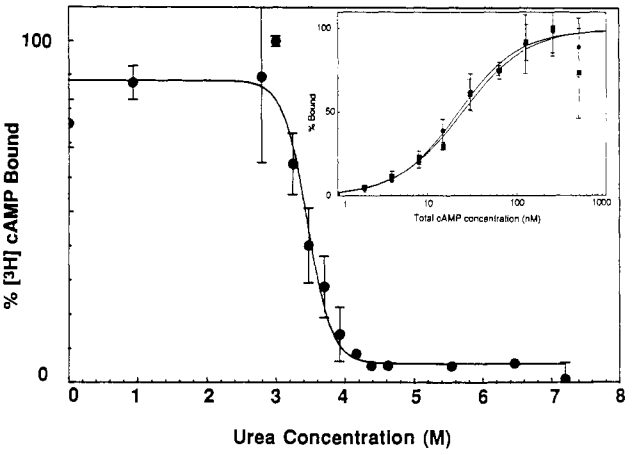

FIGURE 1: [^{3}H]cAMP-binding curve of the regulatory subunit induced by urea. The percentage of [^{3}H]cAMP bound to the regulatory subunit (●) was normalized according to the fully bound native protein. This value corresponds to 2 mol of cAMP bound per R monomer. The amount of [^{3}H]cAMP bound to the recombinant R subunit (10 nM) as a function of increasing urea concentrations was determined as described under Experimental Procedures. The inset is a cAMP-binding curve in the absence (●) and presence (■) of 3 M urea.

Iodide Quenching. Fluorescence measurements were carried out on samples containing the following: regulatory subunit (0.5 μM), increasing concentrations of potassium iodide (0–0.3 M), and selected urea concentrations (0, 3, and 6.8 M). The potassium iodide was dissolved in buffer B, and since I_3^- absorbs in the wavelength region of tryptophyl fluorescence, a small amount of sodium thiosulfate (0.1 mM) was added in order to prevent I_3^- formation (Lehrer, 1971). Additionally, NaCl was included with the potassium iodide solutions to keep the ionic strength constant.

cAMP-Induced Renaturation. In order to determine the effect of cAMP on protein stability, the regulatory subunit was incubated with various concentrations of urea (0–7.5 M) at room temperature for at least 3 h before measuring the fluorescence in a 1-cm quartz cuvette. Excess cAMP (158 μM) then was added to the denatured (or renatured) sample in the cuvette, and the fluorescence measurement was made again. The change in volume due to the addition of cAMP was only 1%. In order to determine the half-maximal concentration of cAMP required to enhance the stability of the regulatory subunit at 5.4 M urea, the fluorescence was measured at increasing cAMP concentrations (0–500 μM) while keeping the protein concentration constant.

RESULTS

cAMP Binding in Urea. The effect of urea on cAMP binding to the type I regulatory subunit is shown in Figure 1. The loss of cAMP-binding activity is half-maximal near 3.5 M urea, indicating that the regulatory subunit is a relatively stable structure. Concentrations greater than 4.5 M urea are required in order to completely abolish cAMP-binding activity. The K_d(cAMP) was also measured in the presence and absence of 3 M urea. The inset of Figure 1 shows that the K_d for cAMP in the absence of urea and in the presence of 3 M urea is still the same, once again emphasizing that even in 3 M urea both high-affinity-binding sites for cAMP are functionally intact.

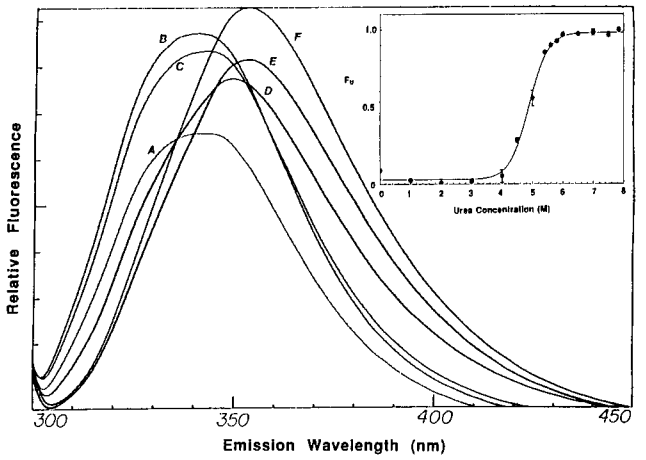

FIGURE 2: Tryptophan fluorescence emission spectra and unfolding curve of the regulatory subunit. Denaturation spectra of the protein in (A) 0, (B) 3, (C) 4, (D) 5, (E) 6, and (F) 7.8 M urea. The samples were excited at 293 nm at 23 °C, pH 7.0. The inset is an unfolding curve of the regulatory subunit induced by urea. The fraction of unfolding (●), F_u, was represented by a fluorescence ratio (353 nm/340 nm) as described in the text. The denaturation fluorescence buffer contained 5 mM MOPS, 0.5 mM EDTA, 100 mM KCl, and 5 mM 2-mercaptoethanol, and the measurements were carried out after a 3-h incubation at various urea concentrations.

