THE BIOPHYSICAL CHEMISTRY OF THE TRANSCRIPTION FACTOR *CREB*

by

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ABSTRACT

The eukaryotic transcription factor CREB (cAMP responsive element binding protein) enhances the expression of several genes in response to increased levels of intracellular cAMP. CREB, a basic-leucine zipper protein, binds to specific DNA elements in the promoter regions of certain cAMP responsive genes and stimulates transcription. CREB is a direct target of the cAMP-dependent protein kinase A (PKA), and phosphorylation by PKA switches CREB from a weak to a strong activator. Both the mechanisms by which phosphorylation activates CREB and by which CREB then increases gene expression are poorly understood. One approach to elucidating these mechanisms of activation is to analyze the molecular structure of CREB.

The purpose of this dissertation is to begin the biophysical and extend the biochemical characterizations of CREB and to test the current models of CREB activation by PKA phosphorylation. Toward this goal I have (i) characterized the secondary structure using circular dichroism; (ii) quantitated the DNA binding properties using a fluorescence anistropy assay; and (iii) studied the tertiary structure using analytical ultracentrifugation. In all studies the PKA-phosphorylated form of CREB was also analyzed and compared to non-phosphorylated CREB. The results argue against two prevalent models of CREB activation: (i) that phosphorylation induces a conformational change in CREB and/or (ii) increases the DNA binding affinity. Instead, phosphorylation by PKA

appears to provide a specific binding site for other proteins such as the coactivator CBP. In addition, a long term goal of this project is to crystallize and solve the three-dimensional structure of the CREB:DNA complex. Toward this aim I have designed and characterized a mutant CREB protein which has improved solubility and is a promising candidate for crystallographic analysis.

INTRODUCTION

Extracellular signals influence the expression of many genes, and an intricate network of signalling pathways exists to transmit messages from the cell surface to the gene. cAMP, for example, mediates various hormone signals, and increased levels of intracellular cAMP stimulates the expression of genes involved in neuroendocrine peptide production (Goodman, 1990), spermatogenesis (Foulkes, *et al.*, 1992) and long term memory (Frank and Greenberg, 1994), among others. Although cAMP signalling involves multiple signal transduction pathways, one pathway is particularly well characterized because of its directness and simplicity. This pathway involves the transcription factor CREB (cAMP Responsive Element Binding Protein) and is initiated at the cell surface when an extracellular ligand binds to a G-protein coupled receptor and stimulates adenylate cyclase to convert ATP to cAMP. Cyclic AMP then binds to the regulatory subunit of protein kinase A (PKA), triggering the release of the active catalytic subunit. The catalytic subunit translocates to the nucleus (Hagiwara, *et al.*, 1993) where it phosphorylates CREB (Gonzalez and Montminy, 1989; Lee, *et al.*, 1990). Phosphorylated CREB is a strong transcriptional activator (Gonzalez, *et al.*, 1991).

The study of cAMP regulated gene expression provides insight into how cells respond to their environment in order to develop, survive and perform their

specific functions. The work presented in this dissertation focuses on events at the downstream end of this signalling pathway, on the processes that immediately surround gene transcription and involve the transcriptional activator CREB. CREB is a DNA-binding protein that stimulates transcription in a tightly controlled manner. By characterizing the biophysical properties of CREB, I aim to elucidate the structure of this protein and to begin to understand, in chemical terms, how a eukaryotic transcription factor can specifically regulate the level of gene expression.

CREB binds the cAMP Responsive Element.

CREB mediates cAMP induced transcription by binding to a specific class of DNA sequences called the cAMP responsive element or CRE (reviewed by (Walton and Rehfuss, 1992)). The CRE was first identified in the promoters of several genes including proenkephalin (Comb, *et al.*, 1986), phosphoenolpyruvate carboxykinase (Short, *et al.*, 1986), c-fos (Gilman, *et al.*, 1986) and somatostatin (Montminy, *et al.*, 1986). The CRE in the somatostatin promoter has been particularly well characterized and is generally considered the canonical CRE sequence (reviewed by (Andrisani and Dixon, 1990)). The somatostatin CRE was defined by making a series of deletions in the 5'-non-transcribed region of the rat somatostatin gene which had been cloned in front of a chloramphenicol acetyltransferase (CAT) reporter gene. A small region was mapped that, when

deleted, resulted in the loss of cAMP induced gene expression in several cell lines (Montminy, *et al.*, 1986; Andrisani, *et al.*, 1987). The insertion of the same DNA element into an otherwise nonresponsive promoter conferred cAMP inducibility suggesting that the CRE acted as an enhancer of gene expression (Montminy, *et al.*, 1986). The CRE had the properties of a traditional DNA enhancer sequence in that it functioned in a distance and orientation independent manner.

The canonical CRE is an eight base pair palindrome (TGACGTCA) which is conserved among a number of cAMP responsive genes (Table I). All of these sequences either consist of the entire canonical eight base pair palindrome or contain the minimal consensus CGTCA region. The CRE sequence is not the only DNA element that can confer cAMP responsiveness to a gene. Other cAMP responsive sequences, such as the AP-2 site (CCCCGAGG), have been identified (Imagawa, *et al.*, 1987). The AP-2 site is unrelated to the CRE and functions through transcription factors that are distinct from CREB. Another DNA enhancer sequence, the phorbol ester response element (the TRE or AP-1 site), is highly related to the CRE (Angel, *et al.*, 1987). This sequence (TGAC_TCA) differs from the CRE by the absence of one base pair. The TRE is unresponsive to cAMP stimulation and does not bind CREB. Rather, the TRE mediates gene expression primarily through mitogen/growth factor response and protein kinase C pathways via the related fos/jun family of transcription factors (Angel and Karin, 1991).

The somatostatin CRE was used to identify and purify the transcription factor CREB (the CRE Binding Protein) (Montminy and Bilezikjian, 1987). The CRE sequence was used to purify CREB conventionally by DNA affinity chromatography (Gonzalez, *et al.*, 1989) and to clone CREB directly by using a radiolabelled CRE oligonucleotide to screen a λgt11 expression library (Hoeffler, *et al.*, 1988). The protein was first cloned from rat (Gonzalez, *et al.*, 1989) and human tissues (Hoeffler, *et al.*, 1988) and is highly conserved across species; there are only three amino acid differences between rat and human CREB. CREB is expressed ubiquitously and was the first protein cloned in a family of related proteins identified by their ability to bind CRE-like sequences (Hai, *et al.*, 1989; Maekawa, *et al.*, 1989) . This group of proteins, which includes ATF-1 (Activating Transcription Factor-1) through ATF-6, have homologous DNA-binding domains, but only CREB, CREM and ATF-1 have so far been shown to be involved in cAMP pathways. These three proteins and their splice variants form a distinct subclass of transcription factors and represent three different genes which show significant conservation of exon structure and amino acid sequence. Because it was the first identified and is perhaps best characterized, CREB is often considered the prototype for this CREB/CREM/ATF-1 family of transcription factors (Fig. 1A).

CREB belongs to the bZIP class of DNA-binding proteins.

The first evaluations of the primary sequence of CREB revealed that it belonged to the basic leucine zipper (bZIP) class of transcription factors (Hoeffler, *et al.*, 1988; Gonzalez, *et al.*, 1989). The bZIP motif includes a basic-charged DNA binding domain and a leucine zipper dimerization domain. Several transcription factors contain this conserved motif including members of the Fos and Jun families, the yeast transcriptional activator GCN4, C/EBP and others. Some representative bZIP amino acid sequences are listed in Table II for comparison.

The basic DNA binding region is rich in arginine and lysine residues which are important for making electrostatic contacts with the DNA phosphodiester backbone. Several conserved residues are contained in this region including an invariant asparagine residue and an invariant cysteine (or serine)-arginine pair. The spacing of these residues relative to the first leucine of the zipper is also invariant (Table II). The CREB/CREM/ATF family contains additional conserved residues including two consecutive alanines and the positioning of several basic residues. This conservation likely reflects the common DNA sequence specificity of this family of leucine zipper proteins.

DNA binding by bZIP proteins requires dimerization which is mediated by the leucine zipper motif (Hope and Struhl, 1987). Dimerization in GCN4 was demonstrated by mixing the wild-type GCN4 protein with a truncated derivative

and examining DNA binding by electrophoretic mobility shift assay (EMSA) (Hope and Struhl, 1987). The mixture generated three protein-DNA complexes: two complexes corresponded to those observed when the proteins were present individually and the third complex, of intermediate mobility, corresponded to the heterodimer. The composition of the complexes was confirmed by two-dimensional gel electrophoresis. A similar assay has been used to demonstrate the heterodimerization properties of different ATF proteins (Hai, *et al.*, 1989; Rehfuss, *et al.*, 1991) and to monitor the effectiveness of CREB bZIP domains engineered with altered dimerization specificity (Loriaux, *et al.*, 1993).

The structural features of the bZIP motif have been extensively studied. The leucine zipper folds as an amphipathic alpha helix which associates as a parallel coiled coil (O'Shea, *et al.*, 1989; Oas, *et al.*, 1990; O'Shea, *et al.*, 1991). The leucine residues form a heptad repeat of amino acids that is arranged with the positions denoted as *abcdefg* (Fig. 2). Leucine residues typically occupy the *d* position and, together with residues in the *a* position, align along one face of the helix forming a hydrophobic surface. The hydrophobic surfaces of two amphipathic helices associate as a parallel coiled coil similar to the coiled coil structures originally described in proteins such as keratin (Cohen and Parry, 1986). Dimer stability is due largely to the hydrophobic residues in the *a* and *d* positions, but charged residues in the *e* and *g* positions of the leucine zipper can

contribute significantly to the specificity of dimerization (Hu, *et al.*, 1993; Loriaux, *et al.*, 1993).

The bZIP domain is largely unstructured in the absence of DNA where electrostatically repulsive interactions between positively charged residues drive the basic region into a more extended, flexible conformation. Specific binding to DNA induces alpha helix formation (O'Neil, *et al.*, 1990; Oas, *et al.*, 1990; Weiss, 1990; Weiss, *et al.*, 1990; O'Neil, *et al.*, 1991; Saudek, *et al.*, 1991). The induced structure concept of the bZIP DNA-binding domain distinguishes it from other DNA binding motifs such as the helix-loop-helix or zinc finger which exist as stably folded domains even in the absence of DNA. The GCN4 bZIP increases from 70% to 95% alpha helix content (Weiss, *et al.*, 1990) in the presence of DNA, whereas the CREB bZIP increases from 43% to 63% alpha helix content (Santiago-Rivera, *et al.*, 1993).

The crystal structures of the bZIP domain of GCN4 bound to the TRE and to the CRE have been solved (Ellenberger, *et al.*, 1992; König and Richmond, 1993). The bZIP binds DNA as a dimer of uninterrupted alpha helices. The coiled coil dimerization interface is oriented almost perpendicular to the DNA axis. There are no kinks or sharp bends in either bZIP monomer, which contrasts one postulate of the original "scissors grip" model for bZIP/DNA binding. This early model, which was otherwise correct, predicted that the invariant asparagine formed an α-helix N-cap and was the position of a bend or kink in the basic region

alpha helix (Vinson, *et al.*, 1989). The crystallography data support the related "induced helical fork" model (O'Neil, *et al.*, 1990). The coiled coil of the leucine zipper dimer bifurcates with a smooth bend in the alpha helix in the direction of the DNA axis allowing the α-helix to traverse the major groove where the protein can make a series of base-specific contacts. GCN4 binds to the TRE site without inducing any DNA bending (Ellenberger, *et al.*, 1992), whereas binding to the CRE site requires DNA flexure (König and Richmond, 1993).

The X-ray structure of c-Fos/c-Jun heterodimeric bZIP domain bound to the TRE has also been recently solved (Glover and Harrison, 1995). The overall structure resembles that of GCN4 bound to DNA. A significant finding of this structure was that the Fos/Jun heterodimer can bind the asymmetric TRE site in either of two orientations related by a 180° rotation about the dyad axis. This finding reflects the indifference of the heterodimer to the orientation of the TRE. The Fos/Jun complex bound to primarily B-form DNA which was bent at most 10° towards the coiled-coil. This lack of bending contrasts electrophoretic mobility data which indicate that both Fos and Jun significantly bend the DNA but in opposite directions (Kerppola and Curran, 1991). The reason for this difference may be because the X-ray structure contained smaller Fos/Jun peptides and a shorter DNA sequence than those used in the DNA bending study. The regions critical for bending may not have been included in the crystallographic structure (Kerppola and Curran, 1995).

Determining the presence and magnitude of directed DNA bending is an important aspect of characterizing protein:DNA interactions, as DNA distortion is one mechanism by which transcription factors can influence the promoter complex. CREB and CREM have recently been reported to bend DNA (de Groot, *et al.*, 1994). Using a circular permutation assay (Wu and Crothers, 1984) de Groot *et al.* showed altered electrophoretic mobilities for CREB- and CREM-DNA complexes depending on the location of a specific binding site on a linearized sequence of DNA. They reported a bend angle of about 73º for both CREB and CREM. Interestingly they reported a 10º increase following phosphorylation by PKA implying that a conformational change occurred in the complex. Unfortunately, these conclusions relied exclusively on the circular permutation assay which does not distinguish between a directed DNA bend and a region of increased flexure. Phasing analysis (Zinkel and Crothers, 1987), in which the flexure is compared in and out of phase with an intrinsically bent DNA sequence, is required to confirm site directed bending. Both circular permutation and phasing analysis were used to measure the bending of a variety of bZIP proteins when bound to TRE or CRE sites (Kerppola and Curran, 1993). This extensive study found that CREB and ATF-1 induced significant variation in DNA complex mobilities as measured by circular permutation analysis suggesting the presence of structural distortions. However, phasing analysis revealed that this distortion was not a result of a directed DNA bend.

An explanation for the variation in mobility of CREB-DNA complexes may come from the results of Paolella *et al.* (Paolella, *et al.*, 1994). This group also used the circular permutation assay in conjunction with phasing analysis to characterize the determinants of GCN4 and CRE-BP1 (or ATF-2, a CREB family member) DNA binding specificity. They found that the CRE sequence, unlike the TRE sequence, had an intrinsic curvature in the absence of protein binding. This curvature was actually straightened following CRE-BP1 binding, implying that CRE-BP1 must introduce a slight bend toward the minor groove upon binding the CRE sequence. Like CREB, CRE-BP1 binds to the AP-1 site only with low-affinity. However, at high protein concentrations binding was detected and a distinct bend toward the minor groove was detected. The authors postulate that the intrinsic flexure of the DNA plays a significant role in binding specificity of CREB family members as well as GCN4. Verification of this model awaits further structural and biochemical characterization of CREB:DNA complexes.

At this point, the majority of structural characterization has been performed on the bZIP domains of GCN4 and on Fos/Jun. Because of the homology between their bZIP domains, GCN4 has often been used as a model for the CREB bZIP region. While the assumption that GCN4 is a good model for CREB structure holds for many of the general characteristics of the CREB bZIP, there are several instances where GCN4 falls short as a model for CREB. GCN4 and Fos/Jun bind both the CRE and the AP-1 with high affinity, whereas CREB is able only to bind

the CRE with high affinity (Paolella, *et al.*, 1994). As mentioned above, the source of this specificity is not completely understood, although it probably involves a combination of the intrinsic flexure of the DNA sequences, the amino acid sequence in the basic region and the sequence of the fork region between the leucine zipper and basic region (Paolella, *et al.*, 1994). Attempts to use the X-ray structure of GCN4 as a model to design a CREB bZIP domain with altered sequence specificity were not successful (M. Loriaux, unpublished results). These modeling attempts underscore the significant differences in the way GCN4 and CREB recognize DNA. Why does CREB bind the CRE and not the TRE? The biophysical characterization of CREB and the eventual three-dimensional structure of the CREB bZIP domain bound to DNA will help answer this question as well as clarify how different bZIP proteins control the very specific interactions of dimerization and DNA binding.

The CREB activation domain contains several functional regions.

The activation domain of CREB encompasses the amino terminal three-fourths of the protein, and contains several regions that are important for both basal and PKA inducible function. These regions have been identified by mutagenesis and deletion analysis. The activity of CREB and the function of individual CREB domains has generally been monitored by *in vivo* transfection assays. There have been two general approaches to these assays, both of which

circumvent endogenous CREB activity. The first approach is to fuse CREB to the yeast Gal4 DNA-binding domain and measure the activity of a Gal4 driven reporter (Lee, *et al.*, 1990; Quinn, 1993). The second approach is to perform the transfection assays in undifferentiated F9 cells which lack a functional cAMP signalling pathway (Yamamoto, *et al.*, 1990; Gonzalez, *et al.*, 1991).

The principle region involved in the cAMP activation of CREB is the kinase inducible domain (KID, (Gonzalez, *et al.*, 1991)) or phosphorylation box (P-Box, (Lee, *et al.*, 1990)). This region contains serine residue 133 which is the site of phosphorylation by PKA (Fig. 1B). Mutation of this residue to alanine, aspartate or glutamate eliminates the cAMP responsiveness of CREB without affecting its basal activity (Lee, *et al.*, 1990; Gonzalez, *et al.*, 1991). Ser133 can also be phosphorylated by the calcium/calmodulin kinases CamKI, CamKII and CamKIV which are likely to regulate CREB function through signal transduction pathways that are distinct from the cAMP pathway (Dash, *et al.*, 1991; Sheng, *et al.*, 1991; Sun, *et al.*, 1995). These pathways are discussed further in the next section. CREB has recently been shown to be a substrate for the glycogen synthase kinase-3 (GSK-3) (Fiol, *et al.*, 1994). GSK-3 is a hierarchical kinase and requires prior phosphorylation at Ser133 to phosphorylate Ser129. Mutational analysis of this site suggests that GSK-3 augments the activation by PKA (Fiol, *et al.*, 1994). The CREB activation domain also contains a number of potential phosphorylation sites for other kinases such as PKC and casein kinase II. Most of

these sites are within the KID, but their role in CREB function not well understood.

Just as phosphorylation has been shown to convert CREB to a strong activator, dephosphorylation has been shown to down-regulate CREB activity (Hagiwara, *et al.*, 1992; Wadzinski, *et al.*, 1993; Wheat, *et al.*, 1994). Hagiwara, *et al.* demonstrated that, following cAMP stimulation, the attenuation of CREB activity in PC12 cells corresponded with dephosphorylation of CREB on Ser133. They attributed this dephosphorylation to protein phosphatase 1 (PP1) (Hagiwara, *et al.*, 1992). Wadzinski, *et al.* demonstrated CREB regulation by dephosphorylation in HEPG2 cells but attributed this regulation to protein phosphatase 2A (PP2A) (Wadzinski, *et al.*, 1993). They found that CREB phosphatase activity in nuclear extracts copurified with PP2A and was completely resolved from PP1. Wheat, *et al*, further defined PP2A as the primary CREB phosphatase (Wheat, *et al.*, 1994). SV40 small t antigen, which binds to nuclear PP2A but not to PP1, protects P-CREB from dephosphorylation in rat liver nuclear extracts and enhances cAMP-stimulated transcription from the PEPCK promoter.

Residues in the KID region other than Ser133 are also important for CREB function. At least two groups have reported that deletion of the DLSSD sequence (residues 140-144) in the KID nearly abolishes CREB function (Lee, *et al.*, 1990; Gonzalez, *et al.*, 1991). Site-directed mutagenesis of Asp140 resulted in 80% loss

of activity whereas site-directed mutagenesis of any of the other residues resulted in a 40-50% loss in CREB activity (Gonzalez, *et al.*, 1991). Both groups speculated that the DLSSD region was important for a PKA-induced conformational change in CREB. Examining the structural details of the entire KID domain will be crucial to understanding its role in PKA-induced activation.

Functional regions outside of the KID domain have been identified by deletion analysis (Fig. 1A). In particular, there are two glutamine rich regions referred to as Q1 and Q2. Several groups found the amino-terminal Q1 domain to contribute to basal, but not PKA-induced CREB activity (Lee, *et al.*, 1990; Gonzalez, *et al.*, 1991; Quinn, 1993), although disagreement existed on the extent of this contribution. Brindle, *et al.* found the Q2 region to be essential for basal function (Brindle, *et al.*, 1993), whereas Quinn demonstrated that both Q1 and Q2 were essential for full basal activity with Q2 contributing more to basal activity than Q1 (Quinn, 1993). In addition to deletion analysis, Quinn demonstrated "gain of function" activity for the independent Q1/Q2 regions and the KID. For example, the KID domain was fused to a Gal4 DNA binding domain (KID-Gal4DBD). This construct was not a transcriptional activator in the absence of PKA stimulation, but became an activator following PKA induction. Similarly, the Q1/Q2 domains conferred a basal stimulation but did not respond to PKA activation. Brindle *et al.* also reported slight "gain of function" activity for KID-Gal4DBD and Q2-Gal4DBD constructs (Brindle, *et al.*, 1993). Both groups noted

that the constitutive region and the PKA-responsive region acted together synergistically.

The importance of the Q regions in basal activity correlates with the activities described for different CREM isoforms (reviewed by (Lee and Masson, 1993; Meyer and Habener, 1993)). CREMα contains an intact KID region but lacks both the Q1 and Q2 regions (Fig. 1). CREMα has no basal activity and does not respond to PKA activation but appears, instead, to be a repressor of CREB function (Foulkes, *et al.*, 1991). On the other hand CREMτ contains both the Q1 and Q2 regions and exhibits both basal and PKA-inducible activity. Similarly, two CREMτ isoforms: CREMτ1 (which contains the Q1 but not the Q2 region) and CREMτ2 (which contains the Q2 and not the Q1 region) are transcriptional activators. The Q2 domain appears to confer a slightly higher activation potential than the Q1 domain (Laoide, *et al.*, 1993). Therefore, these CREM isoforms demonstrate that the Q domains, in addition to being involved in basal activation, contribute to the PKA-inducible activity.

The role of the α-peptide, a 14-amino acid splice insert near the KID that distinguishes CREB341 from CREB327, has been extensively studied, but its function has not been resolved. Yamamoto, *et al.* reported that CREB341 was more active than CREB327 (Yamamoto, *et al.*, 1988), whereas other groups found that the two isoforms were equally active (Berkowitz and Gilman, 1990; Ruppert, *et al.*, 1992; Quinn, 1993). Circular dichroism analysis of the α-peptide showed it

to be α-helical in the presence of 40% TFE. However, TFE can induce α-helix structure in peptide sequences that have a helical propensity, and this induced structure does not necessarily represent a fold which is observed in the native, intact protein (Sönnichsen, *et al.*, 1992). For example, in peptide regions with both β-sheet and α-helical propensity, TFE will most likely induce helix formation. Both the neighboring residues and the environment of the full-length protein have a profound effect on peptide structure. Therefore, the structure and function of the α-peptide awaits further characterization.

Clearly, some discrepancy exists over the functional significance of the different CREB regions. Some of this disagreement is probably due to the different cell systems, protein constructs and assays employed by the different laboratories. However, there is likely some structural interdependence between the different regions, and a characterization of the secondary and tertiary structure of CREB will be necessary to sort out all of its functional domains.

CREB can mediate transcription through non-PKA pathways.

Although isolated as a transcription factor that responds to cAMP signals via direct phosphorylation by PKA, CREB has also been shown to activate transcription in response to other signalling pathways. Inducibility by membrane depolarization and the accompanying increase in intracellular calcium concentrations was mapped to CRE regions in the c-fos (Sheng, *et al.*, 1990) and

proenkephalin (Nguyen, *et al.*, 1990) genes. Depolarization of PC12 pheochromocytoma cells correlated with rapid phosphorylation of CREB on Ser133 (Sheng, *et al.*, 1990) and CREB was subsequently shown to be a calcium activated transcription factor: a GAL4-CREB fusion construct conferred calcium inducibility to a c-fos reporter gene (Sheng, *et al.*, 1991). The proposed mediators of this induction were the calcium-calmodulin protein kinases (CaM kinases) which are activated by the increased calcium concentrations that accompany membrane depolarization. Ser133 was found to be a substrate for both CaM KI and Cam KII in vitro (Dash, *et al.*, 1991; Sheng, *et al.*, 1991). However, in vivo studies with CaM KII and CaM KIV demonstrated that CaM KIV was much more effective than CaM KII in activating CREB in three different cell lines (Sun, *et al.*, 1995). Mutagenesis and phosphopeptide mapping demonstrated that CaM KIV phosphorylates CREB only on Ser133, whereas CaM KII phosphorylates both Ser133 and Ser142. Phosphorylation of Ser142 blocked the activation by CREB in transient transfection assays that would otherwise occur when Ser133 was phosphorylated. Thus, CREB not only provides a point of convergence for cAMP and calcium mediated pathways, but a point at which calcium signals could modulate cAMP stimulated transcription.

CREB has also been shown to be a target of nerve growth factor (NGF) mediated transcription. NGF induction of c-fos was found to require CREB, and NGF treatment of PC12 cells caused a rapid increase in phosphorylation of CREB

on Ser133 (Ginty, *et al.*, 1994). The authors ruled out the involvement of PKA and the CaM kinases in this pathway and identified a novel NGF-inducible, Ras-dependent protein kinase that phosphorylates CREB on Ser133.

