A Stable \(\alpha\)-Helical Domain at the N Terminus of the RI\(\alpha\) Subunits of cAMP-dependent Protein Kinase Is a Novel Dimerization/Docking Motif*

(Received for publication, May 23, 1997, and in revised form, August 29, 1997)

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The RI\(\alpha\) subunit of cAMP-dependent protein kinase is maintained as an asymmetric dimer by a dimerization motif at the N terminus. Based on resistance to proteolysis and expression as a discrete domain in Escherichia coli, this motif is defined as residues 12–61. This motif is chemically, kinetically, and thermally stable. The two endogenous interchain disulfide bonds between Cys\(^{16}\) and Cys\(^{37}\) in RI\(\alpha\) are extremely resistant to reduction even in 8 M urea, indicating that they are well shielded from the reducing environment of the cell. The disulfide bonds were present in recombinant RI\(\alpha\) as well as when the dimerization domain alone was expressed in E. coli, emphasizing the unusual stability of this motif and the disulfide bonds. Although 100 mM dithiothreitol was sufficient to reduce the disulfide bonds, it did not abolish dimerization. In addition, a stable dimer also still formed when Cys\(^{37}\) was replaced with His, confirming unambiguously the original antiparallel alignment of the disulfide bonds. Thus, both in vitro and in vivo, disulfide bonds are not required for dimerization. Circular dichroism of the dimerization domain indicated a high content of a thermostable \(\alpha\)-helix. Based on the CD data, trypsin resistance of the fragment, location of the disulfide bonds, and amphipathic helix predictions, potential models are discussed. A new alignment of the dimerization domains of RI, RII, and cGMP-dependent protein kinase elucidates fundamental similarities as well as significant differences among these three domains.

Many of the proteins that are involved in signal transduction such as protein kinases and transcription factors tend to be modular. Multiple domains with well defined structure and function are assembled into a single polypeptide, thus allowing for the well orchestrated and highly regulated cascade of signaling events.

The regulatory subunits of cAMP-dependent protein kinase have long been recognized as highly modular multifunctional proteins (1, 2). The separate domains and their functional independence were initially established as a consequence of their susceptibility to limited proteolysis. Recombinant approaches have subsequently further defined the features of the isolated domains (3–6). At the amino terminus, a dimerization domain maintains the R subunits\(^{1}\) as an asymmetric dimer. This region is followed by a variable region, often proline-rich and containing multiple phosphorylation sites (7–9). Next is the inhibitor site, an extended segment that resembles a protein substrate and binds to the active site of the catalytic subunit in the absence of cAMP, thus maintaining the complex as an inactive tetramer. Because of its susceptibility to proteolysis in the absence of the catalytic subunit, this region is often referred to as the “hinge” region. At the C terminus are two tandem cAMP binding sites. Upon cooperative binding of cAMP to the R subunit, the active catalytic subunits are released from the holoenzyme complex. Although much is known about the function and structure of the cAMP binding sites, less is known about the dimerization domain (1, 4, 10). In the case of the RII subunit and more recently RI, this dimerization domain is thought to be important for subcellular localization (2, 11).

There are at least four unique gene products in the R subunit family, and all retain the same general domain structure. With the exception of the R subunit in Dictyostelium discoideum (12), all of the R subunits are stable dimers, and it is the amino terminus that is responsible for dimerization. The interaction site between the two protomers is associated with the first 40–60 residues, although the precise boundaries of the dimerization domain differ slightly for RI and RII subunits (13–15). Within the family of R subunits, the sequences at the amino terminus are the most variable. The two general classes are designated as RI and RII, and within each class are at least two variants, \(\alpha\) and \(\beta\) (1). In the RI\(\alpha\) subunit, the dimer region contains two cysteine residues, which form interchain disulfide bonds (13). Since Cys\(^{16}\) is disulfide-bonded to Cys\(^{37}\), the two protomers were predicted to be antiparallel (16).