Fluorescence Properties Associated with Denaturation. In order to study the unfolding of the recombinant regulatory subunit under denaturing conditions, the R subunit (or simply the "protein") was denatured both in urea and in guanidine hydrochloride (GdnHCl). In all cases, except where noted, the protein had all four binding sites saturated with cAMP. The intrinsic tryptophan fluorescence was monitored to follow unfolding of the polypeptide chain. Since the tryptophan residues absorb near 293 nm, the regulatory subunit was excited at that wavelength as described by LaPorte et al. (1980). As seen in Figure 2, at least two distinct stages were seen in the unfolding process. In the absence of urea, the native cAMP-saturated regulatory subunit showed a maximum fluorescence emission at 340 nm. Immediately upon addition of urea, an increase in fluorescence intensity at 340 nm was observed with a maximum value being reached at approximately 2 M urea. The half-maximum concentration of urea required for this initial increase in fluorescence was approximately 1.0 M urea. As the urea concentration increased above 3.5 M, the maximum intensity of the fluorescence at 340 nm not only began to decrease but also a shift in wavelength was observed, indicating that at least one buried tryptophan was being exposed to a new dielectric environment. After the fluorescence intensity reached a minimum near 5 M urea, the fluorescence began to increase slowly with increasing urea concentrations. A maximum intensity at 353 nm was seen at approximately 8 M urea. At this very high concentration of urea, the protein is presumably fully unfolded and has lost most, if not all, of its secondary structure.

Calculation of C_m and ΔG. In order to calculate the C_m (concentration midpoint) associated with the unfolding process, the fluorescence intensity ratio, R (353 nm/340 nm), was used to follow the shift in wavelength since the native form and the denatured form of the wild-type protein displayed maxima at 340 and 353 nm, respectively. A fractional denaturation curve was calculated by using the relationship:

$$F_u = 1 - \left(\frac{R_O - R_D}{R_N - R_D}\right) \quad (1)$$

where F_u is the fraction of unfolding, R_O is the observed intensity ratio at various urea concentrations, and R_D and R_N are the fluorescence intensity ratios of the protein in the presence and absence of 8 M urea, respectively (Tandon & Horowitz, 1990). The denaturation curve seen in the inset of Figure 2 shows this ratio as a function of the urea concentration. It appears that no significant unfolding occurs until the urea concentration reaches 4 M, at which point the transition continues to 6 M urea. The C_m of this transition is approximately 5.0 M urea. When the protein was denatured with GdnHCl, the C_m was near 1.5 M (data not shown). This smaller observed C_m value is not surprising since GdnHCl is a much stronger denaturant than urea.

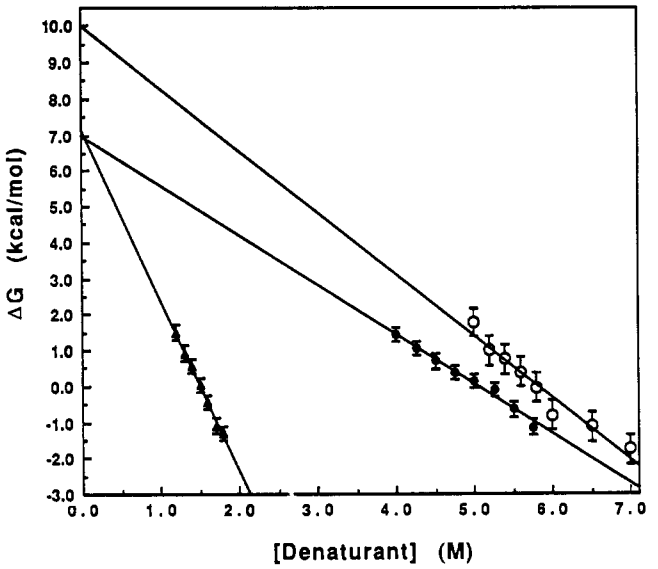

FIGURE 3: Free energy of unfolding, ΔG_D, for the regulatory subunit as a function of denaturant. The ΔG_D values were calculated (as described under Results) from curves similar to the ones seen in Figures 2 and 4. The lines were fitted by a computer linear regression program. The protein was denatured in increasing concentrations of GdnHCl (▲), urea (●), and urea containing 158 μM cAMP (○).