Although CREB contains many potential phosphorylation sites which may help to modulate its function, Ser133 plays a crucial role in integrating numerous signalling pathways. Defining the structure of this region and determining the mechanism of transcriptional activation by phosphorylation of Ser133 will be crucial to our understanding of CREB function.

CREB activates RNA polymerase II transcription by several possible mechanisms.

The initiation of transcription is a tightly regulated process which requires the formation of a multiprotein complex around the promoter. There are several general mechanisms by which activators could affect the assembly of this complex and increase transcription. Activators could (i) change the chromatin structure; (ii) release repression; (iii) increase the rate of complex formation; or (iv) increase the number of complexes formed.

At least seven general factors have been identified which assemble into the basal pre-initiation complex around RNA polymerase II promoters: TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and TFIIJ. There are two main models of how this assembly occurs (reviewed by (Hori and Carey, 1994)). The traditional view is

that these factors assemble in a defined order. TFIID, which includes the TATA-binding protein (TBP) binds to the promoter forming an initial complex. RNA polymerase II in conjunction with TFIIB and TFIIF assemble with TFIID to form the site-selected complex. TFIIH, TFIIE and TFIIJ then bind to form the complete preinitiation complex. This stepwise assembly has been demonstrated in *in vitro* transcription reconstitution assays in the absence of activators. An activator could influence any step in the process by recruiting a particular factor to the pre-initiation complex.

An alternative model for preinitiation complex assembly has recently been suggested based on the discovery of the suppressors of RNA polymerase B complex in yeast (Koleske and Young, 1994). This model proposes that a preformed assembly of RNA polymerase II and a subset of the general transcription factors can enter the pre-initiation complex in one step. The binding is facilitated by TFIID bound to TATA, which positions the complex near the start site, and by activators which recruit the complex to TFIID. In this model, the complex represents a single target with a multitude of potential binding sites for activators. The role of an activator would be to contact any point along this surface and recruit the complex to the promoter.

How does CREB activate gene expression? CREB binds to CRE enhancer sequences that are generally found upstream of the transcription initiation site of RNA polymerase II promoters. Here CREB, at least in some instances, acts as a

weak activator and is responsible for basal, or constitutive, levels of transcription (Xing and Quinn, 1993). Phosphorylation of CREB at Ser133 stimulates a greater than 10-fold increase in this basal expression. How CREB interacts with the general transcriptional machinery, and how this phosphorylation event switches CREB to a strong activator is poorly understood. Early models for CREB activation assumed that different phosphorylation events increased the overall negative charge of the activation domain. In this sense, CREB was thought to behave like the acidic activators of VP16, GCN4 and Gal4. The negatively charged regions of these proteins were thought to form unstructured "acid blobs" or "negative noodles" to recruit binding partners in the preinitiation complex (Sigler, 1988). Substitution of the phospho-serine residue 133 of CREB with the negatively charge residues aspartate or glutamate did not mimic phosphorylation (Lee, *et al.*, 1990; Gonzalez, *et al.*, 1991). This finding suggested that more than the presence of a negative charge was required to create a transcriptional activator and led to a variety of models that relied on a PKA phosphorylation induced conformational change in CREB. One of these models suggested that phosphorylation induced secondary structure in regions near the PKA site such as the DLSSD sequence (Lee, *et al.*, 1990; Meyer and Habener, 1993). Alternatively, PKA phosphorylation may affect the structure or availability of the Q2 domain, thus allowing an interaction with the general transcription complex

that was not previously feasible (Ferreri, *et al.*, 1994). However, no physical data have yet been presented to support these structural models.

Another model for how PKA phosphorylation activates transcription is that phosphorylated CREB has a higher affinity for DNA than non-phosphorylated CREB. Although early reports indicated that CREB and PKA phosphorylated CREB had similar DNA binding affinities (Yamamoto, *et al.*, 1988), later studies reported phosphorylation dependent increases in CRE-binding activity in both *in vivo* and *in vitro* footprinting assays (Merino, *et al.*, 1989; Weih, *et al.*, 1990). A more rigorous electrophoretic mobility shift assay (EMSA) study using purified recombinant proteins indicated that PKA phosphorylated CREB had a higher affinity for lower affinity CRE sequences, such as the tyrosine aminotransferase CRE, but had little affect on the higher affinity CRE sequences, such as the somatostatin CRE (Nichols, *et al.*, 1992). Subsequent dephosphorylation reduced DNA binding. The role of PKA phosphorylation on DNA binding remains controversial as later reports, also using EMSA assays, failed to detect significant differences in DNA binding between CREB and phosphorylated CREB (de Groot, *et al.*, 1993; Anderson and Dynan, 1994). Clearly, a more sensitive and quantitative method is necessary to distinguish whether phosphorylation affects the affinity of CREB for different DNA sequences.

Yet another emerging model suggests that phosphorylation creates a specific binding site for other proteins which then activate gene expression. Such

a candidate protein is CBP (CREB binding protein), a putative coactivator orginally cloned by its ability to specifically bind phosphorylated CREB (Chrivia, *et al.*, 1993). CBP is a 265 Kd nuclear protein which contains several CaMKII phosphorylation sites, a single PKA site, three putative Zn-fingers, a bromodomain and a carboxy-terminal glutamine rich domain. It is not known whether CBP binds DNA directly, but CBP behaves as a transcriptional activator when fused to a heterologous DNA binding domain such as Gal4 (Chrivia, *et al.*, 1993). In transient transfection assays in F9 cells, co-expression of CBP enhances the CREB-mediated gene expression in a dose-dependent manner (Kwok, *et al.*, 1994). Microinjection of CBP antibodies into fibroblasts can inhibit transcription from a cAMP responsive promoter (Arias, *et al.*, 1994). Together, these data suggest that CBP acts as a co-activator of CREB, linking CREB to the general transcription complex.

In addition to communicating with the pol II complex via CBP, CREB may also contact the general transcription machinery directly. Ferreri, *et al.* reported that the TFIID complex, but not TBP alone, supported transcriptional activation by a Q2-Gal4DBD construct. They also reported that Q2 could bind directly to the Drosophila protein dTAF110, albeit with low efficiency. A direct interaction of CREB with mammalian general transcription factors remains to be demonstrated. Nevertheless, CREB is likely involved in multiple interactions that work synergistically to activate transcription.

Thesis Objectives.

The work presented in this dissertation details some of the biophysical chemistry of CREB. The purpose of this work is to gain a better understanding of the molecular structure of CREB and the underlying mechanisms of transcriptional activation by this protein. In particular, there are three specific aims to this project:

1. Begin the molecular characterization of CREB by evaluating its secondary and tertiary structural properties. What does CREB look like?
2. Test the following models of CREB activation by PKA phosphorylation:
   (i) Does phosphorylation induce a conformational change in CREB?
   (ii) Does phosphorylation increase the DNA binding affinity?
   (iii) Does phosphorylation create a specific binding site for other proteins?
3. Establish conditions that will enable X-ray crystallography of the CREB:DNA and CREB-bZIP:DNA complexes.

Three sets of studies were undertaken to accomplish these aims. First, circular dichroism (CD) was used to determine the secondary structure content of full-length CREB and the CREB activation domain. CD was also used to compare the secondary structure of CREB and P-CREB. Second, a fluorescence anisotropy assay was used to quantitate CREB binding to different CRE sequences and to

determine whether phosphorylation affected the DNA binding affinity of CREB. The fluorescence anisotropy assay was also used to monitor the effects of CBP:P-CREB:DNA binding. Finally, analytical ultracentrifugation was used to monitor solubility, measure tertiary properties, and determine the effects of phosphorylation on the tertiary structure of CREB.

TABLE I

Examples of CRE Sequences

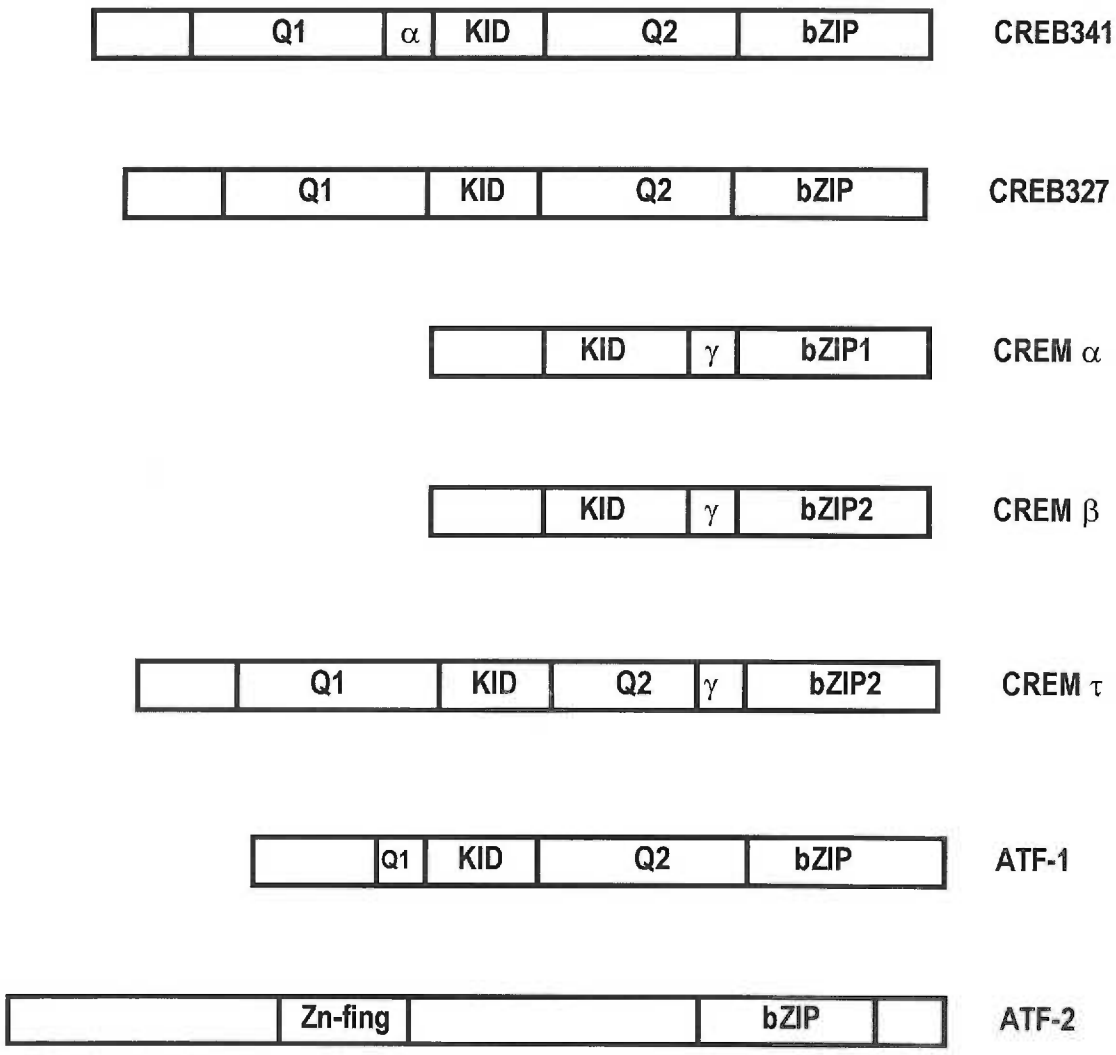
Gene	Sequence	Reference
somatostatin, rat	TGGCTGACGTCAGAGA	(Montminy, *et al.*, 1986)
glycoprotein α- subunit, human	AATTGACGTCATGGGTAA	(Delegeane, *et al.*, 1987)
fibronectin, human	CCCGTGACGTCACCCG	(Dean, *et al.*, 1988)
tyrosine hydroxylase, rat	GCTTTGACGTCAGCCT	(Lewis, *et al.*, 1987)
glucagon, rat	CATTTGACGTCAAAAT	(Philippe, *et al.*, 1988)
vasoactive intestinal peptide, human	TGGCCGTCATACTGTGACGTCTT	(Tsukada, *et al.*, 1987)
tyrosine aminotransferase	TGCAGCTTCTGCGTCAGCGCCAGTAT	(Boshart, *et al.*, 1990)
c-fos, mouse	CCAGTGACGTAGG	(Gilman, *et al.*, 1986)
proenkephalin	GGCCTGCGTCAGCTG	(Comb, *et al.*, 1986)
p-enolpyruvate carboxykinase, rat	CTTACGTCAGAGC	(Short, *et al.*, 1986)

TABLE II

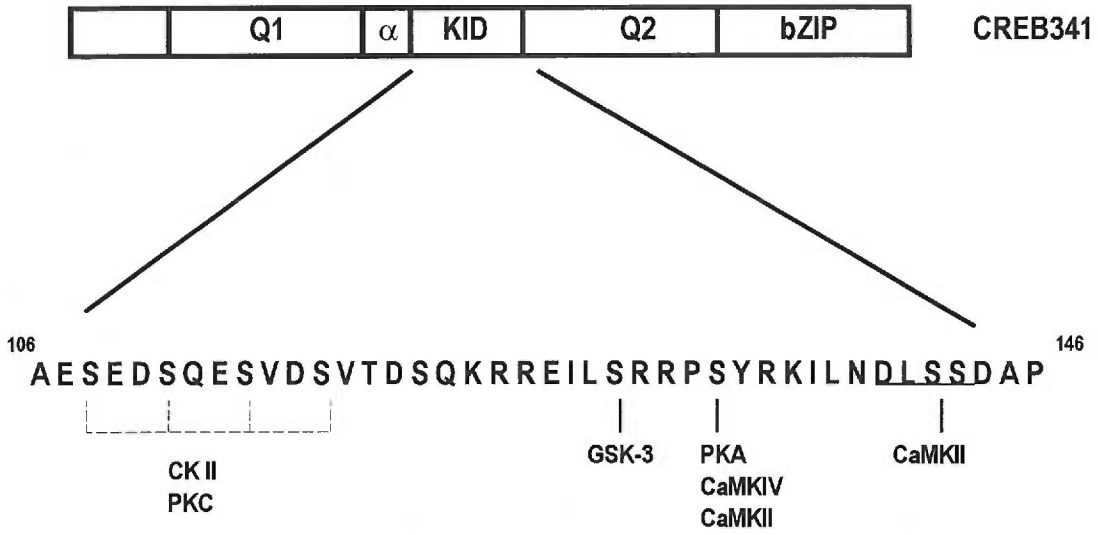
Comparison of bZIP sequences

Protein	Sequence	Reference
	Basic Region / **Leucine Zipper**	
	a d a d a d a d a d	
CREB	A E E A A R K R E V R L M K **N** R E A A R E **C R** R K K K E Y V K C **L** E N R V A V **L** E N Q N K T **L** I E E L K A **L** K D L Y C H K S D	(Hoeffler, 1988; Gonzalez,
CREMI	A E E A T R K R E L R L M K **N** R E A A R E **C R** R K K K E Y V K C **L** E N R V A V **L** E N Q N K T **L** I E E L K A **L** K D L Y C H K A E	(Foulkes, 1989)
CREMII	A E E A T R K R E L R L M K **N** R E A A K E **C R** R K K K E Y V K C **L** E S R V A V **L** E V Q N K K **L** I E E L E T **L** K D I C S P K T D	(Foulkes, 1989)
ATF-1	T D D P Q L K R E I R L M K **N** R E . A R E **C R** R K K K E Y V K C **L** E N R V A V **L** E N Q N K T **L** T E E L K T **L** K D L Y S N K S V	(Hai, 1989)
GCN4	V P E S S D P A A L K R A R **N** T E A A R R **S R** A R K L Q R M K Q **L** E D K V E E **L** L S K N Y H **L** E N E V A R **L** K K L V G E R	(Hope, 1988)
c-Jun	R S Q E R I K A E R K R M R **N** R I A A S K **C R** K R K L E R I A R **L** E E K V K T **L** K A Q N S E **L** A S T A N M **L** R E Q V A Q **L** K Q	(Ryder, 1989)
c-Fos	S P E E E E K R R I R R E R **N** K M A A A K **C R N** R R R E L T D T **L** Q A E T D Q **L** E D E K S A **L** Q T E I A N **L** L K E K E K L E F	(Matsui, 1990)
ATF-2 /CREBP1	N E D P D E K R R K F L E R **N** R A A A S R **C R** Q K R K V W V Q S **L** E K K A E D **L** S S L N G Q **L** Q S E V T L **L** R N E V A Q **L** K Q	(Hai, 1989)
C/EBP	V D K N S N E Y R V R R E R **N** N I A V R K **S R** D K A K Q R N V E T Q Q K V L E **L** T S D N D R **L** R K R V E Q **L** S R E L D T **L** R G	(Landschutz, 1988)

FIGURE 1. Schematic representations of CREB. A. The CREB/CREM/ATF family of transcription factors. The functional regions of these proteins and some of their splice variants are shown for comparison. The CREB/CREM/ATF family of transcription factors is classified by homology in the bZIP domain (Table II) and on the ability to bind CRE-like sequences. The CREB/CREM/ATF-1 group represents of specific subfamily of three highly related genes and their splice variants. ATF-2 (CRE-BP1) is included to illustrate the divergence in this activation domain from the CREB/CREM/ATF-1 family. bZIP: basic leucine zipper domain; Q1 and Q2: glutamine rich regions; KID: Kinase Inducible Domain; α: alpha peptide; γ: gamma exon; Zn-fing: Zn-finger E1A binding domain. B. The KID domain of CREB is expanded and various phosphorylation sites indicated. Solid lines represent sites that have been characterized both *in vitro* and *in vivo*. Dashed lines represent putative phosphorylation sites based on primary sequence analysis.

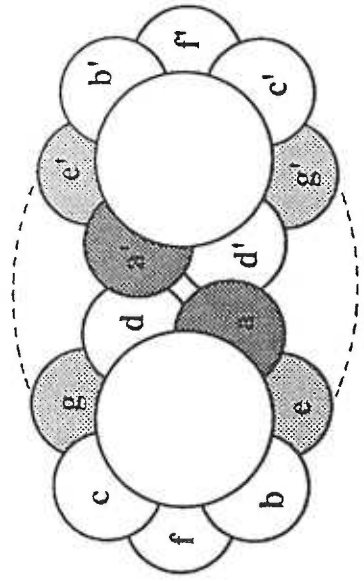


Introduction. Figure 1A.



Introduction. Figure 1B.

FIGURE 2. Helical wheel representation of the CREB leucine zipper. A. The positions of the residues *a-d* are indicated as they align in a parallel α-helix dimer. Residues in the *a* and *d* positions associate along a hydrophobic interface whereas charged residues in the *e* and *g* positions can form electrostatic interactions. B. The residues of the CREB leucine zipper are illustrated. (Reprinted with permission from (Loriaux, *et al.*, 1993)).


A)
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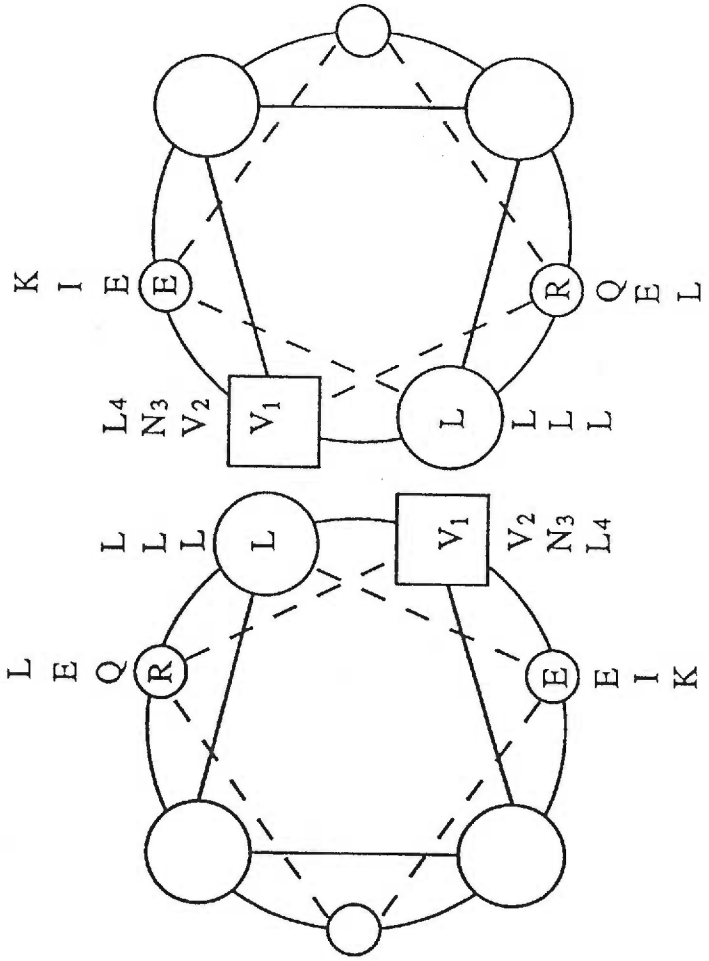
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CHAPTER 1

Circular Dichroism Studies of CREB and Protein Kinase A Phosphorylated CREB

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RUNNING TITLE: Secondary Structure of CREB

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Summary

Using circular dichroism (CD), we have initiated the structural characterization of the eukaryotic transcription factor CREB (cAMP Responsive Element Binding protein). We present a purification scheme for full-length CREB341 and the polypeptide ACT265 (CREB activation domain residues 3-265). A 70°C heat treatment is a critical step in the large-scale purification of these proteins, and we use CD to verify the apparent heat stability of CREB. Deconvolution of the CD spectra reveals a secondary structure content for CREB341 of 37% random coil, 34% β-turn, 9% β-strand and 20% α-helix and a secondary structure content of ACT265 of 52% random coil, 29% β-turn, 16% β-strand and 3% α-helix. Therefore, the presence of the bZIP domain contributes to proper folding of the activation domain. The significant amount of random coil and β-turn structures present in the full-length protein suggests that these structures may be of functional significance. In addition, we used CD to test the model that PKA phosphorylation induces a conformational change in CREB that leads to CREB activation. We show that phosphorylation of CREB341 or ACT265 by the cAMP dependent protein kinase A (PKA) does not alter the CD spectra and therefore the secondary structure content of CREB. Therefore, we suggest that the previously proposed structural changes are unlikely, or, if present, are of a small and local nature undetectable by CD and/or involve a conformational change that does not significantly alter the secondary structure content.

Introduction

The cAMP responsive element binding protein (CREB) belongs to the basic-leucine zipper (bZIP) class of transcription factors and stimulates the transcription of many genes in response to increased levels of intracellular cAMP [1, 2] (reviewed by [3]). CREB can be divided into two functionally and structurally distinct domains: a large N-terminal activation domain and a smaller C-terminal bZIP region which contains a DNA-binding and leucine zipper dimerization domain. Although structural details of various bZIP domains have been well characterized by mutagenesis ([4], reviewed by [5, 6]), circular dichroism [7-9], NMR [10-13] and X-ray crystallography [14-17], little is known about the structures of the activation domains. Physical studies on peptides of the acidic activators GCN4, Gal4 and Vmw65 have been published [18-21] and demonstrate that these peptides are aperiodic or random coil in aqueous solution at neutral pH, although Gal4 and GCN4 readily convert to β-structures by lowering the pH or by adding trifluoroethanol (TFE). The authors suggested that β-sheet is important for activation by these proteins. These findings underscore a key question that remains in the study of transcription factor activation domains: Are there conserved structural motifs that can be identified and classified in these proteins? Comparisons of primary amino acid sequences have revealed some loosely repeated themes including acidic-rich, proline-rich and glutamine-rich

sequences. However, no conserved structure or function has been identified with these regions.

We are studying CREB, a prototype of the CREB/CREM/ATF family of bZIP proteins in order to characterize the structure of a transcription activation domain that includes glutamine-rich regions. CREB also provides a model for studying the structural basis of phosphorylation-dependent transcription activation. Primary sequence analysis and mutagenesis of CREB have revealed several important features of its activation domain. These have recently been reviewed by Meyer and Habener [22] and Lee and Masson [23] and further defined by Quinn [24] and Brindle *et al.*, [25]. A key region in CREB is the series of phosphorylation sites within a serine/threonine-rich region designated the kinase inducible domain (KID) [26] or phosphorylation box (P-box) [27]. Most notable within this domain is Ser133 which can be phosphorylated by both the cAMP-dependent protein kinase A (PKA) and the calcium/calmodulin dependent protein kinases type II and IV [28-30]. Phosphorylation at Ser133 stimulates CREB activity by greater than 10-fold above basal levels [26, 27, 31]. CREB also contains two glutamine-rich sequences, Q1 and Q2, which are responsible for the basal activity of CREB and which are important to the phosphorylation-dependent activity of the KID domain [24, 25, 32].

