Mutations in the Autoinhibitor Site of the Regulatory Subunit of cAMP-dependent Protein Kinase I

REPLACEMENT OF Ala-97 AND Ser-99 INTERFERES WITH REASSOCIATION WITH THE CATALYTIC SUBUNIT*

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Each regulatory (R) subunit of cAMP-dependent protein kinase contains an autoinhibitor site that lies approximately 90–100 residues from the amino terminus. In order to study the importance of this autoinhibitor site in the type I R-subunit for interacting with the catalytic (C) subunit, recombinant techniques were used to replace Ala-97 with Gln, His, Lys, and Arg and to replace Ser-99 with Gly and Lys. All of the mutant proteins having a replacement at Ala-97 showed reduced affinity for the C-subunit ranging from 14- to 55-fold. In general, the decrease in affinity of the Ala-97 mutants for the C-subunit correlated with the increase in size of the side chain. In contrast to wild type R-subunit, where MgATP facilitates holoenzyme formation, MgATP inhibits the reassociation in all of the Ala-97 mutants suggesting that the larger side chains sterically interfere with bound MgATP in the active site of the C-subunit. Whereas MgATP slowed holoenzyme formation, AMP actually accelerated the reassociation of the A97K, A97H (pH 6.0), and A97Q mutants with the C-subunit. Therefore, the side chains of Lys-97, His-97, and Glm-97 can interact either electrostatically or by hydrogen bonding with the phosphate of AMP. This interpretation is reinforced by the fact that the stimulatory effect of AMP on the A97H mutant was pH-dependent. The affinities of the S99G and S99K mutants for the C-subunit were reduced 7- and 24-fold, respectively, suggesting that Ser-99 also may contribute to interactions between the R- and C-subunits.

cAMP-dependent protein kinase is a holoenzyme complex consisting of a regulatory (R) subunit dimer and two catalytic (C) subunits. In the absence of cAMP, the R-subunit dimer associates with the C-subunit with a high binding affinity (1), thus maintaining the C-subunit in an inactive state. The monomeric C-subunit catalyzes the transfer of the γ-phosphate of ATP to peptide or protein substrates with the consensus sequence, Arg-Arg-X-Ser/Thr (for review, see Ref. 2). Initially different isoforms of the holoenzymes were identified by the order in which they eluted from anion exchange resins and were generally categorized as type I and type II (3). The holoenzyme classification is based on the regulatory subunit (4–6).

Several genes encoding different isoforms of the R-subunits have now been cloned (7–13). Despite their diversity, a comparison of the two general types of R-subunit sequences (14, 15) reveals that each isoform retains a conserved and well-defined overall domain structure as summarized in Fig. 1. Two tandem gene-duplicated cAMP-binding domains are located at the carboxy-terminal two-thirds of each polypeptide chain. These cAMP-binding domains are highly conserved in all R-subunits and also show extensive sequence similarity with the cAMP-binding domain of the catabolite gene activator protein in Escherichia coli (16, 17). In contrast to these highly conserved cAMP-binding regions, the amino-terminal third of the protein is quite variable. The first 40–50 residues appear to contain the primary interaction site between the two protomers of the R-subunit dimer for both type I and type II R-subunits (18, 19), although there is little sequence similarity in this region. The major conserved feature in the amino-terminal segment is a proteolytically sensitive hinge region that is essential for interacting with the C-subunit (20).

This hinge region is also the most significant feature distinguishing type I from type II R-subunits. R1β-subunits contain an autophosphorylation site in the hinge region (4), Arg-Arg-X-Ser. Phosphorylation of the Ser can be achieved by an intramolecular event in the tetrameric enzyme, demonstrating that the hinge region occupies the peptide binding site of the C-subunit in the holoenzyme complex, thus preventing access to other substrates (21). In contrast, the R1α-subunits have a pseudophosphorylation site in this region, Arg-Arg-X-Ala/Gly, and type I holoenzymes have a high affinity binding site for MgATP (5). Replacing Ala-97 at the hinge region of R1α with a Ser generates an autophosphorylation site in the R1α-subunit (22). Like the type II holoenzyme, autophosphorylation in the holoenzyme formed with this mutant R-subunit (A97S) is an intramolecular process that does not require dissociation (22). Thus, the side chain of Ala-97 must be in close proximity to the γ-phosphate of ATP in the holoenzyme. This hinge region will subsequently be referred to as the autoinhibitor site.
Mutations in Autoinhibitor Site of R1'-subunit of cAMP-dependent Protein Kinase

