Isoleucine 368 is involved in low-affinity binding of *N*⁶-modified cAMP analogues to site B of the regulatory subunit of cAMP-dependent protein kinase I

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The regulatory (R) subunit of cAMP-dependent protein kinase has a well-defined domain structure including the two in-tandem cAMP-binding sites that constitute the C-terminus of the protein. The N-terminal binding site (A) has a considerably higher affinity for analogues of cAMP that are substituted with bulky and hydrophobic substituents at the 6-amino group of the adenine ring compared to the affinity observed at the second site (B). On the basis of the crystal structure of the catabolite gene activator protein from *Escherichia coli*, molecular modelling of the binding domains suggested that a tyrosine (Y244) in site A could be involved in a high-affinity hydrophobic interaction, whereas a corresponding isoleucine (I368) in domain B could lead to steric hindrance in the binding of bulky *N*⁶-substituted

analogues. Site-directed mutagenesis was used to construct mutations in Y244 and I368. Binding displacement experiments showed that replacing the tyrosine in site A with isoleucine (Y244I) did not affect the interaction of either *N*⁶-substituted or otherwise modified analogues with this site. However, replacing I368 with tyrosine (I368Y) led to a 3–4-fold increase in affinity for those *N*⁶-modified analogues that had a hydrophobic group attached directly or close to the 6-amino molecule. We conclude that I368 is involved in the molecular interaction between binding domain B and the 6-amino group of the adenine moiety of cAMP and that this residue is partly responsible for the reduced affinity of *N*⁶-substituted cAMP analogues for this site.

INTRODUCTION

cAMP-dependent protein kinase (cAPK) is the major allosteric receptor for cAMP in eukaryotic cells (reviewed in [1,2]). The protein consists of several subunits and domains. A homodimer of regulatory subunits (R₂) and two catalytic subunit monomers (C) form the holoenzyme complex R₂C₂. R and C have a high association affinity (*K*_d 10 nM) in the presence of MgATP [3]. The cooperative binding of four cAMP molecules (Hill coefficient 1.6) to the regulatory subunit dimer (R₂) causes a conformational change in each R monomer, which in turn leads to dissociation of the holoenzyme, thus releasing the catalytically active C subunits [4].

There are two types, RI and RII, of regulatory subunit in mammalian tissues. Recent studies have also shown that multiple subforms (α , β) of each R subunit exist [2]. They differ in amino acid sequence, capacity of autophosphorylation and tissue distribution [5–7]. Both main types of regulatory subunit monomer consist of several domains. The N-terminus is the site of inter-subunit interaction. In RI α from bovine cardiac muscle this interaction is mediated by two disulphide bonds between residues C16 and C37 of each chain [8]. The N-terminus is followed by a proteolytically sensitive hinge region which also carries the site of R/C interaction, including the consensus sequence RRXA [5]. The type II regulatory subunit carries the sequence RRXS and is autophosphorylated at the serine residue, indicating that the

hinge region must occupy the peptide binding site of the C subunit in the holoenzyme complex [9]. In bovine RI α , the two in-tandem cAMP binding domains named A and B start at residue 143. The N-terminal site A (residues 143–260) is primarily characterized by a fast exchange of cAMP, whereas the C-terminal site B (residues 260–379) shows a much slower exchange of cyclic nucleotides [10,11].

The two cAMP binding sites also differ with respect to their preferences and relative affinities for analogues of cAMP. In general, modifications at position C-2 or C-8 of the adenine ring favour binding to site B of RI and RII whereas *N*⁶-modifications increase both affinity and relative preference for sites AI and AII [12,13]. Among *N*⁶-modified analogues is a group of compounds with aromatic or bulky hydrophobic substituents directly attached to the 6-amino group of the purine ring that have an unusual high affinity for sites AI and AII. In particular, *N*⁶-phenyl-cAMP shows an 18-fold and 40-fold increase (compared with cAMP) in binding affinity to sites AI and AII respectively, which can be explained only by additional interaction(s) of the aromatic substituent and one or several appropriate amino acid side chain residue(s).

To identify the amino acid residue(s) responsible for the observed differences between domains A and B by *N*⁶-modified cAMP analogues, we used molecular model building of the R subunit binding domains based on the crystal structure of the homologous cAMP-binding protein (CAP) in *Escherichia coli*.