The mechanism of CREB activation by phosphorylation is poorly understood, although several models have been proposed which require the

presence of an allosteric conformational change in the protein. One model suggests that PKA phosphorylation allows a site distal to Ser133, such as the Q2 region, to interact directly with the preinitiation complex [25, 26, 33]. A slightly different model proposes that PKA phosphorylation induces α-helical or β-sheet secondary structures in the regions neighboring the Ser133 site [22, 27]. However, no physical data have been presented that demonstrate a conformational change in CREB.

To begin a biophysical characterization of CREB, we used circular dichroism to study both the full-length protein (CREB341) and a truncated form (ACT265) which spans residues 3-265. This truncated protein contains all sequences important for both basal and PKA-inducible activation and lacks only the bZIP domain. CD is a sensitive measure of secondary structure and is often used to demonstrate conformational changes in proteins following modification or mutagenesis [11, 34]. The CD spectra can also be deconvoluted to determine the contribution of different secondary structures to the signal [35-37]. We demonstrate that the CREB activation domain contains largely β-turn and random coil structures and requires the presence of the bZIP domain for complete folding. We discuss the significance of these findings as they relate to structural motifs in transcription activation domains. Our work further demonstrates that the secondary structure of CREB, as measured by CD, does not change following

phosphorylation by PKA, and, therefore, our results introduce skepticism into models requiring a conformational change for CREB activation.

Experimental Procedures

Plasmids and expression system. Rat CREB341 [2] was subcloned into a Novagen pET expression system using the NcoI and BamHI restriction sites. The NcoI site in CREB encompasses the second ATG of the initial MetThrMet sequence, hence both CREB341 and ACT265 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 plasmids were transformed into *E. coli* BL21(DE3) cells (Novagen). Cells were grown in Luria broth plus 4 g/l glucose at 37°C to an optical density (595 nm) of 0.60 and induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3.5 hours the cells were harvested by centrifugation and resuspended in 10 mM HEPES buffer, pH 7.9 containing 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol (DTT) and 1 mM phenylmethanesulfonyl fluoride (PMSF) for CREB or in 10 mM Tris-HCl buffer, pH 8.0 containing 1 mM EDTA, 1 mM DTT and 1 mM PMSF for ACT265. The bacteria were lysed in a French press at 14,000 psi.

Protein purification. The bacterial extracts of CREB341 were heated to 70°C for 10 minutes and centrifuged for 20 minutes at 15,000 g to remove the particulate fraction. The CREB341 supernatant was purified over a gravity flow DE53 anion exchange column (Whatman) which had been equilibrated in 10 mM HEPES buffer, pH 7.9, containing 5 mM $MgCl_2$, 1 mM EDTA , 5 mM DTT and

30 mM KCl. Proteins were eluted slowly using a step gradient of 30 mM, 70 mM, 120 mM and 200 mM KCl, with CREB eluting at 120 mM KCl. The flow rate was 1 ml/min and each step ranged from 100-400 ml. The anion exchange column separates the full-length protein from contaminating breakdown products and removes the nucleic acid which remains bound to the column. The eluate was concentrated using an Amicon YM30 membrane. Each liter of bacterial culture yields about 2 mg of protein. CREB was greater than 95% of the total protein as estimated by Coomassie based Fast Stain (Zoion) on SDS-polyacrylamide gel electrophoresis.

ACT265 was purified from the bacterial extract without a heat treatment step. The protein was precipitated from the bacterial extract with 10% (wt/vol) ammonium sulfate and the pellet resuspended in 10 mM Tris-HCl buffer, pH 8.0. The resuspended pellet was purified by DE53 column chromatography using a 0-0.5 M KCl gradient and then followed by HPLC sizing column chromatography (Waters Protein-Pak 125). One liter of bacterial culture yields 5 mg of greater than 95% pure protein.

Phosphorylation. CREB341 and ACT265 were phosphorylated using purified catalytic subunit (C-subunit) or purified recombinant C-subunit of PKA (kindly supplied by John D. Scott and Richard A. Maurer, Oregon Health Sciences University). The phosphorylation reaction was carried out in 50 mM MOPS buffer, pH 6.8, containing 50 mM NaCl, 2 mM $MgCl_2$, 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 minutes. To measure the incorporation of phosphate, trace amounts of [γ^{32}P]-ATP were added to the reaction. Percent incorporation was determined by spotting a 5 µl of the reaction onto Whatman glass microfiber filters and washing extensively with 10% trichloroacetic acid (TCA), followed by 5% TCA and then 70% ethanol. The incorporation of γ^{32}P was quantitated in a scintillation counter following the addition of fluor (Beta-max). Assuming one PKA phosphorylation per CREB monomer, phosphate incorporation was consistently stoichiometric. Phosphorylation of CREB by PKA has previously been demonstrated to occur on Ser133 (Gonzalez and Montminy, 1989; Sun *et al.*, 1992). For spectroscopic studies, the ATP was removed by extensive dialysis against the CD buffer.

Circular Dichroism. Purified CREB341 and ACT265 were dialysed into 10 mM potassium phosphate, pH 7.5 and were concentrated to a range of 0.3 to 1.0 mg/ml as determined by amino acid analysis. The CD spectra were taken on a JASCO J-500A spectropolarimeter using a 0.01 cm path length cell (Helma) thermostatted at 20°C. The instrument was calibrated by using (+)-10-camphorsulfonic acid ($\Delta\varepsilon$ = +2.37 M^{-1} 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 JASCO. Spectra and buffer baselines were the average of 10 scans recorded at 0.1 nm intervals. A scanning rate of 10 nm/min and 2 sec time constant was used. Spectra of CREB341 and ACT265 taken at 70°C were an

average of two to four scans. Before spectral deconvolution for secondary structure analysis, spectra were smoothed using the program provided by JASCO and the buffer baseline was subtracted. The CD spectra were deconvoluted for secondary structure content using the variable selection methods described elsewhere [36, 37]. For this analysis combinations from a set of 33 basis spectra (kindly provided by W. Curtis Johnson, Jr., Oregon State University) were used to find those that result in the best fit using criteria described by Compton, *et al.* [35]. All secondary structure values resulting from each combination which met these criteria were averaged to give the final secondary structure values for each experimental spectra.

Results

Overexpression and purification of CREB341 and ACT265. To obtain the large quantities of highly pure protein necessary for biophysical characterization, we developed a bacterial expression and purification scheme for CREB341 (rat CREB residues 3-341). Our purification involves a 10 minute, 70°C heat step followed by chromatography over an anion exchange column. Each liter of bacterial culture yields about 2 mg of greater than 95% pure CREB (Fig. 1A). However, oxidation, aggregation and poor solubility hampered purification efforts as well as CD experiments. These problems stem 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 [13].

To avoid solubility problems and to simplify our structural studies on the activation domain of CREB, we constructed a truncated form by removing the bZIP region (residues 266-341). The ACT265 expression vector was constructed using site-directed mutagenesis to introduce a stop codon in place of Val266. The expression system was the same as for CREB341. Purification did not require a heat treatment and involved an ammonium sulfate precipitation and anion exchange chromatography. One liter of bacterial culture yields about 5 mg of greater than 95% pure ACT265 (Fig. 1B).

CREB341 and ACT265 are heat stable. Boiling cellular extracts is a previously reported method for purifying CREB for use in electrophoretic mobility shift assays (EMSA) [38, 39]. Heating appeared an effective purification step for CREB and several lines of evidence suggested that CREB is stable to this treatment: (i) CREB341 and remains soluble after boiling [38, 39], (ii) heat purified CREB is highly active in the gel shift assay [38, 39]; (iii) heat purified, phosphorylated CREB341 and ACT265 are able to bind specifically to CREB Binding Protein (CBP) [40], and (iv) peptides of the CREB leucine zipper or of its bZIP domain refold after melting (R.G.B., unpublished results; [13]). Therefore, heat-purified CREB retains at least some of its activities (DNA-binding and CBP-binding). However, boiling is an unorthodox method for purifying proteins and can cause irreversible unfolding which may lead to a soluble protein in which some regions have lost their native conformation. The question of whether heat purified CREB refolds completely following heat purification has not been addressed. Drawing functional conclusions on the structural characterization of heat-purified CREB requires that its structural properties in relation to non-heated protein be characterized.

To avoid boiling, which can cause deamidation, we empirically determined the lowest effective temperature for purification of CREB341 and ACT265 to be 70°C. ACT265 was also purified without a heat treatment step. The CD spectrum of non-heat purified ACT265 was determined from 260-180 nm at 20°C. An

aliquot of this protein preparation was heated to 70°C for 10 min and allowed to cool. The CD spectrum from 260-180 nm at 20°C was obtained for this heat-treated activation domain. The spectra of the heated and non-heated protein are compared in Figure 2A. The spectra overlay well with the deviations being typical of the variation seen from experiment to experiment.

We were unable to purify sufficient quantities of spectroscopic quality CREB341 without a heat step. However, we followed the stability of heat purified CREB341 by determining the CD spectrum at 20°C from 260-180 nm. We then raised the temperature of the sample to 70°C and measured the spectrum at this elevated temperature. The sample was cooled slowly back to 20°C and the CD scan was repeated (Fig. 2B). CREB showed little change at 70°C, and after cooling, the spectrum realigned with the original spectrum. This result demonstrates that heat purified CREB undergoes some reversible unfolding at 70°C and returns to its starting structure upon cooling.

To eliminate the remote possibility that the heat-purified CREB had refolded to a heat stable, non-native conformation, we monitored the CD spectra of a CREB mutant, CREB/SER. CREB/SER is a construct in which the three cysteines in the bZIP domain have been substituted with serines and is described in detail in Chapter III. This protein can be purified without a heat step. The CD spectra of CREB/SER matches the spectra of wild-type CREB (Chapter III). The CD spectra of non-heat purified CREB/SER and an aliquot of CREB/SER that has

been heated to 70°C and cooled are presented in Figure 2C. These spectra are superimposible. Therefore, our heat-purification does not interfere with the native structure of CREB.

The CD spectra of CREB and phosphorylated CREB are not significantly different. Phosphorylation of Ser133 is essential for CREB to activate transcription in response to cAMP, however the mechanism of this activation is not understood. We compared the CD spectra of both CREB341 and phosphorylated CREB341 (P-CREB341) as well as ACT265 and phosphorylated ACT265 (P-ACT265) to determine whether phosphorylation induces a change in secondary structure and to test the model that PKA phosphorylation causes a conformational change in CREB. The purified proteins were phosphorylated stoichiometrically with the catalytic subunit of PKA. The far CD spectra of CREB341 and P-CREB341 as well as ACT265 and P-ACT265 are identical (Fig. 3).

In order to futher measure the effects of phosphorylation on CREB structure, we attempted to compare the near UV CD spectra of CREB and phosphorylated CREB. The near UV CD detects changes in the environment of aromatic residues. However, CREB contains few aromatic residues (5 Tyr, 1 Phe, 0 Trp), and due to poor solubility, we were unable to concentrate the protein sufficiently to obtain a near UV CD signal.

The activation domain of CREB contains mostly random coil and β-turn structures. In order to describe the secondary structural characteristics of the CREB activation domain, the CD spectra of CREB341 and ACT265 were deconvoluted and their secondary structure contents obtained using the variable selection method developed by Compton and Johnson [36]. We used a basis set of 33 proteins and were able to reconstruct curves that closely resembled our experimental spectra (Table I). The analysis of secondary structure revealed that CREB341 contains 37% aperiodic or random coil structures, 34% β-turn, 9% β-strand and 20% α-helix. ACT265 contains 52% aperiodic, 29% β-turn, 16% β-strand and only 3% α-helix (Table I). The secondary structure content of the activation domain is higher in random coil and lower in β-turn and α-helix content than would be predicted just from the removal of 76 amino acids of the bZIP domain which have been shown to contain 43% α-helix and 57 % random coil in the absence of DNA (Table I) [13]. The secondary structure content of ACT265, as predicted by subtracting the CD structure content of the bZIP region alone [13] from the CD structure content of the full-length protein, is expected to be 44% β-turn, 31% aperiodic, 12% β-strand and 12% α-helix (Table I). We conclude that although the ACT265 peptide maintains activity as measured by its ability to bind to CBP [40] (J.R.L. unpublished), this domain is not completely folded in the absence of the bZIP region, and in particular, has lost some β-turn structure.

Discussion

We present a straightforward method for purifying CREB341 and ACT265. This purification provides the large yields of highly pure protein which are necessary for extensive structural studies, avoids the use of fusion proteins and does not rely on boiling [38, 39]. We instead demonstrate that a 10 min 70°C heat treatment is sufficient for efficient purification. We also demonstrate that this heat step does not interfere with the native fold of CREB.

The fact that the activation and DNA binding domains of transcription factors are functionally and structurally distinct has been well established (reviewed by [41]) and has also been demonstrated for CREB. CREB functions as an activator in "domain swap" experiments in which the Gal4 DNA binding domain has been fused to the CREB activation domain on either the amino or carboxyl termini [27, 42]. This modularity implies that the native structure of the activation domain is preserved independent of the bZIP domain. However, the secondary structure content of ACT265 as determined by CD shows that the removal of the bZIP domain does cause some loss of secondary structure. Although ACT265 maintains CBP binding function, there is a definite decrease in the expected β-turn and α-helix content and a subsequent rise in random coil content (Table I). This finding argues that the bZIP domain contributes to the folding and stability of the activation domain and suggests that the results of domain-swap experiments require careful interpretation. Although the majority of

activation function may be retained in a chimeric transcription factor, some critical function or protein:protein interaction may be lost.

We also used CD studies to address how phosphorylation by PKA activates CREB. Most current models include significant changes in conformation, the most cited being that phosphorylation at Ser133 induces an allosteric change in CREB which allows a distal domain, possibly the glutamine-rich Q2 domain, to interact with the general transcription machinery [26, 33]. A variation of this model proposes that phosphorylation induces formation of an amphipathic α-helix or β-strand in the KID or an adjacent region thus allowing specific protein-protein interaction [22, 27, 43]. These models are based primarily on mutagenesis studies. Changing the Ser133 phosphorylation site to a negatively charged amino acid such as Glu or Asp, did not mimic the effect of phosphorylation suggesting that more than negative charge, perhaps a conformational change, was required for activation [26, 27, 33]. However, a Glu or Asp would not necessarily substitute for a phosphorylated Ser as the size and charge are not the same.

Further evidence for the proposed conformational change came from a tryptic digest of CREB and P-CREB, which revealed a slight difference in the ratio of different cleavage products [26]. We also performed a series of protease digests and found no major difference in cleavage pattern with trypsin, chymotrypsin or Endo Glu C protease (Appendix I). Although we did detect the same difference in band ratio with the trypsin cleavage, we note that there is a

trypsin cleavage site only 2 amino acids away from the phosphorylation site, and it is likely that a phosphate group would sterically or electrostatically hinder cleavage at this sight and hence slightly alter the kinetics of the reaction. Therefore, the existence of a phosphorylation induced conformational change remains an open question.

We measured the CD spectra of CREB341 and P-CREB341 as well as ACT265 and P-ACT265 to determine whether PKA phosphorylation caused any significant changes in secondary structure. This technique has been used to study phosphorylation induced changes in proteins such as keratin, myelin basic protein and fibrinogen [34, 44, 45]. We found the CD spectra of the phosphorylated forms of CREB341 and ACT265 to be indistinguishable from the CD spectra of the non-phosphorylated forms. This finding argues against the model that PKA phosphorylation induces secondary structure in regions neighboring the PKA site, although we cannot rule out the possibility that such structural changes involve only a few amino acids and are too small to be detectable by CD. It is also possible that a phosphorylation-induced change is more global in nature involving mainly tertiary or quaternary structural changes (e.g. a sliding or a hinge bending motion) that have little effect on the secondary structure. We have continued to pursue these avenues by studying the effects of PKA phosphorylation on DNA binding and sedimentation. However, an emerging model of CREB activation is that the primary role of PKA phosphorylation is not to induce a conformational

change in CREB but to provide a highly specific binding site for coactivators such as CBP [40, 46].

From our CD data we estimated the secondary structure content of CREB by using the variable selection method [36, 37] and a basis set of 33 proteins (provided by A. Toumadja and W.C. Johnson, Jr., Oregon State University). Using such an expanded basis set and including data below 190 nm significantly improves the analysis of β-strand, β-turn and random coil structures [37]. This expanded basis set was necessary to successfully deconvolute the CREB spectra. We obtained the amounts of secondary structure listed in Table I. The structure contents differ somewhat from those predicted from primary sequence or inferred from previous discussions of secondary structure [24] which are much more weighted with β-strand and α-helix. This discrepancy may indicate that certain regions of CREB have a propensity to form periodic structures when bound to other proteins or when within a certain environment.

Our finding that the activation domain of CREB contains largely β-turn and random coil structures is intriguing given the results of recent structural work on several acidic activators. The 79 amino acid activation domain of Vmw65 (or VP16) was shown by NMR and CD to be mostly random coil under physiological conditions [20, 21]. However, primary sequence analysis predicted this region to be α-helix. The CD studies detected some α-helix following the addition of 50% TFE or at low pH [20], although NMR indicated no changes in

structure between pH 5.3 and pH 8.0 or upon the addition of 80% methanol [21]. In similar studies, a 38 amino acid peptide of GCN4 activation domain and a 34 amino acid peptide of Gal4 activation domain were shown by CD analysis to be relatively unstructured under neutral conditions [19]. However, when the pH was lowered to 5.6 these peptides assume primarily β-strand structures.

Together, these findings suggest that β-turn and random coil structures are commonly observed in transcriptional activation domains. In addition, the secondary structures of these regions are sensitive to the enviroment of the surrounding solvent and the accompanying protein regions. These observations have several implications toward the search for structural motifs in activation domains. First, proper folding may require the presence of the surrounding protein sequence. It may, therefore, be difficult to characterize activation domains motifs using only peptides. Second, the ordered secondary structure content may be increased upon specific binding to other proteins or to DNA. This situation would be analogous to the transition from random coil to α-helix that occurs in bZIP domains upon DNA-binding. Finally, the random coil nature of these regions may also have some biological function which may not require the presence of ordered secondary structure. One emerging model proposes that unstructured polymeric domains increase the rate of association between macromolecules by increasing the productivity of the encounter complex (reviewed by [47]). Such a mechanism may be used by the random coil regions of transcription factors to recruit other

proteins to the initiation complex. Primary sequence inspection already reveals that activation domains will not be as strictly defined as DNA binding domains, however, structural analysis appears to be detecting patterns that will lead to models of activation mechanisms.

Abbreviations

The abbreviations used are: CREB, cAMP responsive element binding protein; ACT, CREB activator domain; PKA, cAMP-dependent protein kinase A; C-subunit, catalytic subunit of PKA; CD, circular dichroism; KID, kinase inducible domain; NMR, nuclear magnetic resonance.

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TABLE I.

Secondary structure content of CREB341 and ACT265.

The abbreviations used are: **H**, α-helix; **B**, β-strand; **T**, β-turn; **O**, other structures; **tot**, total structure content.

protein	H	B	T	O	tot
CREB341	.20	.09	.34	.37	1.00
P-CREB341	.22	.06	.32	.40	1.00
ACT265	.03	.16	.29	.52	1.00
P-ACT265	.05	.16	.29	.50	1.00
***Predicted ACT265**	.13	.12	.44	.31	1.00

*Predicted structure contents are based on the CD finding that the 76 amino acid bZIP domain is 43% α-helix and 57% random coil in the absence of DNA [13].

Figure Legends

FIGURE. 1. **Purification of CREB341 and ACT265.** CREB341 and ACT265 were expressed and purified as described in the Experimental Procedures. Various steps are shown as resolved by 12% SDS-PAGE stained with Fast Stain (Zoion). Numbers on the left correspond to the migration of molecular weight markers (MW). A. CREB341: lane 1, uninduced bacterial extract; lane 2, induced bacterial extract; lane 3, extract following 10 min 70°C heat treatment; lane 4, final extract purified over an anion exchange column. B. ACT265: lane 1, uninduced bacterial extract; lane 2, induced bacterial extract; lane 3, particulate fraction from 10% ammonium sulfate cut; lane 4, supernatant from 10% ammonium sulfate cut.

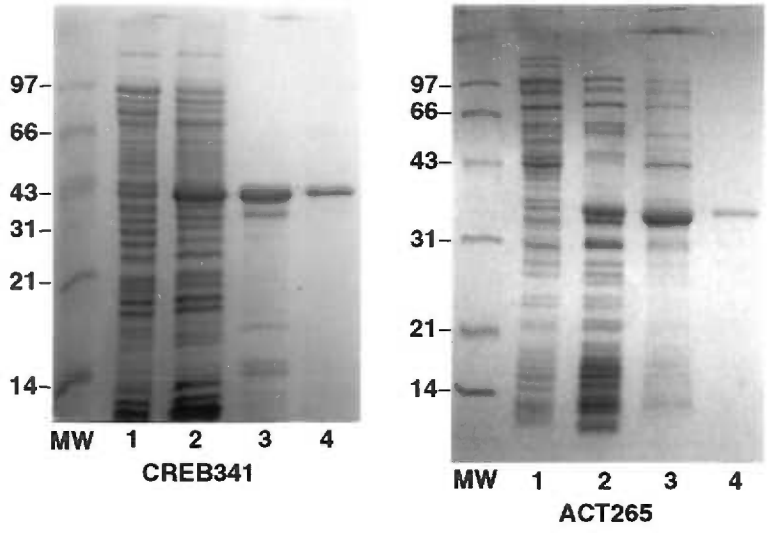
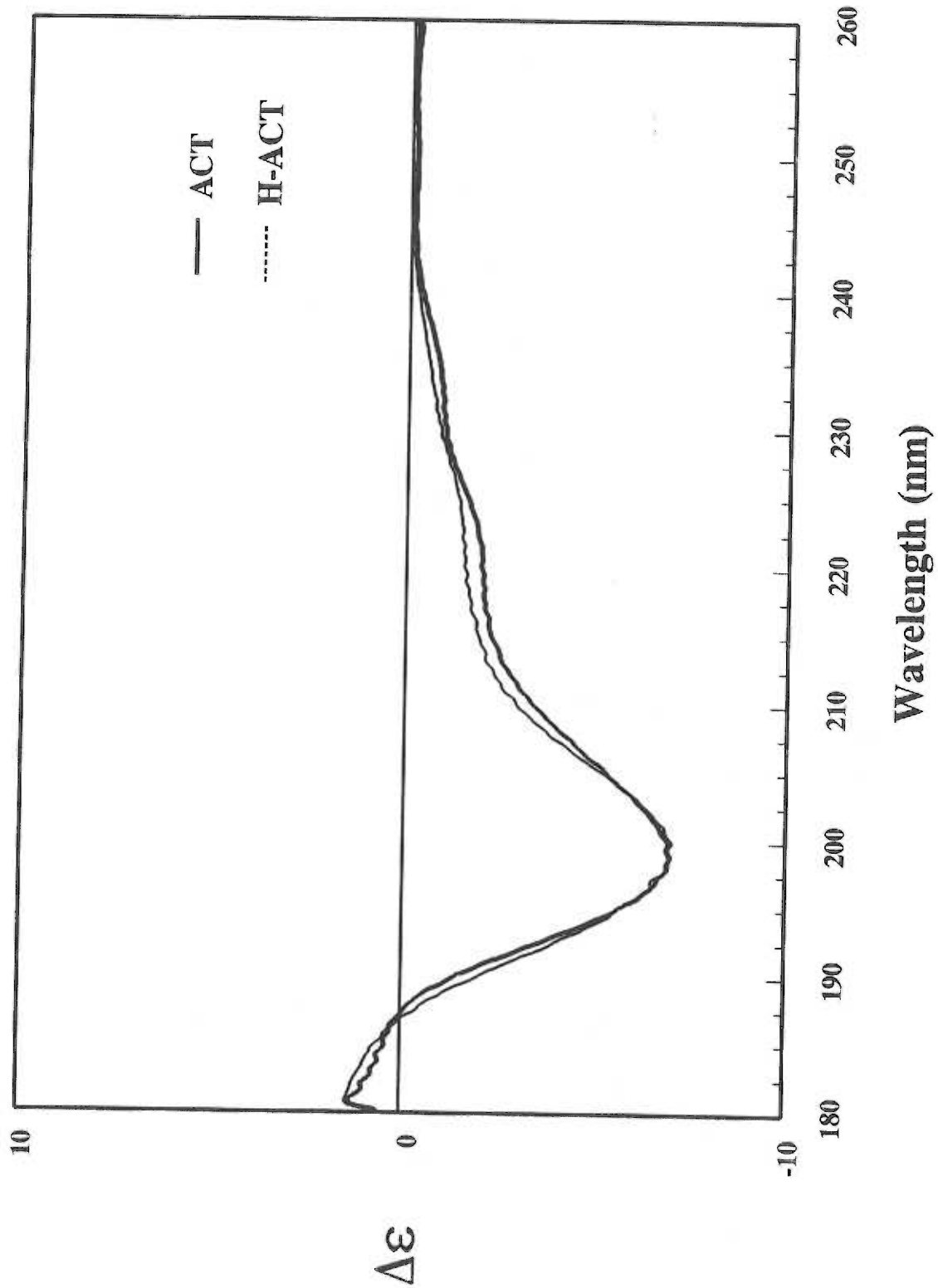
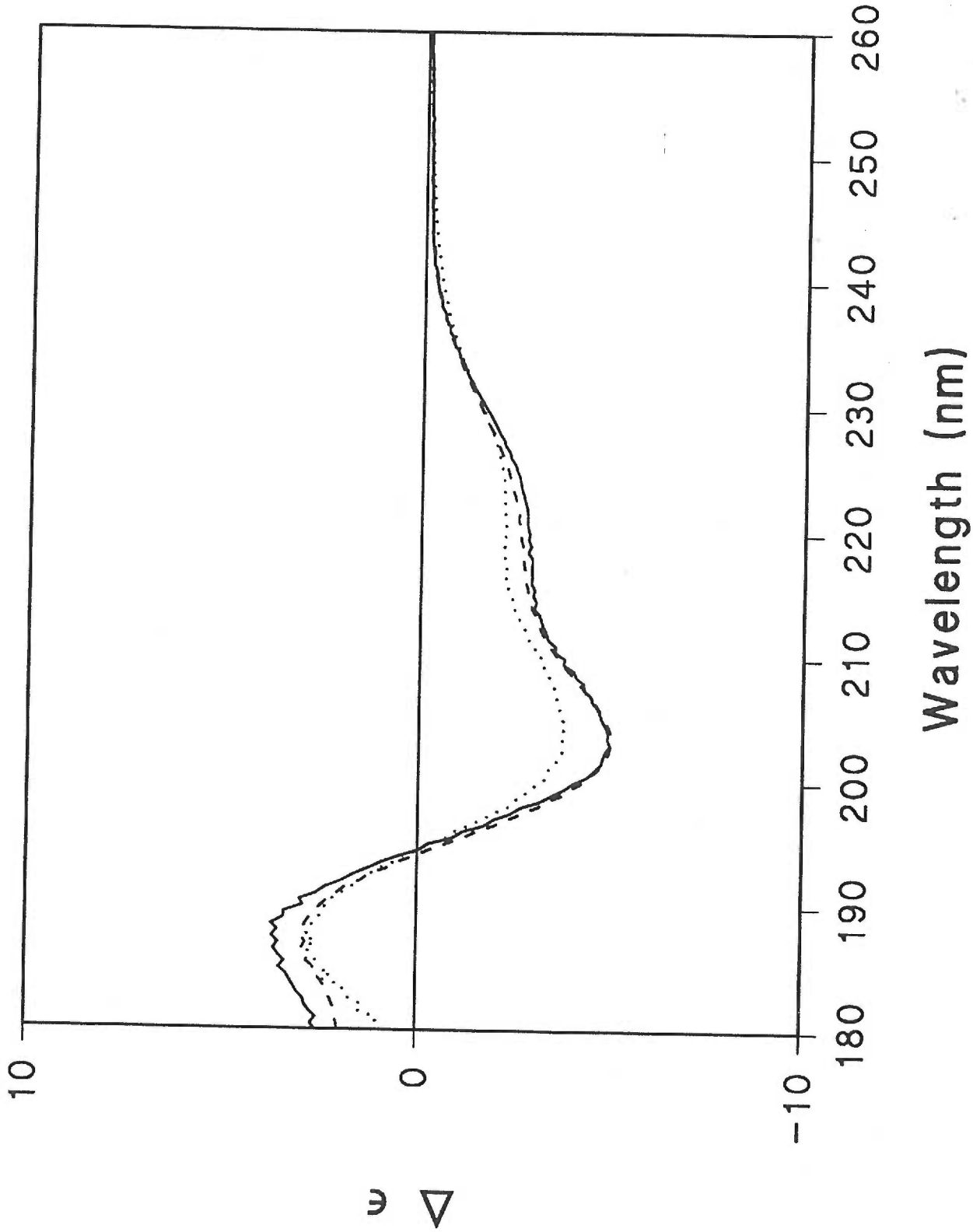