In the case of the RII subunit, the N-terminal dimerization domain has been implicated in a critical biological function, namely interaction with AKAPs (A-kinase anchor proteins) (2, 17–20) While anchoring proteins so far have been associated primarily with the RII subunits, a recent finding shows that a novel family of anchoring proteins recognizes RI as well as RII

\(^{1}\) The abbreviations used are: R subunit, regulatory subunit; RI and RII, type I and II regulatory subunits, respectively; \(\beta\)-ME, \(\beta\)-mercaptoethanol; DTT, dithiothreitol; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)propanesulfonic acid; cAPK, cAMP-dependent protein kinase; cGPK, cGMP-dependent protein kinase; PBS, phosphate-buffered saline; TPC, 1-tosylamido-2-phenylethyl chloromethyl ketone; HPLC, high pressure liquid chromatography.
(11), indicating that the RI subunit is likely to play a role in subcellular targeting of cAPK. Other reports that the RI subunit localizes to the activated T-cell receptor and the neuromuscular junction lend further support to such a role for RI in addition to its role as an inhibitor of the catalytic subunit of cAPK (21, 22).

The RI dimerization domain, expressed as a stable disulfide-bonded, trypsin-resistant fragment, is described here with particular focus on the importance of the endogenous interchain disulfide bonds. Biophysical analysis of the reduced fragment as well as mutagenesis of Cys37 confirmed the antiparallel alignments of the chains and established, furthermore, that disulfide bonds are not required for dimerization in vitro or in vivo. Models of the dimerization/docking domain of RIs are discussed and compared with the RII subunit as well as cGMP-dependent protein kinase.

EXPERIMENTAL PROCEDURES

Expression and Purification of RIs—The bovine RII was expressed and purified as described previously (23).

Mutagenesis, Expression, and Purification of Mutant Proteins—To confirm the importance of the disulfide bonds for dimerization, Cys37 was replaced with His using the in vitro mutagenesis system from Bio-Rad, which is based on the Kunkel method (24) as described previously. The resulting clones containing the mutant DNA were identified by sequencing using the Sanger dideoxy method (25). The mutant double-stranded DNA was transformed into Escherichia coli 222 cells and grown on LB/ampicillin agar plates. Cells were grown and checked for protein expression using SDS-PAGE. The mutant recombinant RII-(C37H) was purified by ion-exchange chromatography on DEAE-cellulose (23).

To determine whether the N-terminal domain could be expressed independently as a stable disulfide-bonded dimer, two stop codons were introduced at Gln26 in the RII subunit as described above. To facilitate purification, the deletion mutant was also fused to a polyhistidine tag in the N-terminal fragment and purified as described previously (23).

Expression and Purification of the N-terminal Peptide (Amino Acids 12–61)—The RII subunit (2 mg/ml) was incubated for 1 h with increasing concentrations of dithiothreitol (DTT) (0–100 mM). Samples were then boiled for 2 min in sample buffer (2.5% SDS, 10% glycerol, and 0.01% bromphenol blue) and subjected to SDS-PAGE. Shag gels (1 mm) were prepared with 10% acrylamide as described by Laemmli (26). In some cases, N-ethylmaleimide (NEM) at a final concentration of 1 mM was added to quench the reaction prior to the addition of the denaturing SDS solution to guarantee that no reduction occurred following denaturation. Samples were also unfolded in the presence of 8 mM urea. RII (2 mg/ml) was dialyzed against 8M urea, 10 mM MOPS (pH 7.0) for 2 h and then incubated with increasing amounts of DTT. The effect of quenching the reaction with NEM prior to the addition of the denaturing SDS solution was also evaluated.

Expression and Purification of the N-terminal Peptide (Amino Acids 12–61) of RII—the Q62stopRI gene was excised from the RII pUC118 vector using EcoRI and subcloned into the pRSETa vector. The recombinant DNA was transformed into E. coli BL21(DE3) competent cells. Expression was tested in the absence and presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside by SDS-PAGE.

Conditions Required for Reduction of Interchain Disulfide Bonds—The RII subunit (2 mg/ml) was incubated for 1 h at 22 °C in a MOPS buffer (20 mM, pH 7.0) containing 100 mM KCl with Pharmacia fast protein liquid chromatography. The retention volume of RII in the absence of DTT was compared with RII that had been pretreated in the MOPS/KCl buffer containing 100 mM DTT.

