tential catalytic clones such as that proposed here as well as novel approaches involving recombinant DNA technologies (Ward et al., 1989; Huse et al., 1989) will all contribute to achieving this goal.

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Effects of Cyclic GMP on the Secondary Structure of Cyclic GMP Dependent Protein Kinase and Analysis of the Enzyme's Amino-Terminal Domain by Far-Ultraviolet Circular Dichroism†

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ABSTRACT: Far-UV circular dichroism spectra of bovine lung cyclic GMP dependent protein kinase (G-kinase) show that the enzyme contains α-helical and β-pleated sheet elements. Binding of cyclic GMP changes the spectra in a way consistent with the induction of β-sheet from random coil. Examination of the amino-terminal sequence of G-kinase indicates the presence of a strongly α-helical segment with several features in common with the leucine zipper motif. We propose that this sequence may be the important part of the dimerization domain of the enzyme. A synthetic peptide corresponding to amino acids 1–39 of G-kinase has a strongly α-helical CD spectrum, supporting the predicted secondary structure of this amino-terminal sequence. In contrast to the native enzyme, a structure reduced in α-helix was found when a constitutively active form of G-kinase, which lacks amino acids 1–77, was studied.

It has been shown that cyclic GMP dependent protein kinase (G-kinase)[1] is one of the major receptor proteins for cyclic GMP [Lincoln and Corbin (1983) and Edelman et al. (1987), reviews]. In most tissues, the levels of G-kinase are relatively low compared with those of cyclic AMP dependent protein

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[1] Abbreviations: G-kinase, cyclic GMP dependent protein kinase; A-kinase, cyclic AMP dependent protein kinase; CD, circular dichroism; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; CAP, catabolite activator protein.

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kinase (A-kinase), but high concentrations are found, for example, in cerebellum (Hofmann & Sold, 1972; Lohmann et al., 1981) and smooth muscle (Kuo, 1974; Ecker et al., 1989). In the latter case a physiological role of the enzyme has been implicated in mediating the relaxant effects of agents which elevate intracellular cyclic GMP levels (Fiscus et al., 1984). Recent studies have shown that in aortic and tracheal smooth muscle G-kinase exists in two different isoforms designated types Iα and Iβ (Lincoln et al., 1988; Wolfe et al., 1989; Wernet et al., 1989). The protein sequences of types Iα and Iβ deduced from cDNAs isolated from bovine tracheal smooth muscle showed that type Iα is identical with the protein sequence of the well-characterized bovine lung G-kinase, whereas the type Iβ isoform differed in its amino-terminal region (Wernet et al., 1989). Both isoforms of G-kinase consist of two identical subunits (Wolfe et al., 1989) and are activated by the binding of 4 mol of cyclic GMP/mol of holoenzyme (Mackenzie, 1982; Corbin & Døskeland, 1983; Wolfe et al., 1987; Landgraf & Hofmann, 1989). From the subunit primary sequence six segments have been derived which correspond to four functional domains (Takio et al., 1984; Heil et al., 1987). The first, amino-terminal segment contains a dimerization region, autophosphorylation sites, and a hinge portion (Monken & Gill, 1980; Aitken et al., 1984; Takio et al., 1984). Subsequent in the primary sequence are the two cyclic GMP binding sites and then three segments involved in substrate-binding and catalytic activity (Takio et al., 1984).

The cyclic GMP binding sites of G-kinase show considerable homology with the cyclic AMP binding sites of A-kinase (Takio et al., 1984). By comparing the primary sequences of these two enzymes with that of the bacterial catabolite gene activator protein (CAP), Weber et al. (1989) have recently predicted a very large similarity between the tertiary structures of the cyclic nucleotide binding domains of the two protein kinases. According to their model, the cyclic GMP binding domain A of G-kinase, which corresponds to the higher affinity binding site, is a largely β-pleated structure with contributions from at least three α-helices and eight strands of β-sheet.

The mechanism by which cyclic nucleotides activate protein kinases is only partially understood. Binding of cyclic AMP to the type II regulatory subunit of A-kinase caused small changes in its observed CD spectrum (Johnson & Wong, 1989), indicating that a conformational change occurred in the regulatory subunit. In addition to the cyclic nucleotide binding sites, the *N*-terminal segment of G-kinase has been implicated in the control of enzyme activity for the following reasons. (a) This region contains the major autophosphorylation sites (Aitken et al., 1984), and autophosphorylation increases the rate of cyclic GMP dissociation from the high-affinity binding site (Hofmann et al., 1985) and increases the affinity of cyclic AMP and other cyclic nucleotide analogues for the high-affinity cyclic GMP binding site (Landgraf et al., 1986). (b) Experiments in which arginine residues of G-kinase were modified by 2,3-butanedione led to the suggestion that an N-terminal portion of the enzyme inhibited its activity in the absence of cyclic GMP (Lincoln et al., 1978). (c) A proteolyzed form of the enzyme, lacking the first 77 amino acids,[2] is constitutively active in the absence of cyclic GMP (Heil et al., 1987). (d) The allosteric effects of MgATP, the binding of cyclic GMP and the effects of high NaCl concentrations on G-kinase are all dependent on the presence of the N-terminal segment (Landgraf & Hofmann, 1989).

