Analysis of the Structural Properties of cAMP-responsive Element-binding Protein (CREB) and Phosphorylated CREB*

(Received for publication, February 6, 1996, and in revised form, March 15, 1996)

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The transcription factor CREB (cAMP responsive element binding protein) is activated by protein kinase A (PKA) phosphorylation of a single serine residue. To investigate possible mechanisms of CREB regulation by phosphorylation, we initiated a structural and biophysical characterization of the full-length, wild-type CREB protein, an altered CREB protein (CREB/SER) in which the three cysteine residues in the DNA-binding domain were replaced with serine residues and a truncated protein (ACT265) which encompasses the entire activation domain of CREB. Circular dichroism (CD) reveals that CREB and CREB/SER have identical secondary structures and contain approximately 20% α-helix, 9% β-strand, 34% β-turn, and 37% random coil structures. PKA phosphorylation does not alter the CD spectra, and therefore the secondary structure, of CREB or of CREB bound to DNA. Protease cleavage patterns indicate that PKA phosphorylation does not induce a global conformational change in CREB. Furthermore, PKA phosphorylation does not change the DNA binding affinity of CREB for either canonical or non-canonical CRE sequences as measured by a fluorescence anisotropy DNA binding assay. Since PKA phosphorylation of CREB results in its specific binding to the transcriptional co-activators CREB-binding protein and p300, we suggest that the PKA activation of CREB occurs by the production of specific, complementary interactions with these proteins, rather than through the previously proposed mechanisms of a phosphorylation-dependent conformational change or increased DNA binding affinity.

Eukaryotic gene transcription employs a number of phosphorylation-dependent mechanisms that regulate the assembly and activity of the transcription machinery. Phosphorylation regulates the nuclear localization, DNA binding affinity, and/or the activation potential of many transcription factors (for review, see Refs. 1–3). Phosphorylation can alter protein function either by introducing an allosteric conformational change in the protein or, more commonly, by allowing (or blocking) specific electrostatic interactions with other molecules. Both of these mechanisms are thought to occur in the regulation of transcription; however, the structural consequences of phosphorylation are poorly understood.

We are investigating both the overall structure and the regulation by phosphorylation of the CREB/CREM/ATF family of transcription factors. These proteins are important for a variety of biological functions, such as neuroendocrine peptide production (4), spermatogenesis (5), and long term memory (6, 7). CREB/CREM/ATF family members share several similarities, including a highly homologous basic leucine zipper (bZIP) DNA-binding domain, an ability to homo- and heterodimerize in specific combinations, a similar gene structure, and the presence of conserved activator regions and phosphorylation sites (for review, see Refs. 8 and 9). The full-length CREB protein, CREB341, used in this study serves as a structural prototype for this family of transcription factors.

CREB consists of an NH2-terminal activation domain and a smaller, COOH-terminal bZIP DNA-binding and dimerization domain. Like most transcription factors, the activation domain of CREB can be further divided into several subdomains of various activation and regulatory functions. CREB has been extensively mapped by both deletion and “gain of function” analysis (Fig. 1A) (10–14). Although the exact roles and boundaries of the different regions vary among cell lines, reporter systems, and laboratories, several general conclusions can be drawn. The activation domain of CREB contains two glutamine-rich regions (Q1 and Q2), which are important to basal activity. The kinase-inducible domain (KID) includes several phosphorylation sites and confers the phosphorylation-induced activity to CREB. CREB activity is directly regulated by PKA (cAMP-dependent protein kinase A) phosphorylation of a single serine residue, Ser133 (10, 11). CREB is also an effector of calcium signaling pathways, and the same serine residue is a substrate for calcium-calmodulin kinase II and IV (15–18).