Abbreviations used: A, N-terminal binding site of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; CAP, cAMP-binding protein; cAPKI and cAPKII, cAMP-dependent protein kinase types I and II; CPK model, Corey–Pauling–Koltun model; DTE, dithioerythritol (2,3-dihydroxybutane 1,4-dithiol); *K*_i, apparent equilibrium inhibition constant; RI α , RI α , RI β and RII β , isoforms of the regulatory subunits of cAMP-dependent protein kinase.

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The modelling suggested to us that a tyrosine in domain A could explain high-affinity binding by stacking interactions between two aromatic rings. For domain B, the corresponding residue was isoleucine, a residue that could be involved in steric hindrance of analogue binding according to the model.

Using oligodirected mutagenesis we introduced complementary mutations in both binding domains to test this explanation. Binding experiments showed that changing the tyrosine in site A did not influence analogue binding, whereas substitution of I368 in site B for tyrosine led to a selective increase in affinity for *N*⁶-modified analogues with aromatic or bulky substituents attached directly or close to the 6-amino group.

EXPERIMENTAL

Materials

Cyclic [5',8-³H]AMP (56 Ci/mmol) and [γ -³²P]ATP (2200 Ci/mmol) were from Amersham International. BSA, PMSF, isopropyl- β -D-thiogalactopyranoside, the phosphate acceptor heptapeptide ('kemptide', LRRASLG) and the nucleotides ATP, cAMP, *N*⁶-benzoyl-cAMP and 8-amino-cAMP were obtained from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). 8-Piperidino-cAMP and *N*⁶-phenyl-cAMP were generously supplied by Dr. J. P. Miller and Dr. R. H. Suva (Life Sciences Division, SRI International, Menlo Park, CA, U.S.A.). The origin and synthesis of the remaining cyclic nucleotides have been described or referred to previously [12]. Antipain, chymostatin, leupeptin and pepstatin were from the Peptide Institute (4-1-2 Ina, Minoshi, Osaka 652, Japan). Enzymes used in DNA manipulations were obtained from either New England Biolabs, Inc., or Boehringer Mannheim and used in accordance with the manufacturer's specifications. All other reagents were of analytical grade.

Site-directed mutagenesis

A full-length bovine RI α -cDNA, lacking the internal *Eco*RI site and cloned into pUC 18, was obtained from Dr. Susan Taylor [14,15]. The *Eco*RI-digested fragment of this plasmid, pUC18-RI α , was isolated from agarose gels. The 1155-bp fragment containing the full-length RI α clone was subjected to mutagenesis as described below. The 19-base oligonucleotides shown in Figure 1 were synthesized on an Applied Biosystems DNA synthesizer, purified on preparative 20% (w/v) polyacrylamide gel, and phosphorylated for use as hybridization probes and DNA primers as described by Zoller and Smith [16]. Standard techniques were used to obtain mutated phage DNAs that were identified by plaque hybridization [17,18].

Isolation of mutant clones

Transformed *E. coli* JM101 was then grown at 37 °C on Luria broth agar plates containing 50 μ g/ml ampicillin. Single-stranded DNA was isolated by the method of Schreier and Cortese [19] and sequenced by the dideoxy method of Sanger et al. [20]. Double-stranded phage DNA was purified from positive mutant plaques by the alkaline lysis method described by Maniatis et al. [21]. The DNA from a single positive phage was then digested with *Eco*RI, and the resulting 1155-bp fragment, which contained the complete sequence coding for the RI α -subunit, was ligated back into the *Eco*RI site of the multiple cloning site of the pUC 18 plasmid. The ligated DNAs were used to transform *E. coli* JM101. Plasmids isolated from individual colonies grown on Luria broth agar plates were checked for correct orientation of inserts. The resulting single-mutation-containing plasmids were pUC-Y244I, pUC-G323E and pUC-I368Y. The fourth plasmid, pUC-R209K, was generously supplied by Dr. Susan

Taylor. These four plasmids were used to construct two double-mutant-containing plasmids.