To analyze the secondary structure of G-kinase, and the effect of cyclic GMP binding on this structure, we have performed CD on the purified holoenzyme. In addition, to investigate the contribution of the N-terminal segment to the secondary structure of G-kinase, we have synthesized a peptide corresponding to amino acids 1–39 of the holoenzyme and investigated its secondary structure. This peptide contains a primary structure motif similar to the leucine zipper recently described as part of a DNA-binding motif in fos, jun, and CREB [see, e.g., Landschulz et al. (1988) and Kouzarides and Ziff (1989)]. Also, we have obtained the CD spectrum of the proteolyzed form of G-kinase which lacks amino acids 1–77. (This form of the enzyme is henceforth referred to as the "constitutively active G-kinase".)

Experimental Procedures

Enzyme Preparation and Preliminary Analysis. G-Kinase was purified from bovine lung according to Hofmann and Flockerzi (1983) with slight modifications. The enzyme was affinity purified on an 8-(2-hydroxyethyl)thio-cAMP Sepharose 4B column and eluted with 20 μM cyclic GMP. The eluted enzyme was concentrated by Ultrafree centrifugation, applied to an AcA-34 gel filtration column (120 cm × 1 cm), and eluted at room temperature with 5 mM Tes, pH 7.0, containing 0.2 mM EDTA, 5 mM benzamidine, 100 mM NaCl, 0.5 mM dithiothreitol, and 1 μg/mL leupeptin. The fractions containing the 150-kDa G-kinase holoenzyme were pooled and dialyzed overnight at 4 °C against 5 L of 10 mM ammonium acetate, 10 mM sucrose, 0.2 mM EDTA, 0.2 mM dithiothreitol, and 1 μg/mL leupeptin. After dialysis the enzyme was concentrated and stored at −70 °C. The constitutively active form of G-kinase was prepared by limited digestion of the affinity-purified holoenzyme with trypsin (Heil et al., 1987) and further purified as described for the native enzyme. Before spectrophotometric analysis, enzymes were dialyzed at 4 °C overnight against 10 mM sodium phosphate and 1 mM dithiothreitol, pH 7, and then centrifuged across a 0.22-μm, low-protein-binding, cellulose acetate filter using Costar Spin-X centrifuge filter units. The filtrate was assayed for protein by using bovine serum albumin as a standard according to Bradford (1976).

Peptide Synthesis. Cyclic GMP dependent protein kinase$_{1-39}$ amide was synthesized by standard solid-phase synthetic techniques using an automated peptide synthesizer employing Boc/Bzl chemistry (Merrifield, 1963; Stewart & Young, 1984) and *p*-methylbenzhydrylamine–resin (0.49 mmol/g of resin). The peptide was deprotected and removed from the resin with the two-step "low–high" HF method (Tam et al., 1983) and purified by gel filtration, cation-exchange chromatography, and preparative C_{18} reverse-phse HPLC. Integration of the analytical HPLC chromatogram (λ = 214 nm) indicated that purity was in excess of 97%. The peptide gave the correct amino acid sequence, as shown by N-terminal amino acid microsequence analysis (automated Edman degradation), on a gas-phase sequencer with TFA/acetonitrile conversion of the amino acids into their PTH derivative. Amino acid analysis after acid hydrolysis gave the expected molar ratios (±8.0%) of the constituent amino acids; the molecular mass by FAB-MS $[MH]^+$ was 4831.5, compared to a calculated value of 4831.4. Prior to CD, the peptide was lyophilized and redissolved in 10 mM sodium phosphate and 1 mM dithiothreitol, pH 7.

Circular Dichroism. CD studies were performed with an Aviv Model 62D spectropolarimeter. Spectra were recorded

[2] Throughout this paper amino acid residues are numbered by ignoring the amino-terminal methionine corresponding to the start codon in cDNA sequences (Wernet et al., 1989; Sandberg et al., 1989). This is in accordance with protein sequencing results (Takio et al., 1984).

Table I: Proportions of α-Helix (fA), β-Pleated Sheet (fB), β-Turns (fT), and Random Coil (fR) in Native, Dimeric Holoenzyme, in the Constitutively Active Form of G-Kinase ("Monomeric"), and in the Peptide Representing Residues 1–39 of G-Kinase Iα Isotype[a]

enzyme	cyclic GMP	temp (°C)	fA	fB	fT	fR
holoenzyme, preparation 1	−	4	0.349	0.211	0.105	0.335
	+	4	0.343	0.328	0.066	0.263
	−	30	0.322	0.262	0.111	0.305
	+	30	0.346	0.292	0.090	0.272
holoenzyme, preparation 2	−	4	0.323	0.318	0.086	0.273
	+	4	0.235	0.583	0.000	0.182
	−	30	0.291	0.402	0.066	0.241
	+	30	0.219	0.614	0.000	0.167
monomeric preparation 1	−	4	0.320	0.327	0.100	0.253
	−	30	0.269	0.444	0.065	0.222
monomeric preparation 2	−	4	0.223	0.609	0.000	0.168
	+	4	0.224	0.594	0.000	0.182
	−	30	0.209	0.605	0.000	0.186
	+	30	0.212	0.594	0.008	0.186
peptide 1–39	−	4	0.802	0.000	0.000	0.198
	−	30	0.750	0.000	0.000	0.250

[a] Measurements were obtained in the absence or presence of a 20-fold molar excess of cyclic GMP, and at 4 or 30 °C, as indicated.

by using enzyme or peptide at 1–1.5 mg/mL, a 1.5-nm bandwidth, 0.5-nm step size, and a time constant of 4 s. Water-jacketed, dichroically neutral, quartz cuvettes, whose path lengths were measured by using an infrared spectrometer (Bree & Lyons, 1956), were used for all measurements. The instrument was calibrated by using (+)-10-camphorsulfonic acid (Tuzimura et al., 1977). A total of 5 scans were averaged for both sample and buffer. After correction of the sample spectrum for solvent contributions, nonlinear regression analysis was used to fit the data.