Models of CREB activation by PKA frequently include structural changes following phosphorylation. The original model proposed that an allosteric conformational change in CREB enables a site distal to Ser133, the Q2 region, to interact directly with the preinitiation complex (11, 12, 19). A related model suggested that PKA phosphorylation induces α-helix or β-sheet secondary structures in the regions neighboring the Ser133 site, thus allowing specific protein-protein interactions (8, 10, 20). A separate model proposed that phosphorylation leads to an increase in DNA binding, at least for the non-canonical class of CRE (cAMP-responsive element) (21–23). However, recent data for CREB activation suggests a different role for PKA phosphorylation, i.e. that phosphorylation simply creates a high-affin-

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*This work was supported by National Institutes of Health Grant DK45423 (to R. H. G.), by the Shriner’s Hospital’s, by the N. L. Tartar Research Fund, and by the Oregon Health Sciences Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviations used are: CREB, cAMP-responsive element-binding protein; CREM, cAMP-responsive element modulator; ATF, activating transcription factor; PKA, cAMP-dependent protein kinase A; bZIP, basic leucine zipper; CRE, cAMP-responsive element; SSRCRE, somatostatin CRE; TATCRE, tyrosine aminotransferase CRE; KID, kinase-inducible domain; Q, glutamine-rich; CBP, CRE-binding protein; DTT, dithiothreitol; HPLC, high performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; Endo, endoproteinase PAGE, polyacrylamide gel electrophoresis; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element.
ity binding site for other proteins. In particular, phosphorylation of Ser\textsuperscript{133} allows the high-affinity binding of CREB to the co-activators CBP (CREB-binding protein) and p300 (24–28). Despite this abundance of models, no structural or biophysical data have been presented that investigate the effect of phosphorylation on CREB structure.

We have initiated a biophysical characterization of CREB to study transcription activation by the CREB,CREM,ATF family of transcription factors. Our goals are, first, to characterize the structure of CREB and, second, to understand the structural mechanism of phosphorylation-dependent activation. Toward this end we have developed purification protocols and designed an altered CREB protein, CREB/SER, with improved solubility. We performed circular dichroism studies to characterize the secondary structure of both the full-length CREB protein and ACT265, a 265-amino-acid polyepitope of the activation domain in which the bZIP domain and a small amino-terminal extension were deleted. Finally, we tested various structural models of phosphorylation-dependent CREB activation by comparing the CD spectra, protease cleavage patterns, and DNA binding affinity of CREB and PKA-phosphorylated CREB.

**EXPERIMENTAL PROCEDURES**

Plasmids and Expression System—Rat CREB341 (29) was subcloned into a Novagen pET15b expression system using the Nco and BamHI restriction sites. The Nco site in CREB341 encompasses the second ATG of the initial Met-Thr-Met sequence, hence all of our constructs are initiated at residue 3. The ACT265 expression plasmid was derived from the CREB341 vector by introducing a stop codon in place of Val266 using site-directed mutagenesis (Promega). The CREB/SER mutant was derived by replacing the cysteine residues 300, 310, and 337 with serine residues using site-directed mutagenesis (Promega). The mutagenesis was performed on a CREB327 DNA that was isolated from a ligation of a hypothetical library and selected into the all-ligation and XbaI sites of p-SELECT (Promega; 30). Human CREB327 and rat CREB341 have identical bZIP amino acid sequences, although they differ slightly in the nucleotide sequence. The substituted bZIP domain of CREB327 was subcloned into the rat CREB341 pET15b expression plasmid using Asp\textsubscript{108} and BamHI sites. The resulting CREB/SER done is a rat CREB341/human CREB327 DNA hybrid, which contains an amino acid sequence identical to rat CREB341 with the three cysteines in the bZIP domain substituted with serines.

The plasmids were transformed into Escherichia coli BL21(DE3) cells (Novagen). Bacteria were grown in Luria broth plus 4 glitier glucose at 37 °C to an optical density (595 nm) of 0.60 and induced with 0.8 mM isopropyl-\textbeta-D-thiogalactopyranoside. After 3.5 h the cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.9, containing 5 mM MgCl\textsubscript{2}, 1 mM EDTA, 5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride for wild-type CREB341 or in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride for ACT265 and CREB/SER. The bacteria were lysed by passage through a French press at 14,000 p.s.i. The bacterial extracts of full-length, wild-type CREB341 were heated to 75 °C for 10 min and centrifuged for 20 min at 10,000 × g to remove the particulate fraction. The CREB supernatant was applied to a DE53 anion exchange column (Whatman) equilibrated at 10 mM Tris-HCl buffer, pH 8.0. The extract was further purified by passage through a French press at 14,000 p.s.i. The protein was eluted by anion exchange chromatography using either DE53 resin (Whatman) or a Mono Q FPLC column (Pharmacia Biotech Inc.). The protein was eluted using a 0–300 mM NaCl gradient. The non-heat-treated protein was further purified over an HPLC sizing column (Waters Protein-Pak 125). One liter of bacterial culture yielded about 5 mg of greater than 95% pure CREB/SER protein (Fig. 1D).