The transition regions of the denaturation curves analyzed above were then used to describe the thermodynamics of unfolding by assuming a simple two-state model. A two-state mechanism can be represented by $F_N + F_u = 1$ where F_N is the fraction of native protein and F_u is the fraction of unfolded protein:

$$F_u/F_N = K_D$$
$$K_D = e^{-\Delta G_D/RT} \quad (2)$$
$$-RT \ln (F_u/F_N) = \Delta G_D$$

where ΔG_D is the free energy of denaturation (Pace, 1986). One simple method of calculating the ΔG for the unfolded protein at zero concentration of denaturant is to assume that the transition region of the unfolding curve is linear with respect to the denaturant concentration. As seen in the inset of Figure 2, these conditions are met for the regulatory subunit. Pace (1986) has suggested that a linear extrapolation method using the equation:

$$\Delta G_D = \Delta G_D^{H_2O} - m[\text{denaturant}] \quad (3)$$

may be used to find $\Delta G_D^{H_2O}$ in the absence of denaturant by plotting ΔG_D values versus the denaturant concentrations in the transition region. When this procedure is applied to the data obtained for the regulatory subunit, the $\Delta G_D^{H_2O}$ for the folded protein was determined to be 7.0 and 7.2 kcal/mol in urea and GdnHCl, respectively (Figure 3).

Renaturation and Effect of cAMP on Unfolding. Although the denaturation of a protein may follow a simple pathway, the renaturation, or refolding, may not be a simple reversal

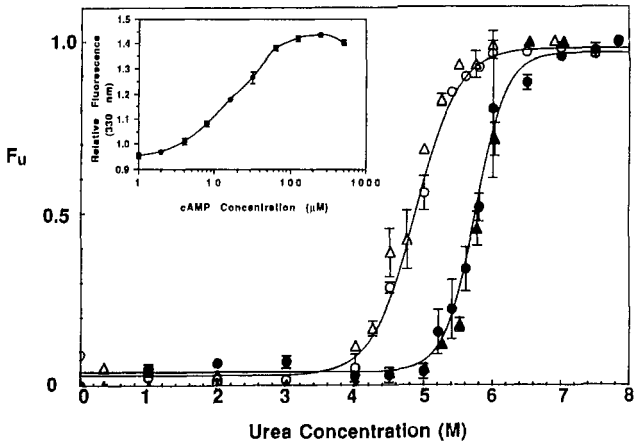

FIGURE 4: Unfolding curve for the denatured and renatured forms of the regulatory subunit in the absence and presence of excess cAMP as a function of urea concentration. The method for determining F_u was carried out as described in the text. The protein (1 μM) was denatured in the absence (○) and presence of excess cAMP (●). Renaturation was carried out in the absence (△) and presence (▲) of excess cAMP. The inset is a plot of the tryptophan fluorescence emission at 330 nm as a function of cAMP concentration when the protein is denatured at 5.4 M urea. The solid lines were fitted by the computer program Graph Pad.

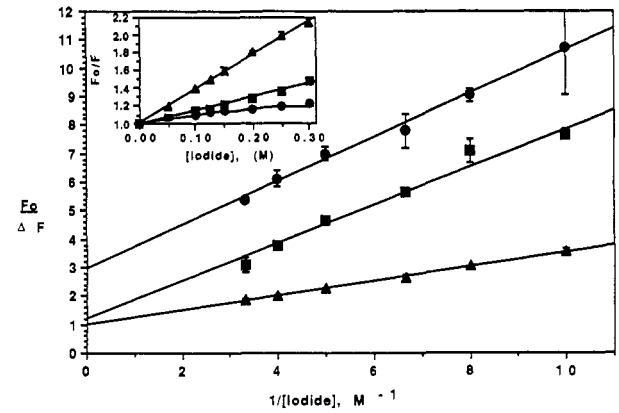

FIGURE 5: Modified Stern–Volmer plot of the regulatory subunit. The tryptophan fluorescence of the regulatory subunit (0.5 μM) was quenched with iodide at 0 (●), 3 (■), and 6.8 M (▲) urea as described in the text. The inset was plotted from the same data. The ionic strength was constant, and the excitation wavelength was 293 nm. The lines were fitted by linear regression.

of the unfolding process. In order to examine the refolding properties, the regulatory subunit was renatured from 7 M urea. The final urea concentration of lowest dilution obtained was 0.35 M urea. At this concentration of urea, the fluorescence spectrum was essentially the same as the one produced by the untreated folded protein (spectra not shown). As seen in Figure 4, the refolding curve overlaps the unfolding curve when the F_u is calculated as described above.