97-
66-
43-
31-
21-
14-
MW 1 2 3 4
CREB341
97-
66-
43-
31-
21-
14-
MW 1 2 3 4
ACT265

FIGURE. 2. **CD spectra of heat treated ACT265 and CREB341.** ACT265 and CREB341 were dialysed into 10 mM phosphate buffer, pH 7.5 and the CD measured from 260-180 nm. Wavelength is represented along the x-axis and molar amino acid ellipticity along the y-axis. A. ACT265: (———) at 20°C without a heat treatment. (-----) at 20°C following a 10 min, 70°C heat treatment. B. CREB341(———) heat purified CREB341 at 20°C. (·····) CREB341 at 70°C. (- - - - -) CREB341 returned to 20°C. C. CREB/SER (———) at 20°C without a heat treatment. (-------) at 20°C following a 10 min, 70°C heat treatment.


ACT
H-ACT
Δε
10
0
-10
180
190
200
210
220
230
240
250
260
Wavelength (nm)


10
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−10
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180
190
200
210
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230
240
250
260
Wavelength (nm)

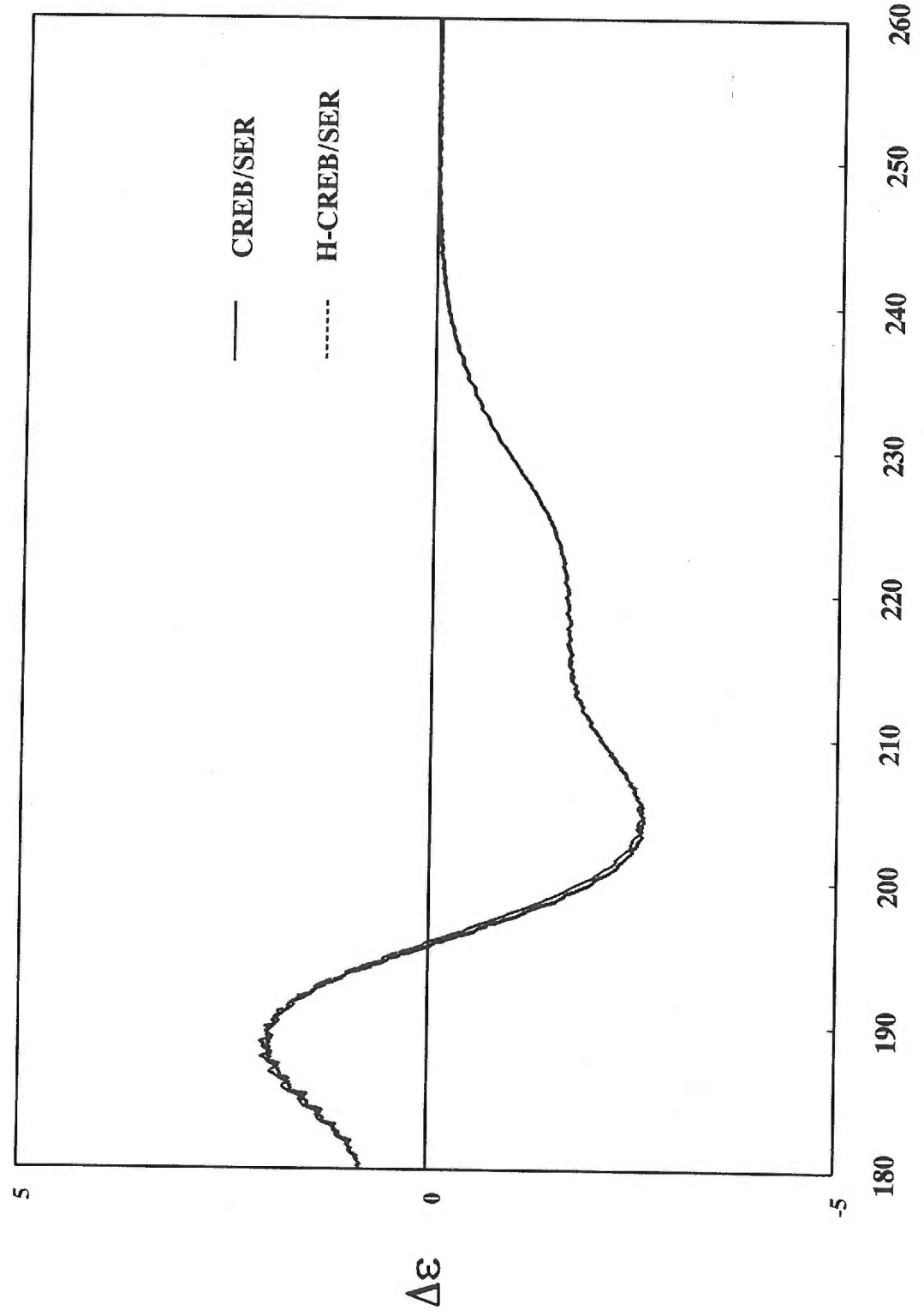
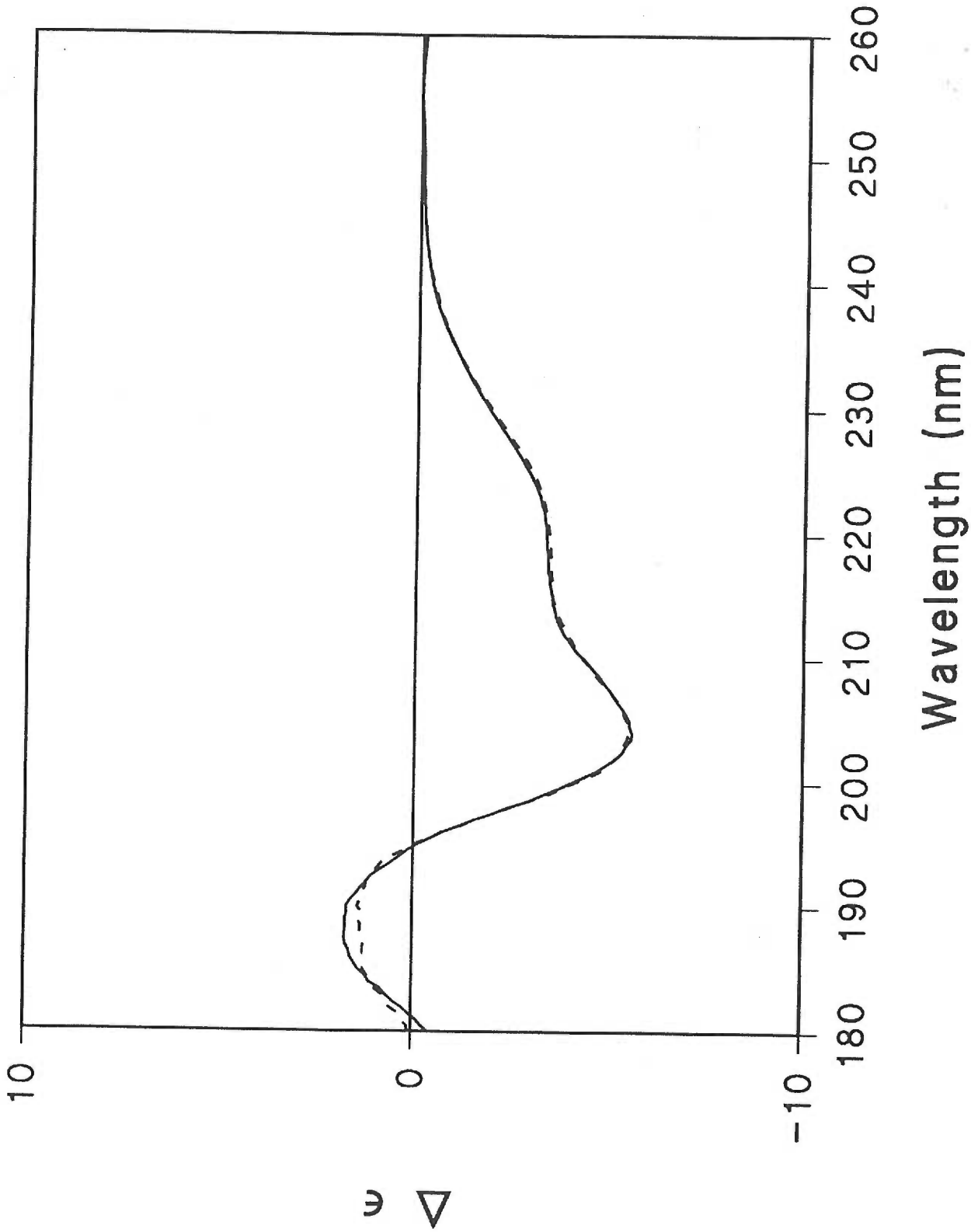

CREB/SER
H-CREB/SER
Δε
5
0
-5
180
190
200
210
220
230
240
250
260
Wavelength (nm)

FIGURE. 3. **CD spectra of phosphorylated CREB341 and ACT265.** CREB341 and ACT265 were phosphorylated by the C-subunit of PKA and dialysed into 10 mM phosphate buffer, pH 7.5. The CD spectra were measured and plotted together with that of non-phosphorylated CREB341 or ACT265. Axes are the same as in Fig. 2. A. (———) CREB341 and (- - - - -) P-CREB341. B. (———) ACT265 and (- - - - -) P-ACT265.


10
0
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180
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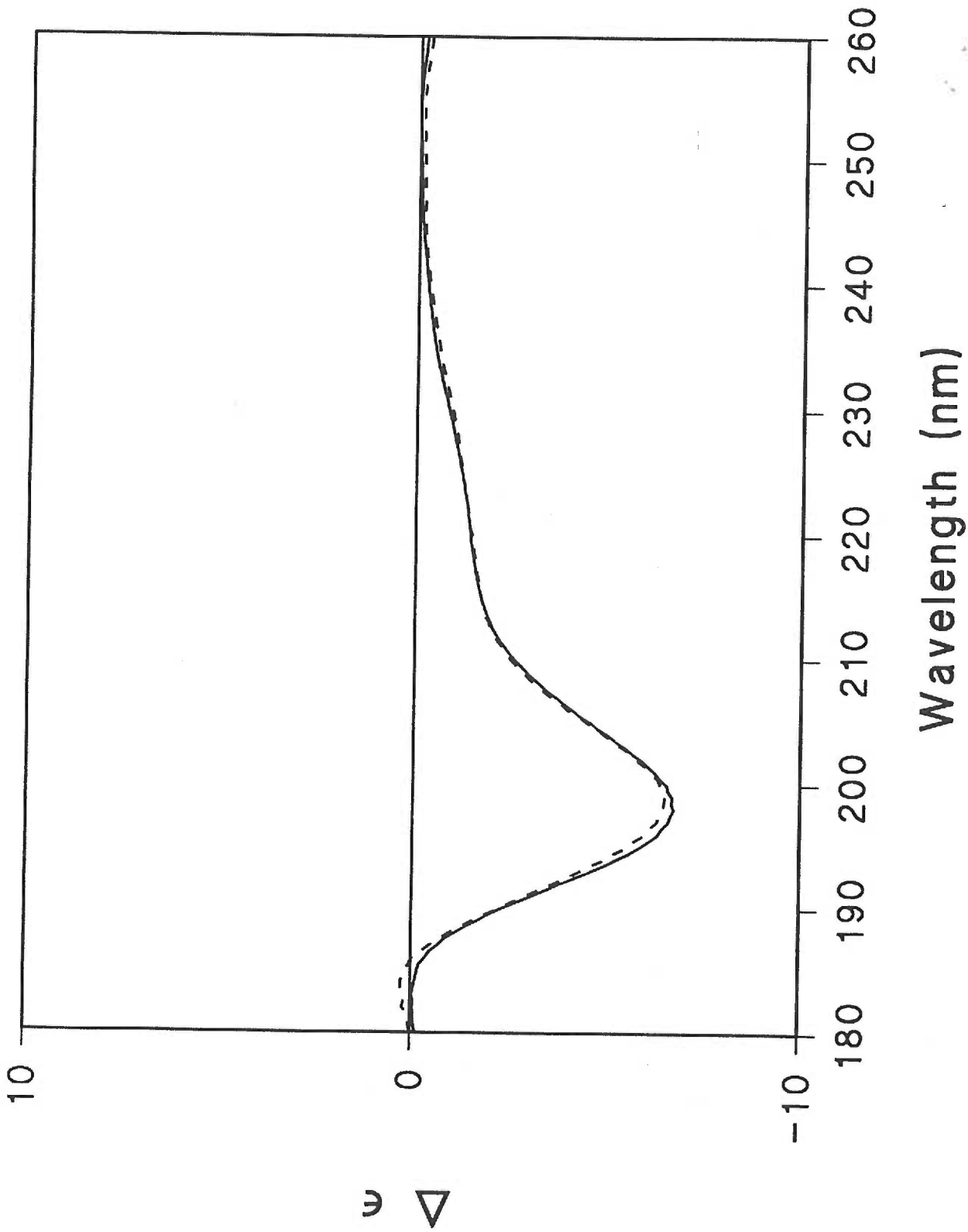
260
Wavelength (nm)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Δ ε
180
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200
210
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240
250
260
Wavelength (nm)

CHAPTER II

CREB and protein kinase A phosphorylated CREB bind DNA with equal affinity

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RUNNING TITLE: Fluorescence anisotropy studies of CREB:DNA binding

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Summary

We have used fluorescence anisotropy to determine whether phosphorylation by protein kinase A (PKA) alters the binding affinity of the eukaryotic transcription factor CREB for different DNA sequences. Binding was assayed by measuring the anisotropy of different fluoresceinated oligonucleotides as a function of CREB concentration. We examined the relative affinities of CREB for the somatostatin CRE, the tyrosine aminotransferase CRE and the related but functionally distinct TRE (AP-1 site). We found a five-fold difference between the equilibrium dissociation constants of CREB for the somatostatin (Kd = 2 ± 1 nM) and tyrosine aminotransferase (Kd = 11 ± 1 nM) CREs. CREB bound to the TRE, but with a much lower affinity (Kd ≥ 200 nM). Phosphorylation of CREB by PKA did not significantly alter the affinity for either the somatostatin or the tyrosine aminotransferase CREs. In addition, the transcriptional coactivator CBP did not influence binding of phosphorylated CREB to either CRE site. Thus, PKA must activate CREB function at a step that is distinct from DNA binding.

Introduction

The cAMP response element, or CRE, confers cAMP responsiveness to the promoters of many genes (reviewed in [1]). CRE sequences have been loosely divided into two classes. The first class generally contains the canonical palindromic TGACGTCA site originally characterized in the somatostatin promoter. The second class of CRE differs from the canonical sequence by at least one base pair or occurs as a split site with several basepairs separating the palindrome. CREs are clearly distinct from the phorbol ester response element (TRE or AP-1 site) which differs from the CRE only slightly (TGACTCA) but is not cAMP-responsive.

CREB, a basic-leucine zipper (bZIP) protein, binds to the CRE and mediates cAMP induced gene expression following phosphorylation by protein kinase A (PKA) on a single serine residue in its activation domain [2]. This phosphorylation event converts CREB into a strong transcriptional activator. The mechanism of this activation is not completely understood, although several models have been suggested (reviewed in [3]). One model proposes that PKA-phosphorylation increases the affinity of CREB for its CRE site. Although initial reports observed that PKA-phosphorylation of CREB did not affect binding to the somatostatin CRE (SSCRE) [4, 5], other groups noticed a cAMP-dependent increase in protein binding to CRE sites [6, 7]. A more extensive study [8] 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. They proposed that the role of phosphorylation was to increase CREB binding to otherwise lower affinity CRE sequences and bring binding to a level observed on the higher affinity CREs such as the SSCRE. Contrary to this conclusion, another recent paper reported that PKA phosphorylation did not affect CREB binding to the HTLV-CRE, a non-canonical class CRE [9]. Clearly, lack of consensus remains over the role of PKA phosphorylation in DNA binding.

One reason for the controversy over the effect of PKA phosphorylation on CREB:DNA binding may be the limitations of the techniques used to measure binding. All of the above mentioned studies relied on footprinting and/or electrophoretic mobility shift assays (EMSA), techniques which readily allow qualitative assessment of DNA binding. However, gel electrophoresis has limitations when used for quantitative assessment of some DNA binding proteins, particularly for bZIP proteins such as CREB which have solubilities that are highly sensitive to binding conditions and are prone to forming higher order aggregates that do not enter the gel matrix [10-12].

Because of the discrepancy among these earlier studies, we have used another method, fluorescence anisotropy, to measure the relative affinities of CREB for both a canonical and non-canonical CRE site and to clarify the role of PKA-phosphorylation on DNA binding. Several bacterial protein:DNA

interactions have been characterized with this technique [13-15]. Fluorescence anisotropy is a direct solution measurement that can be carried out under well-defined, equilibrium conditions. It does not require immobilization of the protein-DNA complex or separation of bound versus free species.

Fluorescence anisotropy offers several other advantages over EMSA for the quantitative study of CREB:DNA interactions. First, it is easy to obtain a large number of data points over several orders of magnitude of protein concentration, thus improving the fit and reliability of mathematical binding models. Second, one can easily vary the binding conditions including ionic strength, pH, and the presence of stabilizing agents such as glycerol or non-ionic detergents. In addition, fluorescence anisotropy can be used to determine the binding constants of multiprotein:DNA complexes such as the binding of CREB binding protein (CBP) to the CREB:DNA complex [16]. Such complexes are often unstable to gel electrophoresis techniques. We have used fluorescence anisotropy to determine whether CBP binding to phosphorylated-CREB affects DNA binding affinity. CBP binds specifically to phosphorylated-CREB and further stimulates transcriptional activation by CREB [16]. CBP binding could indirectly increase the phosphorylated-CREB:DNA binding affinity.

Although EMSA can be used as a quantitative measure of protein-DNA interactions in some instances [17], we have found fluorescence anisotropy to be a more reliable technique for studying CREB-DNA and CBP-CREB-DNA

interactions. In addition to presenting the relative equilibrium dissociation constants of CREB for different CRE sequences, we demonstrate that PKA phosphorylation does not influence DNA-binding either directly or through specific phosphorylated-CREB binding proteins.

Materials and Methods

Protein purification. The rat CREB341 cDNA sequence [18] was subcloned into a Novagen pET expression system. The bacterial extracts of CREB were heated to 70°C for 10 minutes which purifies CREB to about 85% of the total protein. Circular dichroism analysis demonstrates that the recombinant CREB refolds properly after heating (Chapter I). The soluble fraction was purified over a DE53 anion exchange column (Whatman) which had been equilibrated in 10 mM HEPES buffer, pH 7.9 containing 5 mM $MgCl_2$, 1 mM EDTA , 5 mM DTT and 30 mM KCl. Proteins were eluted stepwise with 30 mM, 70 mM, 120 mM and 200 mM KCl, with CREB eluting at 120 mM. Each liter of bacterial culture yielded about 2 mg of 95% pure protein as determined by Coomassie based Fast Stain (Zoion) on SDS-polyacrylamide gel electrophoresis.

CREB phosphorylation. CREB was stoichiometrically phosphorylated with the purified recombinant catalytic subunit of PKA (kindly provided by R. Maurer, Oregon Health Sciences University). The phosphorylation reaction was carried out in 50 mM MOPS buffer, pH 6.8 containing 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT and 1 mM ATP with a molar C-subunit:CREB ratio of 1:100. Samples were incubated at 30°C for 30 minutes. To measure the incorporation of phosphate, trace amounts of [γ^{32}P]-ATP were added to the reaction. Percent incorporation was determined by TCA precipitation followed by quantitation in a scintillation counter.

Oligonucleotides. 5'-fluoresceinated oligonucleotides were purchased from Genosys Biotechnologies and purified by reverse phase HPLC on a Vydac C4 column. The sequences (upper strand only) were:

SSCRE = (5'-CCTTGGC**TGACGTCA**GAGAGAGC-3');

TATCRE = (5'-TGCAGCTTC**TGCGTCA**GCGCCAGTAT-3');

TRE = (5'-AAGCTTGCA**TGACTCA**GACAG-3') (See Fig. 1A.) Only the upper strands were labelled with fluorescein. Oligonucleotides were annealed by combining equimolar amounts of each strand, heating to 95^{o}C and cooling slowly to room temperature. Complete annealing was confirmed by native PAGE. Double stranded oligonucleotides amounted to 95% or greater of the total oligonucleotide present (data not shown).

Fluorescence anisotropy measurements. Fluorescence polarization measurements were collected on an SLM Instruments Model 8000 spectrofluorimeter and on a Beacon Fluorescence Polarization System. Samples in the SLM Model 8000 were excited at 490 nm and emission was measured using 515 nm cutoff filters. Samples in the Beacon system were excited at 490 nm and emission was measured at 530 nm. Equivalent binding curves were obtained with both instruments.