**Protease Digests**—CREB/SER was purified both with and without a heat treatment step. The protein (either with or without a 10 min, 75 °C heat treatment) was precipitated from the bacterial extract with 15% (w/v) ammonium sulfate and resuspended in 10 mM Tris-HCl buffer, pH 8.0. The protein was purified by anion exchange chromatography using either DE53 resin (Whatman) or a Mono Q FPLC column (Pharmacia Biotech Inc.). The protein was eluted using a 0–300 mM NaCl gradient. The non-heat-treated protein was further purified over an HPLC sizing column (Waters Protein-Pak 125). One liter of bacterial culture yielded about 5 mg of greater than 95% pure CREB/SER protein (Fig. 1D).

Phosphorylation—CREB341 and ACT265 were phosphorylated using the purified catalytic subunit (C-subunit) or purified recombinant C-subunit of PKA (kindly supplied by J ohn D. Scott and Richard A. Maurer, Oregon Health Sciences University). The phosphorylation reactions were carried out in 50 mM MOPS buffer, pH 7.0, 5 mM NaCl, 2 mM MgCl\textsubscript{2}, 1 mM DTT, and 1 mM ATP with a molar C-subunit to CREB ratio of 1:100 and was incubated at 30 °C for 30 min. To measure the incorporation of phosphate, trace amounts of \( ^{32}P \) ATP were added to the reaction. Percent incorporation was determined by precipitating 5 μl of the reaction onto Whatman glass microfiber filters and washing extensively with 10% trichloroacetic acid, followed by 5% trichloroacetic acid and finally 70% ethanol. The incorporation of \( ^{32}P \) was quantitated in a scintillation counter following the addition of fluor (Beta-max). Under these conditions, phosphorylation of CREB by PKA had been previously demonstrated to occur only on Ser\textsuperscript{133} (31, 32), and assuming one PKA phosphorylation per CREB monomer, phosphate incorporation was consistently stoichiometric. For spectroscopic studies an excess of ATP was removed by dialysis overnight in the CD buffer.

Circular Dichroism—The purified proteins were concentrated and dialyzed against 10 mM potassium phosphate, pH 7.5. The protein concentration ranged from 0.5 to 1.0 mg/ml as determined by amino acid analysis. The CD spectra were taken on a J asco J-500 spectropolarimeter using a 0.01-cm path length cell (Helma) thermostatted at 20 °C. The instrument was calibrated by using (+)-10-camphorsulfonic acid (\( \Delta \beta = -2.37 \pm 0.01° \text{ cm}^{-1} \) at 290.5 nm and -4.95 at 192.5 nm). Data were collected on an IBM/PC-AT using the IF-500 interface and software provided by J asco. Spectra and buffer base lines were the average of 8–10 scans recorded at 0.1-nm intervals. A scanning rate of 10 nm/min and a 2-s time constant were used. Before spectral deconvolution of secondary structure analysis, the buffer was removed by filtration, the sample was concentrated, and the spectra were smoothed using software provided by J asco. The CD spectra were deconvoluted for secondary structure content using the variable selection methods described elsewhere (33, 34). For this analysis combinations from a set of 33 basis spectra (kindly provided by W. Curtis J ohnson, J r., Oregon State University) were used to find those that result in the best fit using criteria described by Coxcomb et al. (35). All secondary structure predictions in each combination that met these criteria were averaged to give the final secondary structure values for each experimental spectrum. The standard error for each structure value was typically less than 3%.