Secondary Structure Estimation. Estimation of the secondary structure from the CD data, expressed as mean residual ellipticity, was made by using the program PROSEC (*pro*tein *se*conday structure estimator, v2.1, Aviv Associates, Lakewood, NJ) based on the method of Chang et al. (1978). This method uses reference spectra deduced from 15 proteins, the structure of which is known from X-ray crystallography. The CD spectra was calculated from deduced secondary structure using the program BLEND (Aviv Associates, Lakewood, NJ). Chou–Fasman analyses (Chou & Fasman, 1977) were performed by using the program MSEQ (MSEQ, v1.17, University of Michigan Software, Ann Arbor, MI). The amino-terminal α-helix was displayed as a two-dimensional helix representation [modified from Schiffer and Edmundson (1967)].

Results

Characterization of the CD Spectra. The far-UV CD spectrum (190–250 nm) of native G-kinase holoenzyme at 4 °C in the absence of added cyclic GMP is shown in Figure 1. The spectrum has considerable α-helix character (Chen et al. 1972) with a CD maximum at 194 nm and a double CD minimum at 209 and 224 nm. However, the intensity of mean residual molar ellipticity at 194 nm is less than expected for a pure α-helical structure, and the maximum and minima are somewhat broader, indicating the presence of other secondary structural features. The calculated CD spectrum derived from the PROSEC analysis of the experimental data is also shown in Figure 1 and agrees reasonably well with the experimentally derived curve. The computer-assigned secondary structure for the enzyme is given in Table I and is divided approximately equally between α-helix, β-pleated sheet, and turns and random coil. It is difficult to determine β-sheet from CD spectra because of the low intensity of the signal given by this structure relative, especially, to α-helix (Chen et al., 1972). Thus the proportions of β-pleated sheet reported in Table I are more varied than for the other stuctures and should be treated

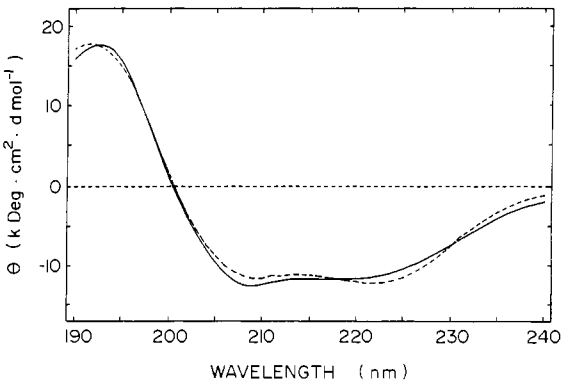

FIGURE 1: Far-UV CD spectrum of G-kinase as determined experimentally (solid line) in the absence of cyclic GMP and at 4 °C, and as calculated from the PROSEC-derived secondary structure determination (dashed line).

cautiously when comparing these values with results from CD specta of other proteins where these have been analyzed by different methods.

Chou–Fasman Analysis. Analysis of the primary sequence of G-kinase (Takio et al., 1984) by the method of Chou and Fasman (1977) gave the results indicated in Figure 2. The overall proportions of secondary structure indicated for this enzyme were α-helix 31%, β-pleated sheet 20%, β-turn 36%, and random coil 13%. These figures were corrected where three or less consecutive amino acids were designated α-helix by the MSEQ program. When such residues were designated to the next most probable structure, the secondary structure proportions became α-helix 24%, β-pleated sheet 25%, β-turn 36%, and random coil 15%. Comparision with Table I indicates that the predicted proportions of α-helix and β-sheet are approximately in accord with the experimental results. However, the proportion of turns is very much larger according to the Chou–Fasman calculations than from the PROSEC-derived data. This discrepancy might be due to two possibilities. First, the method used to interpret the CD spectra is known to give variable accuracies depending on the protein involved (Chang et al., 1978), and in particular the spectrum used to derive random coil is only an average spectrum for the different forms of coil that may exist in solution. A second possibility, which may be relevant in this case, is that the β-turn probability is greatly overestimated by the Chou–Fasman calculations. In the G-kinase sequence, long strings of turn structure are predicted, in many cases not bordered by strands of β-sheet