Construction of double-mutant plasmids

The four plasmids described above contained a single substitution in one cAMP binding site (A or B) of RI. These plasmids were enzymically manipulated to construct new plasmids, each having mutations in both sites A and B. In brief, plasmids pUC-Y244I and pUC-G323E were digested with restriction enzymes *Bsu*36I and *Nsi*I. A 659-bp fragment containing mutant site A from pUC-Y244I and a 3164-bp fragment containing mutant site B plus pUC 18 from pUC-G323E were isolated on a 1% (w/v) agarose gel. These two fragments were treated with DNA ligase, resulting in a double-mutation-containing plasmid, pUC-Y244I/G323E. Similarly, pUC-R209K and pUC-I368Y were treated to obtain the second double-mutant plasmid, pUC-R209K/I368Y.

Expression of single- and double-mutant RI subunits

The four single-mutant RI proteins were expressed by transforming *E. coli* 222 with the four plasmids pUC-Y244I, pUC-G323E, pUC-I368Y and pUC-R209K. For the expression of double-mutant enzymes, *E. coli* 222 was transformed with the ligation mixture of the double-mutant plasmids. Individual colonies were selected by *Bsu*36I and *Nsi*I restriction digests of purified plasmids, which were then sequenced to confirm the presence of two mutations. Colonies from both single and double mutants were screened for the expression of R subunits by either of the following approaches.

(1) Because *E. coli* 222 harbours a mutation in adenylate cyclase, the level of endogenous cAMP in such cells will be low. Therefore cAMP binding capacity could be measured directly in the total cell extracts. Briefly, 1 ml of overnight bacterial culture was centrifuged (6000 *g* for 6 min) and dissolved in 500 μ l of Hepes buffer (15 mM Hepes/NaOH, pH 7.0, 5 mM EDTA, 0.3 mM EGTA). After the addition of 400 μ g of lysozyme, the cell walls were disrupted by a mechanical homogenizer for 10 s. Soluble cell extracts were collected by centrifuging at 9000 *g* for 10 min. For cAMP binding, 50 μ l of solution B (60 mM Hepes/NaOH, pH 7.2, 60 mM EDTA, 1.2 mM 3-isobutyl-1-methylxanthine, 0.2 mM adenosine, 1 mM dithioerythritol (DTE) and 50 μ l of solution E (2.4 μ M cAMP, 97% non-radioactive and 3% ³H-labelled) was added to 250 μ l of supernatant and incubated for 30 min at 37 °C. The binding reaction was quenched by adding 2 ml of 3.8 M (NH₄)₂SO₄. The reaction mixture was then filtered and counted as previously described [22,23].

(2) Expressed mutant R subunits could also be directly observed on polyacrylamide gels containing SDS after electrophoresis [24] of total cell extracts and staining with Coomassie Blue R-250.

Purification of mutant regulatory subunits

Luria broth (20 ml) containing ampicillin was individually inoculated with each single- and double-mutant plasmid in *E. coli* 222 and grown at 37 °C until *D*₅₅₀ reached 0.1–0.5. Luria broth (4 litres) was then inoculated with 10 ml of the original culture and incubated for approx. 16 h. The cells were centrifuged and the resulting pellet was resuspended in lysis buffer (25 mM K₂HPO₄, pH 6.4, 5 mM EDTA, 5 mM EGTA, 10 mM 2-mercaptoethanol). Purification was by the method described previously [22,25] and the purified protein was analysed by SDS/PAGE.

cAMP exchange rates

Measurement of cAMP exchange rates for mutant RI subunits was by the method of Ringheim and Taylor [25]. Briefly, after incubation for 5 min at 0 °C in Hepes buffer (5 mM 2-mercaptoethanol, 60 mM Hepes/NaOH, pH 7.0, 60 mM EDTA, 1 mM DTE, 0.5 mg/ml BSA) containing 1 mM [³H]cAMP, purified proteins (200 nM) were added to 900 µl of non-radioactive exchange buffer (15 mM Hepes/NaOH, pH 7.0, 60 mM EDTA, 0.3 mM EGTA, 1 mM DTE, 5 mM 2-mercaptoethanol, 0.5 mg/ml BSA, 100 µM cAMP, 3.2 M NaCl) at 30 °C. Aliquots were removed at various times, precipitated with ammonium sulphate and filtered as described above [22,23,25].