The binding reactions contained 1 ml of 0.5 nM fluoresceinated oligonucleotide in 25 mM Tris-HCl, buffer, pH 7.6 containing 50 mM NaCl, 5 mM $MgCl_2$, 0.1% triton X-100, 5% glycerol and 10 ug/ml polydIdC. BSA at 1uM

was included in the CREB:DNA experiments to reduce non-specific 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. Prior to each binding assay, fresh DTT to 5 mM was added to the CREB protein and the sample warmed to 60°C for three minutes. This step reduced oxidation-induced aggregation which can interfere with DNA binding [10]. CREB was titrated into the binding mixture over 4-5 orders of magnitude of protein concentration to insure complete binding analysis. Samples were incubated in the fluorimeter for two minutes prior to each measurement to allow thermal and binding equilibrium. Under these conditions, the maximum change in anisotropy occurs within 15-30 seconds (data not shown). Each point on the curves represents the average of four anisotropy measurements with a 10 sec integration time for each reading. The standard deviation for each point was consistently less than 2% of the measured anisotropy value (data not shown).

Data analysis. The curves were fit with a nonlinear least squares regression analysis assuming the simple model [CREBdimer] + [DNA] ↔ [CREBdimer:DNA]. Therefore, the reported equilibrium dissociation constants represent the point of half-maximal change in anistropy. A nonspecific component [Knsp] was included in the model to account for the linear increase in anisotropy at higher protein concentrations.

Results

Binding of CREB to the somatostatin CRE is five-fold greater than to the tyrosine aminotransferase CRE. We used fluorescence anisotropy to measure the relative affinity of CREB341 homodimers for the SSCRE, the TATCRE and the consensus TRE site (Fig. 1A). Representative binding curves are shown in Figure 1B and 1C. The equilibrium dissociation constant (Kd) for the CREB:SSCRE complex was 2 ± 1 nM and the Kd value for the CREB:TATCRE complex was 11 ± 1 nM. Duplicate curves under identical conditions yielded a Kd for the CREB:SSCRE complex of 4 ± 1 nM and a Kd value for the CREB:TATCRE complex of 14 ± 1 nM (data not shown). Binding to the TRE was detectable only at high protein concentrations with a Kd value of about 200 nM.

Phosphorylation of CREB by PKA does not affect DNA binding. A comparison of curves for phosphorylated (P-CREB) and non-phosphorylated CREB bound to the SSCRE and the TATCRE are shown in Figure 2. The Kd value for phosphorylated CREB:SSCRE complex is 2 ± 1 nM, and the Kd value for the P-CREB:TATCRE complex is 11 ± 1 nM. Duplicate curves yielded identical results (data not shown). Thus, the Kd values for the P-CREB:DNA complexes are not significantly different from the nonphosphorylated CREB:DNA complexes. We note that there is a reproducible difference in the total anisotropy change between the CREB: and P-CREB:DNA complex. This difference does not affect the Kd values and is addressed in the discussion.

CBP does not affect CREB:DNA binding. CBP is a coactivator that binds specifically to PKA-phosphorylated CREB [16, 19, 20]. A possible mechanism for activation by CBP could be to increase the affinity of P-CREB for DNA. A similar mechanism has been proposed for the viral protein Tax, which is thought to increase the binding of bZIP transcription factors to the promoter by increasing dimerization [21]. We investigated whether CBP increased the affinity of phosphorylated CREB for either the SSCRE or TATCRE. Either CREB or P-CREB were titrated into solutions of SSCRE or TATCRE which contained saturating amounts (0.5 - 1 uM) of the CREB binding domain of CBP (amino acids 1 - 682 [16, 19]). The greater absolute anisotropy value seen in Figure 3 for the P-CREB complex over the CREB complex is due to the binding of CBP to the phosphorylated form of CREB. The specific binding of CBP increases the size of the P-CREB:CRE complex and therefore increases the anisotropy value.

The equilibrium dissociation constant for the P-CREB:CBP interaction has previously been shown to be approximately 220 nM [16]. CBP does not bind non-phosphorylated CREB at up to micromolar concentrations. The experimental and fitted curves for the phosphorylated and non-phosphorylated CREB:DNA interactions in the presence of the CBP fragment are shown in Figure 3. The Kd for the (CBP:P-CREB):SSCRE complex was 1 ± 1 nM and the Kd for the (CBP:P-CREB):TATCRE complex was 7 ± 1 nM. The Kd of the CREB:SSCRE complex in the presence of CBP was 2 ± 1 nM and the Kd of the CREB:TATCRE

complex in the presence of CBP was 9 ± 1 nM. Therefore, binding of the CREB-binding domain of CBP does not significantly affect the binding of P-CREB to either the SSCRE or TATCRE.

Discussion

We have used fluorescence anisotropy to measure the relative binding affinities of CREB for different CRE sequences and to determine whether PKA phosphorylation affects the affinity of CREB for these sequences. Previous studies have used electrophoretic mobility shift assays (EMSA) to quantify CREB:DNA interactions and have generated some conflicting results [8, 9, 22]. Williams, *et al.* [22] compared the affinities of CREB327 for the SSCRE and the proenkephalin CRE (ENKCRE)[1] [23]. The Kd values were reported as approximately 5 nM for the SSCRE and approximately 30 nM for the ENKCRE, which is a non-canonical site similar to the TATCRE. Nichols, *et al.*, classified these non-canonical sites such as the ENKCRE and TATCRE as "low-affinity" compared to the canonical SSCRE which was classified as a "high-affinity" site [8]. Our equilibrium dissociation constants for CREB341 binding to the SSCRE and TATCRE are of similar magnitude but slightly higher affinity than those originally obtained by EMSA [22]. The slightly higher affinity found by fluorescence anisotropy likely reflects an improvement in sensitivity and accuracy of this method over EMSA. However, these slightly different Kd values could also arise from the small differences between the CRE sequences or CREB isoforms. Our results further suggest that the terminology of high- and low-affinity CRE sites [8] is misleading as the non-canonical sequence still has an

[1]The CREB341 and CREB327 isoforms mRNA splice variants that differ by a 14 amino acid insert of unknown significance.

equilibrium dissociation constant in the low nanomolar range. The non-cAMP responsive TRE, on the other hand, has a Kd around 200-300 nM which is more representative of low-affinity binding when compared to a completely non-specific oligonucleotide sequence in which no binding was detected at up to 500 nM CREB concentration (data not shown).

In addition to comparing binding affinities of CREB for different CRE sequences, we investigated the role of PKA phosphorylation on CREB:DNA interactions. Our data demonstrate that phosphorylation of CREB by PKA does not significantly affect DNA binding affinity to either the canonical SSCRE or non-canonical TATCRE. This observation contrasts a previous report which described a two- to three-fold increase in CREB binding to DNA following PKA phosphorylation [8]. We are not certain of the reason for this discrepancy, however, it may reflect the particular solubility problems of CREB. These problems may be more difficult to control in EMSA than by fluorescence anisotropy. A number of variables may explain the differences between our results and those of Nichols, *et al.*, and we note the following observations: We found a variation in the Kd values from different preparations of protein amounting to approximately ± 3 nM for the SSCRE and ± 6 nM for the TATCRE. We observed that DNA binding activity diminished with prolonged storage of CREB, presumably due to oxidation of the cysteine in the DNA binding domain [10] and aggregation [11]. Some of this activity could be recovered by a three

minute, 60°C heat treatment in the presence of fresh 5 mM DTT. We performed our binding assays in the presence and absence of BSA. We found BSA to reduce the amount of non-specific association that occurred at high protein concentrations. The presence of BSA has also been demonstrated to improve the activity of CREB in gel mobility shift assays [12]. Finally, under the binding conditions presented here (see Materials and Methods) there is little change in binding at 50 mM, 100 mM or 200 mM NaCl. Binding is nearly abolished at 500 mM NaCl as would be expected from the electrostatic nature of protein:DNA interactions. However, in the presence of a minimal buffer containing only fluorescent grade Tris/HCl, pH 8.0 and containing either 50 mM, 100 mM, 200 mM or 500 mM KCl, CREB shows a very sharp salt dependence with maximal binding occurring at 200 mM KCl (Appendix II). This equilibrium dissociation constant agrees well with that presented (Kd = 3 nM +/- 1 nM). Interestingly, P-CREB shows less salt dependence in this minimal buffer giving maximal activity at 100 mM KCl. The sensitivity to ionic strength seems to be primarily a solubility effect since the amount of nonspecific association (Knsp) is minimized under optimal binding conditions. This conclusion is supported by analytical ultracentrifugation experiments (Chapter III).

Although the Kd values for both CREB:DNA and P-CREB:DNA interactions are the same, there was a reproducible difference in the total anisotropy change (A_{max}-A_o) when comparing the CREB:DNA and

P-CREB:DNA binding curves. One explanation for this difference may be that the phosphate somehow quenches the fluorescein label. Alternatively, this difference may reflect a change in rotational correlation time (rotational diffusion) between the two protein:DNA complexes and could indicate a difference in the size or shape of the complex. Such a change might be explained by a conformational change in CREB following phosphorylation as has been proposed [24, 25]. Alternatively, the anisotropy difference could reflect a change in DNA structure such as bending [26, 27] or a DNA-binding dependent conformational change in the phosphorylated protein. We are currently investigating the source of this difference.

We also investigated whether CBP, a transcription coactivator that binds specifically to phosphorylated CREB, affects the P-CREB:DNA interaction. CBP binding could indirectly affect the P-CREB:DNA binding affinity. We found that the CREB binding domain of CBP has no effect on the ability of P-CREB to bind to either the SSCRE or the TATCRE. This finding suggests that CBP acts separately from the DNA binding event and distinguishes CBP from other proteins, such as the viral activator Tax, which have been proposed to increase the DNA binding affinity of bZIP transcription factors [9, 21].

The findings presented here indicate that PKA-phosphorylation does not activate CREB by altering its CRE binding affinity. Rather, the addition of a phosphate at Ser133 may create in CREB the proper stereochemical and structural

scaffold for coactivator binding.

ACKNOWLEDGMENTS

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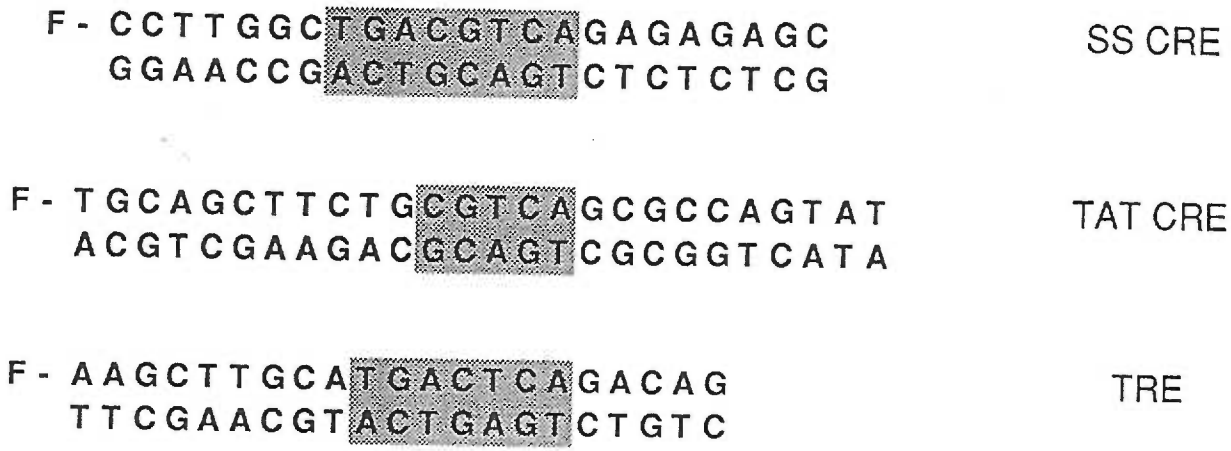
FIGURE 1. Fluorescence anisotropy binding curves of CREB for different CREs. The oligonucleotide sequences used in this study are shown in (A). The normalized change in anisotropy is plotted against CREB concentration. Ao is the anisotropy value of the oligonucleotide in the absence of protein and A is the measured anisotropy value at each concentration point. Both the experimental data points and the fitted curves are shown. An expanded view is given in (B) to illustrate the Kd value. All data points are depicted in (C).


F- CCTTGGCTGACGTCAGAGAGAGC
GGAACCGACTGCAGTCTCTCTCG
SS CRE
F- TGCAGCTTCTGCGTCAGCGCCAGTAT
ACGTCGAAGACGCAGTCGCGGTCATA
TAT CRE
F- AAGCTTGCATGACTCAGACAG
TTCGAACGTACTGAGTCTGTC
TRE

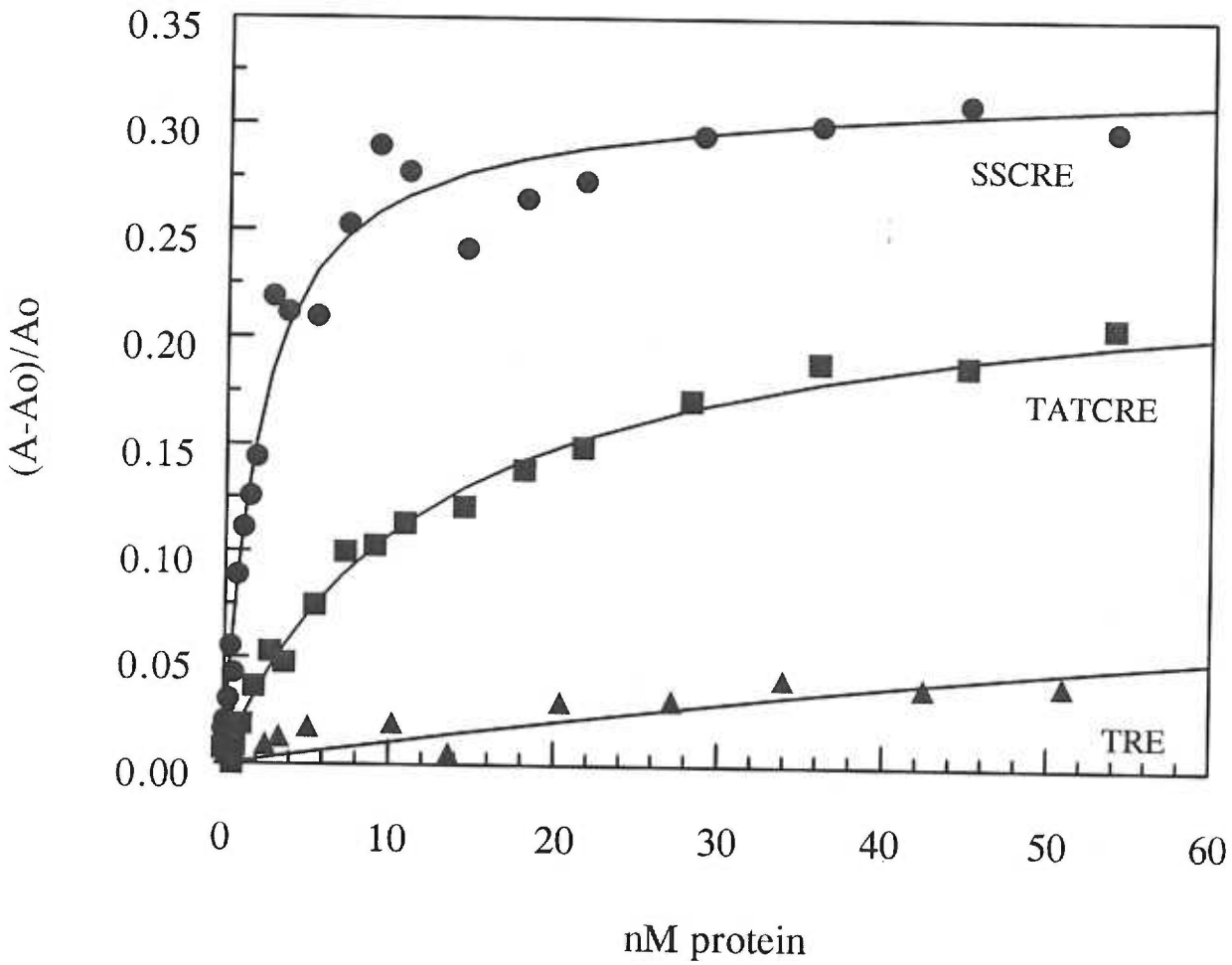


Fig. 1B. Richards, et al.

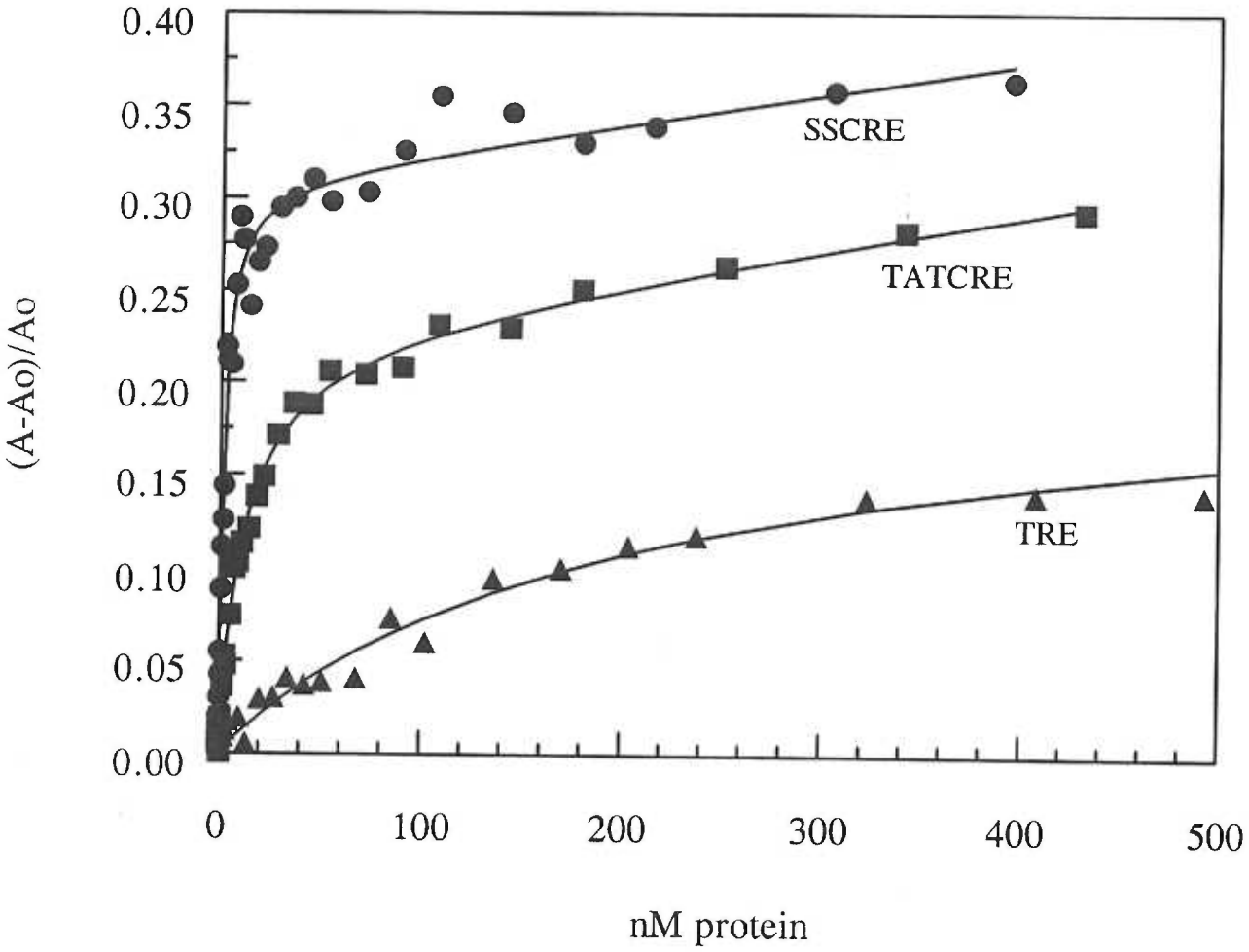


Fig. 1C. Richards, et al.

FIGURE 2. PKA phosphorylation does not affect the binding affinity of CREB for DNA. The experimental and fitted binding curves for CREB and P-CREB with the SSCRE (A) and the TATCRE (B) are shown. Full-scale curves are given in the insets.

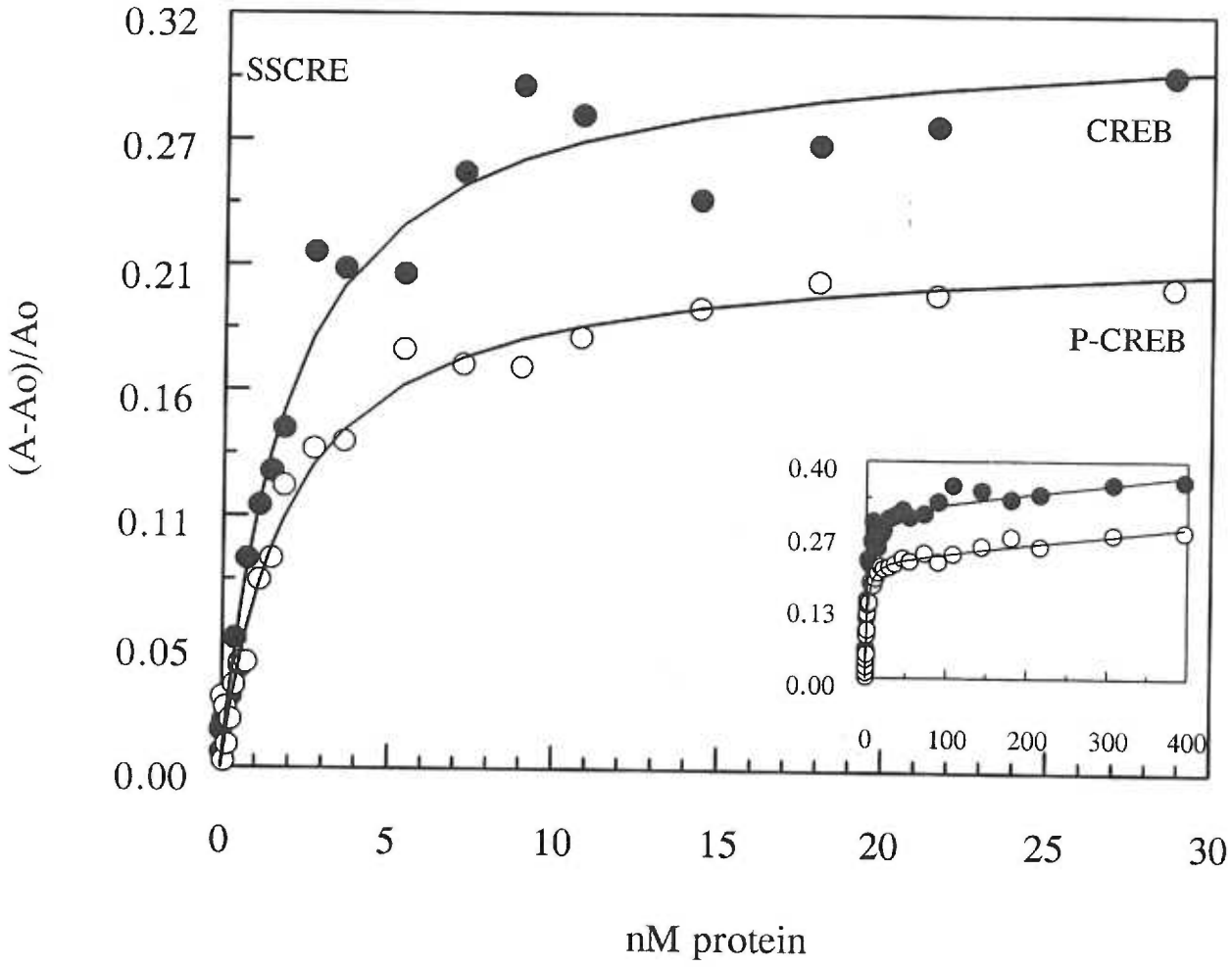


Fig. 2A. Richards, et al.