**EXPERIMENTAL PROCEDURES**

**Materials—**Reagents were obtained from the following sources: γ-[32P]ATP (50 Ci/mmole), α-[32P]ATP (1000 Ci/mmole), Amersham; 2, 6-[3H]cAMP (33 Ci/mmole), ICN; ATP, ADP, AMP, GMP, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride, N-α-tosyl-l-lysin chloromethyl ketone (TLCK), L-1-tosylamido-2-phenyl-ethylchloromethyl ketone (TPCK), Luria broth (L-broth), benzamidine, soybean trypsin inhibitor, leupeptin, phosphonoxypruvate, lactate dehydrogenase, pyruvate kinase, CM-Sepharose, Sigma; membrane filters type HA (0.45 μm), Millipore Corp.; prepacked Sephadex G-25 column, Pharmacia LKB Biotechnology Inc.; NADH, MOPS, G-25 column, Pharmacia LKB Biotechnology Inc.; NADH, MOPS, G-25 column, Pharmacia LKB Biotechnology Inc.; NADH, MOPS. Sequenase DNA Sequencing Kit, United States Biochemical Corp. Enzymes used in DNA manipulations were obtained from Bethesda Research Laboratories.

**Oligonucleotides—**All oligonucleotides were synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego.

**Proteins—**The C-subunit was purified from porcine heart (25). Site-directed Mutagenesis—The mutations were introduced as described (22) except that none of the mutations generated or destroyed a restriction site. The mutant clones were identified by DNA sequence analysis of randomly picked plaques following transformation of the ligation mixture into E. coli JM101.

**Isolation of Wild Type and Mutant Regulatory Subunits—**L-broth (25 ml) containing 100 μg/ml ampicillin was inoculated with colonies expressing either wild type or mutant R1'-subunits and grown at 37 °C until the optical density reached 0.1–0.5. This culture was used to inoculate 2 liters against buffer E at 22 °C. All subsequent procedures were carried out at 4 °C. The cells (400 ml) were centrifuged at 5000 × g for 20 min, and the resulting pellet was resuspended in 30 ml of buffer A (25 mM potassium phosphate (pH 6.5), 5 mM EDTA, 5 mM EGTA, 10 mM β-mercaptoethanol, 10 mM benzamidine, 10 mg/liter TPCK, 10 mg/liter TLCK, 15 mg/liter phenylmethylsulfonyl fluoride, 0.5 mg/liter leupeptin, 10 mg/liter soybean trypsin inhibitor), passed through a French pressure cell twice, and then centrifuged at 12,000 rpm for 20 min. The supernatant was diluted with ice cold water until the conductivity was 0.8 mmhos and then applied to a Whatman DE32 column (85 ml bed volume). After washing the resin with 1 liter of buffer A, the protein was eluted with a linear gradient of buffer B (8 mg/ml potassium phosphate (pH 6.5), 2 mM EDTA, 5 mM β-mercaptoethanol, conductivity 0.8 mmhos, 250 ml) to buffer C (120 mM potassium phosphate (pH 6.5), 2 mM EDTA, 5 mM β-mercaptoethanol, 250 ml).

**Preparation of cAMP-Free Wild Type and Mutant R-Subunits—**The removal of cAMP from R-subunits by urea treatment was accomplished using a modified method of Builder et al. (26). The purified R1'-dimer was dialyzed against buffer D (50 mM MOPS (pH 7.0), 10 mM MgCl2) at 4 °C. Solid urea was added until the final concentration of urea was 8 M (480 mg of urea/700 μl of protein solution). After 30 min at 22 °C, the protein solution was passed over a prepacked Sephadex G-25 column equilibrated with 8 M urea in buffer D to remove the cAMP. The resulting eluate was passed over a second Sephadex G-25 column equilibrated with buffer D to remove the urea. The presence of cAMP-free R1'-subunit was confirmed by [3H]cAMP comparative binding assays of urea-treated R-subunit and non-urea-treated R-subunit at 4 and 30 °C, since cAMP bound to the R-subunit will not exchange with [3H]cAMP at 4 °C.