The protein spectra in the presence of DNA are difference spectra. The CD spectra of the DNA oligonucleotide alone was subtracted from that of CREB bound to DNA. The CD spectra of CREB341 was a 14-base pair, double-stranded palindromic site of the somatostatin CRE (5’-GGCTGACGTTAGCCG-3’), where the consensus CRE site is bold. The nonspecific DNA-binding site was a palindromic 16-base pair consensus oligonucleotide derivative of the E. coli purF operator (5’-ACGGCAAGCTTTCGCG-3’). Protein and DNA were mixed at concentrations of 30 μM each to ensure that all CREB could be DNA bound. Protein Digests—Approximately 10 μg of CREB and C-REB in 25 mM Tris-HCl buffer, pH 8.0, containing 150 mM KCl were digested with 100 ng of trypsin, chymotrypsin, or Endo Glu-C protease (Staphylococcus aurus V8 protease). The samples were incubated from 0 to 10 min for trypsin and chymotrypsin and from 0 to 40 min for Endo Glu-C protease (Fig. 5 and data not shown). The reactions were stopped at various points by the addition of SDS-PAGE loading buffer and boiling for 5 min. The samples were immediately separated by 15% polyacrylamide gel electrophoresis and stained with the Coomassie-based Fast Stain (Zion). Fluorescence Anisotropy Measurements—Fluorescently labeled proteolytic fragments were collected on a PanVerra Baseline Fluorescence Polarization System. Samples were excited at 490 nm, and emission was measured at 530 nm. 5’-Fluoresceinated oligonucleotides were purchased from Genosys Biotechnologies and purified by reverse phase HPLC on a Vydac C4 column. The sequences are presented in Fig. 6A. Only the upper strands were labeled with fluorescein. Oligonucleotides were annealed by combining equimolar amounts of each strand, heating to 95 °C and cooling slowly. Annealing was confirmed by native PAGE, and double-stranded oligonucleotides amounted to 95% or greater of the total oligonucleotide present (data not shown).
The binding reactions contained 1 ml of 0.5 nM fluoresceinated oligonucleotide in 25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 0.1% Triton X-100, 5% glycerol, and 10 mM dithiothreitol. Bovine serum albumin at 1 μM was included in the experiments to reduce nonspecific association. CREB (10–20 μM as determined by amino acid analysis) was serially diluted and titrated into the cuvette such that the total sample volume increased no more than 10%. Binding reactions were performed at room temperature. Samples were incubated in the fluorimeter for 2 min prior to each measurement to allow thermal and binding equilbrium. Under these conditions, the maximum change in anisotropy occurs within 15–30 s. Each point represents the average of four anisotropy measurements with a 10-s integration time for each reading. The standard deviation for each point was consistently less than 2% of the measured anisotropy value.

Data Analysis—The curves were fit with a nonlinear least squares regression analysis assuming a bimolecular model such that the apparent Kₐ values represent the protein concentration of half-maximal DNA binding. A nonspecific component was included in the model to account for the linear increase in anisotropy at higher protein concentrations.

**RESULTS**

Expression and Purification of CREB, ACT265, and CREB/SER—The structural principles of the bZIP dimerization and DNA-binding motif are well characterized; however, little is known about the structure of the full-length CREB protein and, in particular, of the activation domain. The first step in obtaining such information is to develop an overexpression and purification system that provides sufficient quantities of properly folded and active protein. We first developed a bacterial expression and purification scheme for wild-type CREB341. Our purification involves a 10-min, 75 °C heat step followed by anion exchange chromatography. Each liter of bacterial culture yields about 2 mg of greater than 95% pure CREB (Fig. 1B). However, oxidation, aggregation, and poor solubility hampered purification efforts and structural characterization. These problems appear to arise primarily from the bZIP domain, which aggregates as a result of random disulfide bond formation (three of CREB’s four cysteines reside in the bZIP domain), and nonspecific interactions between the basic regions in the absence of DNA (36).

To circumvent solubility problems and to simplify our structural analysis of CREB, we engineered two proteins. The first, ACT265 (residues 3–265), consists of the entire activation domain minus the bZIP DNA-binding domain. Purification did not require heat treatment and included an ammonium sulfate precipitation step and anion exchange chromatography. One liter of bacterial culture yields about 5 mg of greater than 95% pure ACT265 (Fig. 1C).

The second engineered protein consists of full-length CREB in which the three cysteines (Cys³⁰⁰, Cys³¹⁰, and Cys³³⁷) in the DNA-binding domain were substituted with serine residues (Fig. 1A and Fig. 7). There is a fourth cysteine in the activation domain that was not changed (Cys³⁰⁶). This protein, referred to as CREB/SER, has significantly improved solubility as observed by analytical ultracentrifugation. Purification was similar to that for wild-type CREB and ACT265 and was performed both with and without the heat treatment step. One liter of bacterial culture yields about 5 mg of greater than 95% pure CREB/SER (Fig. 1D).