cAMP analogue affinities for binding sites of mutant RI-proteins

The affinity of cAMP analogues for sites A and B was deduced from the abilities of the analogues to compete with [³H]cAMP for binding [12]. Routinely, the incubations (0.05 ml) were performed at 37 °C in 15 mM Hepes/NaOH, pH 7.0, with 0.15 M KCl, 5 mM EDTA, 20 mM 2-mercaptoethanol, 2 mg/ml BSA and 2 mg/ml of a heat-stable protein fraction. Aliquots were removed after 20 and 45 min of incubation. The concentration of each mutant RI protein was 3–6 nM (with respect to cAMP binding sites), and the concentration of [³H]cAMP was 60 nM. Each analogue was tested at 10–15 concentrations covering a 500- to 16000-fold concentration range. Determination of the amount of labelled nucleotide bound to sites A and B of R proteins and the calculation of kinetic constants (K_i values) were as described [26]. The relative affinity is expressed as $K'_i(\text{analogue}) = K_i(\text{cAMP})/K_i(\text{analogue})$, i.e. the ratio between the apparent inhibition constant for cAMP and the analogue.

RESULTS

Construction and expression of mutant plasmids

The two residues that were predicted to interact with N⁶-modified cAMP-analogues, Y244 and I368 of the RI subunit, were selectively changed to Y244I and I368Y respectively, with the synthetic oligonucleotide probes shown in Figure 1. After the M13mp9 clones that contained the mutations had been identified by hybridization and sequencing, the region coding for the R subunit was excised and religated into pUC 18 as described in the Experimental section.

Because R subunits have two different cAMP binding domains that interact with each other during cyclic nucleotide binding and activation [22], we decided to construct double-mutant enzymes with a modification in the second site that greatly reduced or abolished cAMP binding. For binding domain A, substitution of R209 for lysine apparently abolishes high-affinity cAMP-binding [27]. For site B, we introduced another modification (glutamic acid for glycine in G323) that also apparently abolishes cAMP

binding [26]. After identification and sequencing as described above, G323E-containing plasmid was excised and religated into pUC 18.

The double mutants Y244I/G323E and R209K/I368Y were constructed by cutting with appropriate restriction endonucleases (*Bsu*36I and *Nsi*I) and religating the appropriate fragments.

Previous experiments [14] have shown that *E. coli* strain 222 is a better candidate for R subunit expression than strain JM 101. Therefore *E. coli* 222 was transformed with individual mutant plasmids, and transformants were screened for cAMP binding capacity (indicating the level of high-affinity cAMP binding sites) as well as being subjected to SDS/PAGE analysis. Addition of isopropyl-β-D-thiogalactopyranoside caused decreased expression of mutant R subunits. Moreover the proteins accumulated in the largest amounts in the stationary phase of growth. A maximal expression of 2–10 mg of mutant R subunit per litre of culture was estimated on the basis of cAMP binding capacity (results not shown).

Purification of mutant RI subunits

To characterize the mutant proteins, expressed single- and double-mutant RI subunits were partly purified as described in the Experimental section. When harvested cells were disrupted in a French pressure cell, more than 90 % of the expressed RI subunits were found in the soluble fraction. Expressed proteins were subsequently purified by DE-52 cellulose and G-25 gel-filtration chromatography. Up to 30–40 mg of RI protein could be isolated from 4 litres of culture. The resulting proteins migrated as a single band with a molecular mass of approx. 48 kDa in SDS/PAGE gels containing 2-mercaptoethanol. As judged by Coomassie Blue staining, purified proteins were found to be approx. 60–70 % pure. For a few of the mutant enzymes, some degradation occurred during purification. Kinetic experiments, however, revealed no difference between preparations that showed degradation and preparations that did not (results not shown).

cAMP exchange rates

For determining the affinity of selected analogues for sites A and B of mutant R subunits, we had to determine cAMP binding to each of the two sites separately. For wild-type RI subunit, separate binding to one site can be determined by following the exchange rate of cAMP [22]. Site A shows a much faster exchange of cAMP than site B, and the difference is largest with high concentrations of chaotropic salts in the dissociation buffer [22,23]. By following the exchange rate and subtracting cAMP bound to site B from total cAMP binding, it was possible to determine selectively how much cAMP was bound to site A.