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SELEEDFAKILMLKEERIKELEKRLSEKEEEIQELKRKLH40
α ——→ βα ——————————————————→ rt
KCQSVLPVPSTHIGPRTTRAQGISAEPQTYRSFHDLRQAF80
→ ββrrt →rrt →arβrrarart →rrα ——→ β
RKFTKSERSKDLIKEAILDNDFMKNLELSQIQEIVDCMYP120
βaraat ——→α →βt →α →ββααβαββrt →
VEYGKDSCIIKEGDVGSLVYVMEDGKVEVTKEGVKLCTMG160
βrrt ——→arrrt →ββaaat →βaat →ββββrt →
PGKVFGELAILYNCTRTATVKTLVNVKLWAIDRQCFQTIM200
→α →β →rrβrrβαβ ——→ αβaaarβ →αα
MRTGLIKHTEYMEFLKSVPTFQSLPEEILSKLADVLEETH240
rt →βara ——→βrrβ →rrα →βα ——→rrr
YENGEYIIRQGARGDTFFIISKGKVNVTREDSPNEDPVFL280
t →ββt ——→βββrt →ββaat ——→β
RTLGKGDWFGEKALQGEDVRTANVIAAEAVTCLVIDRDSF320
rrrt →α →rraββrββββα →β →rt →
KHLIGGLDDVSNKAYEDAEAKAKYEAEAAFFANLKLSDFN360
•ββββrrart ——→α ——————→βα →rat →
IIDTLGVGGFGRVELVQLKSEESKTFANKILKKRHIVDTR400
ββt ——→αβαββα →rα ——→β →t →
QQEHIRSEKQIMQGAHSDFIVRLYRTFKDSKYLYMLMEAC440
▸aara →ββart ——→β →at →ββα →rt
LGGELWTILRDRGSFEDSTTRFYTACVVEAFAYLHSKGII480
→αβ →at →art →β →α →βββat →β
YRDLKPENLILDHRGYAKLVDFGFAKKIGFGKKTWTFCGT520
βarat →βat →aaββrβaaarββt →β →rrt
PEYVAPEIILNKGHDISADYWSLGILMYELLTGSPPFSGP560
→α →ββrt →aat →β →α →βt ——→
DPMKTYNIILRGIDMIEFPKKIAKNAANLIKKLCRDNPSE600
→βrβ →rrβα →t →aaaraββaaarrt ——→
RLGNLKNGVKDIQKHKWFEGFNWEGLRKGTLTPPIIPSVA640
•rrrt →βα ——→rt →arrt →rrβ →r →
SPTDTSNFDSFPEDNDEPPPDDNSGWDIDF 670
t ——————————→α
```

FIGURE 2: Secondary structure predicted for bovine G-kinase Iα [sequence from Takio et al. (1984) and Wernet et al. (1989)]. α, β, t, and r represent α-helix, β-sheet, β-turn, and random coil, respectively. Where a string of four or more amino acids was predicted to have the same secondary structure, this is represented by an arrow.

(Figure 2). For example, 26 consecutive residues (641–666) virtually at the carboxy terminus of G-kinase were designated as forming a turn structure.

Effect of Cyclic GMP. It has been suggested that the inactive, native G-kinase is activated by binding of cyclic GMP to its binding sites, thereby inducing a conformational change of the enzyme which removes the inhibitory sequence of the amino-terminal domain from the catalytic center. Therefore, the CD spectra of the native G-kinase holoenzyme in the absence or presence of a 20-fold molar excess of cyclic GMP were determined.

In every case, cyclic GMP induced a reduction of the small peak at ~214 nm between the major troughs at 209 and 224 nm (Figure 3A). In addition, the width of the 194-nm peak was enlarged. Other differences between spectra were not reproducible between experiments. Although it is always difficult to interpret such changes in structural terms, the spectrum for β-sheet has a positive peak at ~197 nm and a trough of negative ellipticity at 213–214 nm. Hence the effects on the CD spectra observed as a result of cyclic GMP addition might be explained by an increase in the proportion of β-pleated sheet within the enzyme upon nucleotide binding. The effects of cyclic GMP concluded by PROSEC analysis are given in Table I. Regardless of the enzyme preparation, cyclic GMP caused an increase in β-pleated sheet within the enzyme, largely at the expense of random coil. A reduction in the proportion of β-turns was also observed, to the extent that, in one enzyme preparation, CD owing to β-turns could no longer be detected in the presence of cyclic GMP. Qualitatively the same effect of cyclic GMP was observed when measurements were performed at 4 °C, where the affinity of the enzyme for cyclic GMP is greater (Landgraf & Hofmann, 1989), or at 30 °C. This is not surprising as the enzyme is presumably saturated with cyclic GMP at both temperatures.

Analysis of the Amino-Terminal Segment. Chou–Fasman analysis of the N-terminal residues of G-kinase type Iα in-

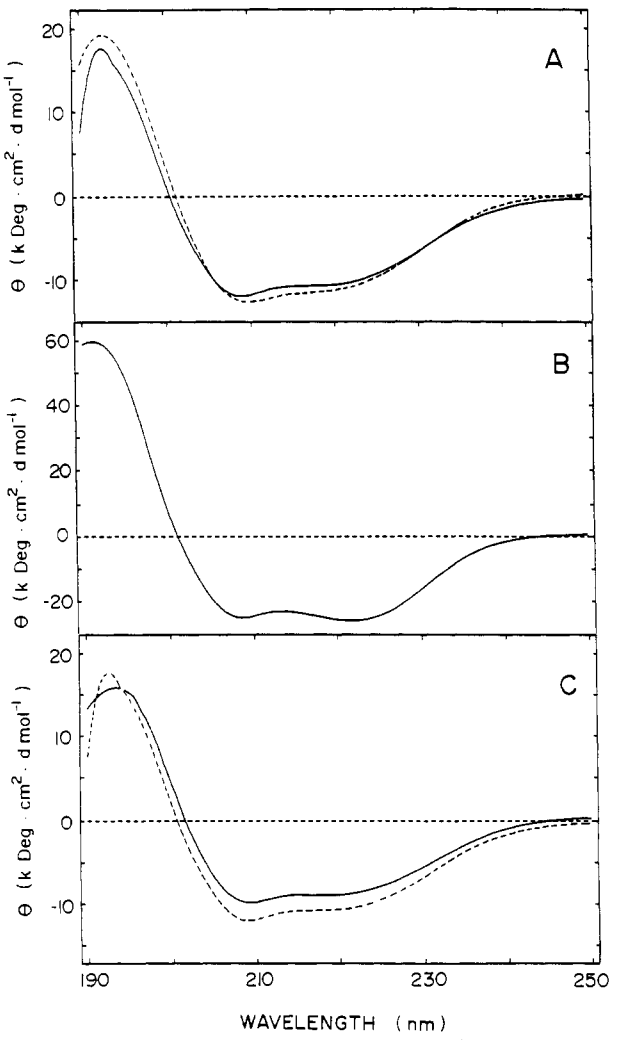
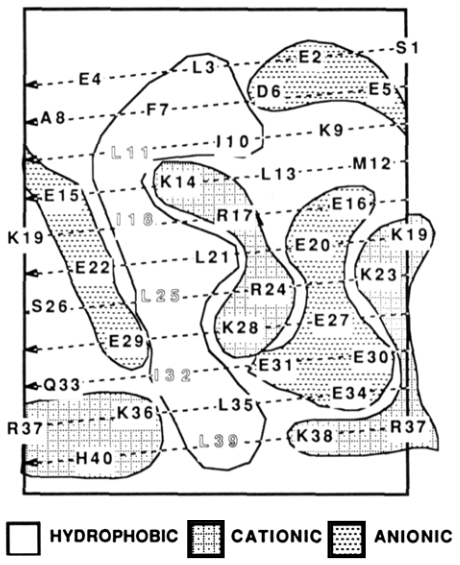


FIGURE 3: Far-UV CD spectra measured at 30 °C for the following: (A) G-kinase in the absence (solid line) and presence (dashed line) of 0.32 mM cyclic GMP; (B) the synthetic peptide representing amino acid residues 1–39 of G-kinase in the absence of cyclic GMP; (C) G-kinase (solid line) or constitutively active G-kinase (dashed line), both determined in the absence of cyclic GMP.

dicated that, with the exception Ile^{10}, the first 38 amino acids are predicted to form exclusively α-helix (see Figure 2). In addition, this sequence would be expected to form a very ordered structure as observed in Figure 4a. This shows the first 40 residues of G-kinase Iα in a two-dimensional helix representation, in which the residues are aligned in appropriate positions on the surface of a hypothetical α-helix. It can be seen that the helix is predicted to have "stripes" of charge or hydrophobicity running down its length. We also noticed that every seventh residue from number 11 to number 53 is a leucine or isoleucine residue (Figure 4b). These features have much in common with the leucine zipper DNA-binding motif and suggest that this region of the enzyme forms the dimerization region of the polypeptide chain (see Discussion). For this reason, and also because the N-terminal segment is known to be very important in regulating G-kinase activity (see the introduction and Discussion), the peptide representing residues 1–39 was synthesized and its structure examined by CD.