**RESULTS**

In order to study the effect of changes in the autoinhibitor site of the R1'-subunit on the capacity of the R-subunit to form a stable complex with the C-subunit, Ala-97 in the R1'-subunit was replaced with Arg, Lys, His, and Gin by site-directed mutagenesis, and Ser-99 was replaced with Gly and Lys. These mutations were designed to determine the steric and charge constraints that are imposed on this segment of the protein. The wild type and mutant R1'-subunits were approximately 95% pure after elution from a DE32 column, with a typical yield of 100–140 mg of protein/4 liters of cells. Fig. 2 shows an SDS-polyacrylamide gel of the total cell extract and
FIG. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified R'-subunits. E. coli 222 was transformed with pUC18 containing either wild type or mutant R' inserts. Wild type (3), A97R (4), A97K (5), A97H (6), A97Q (7), S99K (8), and S99G (9) proteins were purified as described under “Experimental Procedures.” Total cell extract of wild type R' (2) and E. coli 222 (1) are also shown.

FIG. 3. Effect of nucleotides on holoenzyme formation at pH 6.5. Wild type R'-dimer (1.5 μM) and C-subunit (2 μM) were combined and dialyzed in the presence of 100 μM MgATP (O—O) and 1 mM AMP (O—O) and in the absence of nucleotide (O—O). Either A97R (upper panel), A97K (middle panel), or A97Q (lower panel) mutant R'-dimer (1.5 μM each) was also incubated with C-subunit (2 μM) in the presence of 100 μM MgADP (O—O), 100 μM MgADP (O—O), and in the absence of nucleotide (O—O). The percentage activity remaining represents the ratio of catalytic activity present in the absence of cAMP to the activity with a large excess of cAMP at the indicated time.

FIG. 4. Holoenzyme formation of wild type and A97H R' at pH 6.0 (upper panel) and pH 7.9 (lower panel). Wild type R'-dimer (1.5 μM) and C-subunit (2 μM) were combined and dialyzed in the presence of 100 μM MgATP (O—O) and 1 mM AMP (O—O) and in the absence of nucleotide (O—O). A97H dimer (1.5 μM) was also incubated with C-subunit (2 μM) in the presence of 100 μM MgATP (O—O), 100 μM MgADP (O—O—O—O—O), and in the absence of nucleotide (O—O—O—O—O). The percentage activity remaining is the same as in Fig. 3. Holoenzyme formation was complete after 6 h, and it was facilitated significantly in the presence of MgATP. In contrast, none of the mutants reassociated with the C-subunit completely over the 75-h period. For each of the mutant R'-subunits, MgATP actually reduced the rate at which holoenzyme formed compared to the reaction in the absence of nucleotides. This was especially evident with A97R, where no holoenzyme was formed when MgATP was present.

Since this segment of the autoinhibitor site lies close to the γ-phosphate of MgATP in the holoenzyme complex, the effects of MgADP or AMP on facilitating reassociation were investigated. Such an interaction would be particularly relevant in the case of the Lys and Arg substitutions where a positive charge is actually introduced into the autoinhibitor site. The holoenzyme formation between wild type R'-subunit and the C-subunit occurred at a similar rate in the presence of MgADP and MgATP (data not shown). In the case of A97R, MgADP totally abolished reassociation (Fig. 3, upper panel). Compared with no nucleotides, the presence of MgADP did not affect the holoenzyme formation of A97K and A97Q (Fig. 3, middle and lower panel). In contrast to the wild type R'-subunit, where AMP had no effect, AMP actually did enhance the reassociation of A97K and A97Q with the C-subunit (Fig. 3, middle and lower panel).

By replacing Ala-97 with His, it was possible to determine to what extent the nucleotide effect described above was dependent on the charge at residue 97. As seen in Fig. 4, A97H also did not form holoenzyme well compared to wild type R'-subunit. In addition, the stimulatory effect of AMP and the inhibitory effect of MgATP were observed at pH 6.0 (Fig. 4, upper panel) in a manner quite analogous to A97K and A97Q. However, at pH 7.9 where the His presumably has no charge, nucleotides had no effect on holoenzyme formation (Fig. 4, lower panel).