The effect of urea on cAMP binding was indicated in Figure 1. In order to determine whether cAMP stabilizes the protein, unfolding and refolding were carried out in the presence of excess cAMP. This is in contrast to the previous studies which were all carried out on the regulatory subunit with both cAMP-binding sites saturated with cAMP. The regulatory subunit at concentrations of 1 μM was denatured and renatured in urea in the presence of excess cAMP, and the change in tryptophan fluorescence was followed. As seen in Figure 4, a 40-fold molar excess of cAMP over cAMP-binding sites shifts both curves to the right, indicating an increase in protein stability. The C_m changes from 5.0 to 5.8 M urea while the slope of the curve stays constant. A ΔG_D value was calculated for this unfolding curve, and a value of 10 (±1) kcal/mol was determined as seen in Figure 3. This increase of approximately 3 kcal/mol implies that cAMP has a very pronounced influence on the free energy associated with the folded regulatory subunit.

The most significant stabilization occurs in the transition region of the unfolding curve. Since the greatest decrease in the amount of unfolded fraction (F_u) is near 5.4 M urea, this concentration was selected for determining the amount of cAMP needed to produce the enhanced stability. Protein samples (1 μM) were denatured in 5.4 M urea and incubated with increasing concentrations of cAMP before fluorescence measurements were taken as described under Experimental Procedures. The results of this titration with cAMP are presented in the inset of Figure 4. At low concentrations of cAMP, no spectral shift in wavelength is observed. However, at higher concentrations, increasing blue shifts in wavelength (350–340 nm) were noted until an excess level of cAMP is reached (spectra not shown). A midpoint between the two extremes reveals a K_d, apparent equilibrium constant, near 25 μM. This K_d is approximately 12 times the concentration of cAMP needed to saturate a 1 μM concentration of monomeric R^I subunit. The information from these two experiments suggests that (i) the shift in wavelength emission is due to a decreased ability of the protein to bind cAMP under denaturing conditions and (ii) the stability of the protein can be enhanced in the presence of excess cAMP.

Iodide Quenching of the Regulatory Subunit. The fluorescence of a given tryptophan residue in a protein can be quenched by various substances. In particular, iodide ions can quench surface-exposed or accessible tryptophans but cannot penetrate into the interior of a protein (Lakowicz, 1986). As a result, iodide quenching is a useful method for determining the fraction of tryptophan residues that are exposed under different solvent conditions (Lehrer, 1971). The tryptophan fluorescence quenching of the recombinant regulatory subunit was monitored using iodide as a quencher. Iodide quenching was determined under three different denaturing environments (0, 3, and 6.8 M urea) as described under Experimental Procedures. In each case, the ionic strength of the solution was kept constant so that high-salt effects would not be a contributing factor in any observed changes of fluorescence. The protein fluorescence intensity decreased under all conditions, but the extent of quenching was greatest when protein unfolding was induced with 6.8 M urea. A Stern–Volmer equation ($F_o/F = 1 + K[X]$) was applied to the data where F_o/F is the fractional decrease in fluorescence due to a quencher, [X], and K is the Stern–Volmer quenching constant (Lehrer & Leavis, 1978). Since I^- was the selected quencher, a plot of F_o/F vs $[I^-]$ was obtained (see inset of Figure 5). It is obvious from the inset that a nonlinear plot appears when the regulatory subunit is in a folded state. It has been noted that this type of nonlinear behavior represents a heterogeneous system of fluorophores (Lehrer & Leavis, 1978). The 3 M urea plot shows a more linear dependence on the iodide concentration than in the absence of urea, implying that more tryptophans are equally exposed. Moreover, the 6.8 M urea plot is also linear but has the largest slope, or K value, which suggests that the collisional interaction between tryptophan residues and iodide atoms is a more effective process than in no denaturant and 3 M urea.