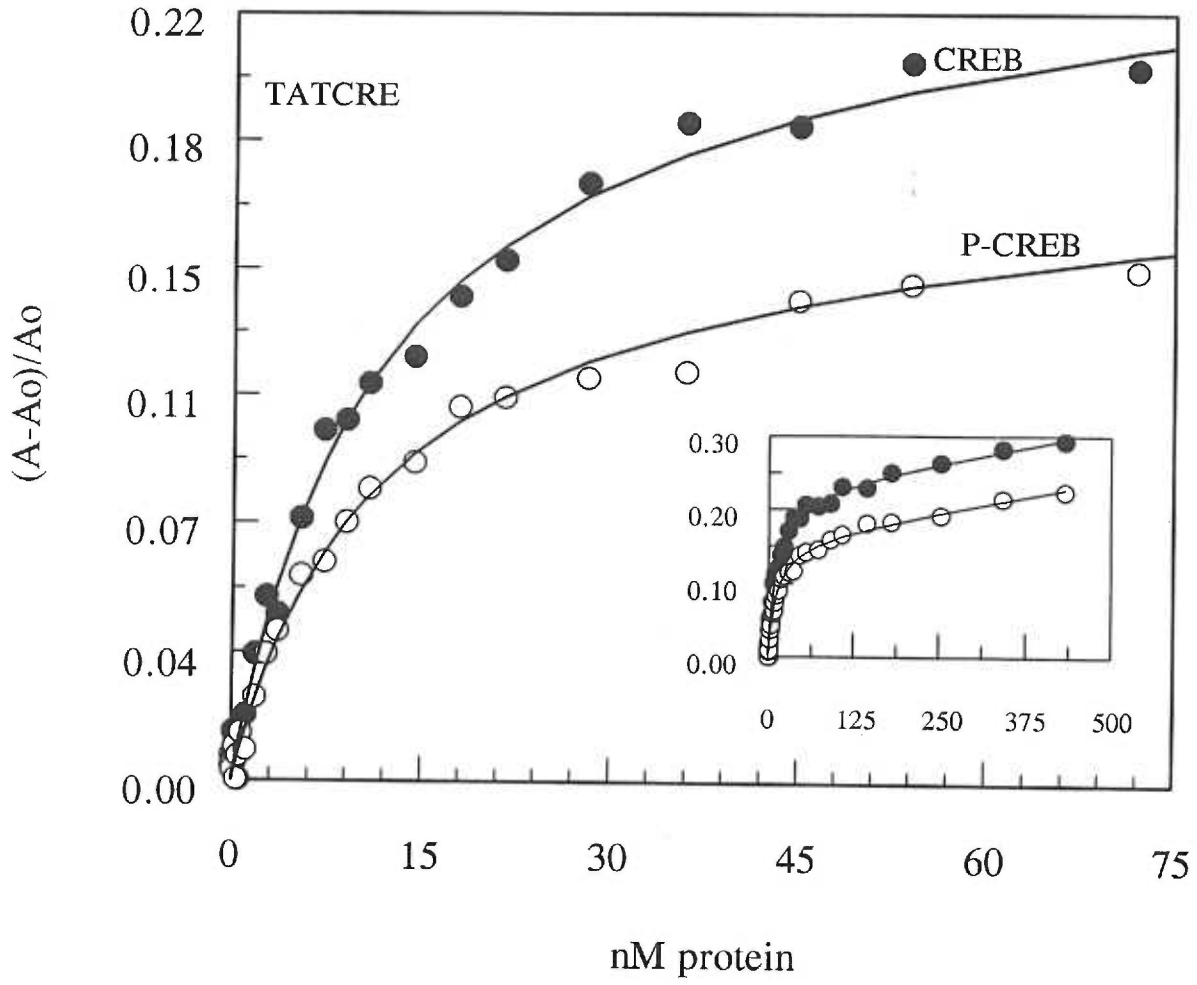


Fig. 2B. Richards, et al.

FIGURE 3. CBP does not affect the binding affinity of P-CREB for DNA. The experimental and fitted binding curves of CREB and P-CREB for the SSCRE (A) and the TATCRE (B) are shown in the presence of saturating amounts of CBP. Full-scale curves are given in the insets.

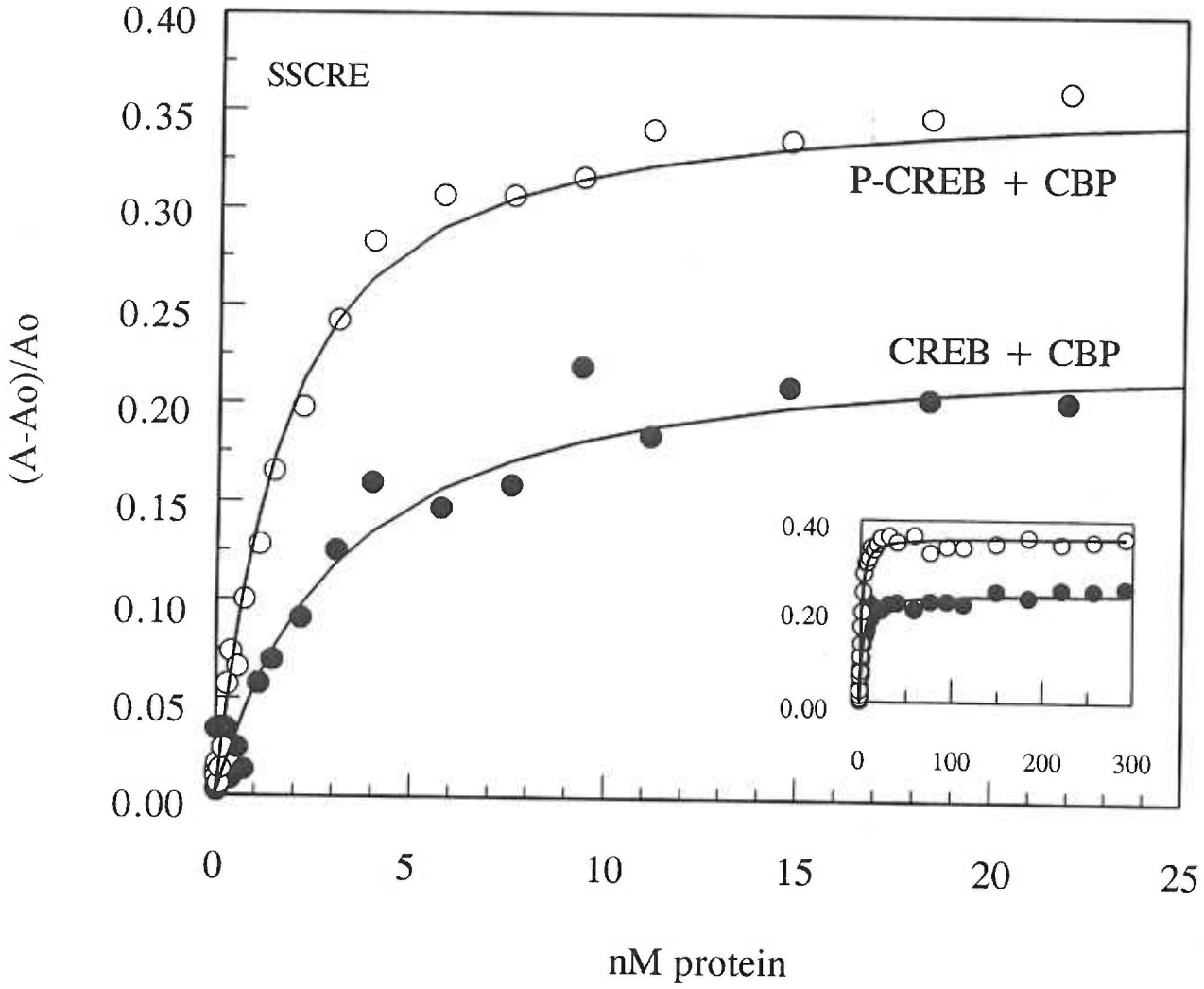


Fig. 3A. Richards, et al.

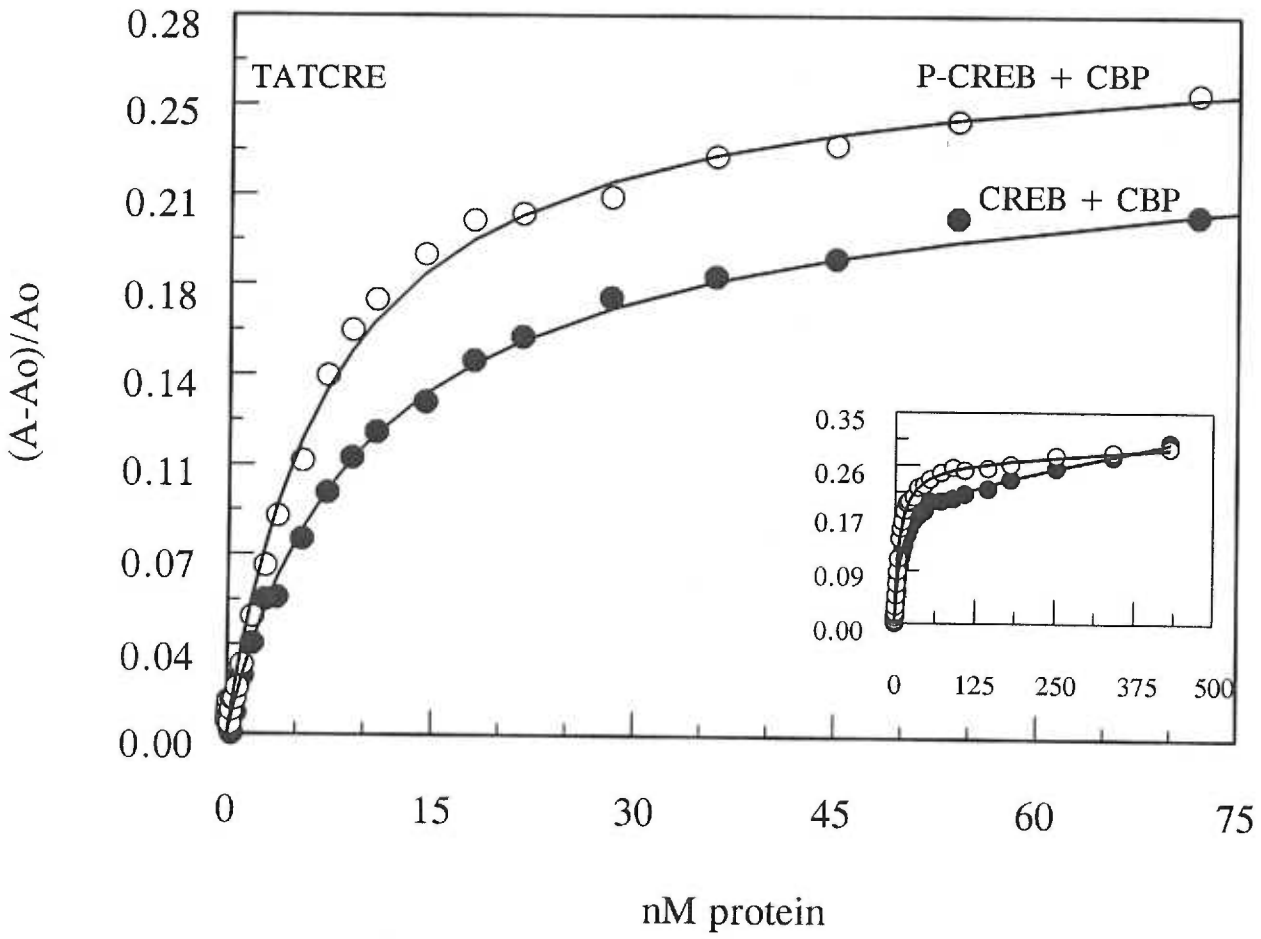


Fig. 3B. Richards, et al.

CHAPTER III

Analytical Ultracentrifugation Studies of CREB and Protein Kinase A Phosphorylated CREB

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RUNNING TITLE: Tertiary structural characteristics of CREB

Summary

Velocity and equilibrium sedimentation studies were performed on the transcription factor CREB to evaluate its tertiary structural characteristics and to compare the structures of CREB and PKA phosphorylated CREB. We tested the model that phosphorylation induces a conformational change in CREB. Wild type CREB is prone to aggregation and was not amenable to analytical ultracentrifugation. This aggregation is largely due to the presence of three cysteines in the bZIP region of the protein which are particularly senisitive to oxidation and disulfide crosslinking. We designed a mutant CREB in which these three cysteines were substituted with serines. We show that this substitution improves solubility and allows for sedimentation analysis. Circular dichroism, DNA binding studies and *in vivo* transfection assays demonstrate that the CREB/SER protein maintains its structural integrity. Velocity sedimention yielded a sedimentation coefficient of $s^{0}_{20,w} = 2.4\ S$ for CREB and $s^{0}_{20,w} = 2.4\ S$ for phosphorylated CREB. Therefore, there is no detectable difference in the tertiary structure of CREB and phosphorylated CREB under these conditions. Calculation of the hydrodynamic parameters of CREB indicates that CREB deviates significantly from an idealized sphere. Equilibrium sedimentation demonstrate that at micromolar concentrations CREB is monomeric in 25 mM Tris/HCl buffer, pH 8.0, containing 100 mM NaCl and dimeric in 25 mM Tris/HCl buffer, pH 7.6, containing 200 mM KCl.

Introduction

Fully, understanding the mechanisms of activated transcription requires a structural knowledge of the proteins involved in this process. Although a wide range of structural data has been gathered on the DNA binding domains of proteins involved in eukaryotic transcription, very little information has been gathered on their activation domains or the full-length proteins. We have undertaken the physical characterization of the full-length transcription factor CREB. CREB (cAMP responsive element binding protein) belongs to the basic-leucine zipper (bZIP) class of transcription factors and is involved in cAMP mediated gene expression (reviewed by [1]). Attempts to crystallize CREB have not yet been successful, and a better understanding of the physical properties of CREB is therefore essential to the progress of elucidating its structural details. One reason structural characterization of CREB has been difficult is because of the tendancy of CREB to oxidize and aggregate, problems which contribute to poor solubility and instability. The three cysteines in the bZIP region were found to be major contributors to these problems. Abate, *et al.* found that oxidation of a conserved cysteine residue in the basic region of fos, jun and CREB reduced DNA binding [2]. In addition, Santiago-Rivera, *et al.* demonstrated that methylation of these cysteines reduces aggregation and improves the solubility of CREB bZIP peptides [3]. In order to improve the solubility of CREB and design a protein that was

more amenable to structural characterization, we replaced the three cysteines in the bZIP region with serine residues. We used analytical ultracentrifugation to confirm the improved solubility of our protein and to characterize the tertiary structure of CREB.

CREB has been proposed to undergo an allosteric conformational change following PKA phosphorylation, and this conformational change is the suggested mechanism by which phosphorylation activates CREB [4, 5]. There is, as yet, no physical evidence to support this model, and we have shown previously that PKA phosphorylation does not change the circular dichroism (CD) spectra of CREB or change its DNA binding affinity (Chapters I and II). However, when performing fluorescence anisotropy assays to measure CREB:DNA binding, we noticed that there was a characteristic difference in the total anistropy change (Amax - Ao) between CREB and phosphorylated-CREB (P-CREB; Chapter II). Since anistropy is a function of molecular shape, one explanation for this difference is that phosphorylation induces a tertiary conformational change CREB that was not detected in our CD study. We reasoned that such a conformational change should be detectable by comparing the sedimentation coefficients of CREB and P-CREB.

Materials and Methods

Expression plasmids and protein purification. 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 cDNA that was isolated from a λgt11 human hypothalamic library and subcloned into the Hind III/Xba I sites of p-SELECT (Promega, [6]). Human CREB327 and rat CREB341 have identical bZIP amino acid sequences, although they differ slightly in the nucleotide sequence. Three separate oligonucleotides with single base mutations in the cysteine codons were used to generate serine substitutions as described in the Altered Sites *in vitro* Mutagenesis System protocol (Promega). The substituted bZIP domain of CREB327 was subcloned into the rat CREB341 pET15b expression plamid (Chapter I) using Asp 718/Bam HI sites (Novagen). The resulting CREB/SER clone is a rat CREB341/human CREB327 cDNA hybrid which contains an amino acid sequence identical to rat CREB341 with the three cysteines in the bZIP domain substituted with serines.

CREB341 and CREB/SER were overexpressed and purified as described in detail elsewhere (Chapter I). Briefly, plasmids were grown in *E. coli* BL21(DE3) bacteria, and protein expression was induced with isopropyl-β-D-thiogalactopyranosid (IPTG). After harvesting, the bacteria were lysed in a French press. The bacterial extracts of CREB341 or CREB/SER were heated to 70°C for

10 min. Circular dichroism studies have demonstrated that CREB is stable to this treatment (Chapter I). The clarified extract was then purified over a DE53 anion exchange column (Whatman) at which point CREB is approximately 95% pure as determined by Coomassie based Fast Stain (Zoion) of SDS-polyacrylamide gel electrophoresis. CREB was stoichiometrically phosphorylated using purified recombinant PKA C-subunit (kindly provided by R. Maurer, Oregon Health Sciences University) as described (Chapter I).

Circular dichroism. The circular dichroism spectra were obtained as described previously (Chapter I). The proteins were dialysed into 10 mM potassium phosphate (pH 7.5). The CD spectra were measured on a JASCO J-500A spectropolarimeter using a 0.01 cm pathlength cell. The reported spectra are the average of 10 scans recorded at 0.1 nm intervals. A scanning rate of 10 nm/min and 2 sec time constant was used. The reported spectra have their buffer baselines substracted and were smoothed using the program provided by JASCO.

DNA binding assays. DNA binding was measured by electrophoretic mobility shift assay (EMSA) and by fluorescence anisotropy. For the EMSA approximately 100 ng of purified protein was preincubated for 15 min at room temperature in a reaction mixture of 10 mM Tris/HCl buffer, pH 7.5, containing 50 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 5 mM dithiothreitol, 1 μg poly dIdC, and 3.9 μg bovine serum albumin. Approximately 15 fmol of ^{32}P-end-labeled somatostatin CRE oligonucleotide were added to the protein

mixture, and the reaction was incubated at room temperature for 15 min. The reactions were loaded onto a pre-run 4% acrylamide gel (29:1) in 45 mM Tris-borate buffer containing 1 mM EDTA (= 0.5X TBE buffer) and electrophoresed at 150 volts for 3 hrs. Dried gels were autoradiographed using Kodak XAR film.

The fluorescence anisotropy DNA binding assays were performed as decribed in Chapter II. Briefly, purified protein was titrated into a solution containing a fluoresceinated oligonucleotide. The sequences (upper strand only) were: SSCRE = (5'-CCTTGGCTGACGTCAGAGAGAGC-3'); TATCRE = (5'-TGCAGCTTCTGCGTCAGCGCCAGTAT-3'); TRE = (5'-AAGCTTGCATGACTCAGACAG-3'). Fluorescence polarization measurements were collected on a Beacon Fluorescence Polarization System. The binding reactions contained 1 ml of 0.5 nM fluoresceinated oligonucleotide in 25 mM Tris-HCl buffer, pH 7.6, containing 50 mM NaCl, 5 mM $MgCl_2$, 0.1% triton X-100, 5% glycerol, 10 μg/ml poly dIdC and 1 μM BSA. Protein (10-20 μM as determined by amino acid analysis) was serially diluted and titrated into the cuvette such that the total sample volume increased by less than 10%. Each point on the curves represents the average of four anisotropy measurements. The standard deviation for each point was consistently less than 2% of the measured anisotropy value. The change in anisotropy was measured and plotted as a function of protein concentration. The curves were fit with a nonlinear least squares regression analysis assuming the model [CREBdimer] + [DNA] ↔

[CREBdimer:DNA]. A nonspecific component was included in the model to account for the linear increase in anisotropy at higher protein concentrations.

Sedimentation. The CREB/SER protein was dialysed into 25 mM Tris/HCl buffer, pH 7.6, containing 100 mM NaCl. Sedimentation properties were measured using double sector cells in a Beckman Model E analytical ultracentrifuge equipped with ultraviolet optics and interfaced to a computer using the ultrascan data acquisition system [7]. Velocity sedimentation was performed at a rotor speed setting of 52,000 rpm and at a temperature from 20-25^{o}C. The inverse sedimentation coefficients, as determined by second moment analysis, were plotted as a function of concentration and extrapolated to zero protein concentration.

Results

The CREB/SER mutant maintains its structural integrity. CREB contains three cysteine residues in the bZIP dimerization and DNA-binding domain. These residues are particularly reactive to oxidation [2] and subsequent aggregation [3]. In order to design a more soluble CREB protein, these cysteine residues were replaced with serine residues using site-directed mutagenesis. To confirm that the cysteine to serine substitutions in CREB did not change the structure of the protein, we compared the secondary structure, DNA binding affinity, CBP binding ability and the *in vivo* activation activity of the CREB/SER mutant to wild type CREB.

We first examined the circular dichroism spectra of wild type CREB and CREB/SER (Fig. 1A; see also Chapter I, Fig 1). The two spectra overlay well with the slight differences being within the range of variation seen between different experiments. The observed differences at 180-190 nm are artifacts due to the higher concentration of CREB/SER and resultant low light signal coming through the protein solution. We conclude that the cysteine to serine substitutions do not affect the secondary structure of CREB.

We next compared the DNA binding affinities of the CREB/SER mutant and wild type CREB by two methods: electrophoretic mobility shift assay (EMSA) and fluorescence anisotropy. The results are presented in Figure 1B and 1C. For the EMSA, approximately equal amounts of the phosphorylated and

non-phosphorylated forms of wild type CREB and CREB/SER were incubated with the somatostatin CRE. The band shifts for the two proteins are indistinguishable, with the phosphorylated CREB/SER mutant showing the same characteristic retardation that the phosphorylated wild type CREB shows.

Quantitation of DNA binding was performed by using a fluorescence anisotropy assay (Chapter II). Wild type CREB and CREB/SER were titrated into a solution of either the somatostatin CRE (SSCRE) or the tyrosine aminotransferase CRE (TATCRE). Equilibrium dissociation constants (Kd) were measured by following the change in anisotropy as protein bound to the DNA. The Kd values are reported in Table I. The values are equivalent for the phosphorylated and non-phosphorylated forms of wild type CREB and the CREB/SER mutant. The CREB/SER mutant also shows the same difference in maximal anisotropy (Amax-Ao) between the phosphorylated and non-phosphorylated forms. Fluorescence anisotropy also confirmed that the CREB/SER mutant binds CBP with an affinity equal to wild type CREB (J. Lundblad, unpublished). Therefore, the serine substitutions do not affect the DNA-binding or CBP-binding activities of CREB.

The sedimentation coefficient of CREB and phosphorylated CREB are indistinquishable. Wild type CREB sedimented nonhomogeneously at low rotor speed indicating the presence of high molecular weight aggregates. The serine substitutions reduced the aggregation and improved the solubility of CREB, thus

enabling analytical sedimentation experiments. Micromolar concentrations of CREB/SER and phosphorylated CREB/SER in 25 mM Tris/HCl buffer, pH 7.6, containing 100 mM NaCl were subjected to velocity sedimentation in a Beckman Model E ultracentrifuge. A representative experiment is shown in Figure 2A. CREB/SER sediments homogeneously as determined by Van Holde-Weischet plot. A plot of 1/*s* versus protein concentration is shown in Figure 2B. Extrapolating the data to zero concentration gives $s^0_{20,w}$ = 2.4 *S* for CREB/SER and $s^0_{20,w}$ = 2.4 *S* for phosphorylated CREB/SER. Therefore, there is no difference in the sedimentation coefficients of CREB and PKA phosphorylated CREB.

CREB association is higly salt sensitive. From the sedimentation coefficient, we calculated the hydrodynamic parameters of CREB. The results were reasonable only when we assumed that CREB was monomeric (Table II). A previous study reported that the dimerization constant for the CREB bZIP domain alone was approximately 20 μM [3]. To verify that CREB is monomeric under our sedimentation conditions, we performed equilibrium sedimentation. Preliminary results gave a molecular weight of about 40 kDa, which is consistent with the molecular weight of the CREB monomer (36,635 Mr). Estimation of the hydrodynamic parameters indicated that CREB has as f/fo of 1.5 and an axial ratio of roughly 1:10. Thus, the CREB monomer deviates significantly from an idealized sphere.

Concomitant DNA binding studies indicated that CREB:DNA binding was highly salt dependent with maximal activity occurring in 25 mM Tris/HCl buffer, pH 8.0, at 200 mM KCl (Appendix II). This salt dependence appeared to be due primarily to protein solubility as evidenced by the amount of non-specific association at high protein concentrations. We therefore repeated our equilibrium sedimentation studies in the presence of 200 mM KCl. Under these conditions, CREB is mostly dimeric at the same concentrations (~10 μM) that it was found to monomeric in the above experiment. Under these new conditions, CREB/SER has a sedimentation coefficient $s^{0}_{20,w}$ = 3.0 *S* (Fig.). Estimation of hydrodynamic parameters indicates that the CREB dimer also deviates significantly from an idealized sphere (Table II).

Discussion

By substituting the three cysteines in the bZIP region with serines, we have constructed a CREB mutant with improved solubility. To verify that these mutations do not alter the structure of CREB, we performed a number of controls. CREB/SER is indistinguishable from wild type CREB in its circular dichroism spectra and in its DNA binding properties. Therefore, the CREB/SER protein provides a useful model for studying the physical properties of CREB.

The sedimentation coefficients of CREB and PKA phosphorylated CREB are indistinguishable. This result indicates that there is no change in overall tertiary structure of CREB and PKA-phosphorylated CREB. Taken in conjuction with our previous findings that PKA phosphorylation does not change the CD spectra, protease cleavage pattern or DNA binding affinity of CREB, these results argue strongly that PKA phosphorylation alone does not change the conformation of CREB. However there are two observed differences between CREB and phosphorylated CREB that have not yet been explained. These differences are the anomalous migration of P-CREB in gel mobility shift assays (Fig. 1B and [8]) and the difference in (Amax-Ao) in the fluorescence anisotropy DNA binding assay (Chapter II). In both these instances CREB is dimeric and bound to DNA. Therefore, the possibility remains that phosphorylation changes the structure of dimeric CREB or of the CREB:DNA complex. Further sedimentation studies of CREB and P-CREB under the conditions in which they are dimeric and in the

presence of DNA will test this hypothesis.