The observed far-UV spectrum for the peptide is shown in Figure 3B, and the calculated secondary structure is given in Table I. As predicted, the CD spectrum indicates that the peptide is predominantly α-helical with an intense peak at ~191 nm and two troughs at ~209 and ~222 nm. Secondary structure analysis showed that the peptide was 75–80% α-helical, depending on temperature, the remaining structure

a).



b).

Iα	SELEEDFAKILMLKEERIKELEK
Iβ	GTLRDLQYALQEKIEELRQRDALIDELELELDQKDE
Iα	RLSEKEEEIQELKRKLHKCQSVLPVPSTHIGPRT
Iβ	LIQKLQNELDKYRSVIRPATQQAQKQSASTLQGE
Iα	T*RAQ61
Iβ	PRTKRQ76

FIGURE 4: (a) 2D helix representation of the first 40 amino acids in G-kinase Iα. Areas of hydrophobicity and anionic and cationic charge are indicated. The residues involved in the heptad repeat are shown with hollow characters. (b) An alignment of the leucine/isoleucine heptad repeat in G-kinase Iα and Iβ amino acid sequences. The leucine or isoleucine residues involved in the heptad repeat are underlined. The asterix indicates the major autophosphorylation site in the Iα isotype.

being random. These results indicate that an α-helical N-terminal domain might exist within G-kinase.

Analysis of Constitutively Active G-Kinase. The constitutively active enzyme lacks the proposed α-helix at the N-terminus, and so CD of this protein might determine whether this helix exists in the intact enzyme. However, the 38 residues of the putative helix form only 5% of the total residues per subunit, and hence removal of this segment will clearly be close to the limit of detection by CD. On the other hand, α-helix gives a strong signal, and so α-helical contents obtained from CD studies are relatively dependable. The results of CD analysis of constitutively active G-kinase are shown in Figure 3C and Table I. The spectrum had a similar overall shape to that of the holoenzyme but had a reduced elliptical intensity at the 194-nm peak and the troughs at 209 and 224 nm. This seems to indicate that there is a reduction in the α-helical content of the constitutively active form compared to the holoenzyme. This qualitative conclusion is supported by the PROSEC analysis for the enzyme, which indicates a small reduction in α-helical content between the holoenzyme and the constitutively active G-kinase. Although small, the difference between the two enzyme forms was observed regardless of temperature or whether cyclic GMP was present, and the magnitude of the difference was the same in each case after averaging results for two preparations. Addition of cyclic GMP to constitutively active G-kinase did not significantly alter its CD spectrum (see Table I).

"Melting" Experiments. To verify that the CD spectra for the G-kinase represented secondary structure within this protein, and to further analyze differences between the holoenzyme and the constitutively active form, melting experiments were performed in which CD spectra were obtained between 200 and 250 nm after raising the sample temperature in 5 °C steps. The results of these experiments are shown in Figure 5. For both proteins, the intensity of the observed elliplicity was slightly reduced for each step from 25 to 40 °C. At 45 °C a change in the form of the spectra occurred, which was more dramatic at 50 °C. In the case of the holoenzyme, a single trough was now seen with minimum elliplicity at ~219 nm. For the constitutively active G-kinase, the trough occurred with a minimum at a slightly higher wavelength (~222 nm), the changes in signal intensity were extremely large, and they were in the opposite direction (reduced elliplicity) to those observed for the holoenzyme. The wavelength of the ellipticity minimum of the "melted" holoenzyme spectrum is typical of β-pleated sheet, but PROSEC was unable to analyze the spectrum in a way that gave sensible results. (The calculated spectrum produced by BLEND from the PROSEC analysis was not the same as the experimental spectrum.) The spectrum produced from the melted, constitutively G-kinase cannot be ascribed to a particular secondary structural feature.

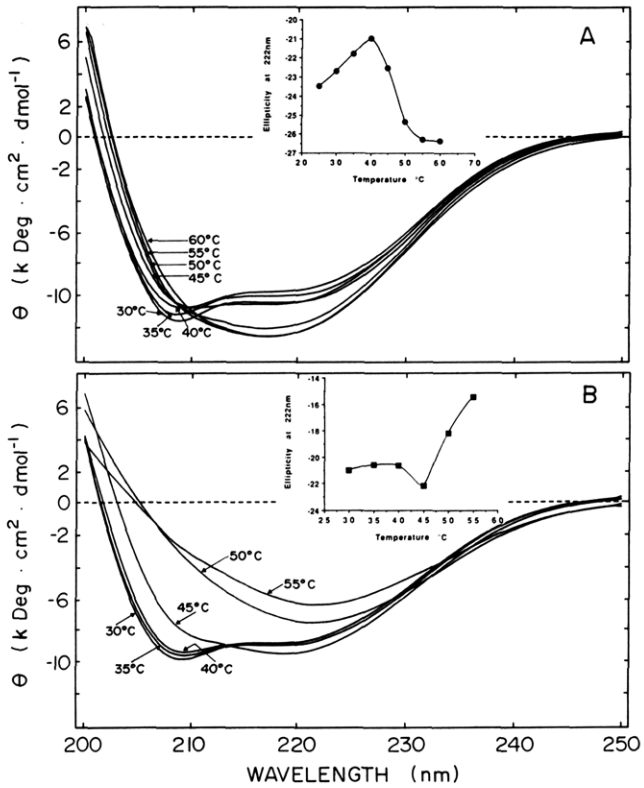


FIGURE 5: CD spectra of the native holoenzyme (A) and constitutively active G-kinase (B) between 200 and 240 nm measured at increasing temperature as shown. The insets show the temperature-induced change in molar residual ellipticity at 222 nm.

DISCUSSION

To the best of our knowledge, this paper reports the first attempt to use a biophysical technique to examine the structure of G-kinase. Analysis by CD is a useful technique for studying the secondary structure of proteins such as G-kinase which are too large for NMR analysis and which have not yet been crystallized. However, the analysis of CD spectra is often difficult as a spectrum from any protein or peptide is the sum of four underlying curves arising from the four basic secondary structure characteristics. In addition, small conformational changes in large proteins may easily be masked because of the