All single- and double-mutant enzymes were saturated with labelled cAMP and the exchange was started by adding excess unlabelled cAMP as explained in the Experimental section. Routinely, experiments were performed at 30 °C with 3.2 M NaCl in the exchange buffer.

Wild-type recombinant RI subunit showed biphasic exchange with half-lives of 95 s and 80 min respectively for sites A and B (Table 1). For both Y244I and I368Y, biphasic exchanges were observed with rate kinetic constants that did not differ from those observed for wild-type protein (Table 1). This indicates that neither of the two mutations introduced disturbs the exchange of cAMP from sites A or B. R209K and G323E, however, showed a rapid monophasic dissociation (half-lives of 14 min and 20 s respectively), indicating that cAMP did not bind to site A (R209K) or site B (G323E) under the experimental

- (a) 5'-A CTC CTC AAT CAT CTT CCG-3'
- (b) 5'-C GAT CTC TTC GAA GTA GTC-3'
- (c) 5'-A CTG CTG GTA GTT GCG CTT-3'

Figure 1 Oligonucleotides used for mutating Y244 to isoleucine (a), G323 to glutamic acid (b) and I368 to tyrosine (c)

Table 1 Exchange rate of cAMP from wild-type and mutant regulatory subunits

Regulatory subunits were saturated with labelled cAMP and then subjected to exchange in a buffer with 100 μ M unlabelled cAMP and 3.2 M NaCl at 30 °C as described in the Experimental section. Aliquots were removed after various times and the remaining nucleotide bound was determined after ammonium sulphate precipitation [22]. Half-times of dissociation are shown for the various enzymes made. A dash means that no binding to the mutated site was detected.

Enzyme	$t_{1/2}$, site A (s)	$t_{1/2}$, site B (min)
Wild-type	95	80
R209K	—	14
I368Y	120	80
R209K/I368Y	—	16
Y244I	85	75
G323E	20	—
Y244I/G323E	15	—

Table 2 Relative affinities and selectivities of cAMP analogues for sites A and B of wild-type regulatory subunits

The affinities of cAMP analogues for site A and B of wild-type regulatory subunits were deduced from the abilities of the analogues to compete with [3 H]cAMP for binding as described in the Experimental section. The relative affinity for a binding site (A, A*, B, B*, where an asterisk denotes a mutated site) is expressed as $K_i(\text{cAMP})/K_i(\text{analogue})$, i.e. the ratio between the apparent inhibition constants for cAMP and for the analogue. The site selectivity (A/B) is the ratio between the affinities for site A and B respectively. Values for wild-type enzyme are taken from Øgreid et al. [11].

cAMP analogue	A	B	Selectivity A/B
cAMP	1.00	1.00	1.00
6-NH-C ₆ H ₅	18	0.48	38
6-NH-t-C ₃ H ₇	4.10	0.063	65
6-NH-C(O)C ₆ H ₅	3.50	0.190	19
6-NH-CH(CH ₃)(CH ₂) ₂ C ₆ H ₅	2.90	0.037	78
6-NH-CH(CH ₃)CH ₂ C ₆ H ₅	2.30	0.056	41
8-Piperidino-cAMP	2.30	0.065	2.90
1- <i>N</i> ⁶ -Etheno-cAMP	0.32	0.11	35
8-NH ₂ -cAMP	0.150	3.90	1/26
8-NH-CH ₃ -cAMP	0.070	3.30	1/47
2-Cl, 8-NH-CH ₃ -cAMP	0.040	6.70	1/168

Table 3 Relative affinities and selectivities of cAMP analogues for sites A and B of mutant regulatory subunits

As for Table 2 except that the results are for single- and double-mutant enzymes with the Y244I substitution. For a direct comparison between wild-type and mutant sites, the ratios A*/A and B*/B have been calculated. Abbreviations used: WT, wild-type; n.d., not determined.