The capacity for the C-subunit to promote the release of [3H]cAMP from the R'-dimer also was measured. The cAMP-free wild type and mutant R-dimers were saturated with [3H]cAMP and then mixed with a 10-fold excess of C-subunit in
the presence of MgATP. Reassociation of the C-subunit and the R'-dimer induced the release of $[^3H]cAMP$ from the ($[^3H]cAMP)_2$-$R'_2$ complex. As shown in Fig. 5, $[^3H]cAMP$ dissociated completely from wild type $R'_2$ within 1 min after mixing with the C-subunit, while all of the R' mutants still had bound $[^3H]cAMP$ after 2 h, indicating that the C-subunit did not reassociate to a significant extent with any R'-mutants over this 2-h period. These data suggest that each of the Ala-97 mutants had a reduced affinity for the C-subunit, although a quantitative measure of the decreased affinity of the different mutants for the C-subunit could not be established under the experimental conditions.

In contrast to measuring the reassociation of the C-subunit with cAMP-saturated R-subunit as seen in Figs. 3 and 4, where holoenzyme formation takes a relatively long time because of the tendency for cAMP to rebind to the R-subunit rather than diffuse out of the dialysis bags, each wild type and mutant R-subunit was first stripped of cAMP and then assayed for its capacity to inhibit C-subunit phosphotransferase activity as described under "Experimental Procedures." Each cAMP-free R'-dimer was preincubated with the C-subunit in the presence of MgATP. The capacity for the C-subunit to phosphorylate peptide substrate was then determined. The inhibition of the C-subunit by each R-subunit is shown in Fig. 6 (upper panel) as a function of $R'_2$-dimer concentration. The concentration of $R'_2$-dimer required for 50% inhibition of C-subunit phosphotransferase activity, the I$_{50}$, was calculated from Hill plots (29). These apparent I$_{50}$ values, summarized in Table I, also indicated that all of the mutant R-dimers had reduced affinity (14- to 55-fold) for the C-subunit, with A97R showing the greatest effect.

Although Ser-99 is 2 residues removed from the actual pseudophosphorylation site, Ala-97, it is invariant in all R'-subunits and is presumably in close proximity to the R-C interaction site. This conserved Ser was replaced with Gly and Lys in order to determine whether it is involved in hydrogen bonding or whether it has steric constraints similar to Ala-97. Holoenzyme formation between the CAMP-saturated R'-subunit and the C-subunit also was measured in the presence and absence of MgATP (Fig. 7). Unlike the Ala-97 mutant R'-subunits previously described, MgATP facilitated holoenzyme formation for both S99G and S99K. S99G reassociated with the C-subunit completely but at a much slower rate than wild type R' (Fig. 7, upper panel). However, S99K only partially inhibited the C-subunit activity after 3 days of incubation (Fig. 7, lower panel). The inhibition of catalytic activity by the Ser-99 mutants also was examined following treatment with urea to dissociate the bound cAMP (Fig. 6, lower panel). Based on their I$_{50}$ value (Table I), S99G and S99K had 7-fold and 24-fold reduced affinity for the C-subunit compared with wild type R', respectively. The MgATP binding properties of the S99G holoenzyme were also determined. This mutant holoenzyme binds MgATP with a high affinity ($K_d = 12.3$ nM), which is indistinguishable from the wild type holoenzyme ($K_d = 12.7$ nM).

**TABLE I**

<table>
<thead>
<tr>
<th>$R'_2$ dimer</th>
<th>I$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.98 ± 0.28</td>
</tr>
<tr>
<td>A97Q</td>
<td>14.1 ± 1.4</td>
</tr>
<tr>
<td>A97H</td>
<td>23.8 ± 1.2</td>
</tr>
<tr>
<td>A97R</td>
<td>31.4 ± 1.4</td>
</tr>
<tr>
<td>A97R</td>
<td>55.6 ± 5.0</td>
</tr>
<tr>
<td>S99G</td>
<td>7.55 ± 0.78</td>
</tr>
<tr>
<td>S99K</td>
<td>24.4 ± 2.4</td>
</tr>
</tbody>
</table>

**FIG. 6. Inhibition of C-subunit activity by wild type and mutant R-subunits.** C-subunit (1 nM) was preincubated for 5 min at 30 °C with varying concentrations of either wild type (○), A97Q (○-○), A97H (○-○), A97K (○-○), A97R (○-○), S99G (○-○), or S99K (○-○) $R'_2$-dimer. Kemptide was added to initiate the reaction. After 10 min at 30 °C, the reaction was stopped, and the phosphorylation of Kemptide was measured. 100% activity corresponds to 2.45 μmol·mg$^{-1}$·min$^{-1}$ phosphotransferase activity.