Further information was obtained by plotting the spectral data using the modified Stern–Volmer equation:

$$\frac{F_o}{\Delta F} = \frac{1}{f_a K[Q]} + \frac{1}{f_a} \qquad (4)$$

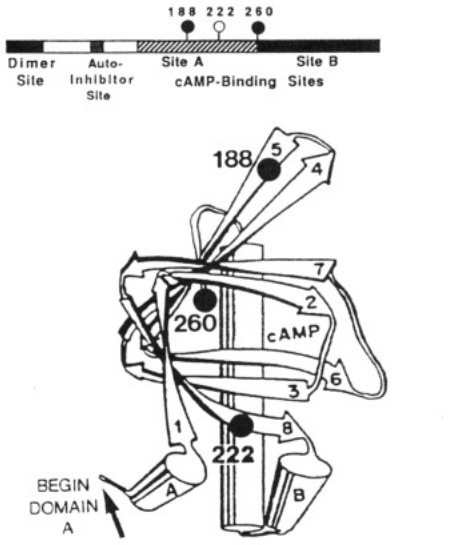

FIGURE 6: Proposed model of the regulatory subunit cAMP-binding domain A. This model was constructed by building the amino acid sequence of the R^I subunit into the crystallographic coordinates of the catabolite gene activator protein (Weber et al., 1987). The predicted locations of the three tryptophan residues are indicated as dark circles. A schematic representation of the general domain structure of the R subunit is indicated above. The locations of these three tryptophans are indicated; Trp-222 which is conserved in both R^I and R^{II} subunits is shown as an open circle.

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 concentration of quencher (Lehrer, 1971). A plot of $F_o/\Delta F$ vs $1/[I^-]$ of the native, partially denatured, and fully denatured forms of the regulatory subunit is seen in Figure 5 which shows that f_a and K values acquired from linear extrapolation are dependent on the urea concentration. Specifically, when the protein is in its native conformation, f_a is 0.33, implying that 33% of the tryptophans or 1 equiv of Trp is accessible to iodide. At an intermediate concentration of urea (3 M), conditions where the R^I subunit is partially denatured, but still fully saturated with cAMP, f_a was 0.83. The simplest interpretation of these data indicates that on average approximately 83% of the total population of tryptophans is exposed when the protein is denatured in 3 M urea. An f_a value of approximately 1.0 was calculated for the fully denatured form of the protein at 6.8 M urea, indicating that under these conditions all three tryptophans are fully accessible to iodide.

Discussion

The type Iα regulatory subunit contains three tryptophan residues (Titani et al., 1984). Two of these, Trp-188 and Trp-222, are contained within the first cAMP-binding domain, site A, while the third is thought to lie at the junction between site A and site B. A model of each cAMP-binding domain was predicted based on the crystal structure of the related protein, the catabolite gene activator protein (CAP) in *E. coli* (Weber et al., 1987). A summary of the overall domain structure of the regulatory subunit and predicted locations of the three tryptophan residues based on this model is shown in Figure 6.

As shown previously by LaPorte et al., binding of cAMP leads to quenching of the inherent tryptophan fluorescence in the cAMP-free type I regulatory subunit (LaPorte et al., 1980). In contrast, the type IIα regulatory subunit contains only one tryptophan and shows no quenching of fluorescence following binding of cAMP (LaPorte et al., 1980). The single Trp in the R^{II} subunit, Trp-226, is homologous to Trp-222 in the type I regulatory subunit. Thus, on the basis of the relatedness of these two proteins, it is reasonable to predict that the quenching of fluorescence seen in the R^I subunit is due to either Trp-188 or Trp-260, or both.

One additional piece of information is available regarding these three tryptophans. Trp-260 is a major site of photoaffinity labeling by $8N_3$-cAMP bound to site A (Bubis & Taylor, 1987). On this basis, it is likely that at least part of the quenching of fluorescence that is seen when cAMP binds to the R^I subunit can be attributed to interactions between the adenine ring of cAMP bound to site A and the indole ring of Trp-260. The results described here used the intrinsic tryptophan fluorescence of the type I regulatory subunit to follow unfolding of the protein in response to both urea and guanidine hydrochloride. Although the detailed mechanism of this unfolding process is complicated, several features are nevertheless apparent.