CREB, ACT265, and CREB/SER Are Heat-stable—Boiling cellular extracts is a previously reported method for purifying CREB for use in electrophoretic mobility shift assays (37, 38). Heating appeared an effective purification step for CREB, and several lines of evidence suggested that CREB is stable to this treatment: (i) CREB remains soluble after boiling (37, 38); (ii) heat-purified CREB is highly active in electrophoretic mobility shift assays (30, 37, 38); (iii) heat-purified phosphorylated CREB binds specifically to CBP (24); and (iv) peptides of the CREB leucine zipper or of its bZIP domain refold after thermal denaturation (36). Therefore, heat-purified CREB retains two critical functions, DNA binding and CBP binding, which are located in two separate structural domains.

Since boiling is an unorthodox method for purifying proteins and can cause irreversible modification, such as deamidation, we empirically determined the lowest effective temperature for purification of both the full-length protein and the truncated activation domain to be 75 °C. ACT265 and CREB/SER were also purified without a heat treatment step, and we used cir-

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2 J. P. Richards, unpublished results.
The activation domain of CREB contains mostly random coil and \( \beta \)-turn structures. To test whether PKA phosphorylation grossly altered the secondary structure of CREB, we compared the CD spectra of both CREB and phosphorylated CREB (P-CREB) as well as of ACT265 and phosphorylated ACT265 (P-ACT265). The purified proteins were phosphorylated stoichiometrically with the catalytic subunit of PKA. The CD spectra of CREB and ACT265 do not change following PKA phosphorylation (Fig. 3).

We extended our CD analysis to examine the spectra of CREB in the presence of DNA. Previous CD studies had shown that DNA binding induced a coil to helix transition in the bZIP peptides of GCN4, CREB, Fos/J un, and C/EBP (39–43); however, the effect of DNA binding on the full-length proteins has not been reported. We examined the CD spectra of full-length CREB and P-CREB bound to DNA for two reasons: first, to determine whether DNA binding induced structural changes beyond the bZIP domain and second, to explore the possibility that any phosphorylation-induced secondary structural changes required DNA binding.

The CD spectra of CREB and P-CREB measured in the presence and absence of DNA are shown in Fig. 4. The CD signals of the oligonucleotides were subtracted from the CREB:DNA spectra. This method assumes that the CD signal of the oligonucleotide does not change or changes little relative to the protein CD signal, and previous studies support this assumption (44–46). There is a small and equal change in the CD spectra of both CREB and P-CREB in the presence of either the specific CRE binding site or a nonspecific oligonucleotide. Therefore, phosphorylation does not induce a DNA binding-dependent change in secondary structure. The DNA-induced change in CD signal represents an increase in \( \alpha \)-helix of approximately 5%, as determined by variable selection (see below). This increase is consistent with the amount of change expected from the CREB bZIP region when it undergoes the coil to helix transition upon DNA binding; a 20% increase in \( \alpha \)-helix was observed in a 72-residue peptide of the CREB bZIP domain (36). DNA binding does not influence significantly the secondary structure of the remaining CREB activation domain.

The Activation Domain of CREB Contains Mostly Random Coil and \( \beta \)-Turn Structures—To examine the secondary structures of the CREB activation domain, the CD spectra of CREB and ACT265 were deconvoluted and their secondary structure contents obtained using the variable selection method developed by Compton and Johnson (33). We used a basis set of 33 proteins, and the results are presented in Table I. The analysis of secondary structure revealed that full-length CREB contains approximately 20% \( \alpha \)-helix, 9% \( \beta \)-strand, 34% \( \beta \)-turn, and 37%...
random coil (Table I). ACT265 contains approximately 3% α-helix, 16% β-strand, 29% β-turn, and 52% random coil. Like several other eukaryotic transcription factors (47), there is a high degree of random coil or aperiodic secondary structures in the CREB activation domain. We find this result interesting, since the ACT265 protein encompasses the entire activation domain and all its subdomains, and phosphorylated ACT265 retains the ability to bind specifically to the co-activator CBP (data not shown).

