One Amino Acid Change Produces a High Affinity cGMP-binding Site in cAMP-dependent Protein Kinase*

(Received for publication, May 16, 1990)

John B. Shabb, Lilly Ng, and Jackie D. Corbin

From the Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Discrimination between cAMP and cGMP is a critical feature of cAMP- and cGMP-dependent protein kinases. An alanine/threonine difference in the cyclic nucleotide-binding sites has been proposed to provide a structural basis for this functional distinction. Site-directed mutagenesis of this alanine to a threonine in a cAMP-binding site of cAMP kinase produced a mutant with markedly increased cGMP affinity as determined by cGMP binding and protein kinase activation assays. Studies of other mutants at this position support the role of the threonine hydroxyl group as the component that enhances cGMP binding activity.

cAMP and cGMP regulate many cellular processes in eukaryotes by activating cAMP- and cGMP-dependent protein kinases (cAMP and cGMP kinases) (1). Although both cyclic nucleotides can activate either enzyme, cAMP kinase has 200-fold greater affinity for cAMP than for cGMP whereas cGMP kinase has the inverse selectivity (2, 3). Structures of the two cAMP-binding sites in each of the mammalian cAMP kinase isozymes types Ia and IIa have been predicted based on homologies with the bacterial cAMP-binding catabolite gene activator protein (4) for which the crystal structure is known (5) (Fig. 1, top). In these models, key arginine, glutamic acid, and glycine residues make contacts with the cyclic nucleotide ribose phosphate moiety. Mutagenesis of these residues has confirmed their importance in the cAMP-binding sites (6–10). Mammalian cGMP kinase binding sites have also been predicted to maintain the same interactions (11). The CAMP and cGMP kinase binding sites are distinguished by an alanine/threonine difference that has been predicted to be critical for determining cAMP/cGMP binding specificity (11). The threonine hydroxyl group in cGMP kinase binding sites is proposed to hydrogen-bond with the guanine 2-amino group of cGMP (Fig. 1, bottom). No such interaction can occur at the corresponding cAMP kinase alanine residue. The importance of this alanine/threonine difference in determining cAMP/cGMP binding selectivity was studied using site-directed mutagenesis to alter one of the two cAMP-binding sites in the regulatory (R) subunit of cAMP kinase.

MATERIALS AND METHODS

Mutagenesis and Expression—The wild-type R1 bacterial expression vector contains cDNA for the bovine testis type Ia R subunit (gift from G. S. McKnight, University of Washington, Seattle, WA) inserted into the polylinker of pUC13 (12). A 323-base pair EcoRI fragment containing part of the B domain coding sequence was subcloned into the polylinker of M13mp8. A synthetic oligonucleotide (5'-CACGTTGGCAGTACGGGGCCG-3') complementary to the cDNA coding strand, except for the underlined bases, was generated to alter the Ala-334 codon (GCA) to one coding for threonine (ACT). Mutagenesis was performed according to a commercially available kit (oligonucleotide-directed in vitro mutagenesis version 2, code RPN1523 (Amersham Corp.)) and confirmed by dideoxynucleotide DNA sequence analysis. The expression vector was reconstructed with the mutated EcoRI fragment and transformed into Escherichia coli strain TG1 cells. One liter of 2 X TY medium (10 g Bacto-tryptone, 10 g Bacto-yeast extract, 5 g NaCl in 1 liter H2O) containing 50 µg of ampicillin/ml was inoculated with early log phase cultures (10 ml, A590 = 0.3) of cells expressing wild-type or A334T R subunits, and incubated at 37 °C with shaking until the A590 was 0.8. Five ml of 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and incubation was continued for another hour. Cells were harvested by centrifugation at 5000 × g for 20 min. Approximately 0.4–0.8 mg of R subunit/liter of culture was expressed under these conditions.