large degree of secondary structure which will not change after, for example, the binding of a ligand. For these reasons CD can make structural predictions, but these must necessarily be verified by other methods, of which X-ray crystallography would undoubtably be the method of choice in this case, once crystals of G-kinase are obtained.

The overall shape of the CD spectrum of the dimeric holoenzyme is reminiscent of an α-helical structure, but more detailed examination showed that the enzyme also contains considerable amounts of β-pleated sheet and of a random coil structure. The addition of cyclic GMP to the enzyme in a sufficient excess to saturate both binding sites caused a small change in the CD spectrum consistent with an increase in the content of β-sheet within the G-kinase. Examination of the primary sequence of the enzyme indicated that, within the N-terminal domain, the first 38 residues would be predicted to form an ordered α-helix (Figures 2 and 4). The synthesized peptide corresponding to these residues was found to be very α-helical, and the constitutively active monomer of G-kinase was found to be slightly less α-helical than the holoenzyme. The latter observation is compatible with the intact enzyme having an α-helix N-terminal domain. However, proteolysis of G-kinase to produce the monomer changes other characteristics of the enzyme, as evidenced by the differences in melting patterns between the holoenzyme and constitutively active form. Hence, although the available evidence is supportive of an α-helical amino-terminal region, an unequivocal designation within the intact enzyme cannot be made at present.

Both the catalytic subunit (Reed & Kinzel, 1984) and the type II regulatory subunit (Johnson & Wong, 1989) gave CD spectra similar in shape to those observed for G-kinase. The average proportions of secondary structure for G-kinase (averaging results for the two preparations and temperatures) are α-helix 32%, β-sheet 30%, β-turn 9%, and random coil 38%. The equivalent proportions concluded for the catalytic subunit of A-kinase were α-helix 49%, β-sheet 20%, and remainder 31% (Reed & Kinzel, 1984). It is difficult to make a direct comparison of the results as the CD spectrum of the holoenzyme of A-kinase has not been reported, but apparently neither protein is dominated by one form of structure. The secondary structure predicted by the Chou–Fasman method is reasonably in accord with the experimentally derived values with the exception of β-turns. This method is much more successful, in general, at predicting the presence of long strings of secondary structure which will tend to predominate when a complete amino acid sequence is considered. Weber et al. (1989) have predicted the secondary structure of the cyclic nucleotide binding sites of G-kinase by homology with the cyclic AMP binding sites of CAP, for which the crystal structure is known. The Chou–Fasman method is in agreement with the assignment of some, but by no means all, of the features of the Weber model. For example, the large α-helix αC (residues 205–226 and 329–350 in the two sites of G-kinase) and the sheet β8 (residues 184–191 and 308–315) are both indicated by the Chou–Fasman method. Other regions are not in agreement, and this is particularly the case for the short sequences of Weber et al. (1989). These differences undoubtedly reflect the weakness of the Chou–Fasman calculations in defining very small regions of secondary structure but may also reflect genuine differences between the kinase and CAP in some cases.

The change in the CD spectra observed when cyclic GMP was added to the G-kinase holoenzyme indicates a conformational change after nucleotide binding. A spectral change was also observed in a CD study of the regulatory subunit of A-kinase after cyclic AMP addition (Johnson & Wong, 1989), but the published spectrum does not extend to sufficiently low wavelengths to allow a valid comparison with Figure 3A. It is perhaps surprising that a small, relatively nonenergized effector molecule can induce observable conformational changes in a protein as large as G-kinase. That this is so suggests that the G-kinase is energetically primed to enter the "active" conformation and that cyclic GMP acts in a manner analogous to the release of a catch on a sprung bolt. Such an analogy also implies that the conformational change induced in the near vicinity of bound cyclic GMP must be under very tight control or random protein movement could supply the necessary energy to release the "catch". In fact, it is difficult to reduce the basal activity of G-kinase (in the absence of cyclic GMP), perhaps indicating that the above analogy is valid. Clearly such questions will only be answered when detailed X-ray crystallographic studies of G-kinase are possible.