cAMP analogue	Mutant enzyme Y244I				Mutant enzyme G323E		Mutant enzyme Y244I/G323E	
	A*	B*	A*/A (WT)	B*/B(WT)	A*	A (WT)	A*	A*/A(WT)
cAMP	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
6-NH-C ₆ H ₅	9.90	0.44	0.55	0.92	12.50	0.72	13	0.72
6-NH-t-C ₃ H ₇	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6-NH-C(O)C ₆ H ₅	2.65	0.22	0.77	1.18	4.52	1.33	4.30	1.22
6-NH-CH(CH ₃)(CH ₂) ₂ C ₆ H ₅	n.d.	n.d.	n.d.	n.d.	3.30	1.14	4.40	1.50
6-NH-CH(CH ₃)CH ₂ C ₆ H ₅	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-Piperidino-cAMP	1.84	0.048	0.80	0.74	3.70	1.60	2.60	1.10
1- <i>N</i> ⁶ -Etheno-cAMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8-NH ₂ -cAMP	0.055	4.60	1.38	0.69	0.25	1.60	0.28	1.87
8-NH-CH ₃ -cAMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Cl, 8-NH-CH ₃ -cAMP	0.066	2.20	0.94	0.67	0.060	1.50	0.030	0.75

conditions used (Table 1). For both double-mutant enzymes, similar rapid monophasic exchanges were observed (Table 1). We also tested cAMP dissociation with lower salt concentration (0.15 M NaCl) in the exchange buffer and observed that site A in general exchanged more slowly, whereas site B exchanged faster (results not shown). The single exception was the R209K enzyme, which did not exchange faster from site B with 0.15 M NaCl in the buffer [27].

We conclude that, under the experimental conditions used, we were able to separately determine cAMP binding to sites A and B of Y244I and I368Y respectively, and that there was no detectable binding to either site A in R209K and R209K/I368Y or to site B in G323E and Y244I/G323E.

Affinity of cAMP analogues for sites A and B of mutant R subunits

The affinities of 10 selected cAMP analogues for sites A and B of mutant enzymes were determined and standardized against the affinities of cAMP for the same sites [$K_i(\text{analogue}) = K_i(\text{cAMP})/K_i(\text{analogue})$]. Table 2 shows the affinities for sites A and B of wild-type RI protein, whereas affinity data for RI mutants Y244I, G323E and Y244I/G323E are shown in Table 3. Table 4 shows the equivalent affinities for mutants I368Y, R209K and I368Y/R209K. The binding constants for wild-type R subunit have been published previously [11] and are shown here to facilitate a direct comparison with mutant enzymes.

For mutant proteins we have indicated a mutated site by an asterisk (Tables 3 and 4). For a direct comparison with the wild-type protein, we show the ratios between the affinity for a mutated and a normal site respectively. Owing to the small amounts of some of the analogues used, we were not able to determine affinities for all mutant enzymes made.

For Y244I, *N*⁶-phenyl-cAMP bound with a relative affinity of 50 % of that for wild-type site A (Table 3). The other compounds tested bound with affinities ranging from 80 % to 140 % of that of wild-type site A. For site B of Y244I, the relative affinities were similar to those previously determined for wild-type enzyme site B.

Enzyme G323E has previously been shown to have no residual binding to site B [26]. This was confirmed by rate-kinetic experiments (Table 1). The relative affinities determined for G323E were close to those previously determined for site A of the wild-type enzyme (Table 3).

Table 4 Relative affinities and selectivities of cAMP analogues for sites A and B of mutant regulatory subunits

As for Table 2 except that results are for single- and double-mutant enzymes with the I368Y mutation. For a direct comparison between wild-type and mutant sites, the ratios A*/A and B*/B have been calculated. Abbreviations used: WT, wild-type; n.d., not determined.

cAMP analogue	Mutant enzyme I368Y				Mutant enzyme R209K/I368Y		Mutant enzyme R209K	
	A*	B*	A*/A(WT)	B*/B(WT)	B*	B*/B(WT)	B*	B*/B(WT)
cAMP	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
6-NH-C ₆ H ₅ -cAMP	16.5	1.84	0.92	3.85	2.30	4.80	0.42	0.87
6-NH-t-C ₄ H ₉ -cAMP	4.60	0.19	1.12	3.00	0.21	3.33	n.d.	n.d.
6-NH-C(O)C ₆ H ₅ -cAMP	4.50	0.57	1.29	3.10	0.66	3.57	0.24	1.29
6-NH-CH(CH ₃)(CH ₂) ₂ C ₆ H ₅ -cAMP	3.20	0.051	1.10	1.37	0.031	0.84	n.d.	n.d.
6-NH-CH(CH ₃)CH ₂ C ₆ H ₅ -cAMP	2.20	0.068	1.00	1.21	0.068	1.21	n.d.	n.d.
1-N ⁶ -Etheno-cAMP	0.59	0.11	1.84	1.00	0.090	0.82	n.d.	n.d.
8-Piperidino-cAMP	1.96	0.043	0.85	0.66	0.085	1.31	0.076	1.17
8-NH ₂ -cAMP	n.d.	n.d.	n.d.	n.d.	2.51	0.65	n.d.	n.d.
8-NH-CH ₃ -cAMP	n.d.	n.d.	n.d.	n.d.	2.20	0.67	n.d.	n.d.
2-Cl, 8-NH-CH ₃ -cAMP	0.028	5.60	0.70	0.84	4.00	0.60	5.30	0.79