Partial Purification—All steps were performed at 0–4 °C. Bacteria were resuspended in 40 ml of KPEM (10 mM potassium phosphate, 1 mM Na2EDTA, 2 mM 2-mercaptoethanol, pH 6.8), repelleted at 4000 × g for 10 min, and stored at −70 °C. Cells were placed in a Bead Beater (Biospec Products) containing a slurry of 40 ml of KPEM and 50 mM benzamidine (KPEMB) and 40 ml of 0.1-M glass beads and homogenized with four 30-s bursts at 1-min intervals. The extract was clarified twice by centrifugation at 12,000 × g for 30 min, adjusted to a conductivity of 6 mMH0, and loaded onto a 5-ml DEAE-Sephadex column equilibrated in KPEM. The column was washed with 50 ml of KPEM, eluted with KPEMB containing 0.2 M NaCl, and 2-ml fractions were collected. One ml of pooled cAMP binding activity was fractionated on a 0.9 × 40-cm Sephadex G-100 column equilibrated with KPEM, 0.2 M NaCl, and 0.5-ml fractions were collected. Fractions containing the leading peak of cAMP binding activity were pooled. This procedure was sufficient to remove endogenous catabolite gene activator protein since it is a dimer of two 22.5-kDa subunits that does not bind to DEAE-cellulose at pH 7.7 (13), as opposed to the dimeric 49-kDa type I R subunit that binds to DEAE-cellulose at pH 6.8 (1). R subunit represented about 0.4–1.4% of the total protein in these preparations, based on [3H]cAMP binding activity.

Other Mutants—A334S and A334G were generated, expressed, and partially purified as described above, except the A334S oligonucleotide was 5'-CACGGTGGCAAGAACCGGGCCG-3', which changes position 334 to a guanine (G). Cyclic Nucleotide Dissociation Assays—About 100 nM R subunit was incubated in the presence of 1 µM [3H]cAMP (1.5 × 105 cpm/µl) (Amersham Corp.) for 45 min at 25 °C in a buffer containing 2 mM NaCl and 0.5 mg/ml histone IIAs as described previously (14). Total [3H]cAMP bound to the enzyme was determined and then a 100-fold molar excess of unlabeled cAMP was added. Incubation was continued at 25 °C, and at each time point the amount of bound [3H]cAMP was determined by processing an aliquot using the potassium phosphate-nitrocellulose filter binding method (14). cGMP dissociation was measured using the same conditions as for cAMP except 20 nM R subunit was incubated with 8 µM [3H]cGMP (9.3 ×
CAMP- and cGMP-binding Sites of Protein Kinases

CAMP-BINDING SITE (CA KINASE)
cGMP-BINDING SITE (cG KINASE)

**FIG. 1.** Predicted protein-cyclic nucleotide contacts for CAMP (C) and cGMP (C) kinases. The models are valid for both fast and slow sites (4, 11). Proposed hydrogen bonds are indicated by dotted lines. The proposed interaction that defines cyclic nucleotide binding specificity is highlighted by the shaded area.

 covert pmol (cpm) (Amersham Corp.). Aliquots were processed by the ammonium sulfate-nitrocellulose filter binding method (15) except that filters were washed with a total of 15 ml of 95% ammonium sulfate in 50 mM potassium phosphate, pH 6.8.

**Protein Kinase Activation Assays**—10 μl containing 0.022 pmol of bovine heart catalytic (C) subunit (16) and sufficient R subunit to inhibit approximately 85% of the total protein kinase activity (0.24-0.33 pmol, determined by [3H]cAMP binding activity) was incubated in the presence of 50 μl (20 mM Tris, pH 7.4, 0.1 mM ATP, 20 mM magnesium acetate, 0.5 mg/ml bovine serum albumin, and 0.1 mM 1-methyl-3-isobutylxanthine) for 10 min at 30 °C to allow association. 10 μl containing 0.5 mM [3H]ATP (1 × 10^6 cpm) (Du Pont-New England Nuclear), 0.5 mg/ml of the synthetic peptide substrate Kemptide (Peninsula Labs), and increasing amounts of CAMP or cGMP were added. Incubation was continued for 10 min at 30 °C, stopped by spotting 50 μl onto phosphocellulose paper, and processed as described previously (17).

**RESULTS AND DISCUSSION**

The CAMP kinase R subunit slow site was chosen for mutagenesis because CAMP dissociation from this site is significantly slower than from the fast site, making it more amenable to biochemical characterization. The codon for Ala-334 in the cDNA for bovine type I R subunit was altered to a codon for threonine (A334T) by oligonucleotide-directed mutagenesis. Expression of wild-type and A334T cDNAs was driven by the IPTG-inducible lac Z promoter of the pUC13 plasmid transformed into the lac repressor overproducing E.