It is not clear where in the G-kinase conformational changes occur after cyclic GMP binding, but two good condidates are in the direct vicinity of the binding site (see above) and in a region close in space to the amino–terminal part of the polypeptide chain. Over the past 10 years a generalized scheme for the control of protein kinases has evolved whereby activity is inhibited in the "inactive" state (in the absence of effector) by the binding of part of the enzymes' protein sequences to their active sites [see Hardie (1988)]. Such inhibitory regions may be autophosphorylated, as is the case of A-kinase regulatory subunit type II (Rangel-Aldao & Rosen, 1976), calcium and calmodulin-dependent protein kinase II (Schworer et al., 1986), and G-kinase (Aitken et al., 1984). In other kinases the "pseudosubstrate" region contains an alanine residue instead of serine or threonine as in A-kinase regulatory subunit type I (Hardie, 1988) and smooth muscle myosin light chain kinase (Pearson et al., 1988). It is assumed that binding of the kinase effector causes a conformational change within a "hinge" region which removes the substrate or pseudosubstrate sequence from the active site, thereby activating the kinase. In G-kinase the hinge region and substrate sequence are located in the N-terminal domain of the enzyme, which is also that part with least homology to A-kinase (Edelman et al., 1987). For this reason, and as the N-terminal domain is known to be very important in the control of G-kinase activity (introduction), it was thought likely that the conformational changes observed on binding of cyclic GMP involve regions close in space to the N-terminus of the G-kinase.

To further ascertain where the cyclic GMP induced conformational change occurs, the effect of cyclic GMP on the CD spectra for constitutively active G-kinase was determined. In contrast to the holoenzyme, no changes in the spectra were observed, and subsequent secondary structure analysis by PROSEC indicated that, in the constitutively active enzyme, β–sheet is not induced by cyclic GMP. In fact, the proportion of β-sheet in the constitutively active enzyme, with or without cyclic GMP, is more reminiscent of the holoenzyme in the presence, than in the absence, of cyclic GMP. This may indicate that removal of the amino-terminal domain results in a conformational reorganization within the enzyme such that β-pleated sheet is increased. It is possible that this "induced" β-sheet is the same structure as is induced in the holoenzyme by cyclic GMP binding, because both enzymes are active in conditions where β-sheet is "induced". Whether in the holoenzyme a conformational change in the amino-terminal domain occurs subsequent to cyclic GMP binding and induces β-sheet in the enzyme, or the amino-terminal portion

is necessary to maintain tertiary structure for cyclic GMP induced β-sheet formation, cannot be concluded from these studies.

Reed and Kinzel (1984) have found that peptide substrates induced β-sheet structure in the catalytic subunit of A-kinase. If the above model for kinase activation is correct, it might be expected that binding of cyclic GMP would remove the autophosphorylation sequence from the active site and, by analogy with A-kinase, *reduce* the β-structure of the enzyme. The fact that reverse was actually observed might be explained in several ways: First, the experiments with A-kinase were performed with isolated catalytic subunit and it may not be possible to observe substrate-induced changes in β-sheet in the holoenzyme. This might be the case, for example, if the increase in β-sheet is too small to detect when the entire protein is present. Alternatively, the induction of β-sheet by cyclic nucleotide and its supression by removal of the autoinhibitory domain may both occur, but the nucleotide effect may be greater. Third, there may be differences between the two protein kinases, although this seems unlikely given the homology between them (Takio et al., 1984; Hanks et al., 1988). It would be interesting to know whether cyclic AMP induces β-sheet in the holoenzyme of A-kinase.

When examining the N-terminal sequence of G-kinase, we noticed that the first 40–50 residues had much in common with the leucine zipper motif (Figure 4), which has recently been described as part of a DNA-binding region in some proteins involved in transcriptional activation (Landschulz et al., 1988). The features of this motif, which is required for dimer formation [see, e.g., Kouzarides and Ziff (1989)], are a periodic heptad leucine repeat in a segment of at least 22 amino acids, a structure compatible with α-helix formation, and, in some cases, the use of hydrophobic residues in the third or fourth position after each leucine (Brendel & Karlin, 1989). Isoleucine residues have also been included in the heptad repeat (White & Weber, 1989). The first 38 amino acids of G-kinase are predicted to be α-helical (Figure 2), a leucine or isoleucine heptad repeat of considerable length occurs, and stripes of charge indicative of charge stabilization exist within the predicted structure (Figure 4a). Also, both isoforms of G-kinase have amino-terminal domains with leucine zipper-type features (Figure 4b), although these sequences are otherwise dissimilar (Wernet et al., 1989; Sandberg et al., 1989). For these reasons we suggest that this region is involved in the dimer formation in G-kinase, although we know of no evidence suggesting that the enzyme is involved in transcriptional activation, and in any case it lacks the "basic motif" necessary for this function [see Kouzarides and Ziff (1989)].