Introduction of the Y244I mutation into site A of G323E did not significantly influence the relative affinities compared with either wild-type site A or with those found in the singly mutated G323E enzyme (Table 3).

For enzyme I368Y, however, we observed that some analogues showed a 3–4-fold enhancement of relative affinity over the wild-type enzyme (Table 4). This was true for *N*⁶-phenyl-cAMP, *N*⁶-*t*-butyl-cAMP and *N*⁶-benzoyl-cAMP, whereas all other analogues, including some *N*⁶-modified analogues, did not show variation in affinities significantly different from the wild-type enzyme.

R209K showed affinities identical with those observed previously for site B of the wild-type enzyme. Introducing the mutation I368Y into R209K, however, confirmed that the substitution of isoleucine for tyrosine increased the relative affinity for those analogues with a benzene ring attached directly to or close to the 6-amino group of the adenine ring and for *N*⁶-*t*-butyl-cAMP (Table 4). All other analogues bound to the mutated site with affinities similar to those previously found in the wild-type enzyme.

DISCUSSION

The present work confirms that I368 in cAMP-binding domain B of RI interacts specifically with hydrophobic substituents directly linked to the 6-amino group of the adenine ring of cAMP.

Basic knowledge about the architecture of the cAMP binding sites was originally derived from the 2.5 Å crystal structure of CAP from *E. coli* [28]. Both cAMP-binding domains A and B of R subunits show extensive sequence homology with the cAMP binding domain of CAP and thus a three-dimensional model for each cAMP binding site has been constructed [29]. The general features of this model (using bovine RI α) include: (1) an invariant arginine (R209 in site A, or R333 in site B) that in CAP chelates to one exocyclic oxygen of the phosphate moiety; (2) an invariant glutamic acid (E200 and E323 in sites A and B respectively) that in CAP hydrogen-bonds to the 2'-hydroxy group of cAMP; and (3) interaction of Y371 at the inward-facing surface of a long α -helix with the adenine ring of cAMP in site B. The validity of this model as a general framework for the folding of the polypeptide chain in both cAMP binding sites was later confirmed by photoaffinity labelling experiments [30,31] and by isolating forms

of the R subunit that are defective in their cAMP binding properties [26,32].

To address more specifically the question of which residue(s) are important for adenine-ring interactions, we constructed a complete Corey–Pauling–Koltun (CPK) atomic model of RI site B based on the CAP model. Site A was constructed by remodelling site B (substituting some residues that were different between the two sites). According to these models, each cAMP binding site consists of two clearly distinguishable super-secondary structural elements. First, there is a barrel of β -sheets that accommodate the invariant residues essential for recognizing the phosphate (R209/R333) and ribose (E200/E323) of cAMP. Secondly, the β -barrel is flanked at the C-terminus by a helix–loop–helix motif. The second helix (C-helix) is rather extended (27 Å) and displays an amphipathic character (Figure 2). The amphipathic region of the C-helices for site A and B differ in length. Amphipathicity was found for the helix of site B (Figure 2, right) between residues 362 and 379 (18 residues equal four helix turns) and for site A between residues 241 and 252 (3.5 helix turns) (Figure 2, left). A hydrophobic region faces the interior of the protein, which is the barrel structure. The outside of the C-helix is fairly hydrophilic but lacks the characteristic pairs of acidic and basic residues.