To determine the molecular weight of the dimerization domain, RI(12–61), the protein was loaded onto the Superdex 75 HR 10/30 column at an initial concentration of 1 mg/ml. The column was calibrated using the Pharmacia calibration kit.

Circular Dichroism—The lyophilized dimerization domain, RII(12–61), (0.14 mg) was redissolved in 1 ml of 25 mM potassium phosphate and 2 mM EDTA, pH 6.8, and placed in a cuvette with a 0.2-cm path length. Circular dichroism measurements were made on an AVIV CD spectropolarimeter. The spectrum was scanned from 300 to 200 nm at the temperatures indicated. The mean residue ellipticity, [θ], was calculated as θ/C, where θ is the ellipticity in degrees, l is the cell path length in cm, and C, is the concentration of amino acid residues in dml/hiter.

Prediction of Secondary Structure—To further analyze the secondary structure of the dimerization domain, the sequence of the trypsin-resistant segment was analyzed using the CDESTIMA computer program, which combines the CD data with the Chou-Fasman algorithm (27).

RESULTS

Conservation of Interchain Disulfide Bonds in Recombinant RIs—When the RII subunit of cAPK was purified from mammalian tissues, the two proteomes of the dimer were stoichiometrically cross-linked by interchain disulfide bonds (13). No significant amount of monomer was observed in the absence of β-ME. As seen in Fig. 1, when the RII subunit and a deletion mutant, RI(Δ260–379), were overexpressed in E. coli, the proteomes were also totally cross-linked by interchain disulfide bonds just as was observed for the full-length mammalian RII subunit. To confirm that these disulfide bonds were not formed as an artifact during purification, the total extract was lysed directly in SDS-PAGE buffer. Under these conditions, the two proteomes were still stoichiometrically linked by interchain disulfide bonds. No monomorphic RII subunit was observed.

Stability of the Endogenous Disulfides in the RI Dimer—The stability of these interchain disulfide bonds in RII was assessed first by defining the conditions required for reducing the disulfide linkage. The RII dimer was incubated for 1 h at room temperature with increasing concentrations of DTT. Reduction was monitored by nonreducing SDS-PAGE. Excess DTT was quenched by adding 1 M NEM prior to adding the sample to the SDS denaturing solution. As seen in Fig. 2A, significant amounts of monomer were only observed when the...
monomer was observed at DTT concentrations of 5 mM; however, this most likely occurred after the addition of the SDS denaturing buffer (Fig. 2C).

The above results indicated that the disulfide bonds in the RI α dimer were extremely resistant to reduction. Even when the rest of the molecule was exposed to 8 M urea, a condition sufficient to unfold the cAMP-binding domains (28), the disulfides in the dimerization domain remained protected, suggesting that the secondary structure in the disulfide-bonded segment was still intact.

Expression of the Dimerization Domain—To further probe the stability and structural features of this dimerization motif, recombinant techniques were used to overexpress a deletion mutant of RI α that contained only the dimerization region. The N-terminal fragment was overexpressed and purified using the cobalt-agarose affinity resin.

Fractions containing His6-RI-(1–61) were then pooled and digested with TPCK-treated trypsin. The tryptic peptide corresponding to residues 12–61 was purified by HPLC as described under “Experimental Procedures.” Fig. 3 shows the purified fusion protein as well as the final trypsinized peptide analyzed on SDS-PAGE under reducing as well as nonreducing conditions. The apparent molecular mass for the monomer was 9.0 kDa. In the absence of β-ME, the purified fragment ran with an apparent molecular mass of 18 kDa. Thus, the peptide alone, like the intact RI α, is a stable disulfide-bonded dimer. The tryptic peptide was analyzed by analytical gel filtration column in KMOPS buffer (20 mM MOPS, 100 mM KCl, pH 6.5). Under these conditions, the apparent molecular mass was calculated to be 17 kDa based on constructed calibration curves.