For example, the fluorescence intensity of the unfolded protein is greater than the intensity for the native form. It has been noted that this type of behavior is opposite to what is observed in most proteins (Hecht et al., 1990). The unfolding process itself can be divided into at least two general steps. The first step is associated with an increase in fluorescence at 340 nm and occurs at levels of urea below 2–3 M. This low level of urea may abolish the cAMP-induced quenching of fluorescence but leaves the cAMP-binding sites functionally intact since the K_d(cAMP) is similar in the absence and presence of 3 M urea. The second step, presumably representing the complete unfolding of the polypeptide chain, is associated with a shift in the maximum fluorescence from 340 to 353 nm. This occurs at a relatively high concentration of urea (C_m = 5.0 M). Furthermore, loss of cAMP-binding activity precedes the shift in wavelength. The loss of cAMP-binding activity also does not appear to occur in a stepwise manner, indicating that both sites loose function more or less simultaneously. Cyclic AMP also stabilizes the regulatory subunit during the latter stage of unfolding and makes it more resistant to denaturation. Finally, the unfolding of the regulatory subunit appears to by fully reversible both in the presence and in the absence of cAMP.

The iodide quenching is consistent with a model that has approximately 1 equiv of Trp exposed in the native regulatory subunit. The quenching constant values obtained from the modified Stern–Volmer plots can be utilized to make some qualitative comparisons about the quenching equilibrium between iodide and the tryptophan residues. For example, quenching constant values, or slopes, of the 0 and 3 M urea plots of the regulatory subunit appear almost parallel in Figure 5. These two similar quenching constants may be due to the possibility that the partially folded regulatory subunit may still have one buried tryptophan. This fact is further supported by comparing these slopes to the one for the fully denatured protein. A much smaller slope for the unfolded form of the regulatory subunit means a higher quenching constant, i.e., a larger population of tryptophan residues are quenched with iodide. Additionally, at 3.0 M urea, more than two tryptophans become accessible, and this increase in accessibility of the tryptophans to iodide quenching is concomitant with the loss of the quenching that is associated with cAMP binding to the native protein. Thus, under these conditions, although cAMP still binds with high affinity, its orientation with respect to Trp-188 and/or Trp-260 is significantly different. At a concentration of 7 M urea, all three tryptophans are fully accessible to iodide. Selected mutations of each Trp or, al-

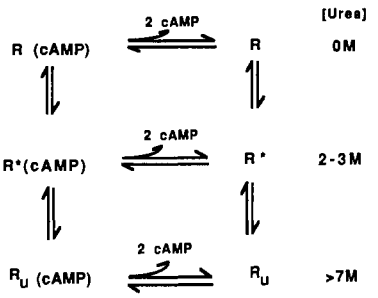

FIGURE 7: Schematic diagram illustrating the unfolding of the regulatory subunit. The concentration of urea is correlated with each stage of unfolding where R = the native state, R* = an intermediate state, and R_u = the unfolded state.

ternatively, chemical modification of the tryptophans in the presence and absence of urea will be required before cAMP-induced quenching and iodide accessibility can be correlated with specific tryptophan residues.

A minimal scheme showing the unfolding of the regulatory subunit is shown in Figure 7. For simplicity, the regulatory subunit is shown as a monomer since no cooperativity is seen for cAMP binding to the two protomers in the dimer (Døskeland & Øgreid, 1984; Ringheim & Taylor, 1990). In the absence of urea, cAMP binds very tightly with a K_d of 10 nM. Under these conditions in the cell, the free regulatory subunit exists almost exclusively as the fully liganded species, $R(cAMP)_2$. In the presence of 8 M urea, the polypeptide chain is in a fully unfolded conformation indicated as R_u. The regulatory subunit also exists in at least one intermediate conformational state, R*, at concentrations between 2 and 3 M urea. Under these conditions, cAMP still binds with high affinity as evidenced by a similar K_d; however, no quenching of fluorescence is associated with cAMP binding. In the presence of excess cAMP, the equilibrium going from R_u to R* is shifted toward $R^*(cAMP)_2$. At this point, we can say nothing about the order of binding or release of cAMP with respect to site A and site B under these various denaturing conditions.

Since the tryptophans in the type I regulatory subunit are associated primarily with cAMP-binding site A, it is possible that the observed unfolding represents the selective unfolding of site A and is relatively independent of site B and the amino terminus. Previous results based on limited proteolysis and more recently on the construction of specific deletion mutants indicates that each cAMP-binding domain can retain high-affinity cAMP binding independently (Ringheim et al., 1988). In order to resolve whether the two cAMP-binding sites unfold sequentially, independently, or concomitantly, it will be necessary not only to characterize some of these truncated forms of the regulatory subunit but also to construct specific mutant forms of the regulatory subunit where tryptophans are selectively introduced into site B and into the amino terminus.

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