The residue previously shown to be important for adenine binding of cAMP (Y371) is located at the hydrophobic side of the C-helix (Figure 2, right). In CAP, the corresponding residue is T127, which hydrogen-bonds to the adenine *N*⁶-amino group [28]. From cAMP-analogue mapping studies, it is known that no hydrogen-bonds are involved in recognizing and binding the adenine moiety of cAMP [12,33,34]. Those studies revealed a strong hydrophobic character of the adenine binding pocket.

The exact geometrical position of the helix with respect to the adenine ring is ambiguous, however. According to the CAP model, Y371 approaches the adenine ring but the residue is located one helix turn below with respect to T127 of CAP. Model building with CPK atoms also revealed that cAMP can more easily be accommodated by moving the helix one turn upward to bring Y371 and the adenine ring into closer contact. Another model prediction suggests that site A actually moves the C-helix three turns upwards [35].

To test the validity of our model, we analysed the mutants I368Y and Y244I with binding of cAMP analogues modified at various positions of the adenine ring and substituents of various

Figure 2 C-helices of binding site A (A) and B (B)

The amphipathic character is shown for the C-helices of cAMP binding sites A and B. Hydrophobic residues facing the front of the helix are encircled. The helices extend from residues 241–260 and 361–379 for sites A and B respectively.

lengths and bulkiness. N^6 -phenyl-cAMP, N^6 -benzoyl-cAMP and N^6 -t-butyl-cAMP showed a significant increase in affinities (3–4-fold) for the I368Y mutant compared with the affinities for wild-type site B (Table 4). The increase in affinities was similar for mutant enzymes that had a non-mutated site A as well as a site A where cAMP binding could apparently not occur (R209K, Table 4). This additional affinity was only observed with analogues that had hydrophobic or aromatic substituents directly attached to the N^6 atom. None of N^6 -[(1-methyl,3-phenyl)-propyl]-cAMP, N^6 -[(1-methyl,2-phenyl)ethyl]-cAMP or 1, N^6 -etheno-cAMP showed affinities that differed from those found for wild-type site B (Table 4). No variance in affinities was observed for otherwise modified cAMP analogues.

The results obtained clearly correspond to predictions made with the CAP model for site B. Obviously, I368 is an important residue within the binding pocket of cAMP. Our initial suggestion was that I368 might show steric hindrance for N^6 -substituted cAMP analogues and thus explain the decreased affinity observed for this site. The results show that I368 is directly involved in building that part of the binding pocket that provides the micro-environment necessary for recognizing the adenine moiety of cAMP. Because the effect of the mutation is lower than expected (3–4-fold) compared with the wild-type site A (18-fold), other, so far unidentified, residues must be involved in the interaction with the phenyl group or the t-butyl substituent. The results obtained with other N^6 -modified cAMP analogues, where the aromatic ring is further distant (Table 4), support this idea.

In contrast, the corresponding mutation in site A did not follow the predictions made. Thus N^6 -modified cAMP analogues with bulky or aromatic substituents still showed a high affinity for the mutated site (Y244I) (Table 3). This apparent contradiction can be explained as follows: every model deduced from the CAP structure was originally based on the co-ordinates for cAMP binding site B [29]. As observed in the present study, the direct application of this model to site A has not always been successful. For example, 8- N_3 -cAMP labelling of the wild-type regulatory subunit identified Y371 of site B as being in close proximity to the adenine ring. The corresponding residue, F247 in site A, was not labelled but rather W260 [29]. By using a deletion mutant (Δ 260–379) that had the entire B domain removed, residue Y244 (one turn down the helix) was labelled by the azido probe [30].

The CPK-derived model for site A predicted that Y244 was in close proximity to the adenine ring of cAMP. Because our experiments verified the predictions made for site B but not for site A, we conclude that residue Y244 does not necessarily contribute to the high-affinity binding of N^6 -phenyl-substituted cAMP analogues to site A. These results, as well as the inability of 8- N_3 -cAMP to label F247 or F244 when site B is present, indicate that site B is disturbing the geometry of site A to an extent that makes it difficult to predict structural details of site A on the basis of the CAP model.

Molecular modelling and model-building of the binding sites, followed by engineering of mutants by site-directed mutagenesis, have unravelled some of the molecular mechanisms responsible for site-selective analogue binding in site B. When combined with analysis of the three-dimensional structure of the R subunit [36], such studies should provide further insights into the molecular interactions underlying the action of cAMP.

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