Importance of the Disulfide Bonds for Dimerization—When the RI α dimer was analyzed by gel filtration in the absence of DTT, it eluted as an asymmetric dimer with a Stokes radius of 46.3 Å (29). Pretreatment of the RI subunit with 100 mM DTT for 1 h was sufficient to achieve complete reduction of the disulfide bonds as shown by SDS-PAGE (Fig. 2). When this protein was loaded onto the gel filtration column equilibrated with 100 mM DTT in the running buffer, the elution volume was unchanged, indicating that the dimer was intact although the interchain disulfide bonds were no longer present.

Antiparallel Alignment of the Protomers—To confirm that the chains were aligned in an antiparallel arrangement and to further determine whether the disulfide bonds were necessary for a stable dimer, Cys37 was replaced with His. This Cys37 → His mutant migrated on SDS-PAGE in the absence or presence of DTT as expected. Even when the rest of the molecule was exposed to 8 M urea, a condition sufficient to unfold the cAMP-binding domains (28), the disulfides in the dimerization domain remained protected, suggesting that the secondary structure in the disulfide-bonded segment was still intact.

Circular Dichroism of the Isolated Dimerization Domain—The CD spectrum for the RI α-(12–61) dimerization domain (Fig. 5) showed two minima at 205 and 222 nm, indicating a
Dimerization Domain in the RIA Subunit of cAPK

The RIA subunit of cAPK is maintained as a dimeric protein by a dimerization domain at the N terminus. As demonstrated here, this domain is mostly helical and is chemically, kinetically, and thermally stable. Although disulfide bonding is rare in intracellular proteins, the RIA subunit is an exception, since the full-length mammalian and recombinant proteins expressed in E. coli were both found exclusively as disulfide-bonded dimers. Even when deletion mutants of the dimerization domain alone were expressed in E. coli, the two chains were still disulfide-bonded. The disulfide bonds were, furthermore, extremely resistant to reduction, even in the presence of 8 M urea, suggesting that these disulfide bonds are buried and shielded from solvent. The boundaries of the trypsin-resistant core that define this stable dimerization domain are indicated in Fig. 6.

Although the disulfide bonds were chemically stable, once reduced, the protein did not dissociate into monomers. It remained as a dimer with a Stokes radius that was indistinguishable from that of the wild type protein. To determine whether the disulfide bonds were required for dimerization in vivo, a mutant protein was engineered where one of the two cysteines was replaced with His. This mutation, C37H, was sufficient to abolish disulfide bonding, confirming the antiparallel alignment of the two chains that was shown previously by peptide mapping (16). This mutation was not, however, sufficient to prevent dimerization. Thus, like the RI subunits that lack interchain disulfide bonds, the RIA subunit is intrinsically a stable dimer, independent of the interchain disulfide bonds.

In the absence of a high resolution structure, the structural features of this dimerization domain were probed by CD, which indicated a high degree of a thermostable α-helix. Based on the CD spectrum of the isolated dimerization domain, the proteolytic resistance of the fragment, the resistance of the disulfide bonds to reduction, and secondary structure predictions, it is possible to propose a model for the tertiary structure of the dimerization motif, RIA-(12–61). The circular dichroism coupled with secondary structure prediction algorithms was used as a guide for localizing potential amphipathic helices. Residues 45–59, preceded by prolines, are predicted to be a well defined amphipathic α-helix. The N-terminal portion is predicted to contain an additional α-helix as well as two β-strands. If the segment between Cys45 and Cys37 is predominantly helical, then the two chains must be antiparallel, to accommodate interchain disulfide bonding between Cys16 and Cys37. This would mean that the predicted helices at the C terminus would also be antiparallel, yielding a four-helix bundle motif. However, if the segment between Cys45 and Cys37 contains both α-helix and β-strand, the predicted C-terminal helix could be parallel, antiparallel, or not interacting at all. From the information described here, we know only that the residues within amino acids 16 and 37 are shielded from the solvent based on the stability of the disulfides and the protease resistance of the fragment.