Equilibrium sedimentation revealed a number of interesting features of CREB. In 25 mM Tris/HCl buffer, pH 7.6, containing 100 mM NaCl, CREB exists as monomer and, as concentration is increased, higher order aggregates; dimer is not detectable. However, in 25 mM Tris/HCl buffer, pH 8.0, containing 200 mM KCl, the presence of dimer is clearly indicated. For the moment we are assuming these differences are due to the concentration of salt and not the type of salt (NaCl vs KCl) or pH (7.6 vs 8.0). However, we have not yet investigated all the variables. Van Holde-Weischet plots of velocity sedimentation experiments indicate that under the latter conditions CREB is a non-homogeneous mixture and deconvolution of the equilibrium data suggest the presence of monomer, dimer and tetramer. Analysis of these data are in progress.

Acknowledgements

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TABLE I

Equilibrium dissociation constants for CREB and various DNA sequences. The Kd values for wild type CREB and CREB/SER were determined by fluorescence anisotropy binding assays.

	SSCRE	**TATCRE**
CREB	2 nM	11 nM
P-CREB	2 nM	11 nM
CREB/SER	3 nM	10 nM
P-CREB/SER	6 nM	16 nM

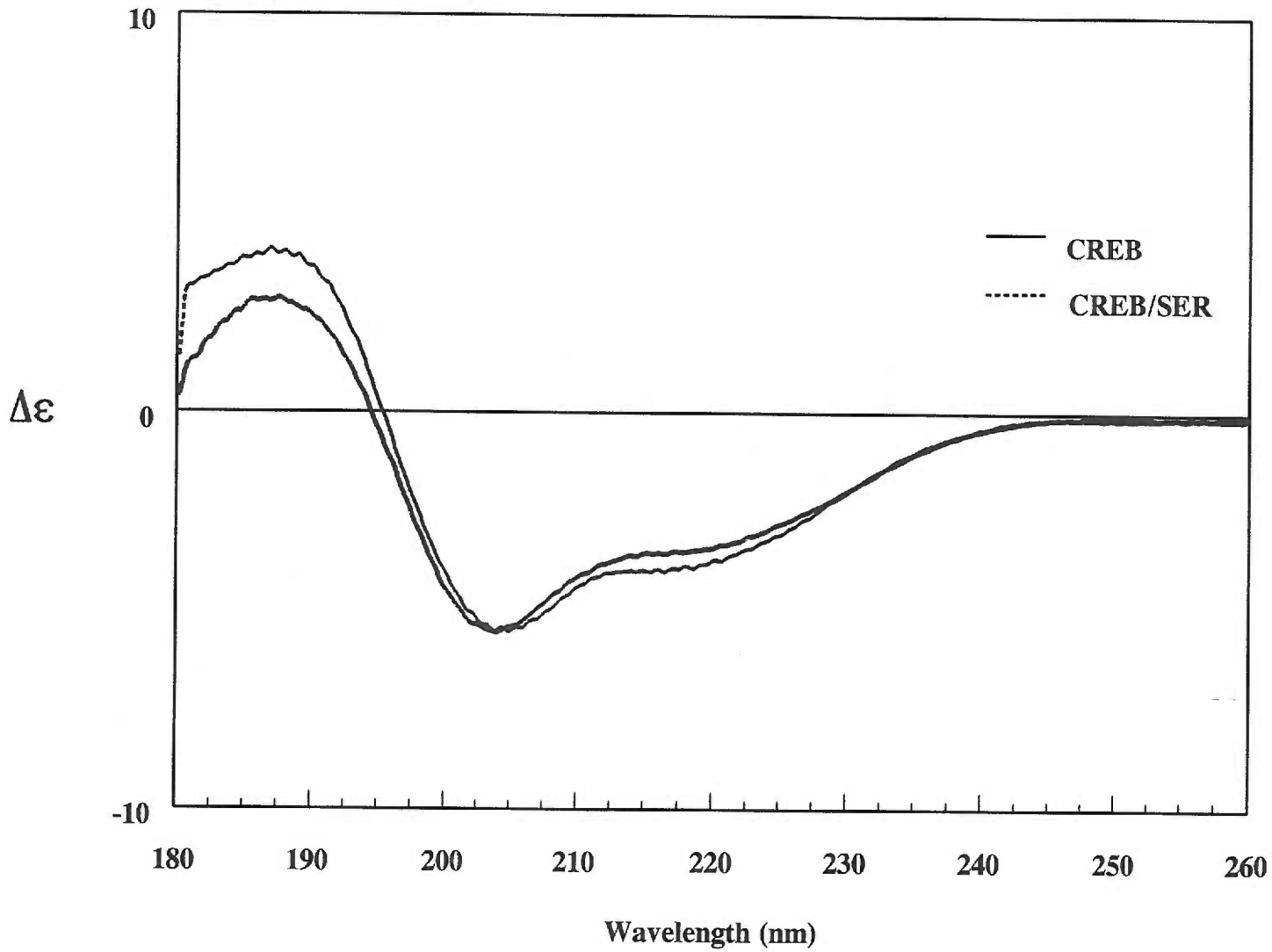
TABLE II

Hydrodynamic Parameters of CREB.

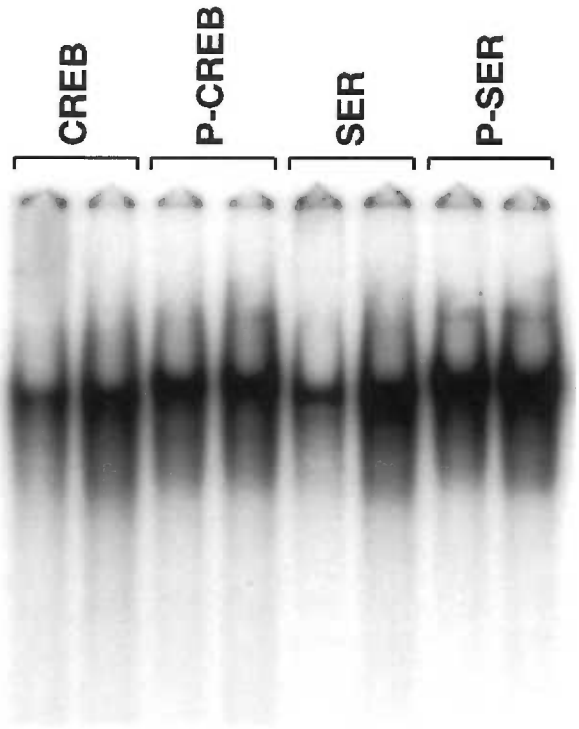
Property	**100 mM NaCl**	**200 mM KCl**
Mr*	~ 40, 000	~ 80,000
$s^{0}_{20,w}$	2.4	3.0
f/fo	1.5	1.8

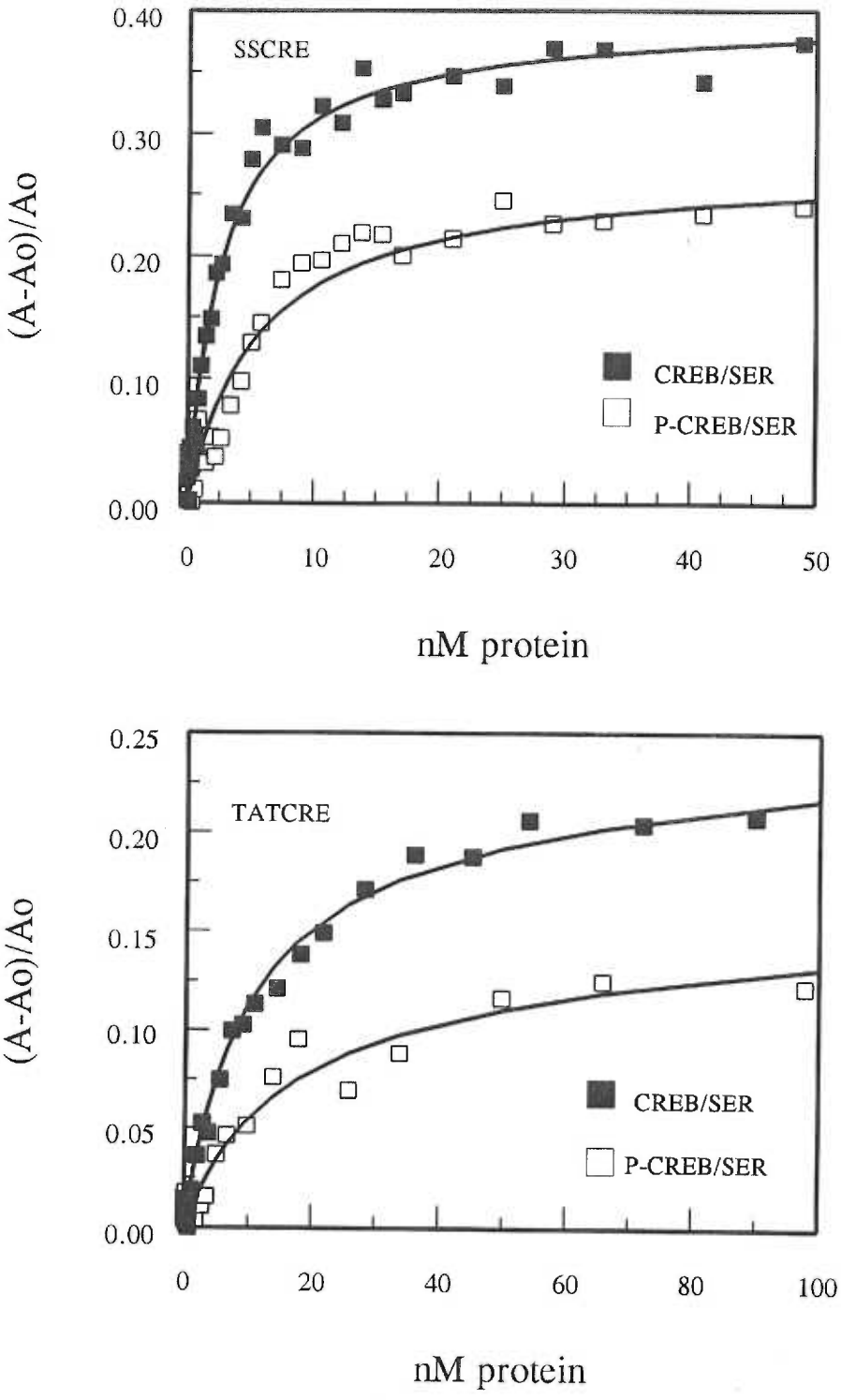
* Mr calculated from equilibrium runs assuming a homogeneous species.

FIGURE 1. CREB/SER maintains the properties of wild type CREB. A. Circular dichroism of wild type CREB and CREB/SER. The CD spectra of CREB and CREB/SER from 260-180 nm are presented. The proteins are in 10 mM phosphate buffer, pH 7.5. Wavelength is represented along the x-axis and molar amino acid ellipticity along the y-axis. (———) wild type CREB. (--------) CREB/SER. B. EMSA of the PKA-phosphorylated and non-phosphorylated forms of CREB and CREB/SER. 50 and 100 ng of each protein bound to the SSCRE are shown as labelled. C. Fluorescence anisotropy binding curves of the phosphorylated and non-phosphorylated forms of CREB and CREB/SER bound to the SSCRE or TATCRE are shown. The protein concentration is plotted against the normalized anisotropy value where Ao is the anisotropy of the DNA in the absence of protein and A is the value at a given protein concentration. Both the experimental and fitted curves are shown.



Chapter III. Figure 1A.

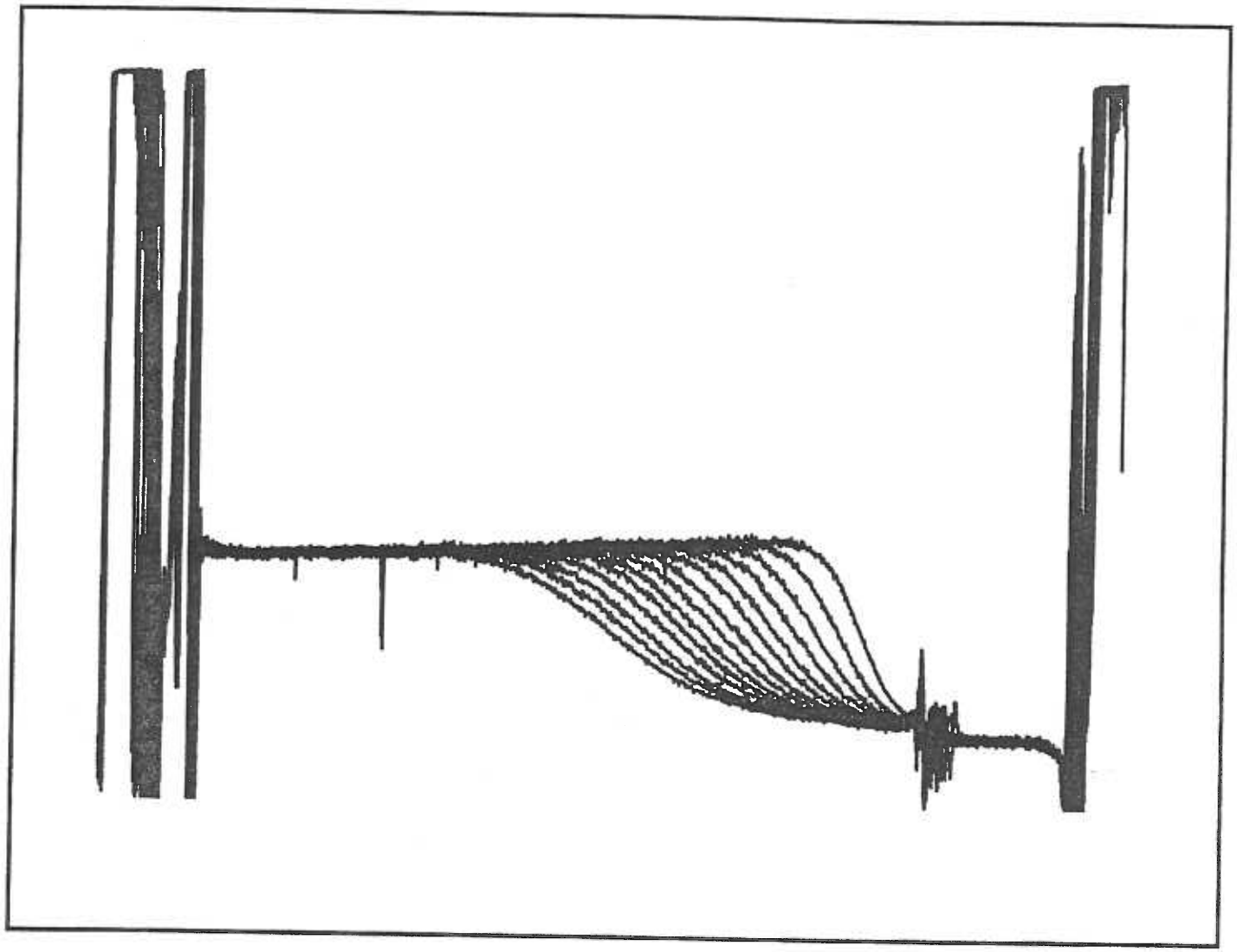

CREB
P-CREB
SER
P-SER

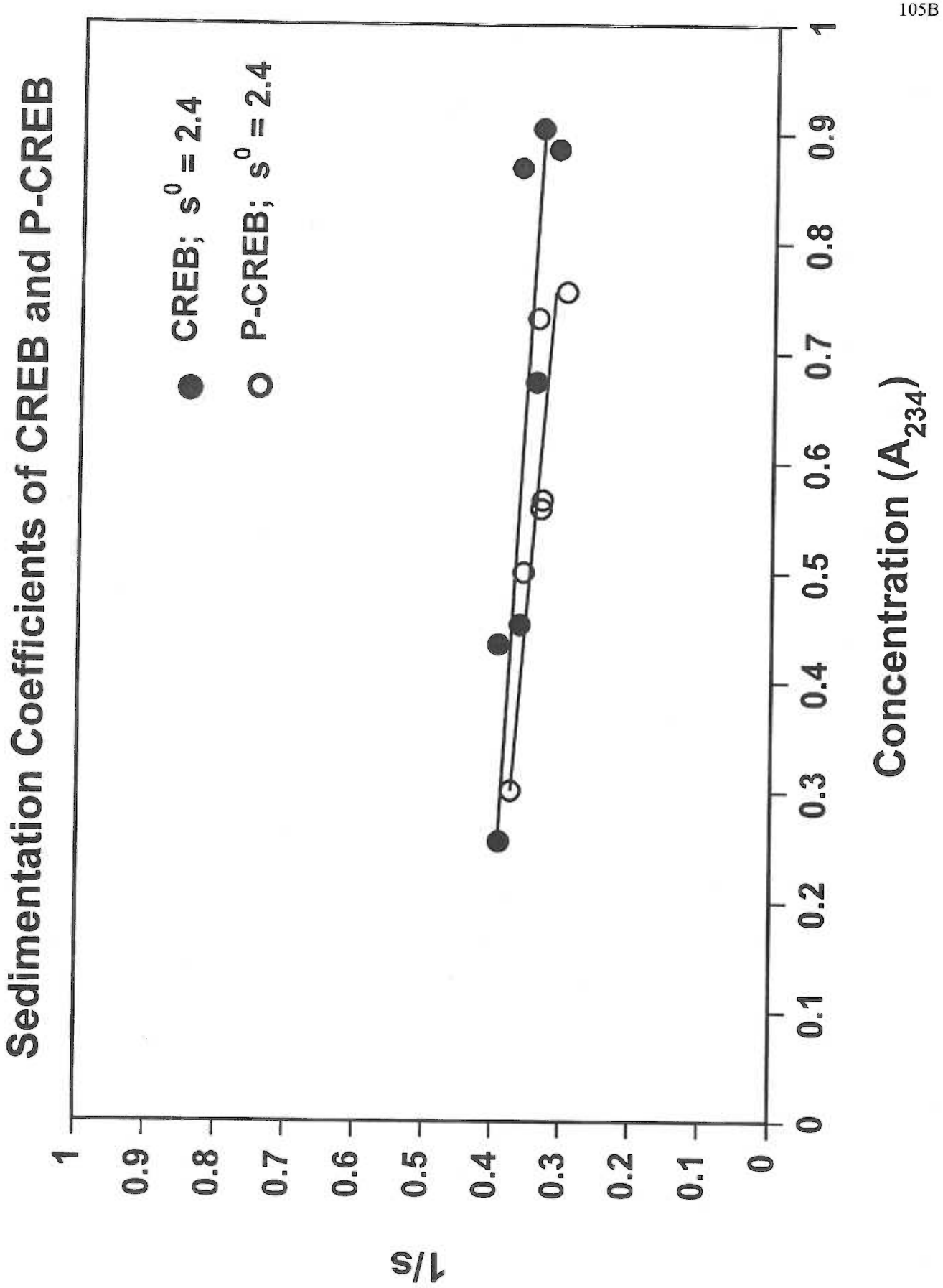


Chapter III. Figure 1C.

FIGURE 2. Analytical sedimentation of CREB/SER and PKA-phosphorylated CREB/SER. A. A representative velocity sedimentation experiment of CREB/SER is profiled. The x-axis represents the distance, *r*, from the center of rotation, and the y-axis depicts the protein concentration as represented by absorbance at 234 nm. Each curve represents a scan taken at a different time point, *t*. B. The inverse of the calculated sedimentation coefficients (1/s) for CREB/SER and P-CREB/SER are plotted as a function of protein concentration as represented by absorbance at 234 nm. The points were fit to a linear function and the sedimentation coefficient at zero protein concentration was extrapolated ($s^{0}_{20,w}$).

Velocity Sedimentation of CREB




Sedimentation Coefficients of CREB and P-CREB
CREB; s^0 = 2.4
P-CREB; s^0 = 2.4
1/s
Concentration (A_{234})
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1

DISCUSSION

The purpose of this dissertation is to begin the biophysical characterization of the mammalian transcription factor CREB. The structural characterization of CREB is important for several reasons:

(i) CREB is a well-studied member of the CREB/CREM/ATF family of transcription factors and, as such, serves as a prototype or model for these proteins.

(ii) The basis for specificity in bZIP dimerization and DNA recognition is not completely understood. For example, the CREB bZIP is homologous to the yeast GCN4 bZIP, yet these two peptides have distinctly different DNA binding specificities. Determining the principles that govern dimerization and DNA recognition is important, from a biological standpoint, for predicting and identifying dimerization partners for various bZIP family members and their cognate DNA control elements. From the standpoint of protein engineering, biophysical characterization of bZIP proteins is important because these structures are candidates for engineered peptides that may be introduced into cells to regulate gene expression. The ability to design zippers with specific dimerization and DNA binding properties may one day be of therapeutic use.

(iii) Little is known about the structure of the transcription factor activation domains and how these domains regulate gene expression. Are there conserved

activation domain motifs as there are conserved DNA binding motifs? The existence of activation domain motifs is suggested by patterns identified in primary sequences such as glutamine-rich regions, acidic activation domains and proline-rich regions. These recurring patterns are not as strictly conserved as the DNA binding domains and have not yet been shown to represent any conserved structure. The existence of motifs remains one of the most interesting questions in activation domain structure. CREB contains two glutamine-rich regions, and a detailed structural analysis of CREB may help identify essential properties of this motif as well as identify specific regions of the protein worth targeting for further functional analysis.

(iv) CREB provides an excellent model for studying the mechanism of activation by phosphorylation. Phosphorylation on residue Ser133 by PKA or other kinases switches CREB from a weak to a strong transcriptional activator. Some of the models that have been proposed for this activation include phosphorylation-induced conformational changes in CREB (Gonzalez, *et al.*, 1991; Meyer and Habener, 1993), increased DNA-binding affinity (Nichols, *et al.*, 1992), or creation of a specific protein binding site (Chrivia, *et al.*, 1993).

The first step in the structural analysis of any protein is to develop a scheme to obtain milligram quantities of highly pure protein. Protocols were developed for overexpressing and purifying three CREB constructs using a bacterial system.

The proteins used for this work are: (i) CREB341, the full-length, wild-type protein; (ii) ACT265, the N-terminal activation domain of CREB341 missing only the bZIP domain; and (iii) CREB/SER, the full-length CREB341 in which the three cysteines in the bZIP domain have been replaced with serine residues.

The characterization of the structural and biochemical properties of CREB began with circular dichroism (CD) analysis. This technique was chosen to answer the following questions: (i) Can the apparent heat stability of CREB be confirmed by physical techniques? (ii) What is the secondary structure content of CREB? (iii) Does PKA phosphorylation change the secondary structure content of CREB? CD was also used to confirm that the Cys to Ser mutations in CREB/SER did not change the protein structure.

A number of previous studies used CREB purified by boiling for studying DNA binding (Hoeffler, *et al.*, 1991; de Groot, *et al.*, 1994). Boiled CREB maintains its ability to bind DNA as measured by EMSA. Retention of DNA binding activity is strong evidence that the bZIP domain is structurally intact, however, this assay does not address the effects of boiling on the activation domain or on processes such as deamidation that might interfere with structural analysis, particularly crystallography. The results of the CD studies demonstrated that CREB, ACT and CREB/SER are heat stable. No previous studies directly addressed the effects of heating on CREB structure. Since boiling might cause deamidation and no simple assay exists to measure this effect, our heat treatment

was only to 70°C. This temperature was adequate for purification and is less likely to cause deamidation. Although I was unable to purify wild type CREB to a level suitable for CD without using the heat step, I was able to purify both ACT265 and CREB/SER without a heat treatment. The CD spectra of both the heated and non-heated samples of these proteins are the same, demonstrating that the protein refolded properly after heating (Chapter I, Fig. 1). Therefore, a 10 min, 70°C heat treatment is an appropriate step for purifying CREB for structural characterization.

From the CD spectra the secondary structure content of these proteins was calculated using the variable selection method and a basis set of 33 proteins (Compton and Johnson, 1986; Manavalan and Johnson, 1987). The full-length CREB contains 37% random coil, 34% β-turn, 9% β-strand and 20 % α-helix. The presence of β-turn and random coil structures is an emerging theme in the search for activation domain motifs. Small peptides of the activating regions from Gal4 and GCN4 were shown to be either random coil or, under certain conditions, β-rich (their analysis did not actually distinguish between β-strand and β-turn structures) (Leuther, *et al.*, 1993; Van Hoy, *et al.*, 1993). The results presented here represent one of the first structural characterizations performed on a full-length eukaryotic transcription factor where activation domain structure can be studied in the context of the native protein. CREB contains less α-helix and β-sheet than predicted from analysis of the amino acid sequence (Quinn, 1993).