**FIG. 2.** Detection of partially purified wild-type and A334T R subunits. 80 nM (10 μl) Sephadex G-100 fractionated wild-type or A334T R subunit was incubated with 1.5 μM (1 μCi) 8-azido-[3H]cAMP (ICN) for 30 min on ice in the dark, exposed to UV light for 30 min, subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel, and autoradiographed (25). Native R is the purified bovine lung type I R subunit.

cGMP dissociation rates were measured for the wild-type and A334T R subunits (Fig. 3, bottom). Dissociation of [3H]cGMP from the wild-type R subunit was too rapid to distinguish its fast and slow components at 25 °C, consistent with the low affinity of cGMP for this protein. However, two components were readily discernible for A334T. Dissociation of [3H]cGMP from A334T was 2 orders of magnitude slower (Table I) than that from the wild-type R subunit (Fig. 2).

**RESULTS AND DISCUSSION**

The CAMP kinase R subunit slow site was chosen for mutagenesis because CAMP dissociation from this site is significantly slower than from the fast site, making it more amenable to biochemical characterization. The codon for Ala-334 in the cDNA for bovine type I R subunit was altered to a codon for threonine (A334T) by oligonucleotide-directed mutagenesis. Expression of wild-type and A334T cDNAs was driven by the IPTG-inducible lac Z promoter of the pUC13 plasmid transformed into the lac repressor overproducing E.

**RESULTS AND DISCUSSION**

The CAMP kinase R subunit slow site was chosen for mutagenesis because CAMP dissociation from this site is significantly slower than from the fast site, making it more amenable to biochemical characterization. The codon for Ala-334 in the cDNA for bovine type I R subunit was altered to a codon for threonine (A334T) by oligonucleotide-directed mutagenesis. Expression of wild-type and A334T cDNAs was driven by the IPTG-inducible lac Z promoter of the pUC13 plasmid transformed into the lac repressor overproducing E.

**RESULTS AND DISCUSSION**

The CAMP kinase R subunit slow site was chosen for mutagenesis because CAMP dissociation from this site is significantly slower than from the fast site, making it more amenable to biochemical characterization. The codon for Ala-334 in the cDNA for bovine type I R subunit was altered to a codon for threonine (A334T) by oligonucleotide-directed mutagenesis. Expression of wild-type and A334T cDNAs was driven by the IPTG-inducible lac Z promoter of the pUC13 plasmid transformed into the lac repressor overproducing E.
CAMP- and cGMP-binding Sites of Protein Kinases

Fig. 3. Wild-type and A334T R subunits are distinguished by their cGMP dissociation characteristics. [3H]cAMP and [3H]cGMP dissociation curves for each R subunit were measured as described under "Materials and Methods." B_0 = total amount of [3H]-labeled cyclic nucleotide bound to the R subunit. B = amount of bound [3H]-labeled cyclic nucleotide remaining at time (t) after addition of a 100-fold molar excess of unlabeled cyclic nucleotide. At 50% saturation with [3H]-labeled cyclic nucleotide, In(B/B_0) = -0.69. This is a representative experiment.

Fig. 4. A334T holoenzyme is activated at lower concentrations of cGMP than is wild-type CAMP kinase. Protein kinase activity of native C subunit associated with recombinant R subunit was measured in the presence of increasing concentrations of cyclic nucleotide as described under "Materials and Methods." 100% protein kinase activity was determined in the presence of R subunit at concentrations of cyclic nucleotide sufficient to attain a maximum activity that was consistently higher than an equal amount of free C subunit, possibly because of a stabilizing effect of R subunit on the C subunit. Hill plots (insets) were plotted to determine K_m values (x intercept). γ is the fraction of protein kinase activity at a given concentration of cyclic nucleotide relative to maximally activated protein kinase. This is a representative experiment.

Table I

| R subunit | Slow site t, | nM | cAMP | cGMP | cAMP | cGMP |
|-----------|--------------|----|------|------|------|------|
| Wild type | 27 ± 4       | 0.55 ± 0.05 | 0.021 ± 0.002 | 4.1 ± 0.4 |
| A334T     | 30 ± 6       | 63 ± 9     | 0.028 ± 0.002 | 0.25 ± 0.02 |
| A334S     | 23 ± 2       | 27 ± 3     | 0.033 ± 0.002 | 0.85 ± 0.1  |
| A334G     | 4.8 ± 0.1    | 0.33 ± 0.08 | 0.13 ± 0.01 | 9.7 ± 0.1  |

Values for cAMP and cGMP slow site dissociation t, values are ± S.E., n = 3, and were determined from results like those in Fig. 3. K_m values are ± S.E., n = 4, and are calculated from results like those in Fig. 4.