The stability of the RIA dimeric domain is surprising but not unique for helical dimerization motifs. For instance, many DNA-binding proteins have a unique α-helical region that contains a characteristic heptad repeat of leucine residues referred to as a leucine zipper. The motif is found in transcription factors (i.e. GCN4) and in nuclear transforming oncogene products such as Fos, Jun, and Myc (30). The stability of these domains has been addressed thermodynamically (31) and in fact correlates with the remarkable thermal stability we observe for the RIA dimerization domain. Despite the similarity of the RIA dimerization domain in terms of stability with other known dimerization domains, we believe that the RIA dimerization domain is a novel and unique motif. Further experiments are under way to probe this domain structurally.

Is this dimerization motif in RIA conserved in other related members of this enzyme family? Two other members of this family, cGMP-dependent protein kinase (cGPK) and the RII subunits, are both stable dimers. The N-terminal sequences of

**FIG. 4.** Gel filtration of wild type RIA and mutant RIA-(C37H).

Wild type RIA (200 μl; solid lines) and mutant RIA-(C37H) (dashed lines) were chromatographed on a Superdex 75 HR 10/30 column. The inset shows a 15% SDS gel. Lane 1, wild type RIA (25 mM DTT); lane 2, wild type RIA (50 mM DTT); lane 3, wild type RIA (100 mM DTT); lane 4, RIA-(C37H) (0 mM DTT); lane 5, RIA-(C37H) (100 mM DTT).

High content of α-helix. The α-helicity of the peptide was monitored over a wide pH range. The peptide was stable to pH changes and still maintained a considerable amount of secondary structure at extremes of pH (2 and 12).

Based on the amino acid sequence of the RIA N-terminal domain, it is speculated that hydrophobic interactions are critical in mediating interfacial packing at the dimeric interface. To test this hypothesis, CD spectra of the N-terminal peptide were taken at 100 mM and 1 M potassium fluoride. As shown in Fig. 5B, at higher ionic strength there is an increase in the α-helicity of the peptide, confirming that, in fact, hydrophobic interactions are important.

The thermal stability of the RIA-(12–61) fragment was also monitored by CD. The spectra were scanned from 300 to 200 nm, and the temperature was increased from 0 to 92 °C. CD spectra at selected temperatures are shown in Fig. 5C. From 0 to 62 °C, there was a gradual loss of ellipticity, yet at both temperatures, there was still defined α-helical structure. A spectrum typical of a random coil was not observed even at 92 °C, indicating that secondary structure was still present. A complete temperature curve is shown in Fig. 5C. After RIA-(12–61) was incubated at 92 °C, it was allowed to cool to room temperature (25 °C). The spectrum of the refolded RIA-(12–61) was very similar to the original spectrum at 25 °C, indicating that the partial unfolding of dimeric fragment was fully reversible.

**Secondary Structure Prediction**—Based on the CDESTIMA program, which couples the CD data with a Chou-Fasman algorithm, the RIA-(12–61) dimeric fragment was predicted to be approximately 40–50% helical at 0 °C (Table I). Boundaries of the predicted helices are indicated in Fig. 6.

**DISCUSSION**

The RIA subunit of cAPK is maintained as a dimeric protein by a dimerization domain at the N terminus. As demonstrated here, this domain is mostly helical and is chemically, kinetically, and thermally stable. Although disulfide bonding is rare in intracellular proteins, the RIA subunit is an exception, since
all three proteins are compared in Fig. 7. The RII subunits, like cGPK and the RI subunits, are stable dimers, although they do not have interchain disulfide bonds. Previous alignments of the RI and RII subunits were based on the obvious sequence similarities in the cAMP-binding domains and in the autoinhibitor sites (32, 33). Similarities in these regions are extensive, with the major difference being that the RI subunits are typically about 20 residues shorter than the RII subunits due to a truncation of cAMP-binding domain B. Based on this original alignment, the sequence similarities in the amino terminus were not initially apparent (16). However, if the two molecules are aligned as shown in Fig. 7, where the predicted functional dimerization domains of RI and RII are aligned, similarities in the dimerization domains can also be recognized. For example, the type II holoenzyme, but not free RII, was susceptible to proteolysis at Arg45, and removal of these first 45 residues was sufficient to eliminate dimerization of this protein (14). This suggests that the region flanking Arg45 in the RII subunit must be on the surface, since it is accessible to trypsin. This protease-
sensitive region is in the exact location as the protease-accessible site in the RI subunit (Ser12-Lys61) when the sequences are aligned as shown in Fig. 7. When the first 50 residues from the RII subunit were subjected to the CON22 program, which predicts secondary structure based on Chou-Fasman and Garnier algorithms (27, 34), one major a-helical region was identified, residues 27–45. In the alignment shown in Fig. 7, this segment now aligns with the predicted carboxyl-terminal helix in the dimerization domain of RII.