**DISCUSSION**

The activity of cAMP-dependent protein kinase is regulated by the reversible binding of the R-subunit to the C-subunit, and it has been shown that the autoinhibitor site of the R-subunit is required for recognition by the C-subunit. As summarized in Table II, the autoinhibitor site contains an amino acid sequence similar to those found in C-subunit substrates and inhibitors (32-34). Evidence for the essential nature of the autoinhibitor site for recognition by the C-subunit comes from limited proteolysis (35). The amino terminus can be cleaved up to the two key arginines in the autoinhibitor site without significantly impairing R-C interaction. Cleavage just
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Beyond the 2 arginines, however, yields a monomeric cAMP binding protein that no longer forms a tight complex with the C-subunit (36). As seen in Table II, a primary distinguishing feature of the type I and type II R-subunits is that Rᵢ-subunits contain an autophosphorylation site in this region, while Rᵢ-subunits have a pseudophosphorylation site (Table II). Autophosphorylation of the Rᵢ-subunit at this site can occur as an intramolecular event in the holoenzyme complex and does not require dissociation of the R- and C-subunits (21), indicating unequivocally that the autoinhibitor site actually occupies the substrate binding site of the C-subunit in the holoenzyme complex. When Ala 97 in the autoinhibitor site is changed to Ser, the mutant holoenzyme also can be autophosphorylated at this Ser by an intramolecular mechanism (22), suggesting that the side chain of residue 97 in the autoinhibitor site is close to the γ-phosphate of ATP in the MgATP-holoenzyme complex.

Recombinant techniques were used here to characterize the reversible binding of the Rᵢ-subunit to the C-subunit. In order to further probe the steric and charge constraints at the 97 position (Table II), Ala-97 in the Rᵢ-subunit was replaced with Arg, Lys, His, and Gln. The comparative sizes of side chains at position 97 in the wild type and mutant proteins are shown in Fig. 8. The mutant protein with the largest side chain substitution, A97R, had the highest IC₅₀ value or the lowest affinity for the C-subunit (Table I). Overall, the IC₅₀ values of the Ala-97 mutants increased as the size of the side chains increased, indicating that not much space exists at the active site of the C-subunit to accommodate side chains larger than Ala. Previous results where Ala-97 was replaced with Ser are also consistent with these findings. Like the above mutants, the A97S R-subunit formed holoenzyme more slowly than the wild type R-subunit, and phosphorylation of Ser-97 slowed holoenzyme formation even further (22).

While the IC₅₀ value measures the affinity of the C-subunit for cAMP-free R-subunit, the formation of holoenzyme in vivo involves reassociation of the C-subunit with cAMP-saturated R-subunit (26). Holoenzyme formation for the wild type Rᵢ-subunit is facilitated by MgATP, presumably because the binary complex of MgATP and the C-subunit has a higher affinity for the cAMP-saturated R-subunit than does the free C-subunit (37). In contrast, reassociation of all of the cAMP-saturated mutant R-subunits with the C-subunit was inhibited by MgATP (Figs. 3 and 4). The rate of holoenzyme formation correlated well with the size of the side chain at residue 97 (Figs. 3 and 4), where mutants with smaller side chains had a greater capacity to form a stable complex with the C-subunit. These results are very likely due to steric effects, where MgATP in the active site of the C-subunit partially obstructs the binding of the mutant R-subunits with larger side chains at residue 97. In the case of A97H, holoenzyme formation formed very poorly compared to wild type R-subunit under conditions where the His side chain presumably has a positive charge (pH 6.0) and at pH 7.9, where it should be neutral (Fig. 4). Thus, it appears that steric factors are a greater impediment to holoenzyme formation than the presence of a positive charge in these mutant R-subunits.