What is the significance of this discrepancy? The DNA-binding domain of CREB and other bZIP proteins exists largely as random coil in the absence of DNA and becomes α-helical upon DNA-binding (O'Neil, *et al.*, 1990; Oas, *et al.*, 1990; Weiss, 1990; Weiss, *et al.*, 1990; O'Neil, *et al.*, 1991; Saudek, *et al.*, 1991; Santiago-Rivera, *et al.*, 1993). Regions in the activation domain may also adopt this "induced structure" model. The random coil regions may have the propensity to form the ordered structures suggested by their primary sequence when bound to an appropriate partner. One future direction is to use CD to study the effects of dimerization and DNA binding on the secondary structure of the full-length protein and to study the structural effects of CBP binding on the activation domain. An alternative explanation for the significant presence of random coil regions is that they may represent a functional motif. One proposed role for random coil structures is that they can increase the rate of association between macromolecules by increasing the productivity of the encounter complex (reviewed by (Pontius, 1993)). That is, they can increase the association rate of protein:protein or protein:nucleic acid interactions. The secondary structure analysis of CREB suggests that both ordered secondary structures (primarily β-structures) and more flexible random coil regions are important for activation domain structure. The next step will be to assign these structures to the different regions of CREB.

The CD study also reveals that the activation domain loses some structure when the bZIP domain is removed (Chapter I, Table I). The significance of this finding is two-fold. First, the biological activity of many activation domains, including that of CREB, is often characterized by domain swap experiments in which all or part of the activation domain is fused to a heterologous DNA-binding domain (Ransone, *et al.*, 1990). Although activation domains retain much of their activity in such cases, some critical function may be lost. For example, if the region immediately adjacent to the bZIP (in CREB, the Q2 domain) depends on the bZIP for proper structure, its function may be altered in domain swap experiments. A second reason why the loss of structure in ACT265 is important is that phosphorylated-ACT maintains its ability to bind to CBP (Chrivia, *et al.*, 1993). Either structure is not lost in the regions required for CBP binding or there are few structural requirements for initiating this binding interaction. This explanation would agree with the induced-structure upon protein binding model.

Using CD, no changes in secondary structure were detected either upon phosphorylation of CREB or ACT. Because ACT is a smaller protein than CREB, even small changes in secondary structure, those affecting 5-10 % of the residues, should be detectable in the ACT peptide. We can therefore rule out at least some versions of the conformational change upon PKA phosphorylation models. In particular, PKA phosphorylation is unlikely to induce α-helix or β-sheet structures in regions adjacent to Ser133 (Meyer and Habener, 1993).

The second set of studies performed towards characterizing the biophysical and structural properties of CREB was a quantitative analysis of DNA binding using a fluorescence anisotropy assay. The purpose of initiating this study was two-fold. First, we wanted to develop a way to measure and compare the binding properties of CREB for different DNA sequences. A quantitative analysis of DNA binding *in vitro* will help elucidate the contribution of different CRE sites that occur *in vivo* and better define the contributions toward specificity of the different basepairs in the CRE. A quantitative analysis will also aid future efforts of CREB:DNA crystallization by defining the optimal DNA sites for subsequent crystallization experiments. The second reason for a quantitative analysis of DNA binding is to compare the DNA-binding affinities of CREB and P-CREB for different CRE sequences. One model for phosphorylation-induced activation proposes that phosphorylation increases DNA binding, at least to certain "low-affinity" binding sites (Nichols, *et al.*, 1992). We demonstrated that, in addition to not being "low-affinity" sites, phosphorylation did not affect binding to any of the CREs tested. Therefore, the mechanism of CREB activation by phosphorylation is unlikely to be by increasing the DNA binding affinity.

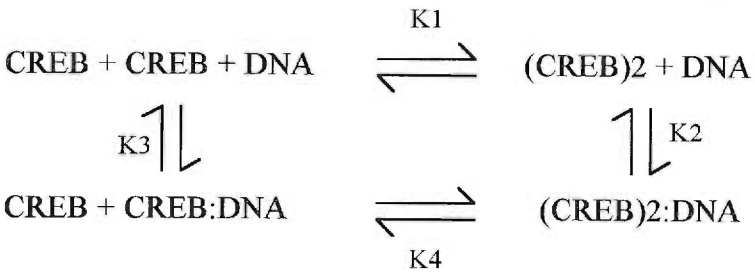
Relevant to our fluorescence anisotropy binding studies is a recent report by Anderson and Dynan (Anderson and Dynan, 1994) which reported the Kd of CREB for the HTLV-CRE, a non-canonical class CRE, using EMSA. In that

work the authors analyzed their results with a two-step binding model assuming the pathway

$$\text{CREB} + \text{CREB} + \text{DNA} \overset{Kd_1}{\leftrightarrow} (\text{CREB})_2 + \text{DNA} \overset{Kd_2}{\leftrightarrow} (\text{CREB})_2\text{:DNA}.$$

This model assumes that the $\text{CREB}_{\text{monomer}}$:DNA complex does not contribute to binding. The reported equilibrium dissociation constants were Kd_1 (CREB dimerization) = 7 nM and Kd_2 (DNA binding) = 60 nM. Although the authors may be correct in looking toward cooperative models for CREB:DNA binding, there are several shortcomings to these results. First, although they attempted a quantitative binding analysis, each binding curve represents less than ten data points (approximately 6) and the range of protein titration encompasses only about two orders of magnitude. Second, there is a high degree of variability in their results making it difficult to distinguish between simple and cooperative binding. Third, their modeled values for the Kd of CREB dimerization are at odds with those previously reported for CREB-like bZIP domains. The CREB bZIP domain has a reported Kd ~ 20 μM (Santiago-Rivera, *et al.*, 1993). The full-length yeast GCN4 protein, which has a bZIP domain homologous to that of CREB, has a reported equilibrium dissociation constant for dimerization of Kd = 12 μM and the C/EBP bZIP domain has a Kd = 17 μM (O'Neil, *et al.*, 1991). These findings underscore a major paradox of bZIP protein:DNA interactions. DNA binding by bZIP proteins is generally assumed to require dimerization. Yet, the Kd of

dimerization is on the order of 10 μM (for bZIP domains with only 4 heptad repeat leucine zippers) and DNA binding is readily observed at nanomolar concentrations of protein where virtually no dimer exists. Cooperative DNA binding could explain these results. We are unable to distinguish between a simple and cooperative binding model at this point with our data. However, we are working on establishing binding conditions at lower DNA concentrations to increase the sensitivity of our fluorescence anisotropy binding assay at low protein concentrations, and we are designing mathematical fitting models that can explore all possible binding pathways. We are currently trying to investigate the potential for a $(CREB)_{monomer}$:DNA interaction and measure the Kd of full-length CREB dimerization. Our aim will be to determine all of the equilibrium dissociation constants in the following binding scheme and determine the probable pathway of CREB:DNA association.



As a follow-up to characterization of CREB by CD, analytical ultracentrifugation studies were performed on CREB and P-CREB. The goal of the sedimentation analysis was to determine whether there was a tertiary

conformational change in CREB following PKA phosphorylation which was therefore by CD analysis. These studies also allow characterization of the hydrodynamic or shape properties of CREB. Wild-type CREB was not appropriate for the analytical ultracentrifugation studies. A significant percentage of protein sedimented at speeds as low as 10,000 rpm, indicating the presence of aggregation. Although some of this aggregation was reduced by addition of 5 mM DTT and 150 mM NaCl, we were still unable to perform sedimentation analysis. In order to circumvent the aggregation problem, I designed a mutant in which the three cysteines in the DNA binding region were replaced with serines. Santiago-Rivera, *et al.* had already demonstrated that modification of these cysteines reduced, but did not eliminate aggregation of the CREB bZIP domain (Santiago-Rivera, *et al.*, 1993). The CREB/SER mutant was structurally and biochemically intact as shown by CD analysis, gel shift and fluorescence anisotropy. Sedimentation analysis demonstrated that these substitutions were highly effective in reducing aggregation. We were able to perform both velocity and equilibrium sedimentation analysis.

Our first results from velocity sedimentation showed that both CREB/SER and P-CREB/SER sedimented indistinguishably with $s^{0}_{20,w} = 2.4\ S$. Therefore, PKA phosphorylation does not change the overall tertiary structure of CREB, and a conformational change in CREB following PKA phosphorylation is unlikely. One possibility that remains to be investigated is whether there is a conformational

change in the CREB:DNA complex following phosphorylation. This possibility will be investigated by CD and analytical sedimentation.

Estimation of the hydrodynamic properties from the $s^{0}_{20,w}$ values suggested that CREB was monomeric under our conditions (10 uM protein in 25 mM Tris/HCl, pH 7.6, 100 mM NaCl). The presence of monomer was confirmed by equilibrium sedimentation analysis. At higher protein concentrations (30 μM) we observed both monomer and a large degree of higher order species (> dimer) that interfered with analysis. Coincident with these studies we observed that DNA binding activity was salt dependent and was optimal in 25 mM Tris/HCl buffer, pH 8.0, containing 200 mM KCl. Under these conditions, CREB is dimeric at 10 uM. Therefore the association state of CREB is highly sensitive to ionic strength. We are currently continuing sedimentation analysis under these new conditions to study the dimerization properties of CREB and determine the effects of DNA binding on CREB and P-CREB tertiary structure.

CONCLUSIONS

Although there is clearly much to be done toward a complete structural analysis of CREB, at this stage, a few general conclusions can be drawn:

1. PKA phosphorylation does not change the secondary structure, protease cleavage pattern or tertiary structure of CREB. Therefore, a phosphorylation-induced conformational change is unlikely to be the mechanism of activation.

2. PKA phosphorylation does not change the binding affinity of CREB for DNA, and this, too, is an unlikely mechanism of activation by phosphorylation.

3. PKA phosphorylation does create a specific binding site for CBP and possibly other proteins (Chrivia, *et al.*, 1993; Kwok, *et al.*, 1994). The role of PKA phosphorylation in CREB activation may be to provide the proper stereochemical scaffold for coactivator binding.

4. β-turn and random coil structures are prominent features of full-length CREB and may represent recurring structural motifs in transcription factor activation domains.

5. The improved solubility of CREB/SER makes this protein a promising candidate for X-ray crystallography.

CREB has been a challenging, and therefore intriguing, protein to characterize structurally. A few of the unusual properties of CREB warrant special mention. (i) CREB can be boiled with little apparent effect on structure or

function. The heat stability and secondary structure analysis suggest a highly flexible protein. (ii) A basis set of 33 proteins (the largest currently available) was required to analyze the CREB CD spectra. Therefore, CREB is *unlike* other proteins of known structure. (iii) Sedimentation reveals that both monomeric and dimeric CREB deviate significantly from an idealized sphere and have axial ratios exceeding 1:10. Such an elongated molecule requires strict folding. The paradox is that CREB appears simultaneously to be both highly structured and highly unstructured.

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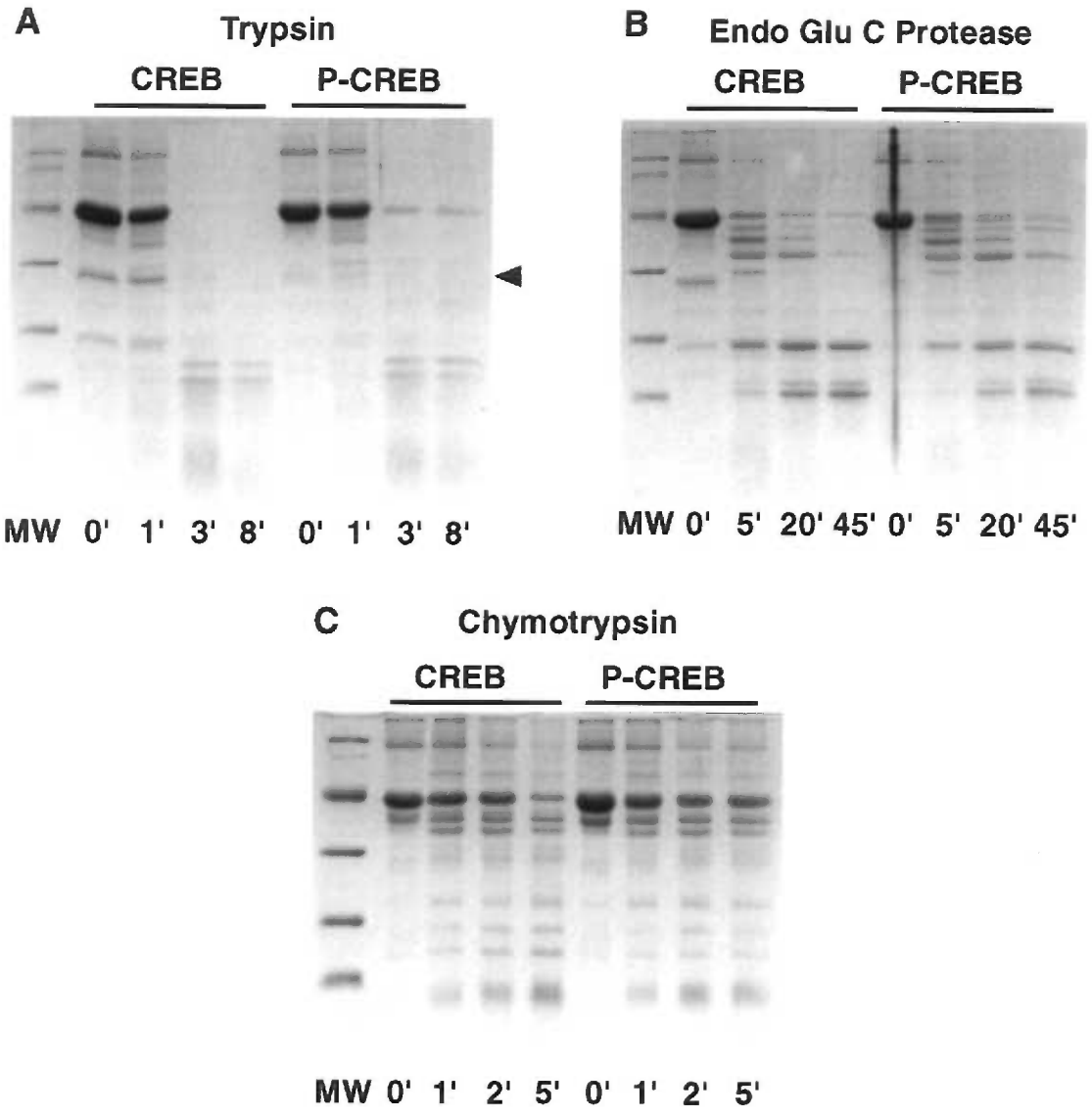
APPENDIX I

FIGURE 1. Protease digest of CREB and P-CREB. Approximately 10 ug of CREB and P-CREB in 10 mM PO_4 buffer, pH 7.5, were digested with 100 ng of either A. trypsin; B. chymotrypsin; or C. Endo Glu C protease. The samples were incubated for the times indicated and the reactions were stopped by the addition of SDS-PAGE loading buffer and boiling for 5 min. The samples were immediately separated by 15% polyacrylamide gel electrophoresis in a Hoeffer 15 cm gel apparatus.

No clear differences in the cleavage pattern are detectable between CREB and P-CREB. We did notice a reproducible increase in intensity of one band in the trypsin digest that is more pronounced in CREB than in P-CREB (located slightly above the arrow). This difference is difficult to visualize because of the faintness of the bands and the presence of a contaminating species in the CREB sample. There are trypsin cleavage sites two and three amino acids away from the phospho-serine and it is possible that the phosphate group interferes with the kinetics of cleavage. Contrary to the interpretation of a similar digest in a previous report (Gonzalez, *et al.*, 1991), we do not think this assays supports the model of a conformational change in CREB following PKA phosphorylation.


A
Trypsin
CREB
P-CREB
MW 0' 1' 3' 8' 0' 1' 3' 8'
B
Endo Glu C Protease
CREB
P-CREB
MW 0' 5' 20' 45' 0' 5' 20' 45'
C
Chymotrypsin
CREB
P-CREB
MW 0' 1' 2' 5' 0' 1' 2' 5'

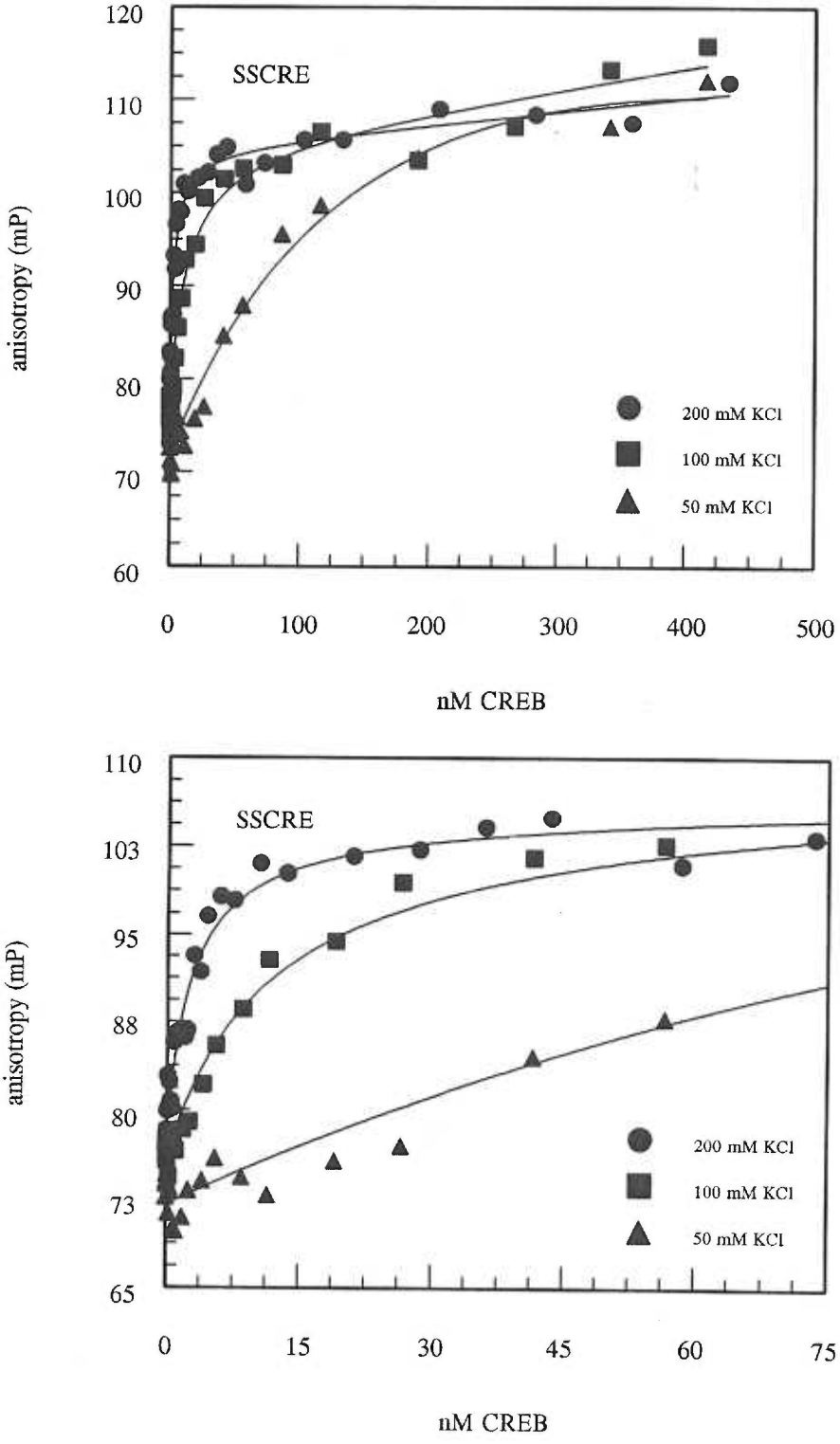
APPENDIX II

TABLE I
Dissociation constants of CREB and P-CREB
in 25 mM TRIS/HCl, pH 8.0.

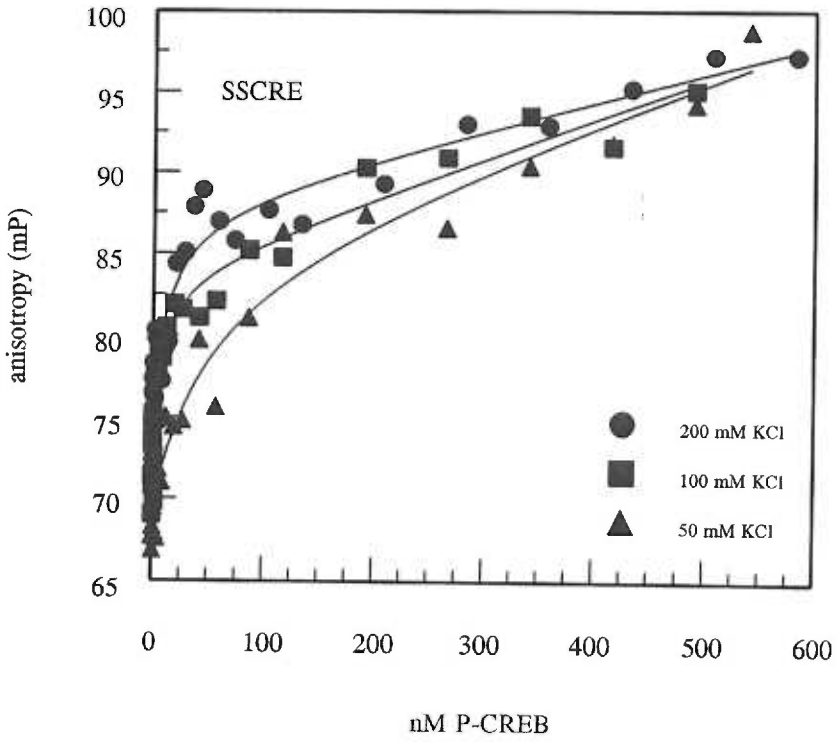
Protein	ionic strength	Kd (nM)
CREB	50 mM KCl	~ 200
	100 mM KCl	11 +/- 2
	200 mM KCl	3 +/- 1
P-CREB*	50 mM KCl	47 +/- 30
	100 mM KCl	7 +/- 4
	200 mM KCl	14 +/- 6

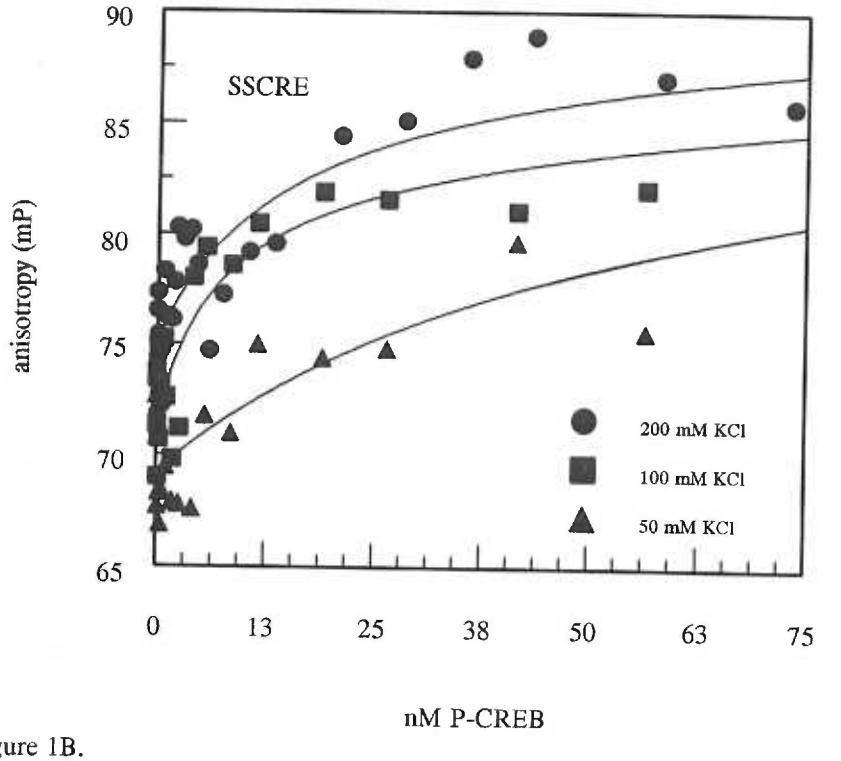
* Amount of error in calculated Kd reflects the poorer fit of the data to the current binding model.

FIGURE 1. Salt dependence of CREB and P-CREB on DNA binding. Fluorescence anisotropy DNA binding curves performed in 25 mM fluorescent grade Tris/HCl buffer, pH 8.0 containing the indicated amount of KCl are shown for A. CREB and B. P-CREB bound to the SSCRE. The anisotropy values represent the non-normalized data given in milli-polarization units (mP). The calculated Kd values are shown in Table I. Binding reactions were performed as described in Chapter II. Both the full-scale and expanded-scale views are shown.



APPENDIX II. Figure 1A.





APPENDIX II. Figure 1B.