Protease Cleavage Patterns of CREB and P-CREB—CD is a powerful technique for comparing the secondary structure of modified or mutated proteins, but there are several types of conformational changes, such as the movement about a hinge, that might go undetected in a CD spectra. Proteins that undergo global conformational changes often demonstrate changes in their protease cleavage patterns. We further investigated the effects of PKA phosphorylation on CREB structure by comparing the protease cleavage patterns of CREB and P-CREB when partially digested by trypsin, chymotrypsin, and Endo Glu-C protease. Some representative digests are presented in Fig. 5. We note the difficulty in interpreting these patterns, because CREB is particularly sensitive to protease cleavage. This protease sensitivity corroborates the prevalence of unstructured regions. Nevertheless, we did not detect any significant differences in the protease cleavage patterns of CREB and P-CREB. The number and molecular weight of the digested bands for each protease are the same in both proteins, and in the absence of any qualitative change in cleavage pattern, we see no evidence for a phosphorylation-induced conformational change in CREB.

CREB and P-CREB Bind DNA with Equal Affinity—A number of studies have addressed the effect of PKA phosphorylation on DNA binding. Initial reports observed that PKA phosphorylation of CREB did not affect binding to the somatostatin CRE (SSCRE), the canonical, 8-base pair sequence (48, 49). However, other groups noticed a CAMP-dependent increase in CREB binding to some of the non-canonical CRE sites (21, 22). A more extensive study (23) indicated that PKA phosphorylation significantly increased the binding of CREB to the tyrosine aminotransferase CRE (TATCRE) and other non-canonical sites, but had less of an effect on the canonical SSCRE. Phosphorylation was proposed to increase CREB binding to the non-canonical "low-affinity" CRE sequences and bring binding to a level observed for the "high-affinity" CRE sequences such as the SSCRE. Contrary to this conclusion, a recent paper
reported that PKA phosphorylation did not affect CREB binding to the HTLV-CRE, a non-canonical class CRE (50). Lack of consensus remains over the role of PKA phosphorylation in DNA binding.

We used a fluorescence anisotropy DNA binding assay to measure the relative affinity of CREB and P-CREB homodimers for various CRE sequences. Fluorescence anisotropy is a direct solution technique used for monitoring protein-DNA and protein-protein interactions (25, 51–54). We measured the apparent equilibrium dissociation constant, $K_d$, of CREB and P-CREB for the somatostatin CRE (SSCRE), the tyrosine amino transferase CRE (TATCRE), and the consensus TRE (12-O-tetradecanoylphorbol-13-acetate-responsive element or AP-1 site; Fig. 6). The TRE sequence is similar to the CRE, but it does not confer CAMP responsiveness and does not bind CREB with high affinity. The apparent $K_d$ values were calculated as the protein concentration of half-maximal DNA binding. The apparent $K_d$ for the CREB-SSCRE complex is $2 \pm 1 \text{ nM}$, and the $K_d$ for the CREB-TATCRE complex is $11 \pm 1 \text{ nM}$. The $K_d$ for the P-CREB-SSCRE complex is $2 \pm 1 \text{ nM}$, and the $K_d$ for the P-CREB-TATCRE complex is $11 \pm 1 \text{ nM}$. Binding to the TRE occurs with a $K_d$ value of approximately $200 \text{ nM}$. Phosphorylation does not change the DNA binding affinity of CREB for either canonical or non-canonical CRE sequences.

**DISCUSSION**

We are studying the phosphorylation-dependent activation of the CREB/CREM/ATF family of transcription factors and have initiated the structural and biophysical characterization of CREB, the prototypic family member. CREB contains both glutamine-rich and phosphorylation-dependent activation motifs. We have examined the activation domain alone and in the context of the full-length protein and have tested some of the proposed structural mechanisms for activation by PKA phosphorylation.

We first developed straightforward methods for purifying full-length CREB and the truncated ACT265. These purifications provide the yields of pure protein necessary for structural studies, avoid the use of fusion proteins, and do not rely on boiling (37, 38). Furthermore, we demonstrate that a 10-min, 75°C heat treatment is sufficient for the initial step of our purification protocols and that this heat step does not alter permanently the secondary structure of CREB. An advantage of this heat treatment is that it reduces, although it does not eliminate, proteolytic breakdown. In addition, we demonstrate that the substitution of the three cysteines in the DNA-binding domain with serines yields a full-length CREB protein that is more soluble and more readily purified in quantities sufficient for biochemical and biophysical studies. These cysteine to serine substitutions do not change the secondary structure or DNA binding properties of CREB.