The mutant R-subunits that might introduce a charge near the α- or β-phosphate of the bound nucleotide in the C-subunit could have the potential to ion pair with smaller nucleotides such as AMP and ADP. In this way, reassociation of the mutant R- and C-subunits could be facilitated by the presence of AMP and MgADP, even though these nucleotides do not play this role in the formation of wild type holoenzyme. Three of the mutants, A97K, A97H, and A97R, introduce a positive charge. Gln is not charged but does have the potential for hydrogen bonding. When Ala-97 is changed to Lys or Glu, holoenzyme formation is accelerated in the presence of AMP; however, AMP does not have any effect on the holoenzyme formation of A97R (Fig. 3). Perhaps the most revealing mutant is A97H, because its side chain can exist in either a protonated or nonprotonated state. With this mutant, AMP stimulated holoenzyme formation at pH 6.0, but not at pH

![Fig. 7. Holoenzyme formation of wild type Rᵢ, S99G (upper panel), and S99K (lower panel) at pH 6.5.](image)

Time(h)

% Activity Remaining

0 20 40 60 80 100

S99G

S99K

TABLE II

Sequences of the autoinhibitor sites in various regulatory subunits

<table>
<thead>
<tr>
<th>Nomenclature for autoinhibitor site</th>
<th>n⁻²</th>
<th>n⁻¹</th>
<th>n⁻⁰</th>
<th>n⁰</th>
<th>n¹</th>
<th>n²</th>
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<tbody>
<tr>
<td>RI (bovine skeletal muscle, Ref. 14)</td>
<td>Arg</td>
<td>Arg</td>
<td>Gly</td>
<td>Ala</td>
<td>Ile</td>
<td>Ser</td>
</tr>
<tr>
<td>RI (mouse brain, Ref. 8)</td>
<td>Arg</td>
<td>Arg</td>
<td>Gly</td>
<td>Gly</td>
<td>Val</td>
<td>Ser</td>
</tr>
<tr>
<td>RI (Dictostelium discoideum, Ref. 11)</td>
<td>Arg</td>
<td>Arg</td>
<td>Gly</td>
<td>Ala</td>
<td>Ile</td>
<td>Ser</td>
</tr>
<tr>
<td>RI (Drosophila, Ref. 41)</td>
<td>Arg</td>
<td>Arg</td>
<td>Gly</td>
<td>Gly</td>
<td>Val</td>
<td>Ser</td>
</tr>
<tr>
<td>RI (Chromobactis elegans, Ref. 13)</td>
<td>Arg</td>
<td>Arg</td>
<td>Thr</td>
<td>Gly</td>
<td>Ile</td>
<td>Ser</td>
</tr>
<tr>
<td>RI (yeast, Ref. 12)</td>
<td>Arg</td>
<td>Arg</td>
<td>Thr</td>
<td>Ser</td>
<td>Val</td>
<td>Ser</td>
</tr>
<tr>
<td>RI (bovine cardiac muscle, Ref. 15)</td>
<td>Arg</td>
<td>Arg</td>
<td>Val</td>
<td>Ser</td>
<td>Val</td>
<td>Cys</td>
</tr>
<tr>
<td>RI (rat skeletal muscle, Ref. 9)</td>
<td>Arg</td>
<td>Arg</td>
<td>Val</td>
<td>Ser</td>
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<td>Cys</td>
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<td>RI (mouse brain, Ref. 9)</td>
<td>Arg</td>
<td>Arg</td>
<td>Val</td>
<td>Ser</td>
<td>Val</td>
<td>Cys</td>
</tr>
<tr>
<td>RI (rat ovarian granulosa cells, Ref. 10)</td>
<td>Arg</td>
<td>Arg</td>
<td>Ala</td>
<td>Ser</td>
<td>Val</td>
<td>Cys</td>
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7.9 (Fig. 4). Therefore, the A97H mutant reinforces the hypothesis that the side chain of residue 97 probably stretches deep enough into the ATP binding site of the C-subunit to interact either electrostatically or by hydrogen bonding with the phosphate of AMP.

When MgATP is bound to the C-subunit, interaction with the mutant protein containing the bulkiest side chain, A97R, is essentially abolished. In vivo, one would predict that the free C-subunit always exists as a binary complex with either MgATP or MgADP. Thus, this mutant R-subunit, A97R, should act in vivo as an essentially irreversible inhibitor or "sink" for cyclic nucleotide, since cAMP release is mediated by interaction with the MgATP-C-subunit complex (37).