These results are consistent with a very high affinity binding of cGMP to the A334T slow site. A334T may surpass cGMP kinase in this respect for two reasons. First, the cAMP-binding sites of CAMP kinase may be inherently better designed for cyclic nucleotide binding, and the addition of a strong positive cGMP binding determinant in A334T might make it a more ideal cGMP site than either site in cGMP kinase. Second, interaction between the CAMP kinase R and C subunits is known to cause at least a 200-fold increase in the exchange rate of CAMP (19). This same phenomenon may also occur with cGMP kinase, but while cyclic nucleotide dissociation from the R subunit can be measured in the absence of C subunit interaction, this cannot be effectively accomplished with cGMP kinase, which has fused regulatory and catalytic components.
Protein kinase activation constants ($K_a$) were also measured as another indication of relative cyclic nucleotide binding affinities. An 11-12-fold molar excess of either wild-type or A334T R subunits was required to inhibit 85% of native bovine heart cAMP kinase C subunit activity. The resultant inactivated holoenzymes were used to determine the $K_a$ values for cAMP and cGMP (Fig. 4). $K_a$ determinations were not greatly affected by the excess R subunit since a 5-fold increase of R subunit over the standard amount elevated the apparent $K_a$ for cAMP or cGMP by only 25%. Wild-type and A334T holoenzymes had similar $K_a$ values for cAMP, which were within the reported range of 0.008-0.06 μM (8, 10, 20–22). In contrast, cGMP $K_a$ values indicated that A334T had a 16-fold higher affinity for cGMP (Table I). Although this difference was substantial, it was less than the 115-fold difference between their slow site cGMP dissociation rates. The $K_a$ represents an average of cyclic nucleotide affinities for the slow (mutated) and fast (nonmutated) sites (21) and could account, in part, for this discrepancy. Thus, the lower $K_a$ for cGMP of A334T relative to wild-type R subunit was largely due to a marked decrease in the rate of dissociation of this cyclic nucleotide from the slow site of the mutated protein.

The binding affinity of cGMP for a CAMP kinase depended upon the amino acid that occupied position 334 (Table I). Substitution of a serine (A334S) produced a slow site dissociation rate for cGMP that was 50-fold slower than that of the wild-type slow site, but half that of A334T. The intermediate cGMP binding activity was also reflected in the $K_a$ values of this mutant. Since serine also has a hydroxyl group, its interaction with cGMP may be similar to that of threonine, albeit with a slightly reduced affinity. Like A334T, A334S did not show an appreciable change in its interaction with cAMP relative to the wild-type protein. On the other hand, substitution of Ala-334 with glycine (A334G) was deleterious to both cGMP and cAMP binding. This may be due to the flexible nature of glycine in the peptide backbone, which could allow conformational distortion of the slow site. Decreased cAMP interaction of this mutant extended to the fast site, even though the mutation was only in the slow site (fast site $t_c$, in min ± S.E., n = 3: wild type = 3.8 ± 0.32; A334G = 1.3 ± 0.15). This observation was consistent with other studies which demonstrated that mutations in one R subunit binding site affect the CAMP binding properties of the nonmutated site (8–10).

Other studies of CAMP kinase CAMP-binding site mutants have identified amino acid residues that, when changed, either decreased or altogether eliminated CAMP binding (6–10, 22). In contrast, the mutations at Ala-334 provide the first examples of either an increase in cyclic nucleotide interaction or an alteration in the specificity of CAMP kinase in a predictable amino acid-dependent manner. Site-directed mutagenesis of this amino acid residue further supports the importance of hydrogen bonding potential near the 2-position of the purine moiety for determining cAMP/cGMP binding specificity of cyclic nucleotide-dependent protein kinases. The alanine/threonine difference described here was an apparently critical step during the evolutionary divergence of these two kinases. Together with changes in the catalytic domains, the mutation would explain the emergence of separate physiological functions for CAMP and cGMP kinases.