The original models of CREB activation included significant conformational changes in response to phosphorylation. These models were based primarily on mutagenesis studies in which the Ser$^{133}$ was replaced with the charged residues glutamate or aspartate (10, 19, 31). These substitutions did not mimic phosphorylation, and the conclusion was that a conformational change, rather than increased negative charge, was required for phosphorylation-dependent activation. We measured the CD spectra of full-length CREB and P-CREB as well as of ACT265 and P-ACT265 to determine whether PKA phosphorylation caused any significant changes in secondary structure. Circular dichroism has been used to study phosphorylation induced changes in proteins such as keratin, myelin basic protein, and fibrinogen (55–57). We found the CD spectra of the phosphorylated forms of CREB and ACT265 to be indistinguishable from the CD spectra of the non-phosphorylated forms. This finding argues that PKA phosphorylation alone does not significantly alter the secondary structure of CREB.

DNA binding has been shown to induce a coil to helix transition in bZIP peptides (36, 39, 41–43, 58). However, the presence of this change in the full-length protein, or the effects of DNA binding on regions outside of the DNA-binding domain, have not been addressed. We measured the CD spectra of CREB bound to both a high affinity somatostatin CRE site as well as to a nonspecific oligonucleotide that encompasses the E.

![Fig. 6. Fluorescence anisotropy DNA binding curves of CREB and P-CREB for different CRE sequences.](image_url)
coli purF operator. We observed a small but reproducible change in the CD spectra of the CREB-DNA and P-CREB-DNA complexes with a definite shift in the α-helical peaks that occur at 208 and 222 nm. Phosphorylation did not affect the CREB-DNA spectra. Using variable selection, we determined that DNA binding resulted in an approximately 5% increase in α-helix content. This increase in α-helix accounts for the induced structure in the bZIP domain and indicates that DNA binding does not affect CREB secondary structure beyond the bZIP domain.

We measured the CD spectra of CREB and P-CREB in the presence of a nonspecific oligonucleotide, which is the usual control for monitoring specific DNA binding-induced changes. Previous studies on peptides of bZIP domains detected a greater increase in α-helix content in the presence of specific DNA than with nonspecific DNA site (36). We observed a similar increase in α-helix content with both the specific and nonspecific oligonucleotides. We explain this observation by noting that under our experimental conditions (10 mM phosphate, pH 7.5, 30 μM protein, and 30 μM DNA), CREB-DNA binding is strongly favored even to the non-consensus oligonucleotide. Using such an expanded basis set and including data below, we can deconvolute successfully the full-length CREB spectra, the secondary structure of which is dominated by β-turn and random coil. The secondary structure content is presented in Table I, but differs somewhat from that calculated from primary sequence analysis (13), which contains a higher β-strand and α-helix content. We also estimated the secondary structure content from the primary amino acid sequence (59) and present a predictive outline of secondary structures in Fig. 7. Although such a speculative map should be interpreted with caution, it does correlate reasonably with the CD data and provides some insight into the probable locations of the secondary structure elements of CREB. For example, the Q2 region has a high probability of being largely β-strand, whereas the KID region, like acidic activation domains (60–63), appears to be less structured. The CD data indicate that PKA phosphorylation alone is not sufficient to induce significant secondary structure in the KID; however, phosphorylation enables CREB to bind specifically to other proteins, and such interactions may stabilize or induce secondary structure in this region. This model remains to be tested. The secondary structure content of the activation domain, ACT265, is higher in random coil and lower in β-turn and α-helix content than might be predicted just from the removal of 76 amino acids of the bZIP domain. CD analysis of the bZIP domain alone demonstrated that this region contained approximately 43% α-helix and 57% random coil in the absence of DNA (36). These values can be used to predict the structure content of the activation domain by subtracting the bZIP secondary structure content from the secondary structure content of the full-length protein. By this method, the predicted secondary structure content of ACT265 is about 13% α-helix, 12% β-strand, 44% β-turn, and 31% random coil. Therefore, the ACT265 polypeptide appears to have lost some ordered secondary structure, namely β-turn and α-helix, when compared with the values presented in Table I. This loss of structure could be a result of unfolding around the site of truncation, or it could indicate that the bZIP domain influences the structure or stability of the activation domain.