Yeast contains three C-subunit isozymes and a single gene, BCY1, encoding for the R-subunit. The yeast R-subunit has a domain structure similar to the mammalian R-subunit, with two tandem cAMP binding sites and a Ser at the hinge region. Kuret et al. (29) reported that mutations at the autophosphorylation site, Ser-145, altered the affinity of the yeast R-subunit for the yeast C-subunit. Replacement of Ser-145 with smaller Ala or Gly created mutant R-subunits with higher affinities for the yeast C-subunit. When Ser-145 was changed to larger residues such as Lys, Thr, Glu, and Asp, the mutant R-subunits became worse inhibitors of the yeast C-subunit. It was concluded that a small amino acid at position 145 facilitated R-C interaction. This conclusion is consistent with the results here that large residues at the n° position of the R-subunit interfere with holoenzyme formation. However, the IC_{50} values of the wild type and mutant R'-subunits here are lower than those measured for the yeast R-subunit. The apparent differences in IC_{50} between the yeast and bovine R'-subunits may be due to the way in which the R-subunits interact with their respective C-subunit. Kinetic studies showed that the mammalian C-subunit had a K_{m} value for Kemptide that was 20-fold lower and a IC_{50} value for the protein kinase inhibitor that was 30-fold lower than the comparable kinetic constants for the yeast C-subunit (38). Since Kemptide and the protein kinase inhibitor both have the sequence, Arg-Arg-X-Ser/Ala, which is similar to the autoinhibitor sites in the R-subunits, the lower IC_{50} values for the bovine R'-subunit proteins may reflect a greater capacity for the mammalian C-subunit to bind these particular substrates and inhibitors compared to the yeast C-subunit. In addition, the studies in this paper showed that the side chain of amino acid 97 in R' could have either beneficial or detrimental interactions with the bound nucleotides in the holoenzyme complex.

Chemical modification of the R'-subunit with iodosacetic acid identified Cys-97, which is 2 residues beyond the autophosphorylation site (the n° position), as potentially being involved in the interaction with the C-subunit (23). Cross-linking of the R'-subunit with the C-subunit also demonstrated that Cys-97 could form an interchain disulfide bond with Cys-199 of the C-subunit (24), which was shown independently to be close to the active site (23, 39, 40). In the R'-subunit, Ser-99 occupies the equivalent position as Cys 97 in the R'-subunit. As seen in Table II, this Ser is conserved in all the R'-subunits, and thus it was suggested that it may play an essential role in R'-function. Therefore, Ser-99 in bovine R' was subjected to site-directed mutagenesis to examine its role in R-C interaction.

The IC_{50} values of S99G and S99K were 7- and 24-fold higher than wild type R' (Table I), respectively, indicating that neither could form a complex with the C-subunit as well as the wild type R'-subunit. The effect of those mutations on reassociation with cAMP-saturated R-subunit was even more pronounced, particularly in the absence of MgATP. The S99G mutant, for example, inhibited the C-subunit activity completely in the presence of MgATP but formed holoenzyme worse than any of the Ala-97 mutants in the absence of MgATP (Fig. 7, lower panel), suggesting that large side chains at the residue 99 position may introduce steric problems at the R-C interface. The fact that MgATP facilitated holoenzyme formation for both Ser-99 mutants compared to the absence of nucleotide, and that wild type holoenzyme and S99G holoenzyme had the same affinity for MgATP (K_{d} = 12 nM), indicate that Ser-99 does not interfere with MgATP binding in the holoenzyme complex. This is in clear contrast to mutations at position 97, where MgATP sterically inhibited holoenzyme formation. As seen in Table II, either Ala, Gly, or Ser is found in all the R-subunits at the position corre-
the steric constraints seem to be substantial because very little space exists in the holoenzyme complex to accommodate amino acid residues with large side chains.

...responding to Ala-97 in bovine R', and substituting any larger amino acid for Ala-97 adversely affects binding to the C-subunit. Thus, at the position in the R' autoinhibitor site, any amino acid for Ala-97 adversely affects binding to the C-subunit. Thus, at the amino acid for Ala-97 adversely affects binding to the C-subunit. Thus, at the amino acid for Ala-97 adversely affects binding to the C-subunit.

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**REFERENCES**