Acknowledgments—We appreciate the helpful suggestions of Drs. Terry Woodford, Luis Guerra-Santos, and Irene Weber and the critical reading of this manuscript by Drs. Irene Weber, Tom Soderling, and Sharron Francis.

REFERENCES
1. Beebe, S. J., and Corbin, J. D. (1986) in The Enzymes (Boyer, P. D., and Krebs, E. G., eds) Vol. XVII, pp. 43–111, Academic Press, New York.
2. Deskeland, S. O., Øgreid, D., Ekanger, R., Sturø, P. A., Miller, J. P., and Suva, R. H. (1985) Biochemistry 24, 1094–2001.
3. Corbin, J. D., Øgreid, D., Miller, J. P., Suva, R. H., Jastorff, B., and Deskeland, S. O. (1986) J. Biol. Chem. 261, 1208–1214.
4. Weber, I. T., Steitz, T. A., Bubis, J., and Taylor, S. S. (1987) Biochemistry 26, 343–350.
5. Weber, I. T., and Steitz, T. A. (1987) J. Mol. Biol. 198, 311–326.
6. Bubis, J., Neitzel, J. J., Saraswat, L. D., and Taylor, S. S. (1988) J. Biol. Chem. 263, 9668–9673.
7. Øgreid, D., Deskeland, S. O., Gorman, K. B., and Steinberg, R. A. (1988) J. Biol. Chem. 263, 17397–17404.
8. Steinberg, R. A., Russell, J. L., Murphy, C. S., and Yphantis, D. A. (1987) J. Biol. Chem. 262, 2664–2671.
9. Kuno, T., Shuntoh, H., Sakaue, M., Saijoh, K., Takeda, T., Fukuda, K., and Tanaka, C. (1988) Biochem. Biophys. Res. Commun. 153, 1244–1250.
10. Woodford, T. A., Correll, L. A., McKnight, G. S., and Corbin, J. D. (1989) J. Biol. Chem. 264, 13321–13328.
11. Weber, I. T., Shabb, J. B., and Corbin, J. D. (1989) Biochemistry 28, 6122–6127.
12. Wolfe, L., Woodford, T. A., Francis, S. H., and Corbin, J. D. (1990) in Activation and Desensitization of Transducing Pathways (Konijn, T. M., Houslay, M. D., and Van Haastert, P. J., eds) NATO ASI Series, Vol. H 44, pp. 133–148, Springer-Verlag, Berlin.
13. Anderson, W. B., Schneider, A. B., Emmer, M., Perlman, R. L., and Pastan, I. (1971) J. Biol. Chem. 246, 5929–5937.
14. Rannels, S. R., and Corbin, J. D. (1986) Methods Enzymol. 99, 168–175.
15. Deskeland, S. O., and Øgreid, D. (1988) Methods Enzymol. 159, 147–160.
16. Flockhart, D. A., and Corbin, J. D. (1984) in Brain Receptor Methodologies (Marangos, P. J., Campbell, I. C., and Cohen, R. M., eds) Part A, pp. 209–215, Academic Press, Orlando, FL.
17. Roskoski, R., Jr. (1983) Methods Enzymol. 99, 3–6.
18. Rannels, S. R., and Corbin, J. D. (1980) J. Biol. Chem. 255, 7050–7058.
19. Chau, V., Huang, L. C., Romero, G., Biltonen, R. L., and Huang, C. A. (1980) Biochemistry 19, 924–928.
20. Kuno, T., Shuntoh, H., Takeda, T., Ito, A., Sakaue, M., Hirai, M., Ando, H., and Tanaka, C. (1989) Eur. J. Pharmacol. 172, 263–271.
21. Øgreid, D., Deskeland, S. O., and Miller, J. P. (1983) J. Biol. Chem. 258, 1041–1049.
22. Bubis, J., Saraswat, L. D., and Taylor, S. S. (1988) Biochemistry 27, 1570–1576.
23. Robinson-Steiner, A. M., Beebe, S. J., Rannels, S. R., and Corbin, J. D. (1984) J. Biol. Chem. 259, 10596–10605.
One amino acid change produces a high affinity cGMP-binding site in cAMP-dependent protein kinase.

J B Shabb, L Ng and J D Corbin

J. Biol. Chem. 1990, 265:16031-16034.

Access the most updated version of this article at http://www.jbc.org/content/265/27/16031

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/27/16031.full.html#ref-list-1