We performed protease digests to explore the possibility of a global conformational change following phosphorylation that would not have been detected in our CD studies. A previous argument for a phosphorylation-induced conformational change came from a tryptic digest of CREB and P-CREB, which revealed a slight difference in the ratio of two different cleavage products (11). Sequencing assigned the NH2 termini of these tryptic peptides to be residues 125 and 135, sites flanking the phosphorylated Ser133. We note that the phosphate on Ser133 would likely hinder sterically or electrostatically the cleavage at one or both of these sites and thereby alter the kinetics of the reaction. We performed a series of protease digests of CREB and P-CREB with trypsin, chymotrypsin, or Endo Glu-C protease and found no significant difference in cleavage pattern. We emphasize that in all of our partial proteolysis patterns, the number and molecular weight of bands were the same for both CREB and P-CREB. Under careful inspection we, too, could detect slight differences in band intensities; however, in the absence of any clear qualitative change, we do not consider this evidence for a conformational change in CREB.

Finally, we tested mechanisms of phosphorylation-dependent CREB activation by comparing the binding affinities of CREB and P-CREB for various DNA sequences. The previously reported data that phosphorylation increased DNA binding could support the model of a phosphorylation-induced conformational change, since the phosphate on Ser133 must somehow influence the activity of the DNA-binding domain located at the COOH terminus of the protein. The lack of consensus over the effects of phosphorylation on DNA binding may be due to the limitations of the techniques used. Previous studies relied on footprinting and/or electrophoretic mobility shift assays. However, gel electrophoresis has limitations when used for quantitative assessment of some DNA-binding proteins, particularly for bZIP proteins such as CREB that have solubilities that are highly sensitive to binding conditions and are prone to forming higher order aggregates that do not enter the gel matrix (36, 64, 65). We used a fluorescence anisotropy DNA binding assay to measure the relative binding affinities of CREB for both a canonical and non-canonical CRE site and to clarify the role of
PKA phosphorylation on DNA binding. Fluorescence anisotropy, a direct solution measurement, can be carried out under a variety of well-defined equilibrium conditions and does not require immobilization of the protein-DNA complex or separation of bound versus free species (51–54). Our data demonstrate that phosphorylation of CREB by PKA does not alter the DNA binding affinity for either the canonical SSCRE or the noncanonical TATCRE. In addition, our data suggest that the previous assessment of high-affinity versus low-affinity binding sites, in respect to the SSCRE and TATCRE (23), may not be accurate. The difference in apparent Kd between the SSCRE and the TATCRE is less than 10-fold, whereas CREB binds to the TRE and other nonspecific sequences 100-1000-fold less efficiently.

In conclusion, we show PKA phosphorylation alone does not alter the secondary structure, protease cleavage patterns, or DNA binding affinity of CREB. These results argue against models of phosphorylation-induced structural or conformational changes in CREB. Our data do support a model that PKA phosphorylation creates the proper electrostatic and structural scaffold for specific co-activator binding. However, we emphasize that these models need not be mutually exclusive, since phosphorylation enables co-activator binding and may prepare CREB for structural changes that could occur in conjunction with co-activator binding. The next step, biophysical evaluation of P-CREB-co-activator complexes, will further test the structural models of activated transcription.

Acknowledgments—We thank Cynthia Bohan and Jay Gambe for the amino acid analysis, Drs. W. Curtis, J. Johnson Jr. and Araz Tountzadik (Oregon State University) for their updated version of the variable selection program for CD analysis, Drs. J. John D. Scott and Richard A. Maurer (Oregon Health Sciences University) for providing PKA C-subunit, and members of the Goodman and Brennan laboratories for helpful comments.

Note Added in Proof—While this manuscript was under review, a report by Parker et al. (1996) Mol. Cell. Biol. 16, 694–703 concluded similarly that PKA phosphorylation of CREB does not induce a conformational change in its unstructured activation domain, but rather immobilization of the protein-DNA complex or separation of bound versus free species (51–54). Our data demonstrate that phosphorylation of CREB by PKA does not alter the DNA binding affinity for either the canonical SSCRE or the noncanonical TATCRE.