Within the N-terminal domain of the G-kinase type Iα holoenzyme is a cystine residue often stated to be involved in dimerization. However, the evidence for a disulfide bond between the subunits is not definitive. Evidence for the bond was found in gel studies of the enzyme by Monken and Gill (1980), but the cystine was not detected in proteolysis studies in the absence of reducing agents (Takio et al., 1983). It is unclear whether the enzyme used in the former case was oxidized or in the latter case was reduced. However, sulfide titration studies with 5,5′-dithiobis(2-nitrobenzoic acid) suggest that native G-kinase may be oxidized in vitro at Cys^{42} within the dimerization domain since only 10 of 11 cysteine residues per subunit are titratable (W. Landgraf, unpublished observation). The fact that a cystine can form within the enzyme (whether or not it is present in vivo) suggests that the Cys^{42} residues are close in the holoenzyme and that if the proposed amino-terminal helices are involved in dimerization, they must lie parallel to each other in the holoenzyme dimer. It has recently been found that the 1β isotype of G-kinase, which is also dimeric (Wolfe et al., 1989), does not contain cysteine in the dimerization region (Wernet et al., 1989), indicating that other features of the kinase are able to induce dimerization. The presence of a region with the characteristics of a dimerization site and the at best questionable role of the disulfide bridge in this site would seem to indicate that the presence of Cys^{42} alone is not the only requirement for dimerization in vivo. This suggestion could in principle be tested by site-directed mutagenesis studies.

In accordance with the secondary structure predictions, the peptide representing residues 1–39 was found to exist in a predominantly α-helical structure, although we do not know if it is monomeric or dimeric in solution and experiments are in progress to answer this question. In addition, the constitutively active form of G-kinase, which is monomeric and lacks residues 1–77, has a reduced α-helical structure relative to the native holoenzyme. However, other differences exist between the structures of the proteolyzed and native forms of G-kinase, as was most clearly shown in the melting studies in which the effects of temperature were qualitatively different for the two forms. This probably indicates that the N-terminal segment and/or subunit association is also important for stabilizing the structure of other regions of the protein.

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Cytoplasmic and Nuclear Distribution of Casein Kinase II: Characterization of the Enzyme Uptake by Bovine Adrenocortical Nuclear Preparation†

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ABSTRACT: Casein kinase II (CK II) is a ubiquitous protein kinase that has been found in both nuclear and soluble subcellular fractions and whose precise cellular functions and mechanisms of control remain to be clarified. Using immunocytochemical localization, it was observed that the intracellular distribution of CK II exhibited a striking shift toward an increased nuclear concentration during active proliferation of bovine adrenocortical cells in primary culture. The interaction of CK II with purified adrenocortical cell nuclear preparation was thus examined in vitro. CK II was found to rapidly associate with nuclei in a temperature-dependent and saturable process, resulting in a tight binding of the kinase to nuclear components, as shown by various extraction procedures. This association resulted in a concentration of the kinase in the nuclear preparation about 100-fold that in the medium and exhibited two types of binding sites with K_a of 10^9 and 10^7 M^{-1}, respectively. The nuclear CK II uptake was dependent upon the presence of ATP and was stimulated by a kinase activator such as spermine, although the enzyme activity did not appear to be required for the process. These observations would be in line with a pore-mediated, energy-dependent nuclear uptake of the kinase. Since a number of potential nuclear CK II targets have been reported, including the oncoprotein myc, it is suggested that the nuclear translocation of the kinase as characterized in vitro may have a biological significance in living cell, especially in the control of nuclear activities related to cell proliferation and the mechanism of action of growth factors.

Casein kinase II (CK II) is an ubiquitous enzyme belonging to the threonine–serine protein kinase family (Edelman et al., 1987; Meggio et al., 1984). It was also named casein kinase G (CKG) due to its ability to use GTP as well as ATP as phosphoryl donor (Cochet et al., 1980; Cochet & Chambaz, 1983b), phosvitin kinase, glycogen synthase 5 kinase, casein kinase TS, PC 0.7 kinase, troponin T kinase, and protein kinase NII (Edelman et al., 1987). The enzyme has been purified from various tissues and shown to be an oligomeric protein made of two different subunits, with an $\alpha_2\beta_2$ stoichiometry

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