The dimerization domain in RIIα has a unique functional role. Within this segment is an “anchoring” motif that enables the RII subunit to bind with high affinity to other proteins such as the brain protein, p75 (35), and the microtubule assembly protein 2 binding (39). It was concluded that dimerization was required for anchoring protein interactions, and that residues 1–14 are an essential part of the dimerization domain that are required for microtubule assembly protein 2 interaction. Residues 1–79 of the RII subunit, for example, are required for microtubule assembly protein 2 interaction, and removal of the first 10 residues was sufficient to abolish microtubule assembly protein 2 binding (39). It was concluded that dimerization was required for anchoring protein interactions and that residues 1–14 are an essential part of the dimerization domain in the RIIα subunit (15, 40, 41). Indirect evidence that residues 1–30 in the RIIα subunit have an amphipathic surface comes from the results of Carr et al., who concluded that the RIIα dimer interacts with anchoring proteins (microtubule assembly protein 2, P150, and two thyroid proteins) that contain a 14-residue amphipathic helix (42, 43). Scott and co-workers (2, 18) have identified a whole family of proteins that contain a 14-residue amphipathic helix (42, 43).

In cGMP-dependent protein kinase, the regulatory and catalytic domains are fused into a single polypeptide chain. Like cAPK, the two protomers are joined covalently, but there is only one interchain disulfide bond (45). An amino-terminal, 39-residue peptide of cGMP-dependent protein kinase was isolated, and, based on circular dichroism and NMR analysis, was also found to be predominantly a-helical (46). cGMP-dependent protein kinase contains a leucine/isoleucine heptad repeat consistent with a leucine zipper motif (47). Although cAMP-dependent protein kinase and cGMP-dependent protein kinase have similar regulatory domains, the RIIα dimerization domain does not contain an exact leucine/isoleucine heptad repeat like cGPK. In addition, there are prolines in the middle of the RIIα dimerization domain. In theory, the leucine zipper in cGPK could be parallel or antiparallel; however, the single disulfide bond involving a Cys at the end of the dimerization motif in the a isof orm of cGPK makes it likely that this segment of the helical dimerization motif is parallel in cGPK (48). In contrast, the linking of Cys189 in one chain with Cys87 in the other chain mandates that this amino-terminal segment of the dimerization motif must be antiparallel in RIIα. Thus, although both related proteins have a stable helical dimerization motif, there are some striking differences. The orientation of the rest of the molecule, in each of the dimerization domains discussed above, depends critically on the directionality of the C-terminal helix in each of them. This detailed comparison of the N-terminal domains of RI, RII, and cGMP-dependent protein kinase emphasizes that there are some fundamental similarities and differences among the three proteins, although all contain a well defined motif for maintaining a dimeric aggregate state. Ultimately, high resolution structures are necessary to define these differences.

Acknowledgments—We thank Siv Garrod at University of California San Diego (UCSD) for technical assistance in peptide isolation and sequencing, Patricia Jennings in the Department of Chemistry and Biochemistry (UCSD) for guidance in CD instrumentation, and Steve Smith at UCSD for technical services in amino acid analysis.

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FIG. 7. Sequence comparison of cGMP-dependent protein kinase and the RI and RII subunits of cAMP-dependent protein kinase. The similar hydrophobic acids are highlighted. The leucine/isoleucine heptad repeat in cGPK is marked with dots. The dimerization domains in these three proteins are enclosed in boxes. The cysteine pairs that are involved in disulfide bond linkage are shown with arrows.
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