8-Substituted cAMP Analogues Reveal Marked Differences in Adaptability, Hydrogen Bonding, and Charge Accommodation between Homologous Binding Sites (AI/AII and BI/BII) in cAMP Kinase I and II†

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ABSTRACT: cAMP analogues, systematically substituted at position 8 of the adenine moiety (C8), were tested quantitatively for binding to each cAMP interaction site (A and B) of the regulatory subunits of cAMP-dependent protein kinase type I (RI) and II (RII). Site AII did not accommodate cAMP analogues with any bulk at position 8, whereas site AI accepted even bulky 8-substituents. This implies that the narrow, buried pocket of site AI facing position C8 of cAMP in the RI-cAMP crystal [Su, Y., Dostmann, W. R., Herberg, F. W., Durick, K., Xuong, N. H., Ten Eyck, L., Taylor, S. S., and Varughese, K. I. (1995) *Science 269*, 807−813] must undergo considerable conformational change and still support high-affinity cAMP analogue binding. The B sites of RI and RII differed in three respects. First, site BI had a lower affinity than site BII for cAMP analogues with hydrophobic, bulky 8-substituents. Second, site BI had a preference for substituents with hydrogen bonding donor potential close to C8, whereas site BII had a preference for substituents with hydrogen bonding acceptor potential. This implies that Tyr^{371} of RI and the homologous Tyr^{379} of RII differ in their hydrogen bonding preference. Third, site BI preferred analogues with a positively charged amino group that was an extended distance from C8, whereas site BII discriminated against a positive charge. The combined results allow refinement of the cAMP binding site geometry of RI and RII in solution, and suggest design of improved isozyme-specific cAMP analogues.

Cyclic AMP (Figure 1) is used by nature in several ways to promote signaling. In enteric coliform bacteria, cAMP regulates gene expression through binding to a catabolite repressor protein (*2*). In eukaryotes, cAMP binds to and activates the cAMP-dependent protein kinase (cAK) and the newly discovered cAMP-activated guanine nucleotide exchange factors (*3*−*5*). Other receptors for cAMP include the cyclic nucleotide-gated olfactory ion channel (*6*) and the chemotactic cAMP receptor of *Dictyostelium discoideum* (*7*, *8*). Despite the multitude of cAMP receptors, cAK isozyme I and II mediate most known effects of cAMP in mammalian cells (*9*, *10*). The mammalian isozymes are tetramers composed of a regulatory subunit (R) dimer and two catalytic subunits (C).[1] RI and RII carry two in-tandem cAMP binding sites termed AI and BI, and AII and BII, respectively. Cyclic AMP must bind to both site A and site B to induce efficient activation of cAK under physiologically relevant conditions (*9*, *10*). Each R subunit exists in two genetic forms, α and *β*, which differ very little in the cAMP binding domains (*11*).

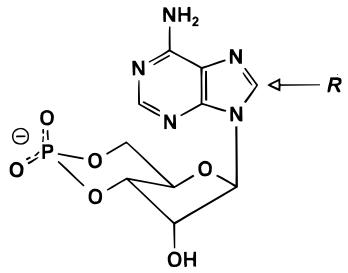

FIGURE 1: Chemical structure of the cAMP molecule. The arrow points to the C8 position, in which various substituents (R) were introduced to produce the compounds (**1a**−**i**, **2a**−**e**, and **3a**−**g**) that were studied.

The recent crystal structure elucidation of (Δ1−91)RIα with cAMP liganded to both site AI and site BI (*1*) supported and explained the finding from earlier studies, based on cAMP analogue binding to RI, that site AI differed from site BI by generally accepting N^6-substituents of the adenine ring of cAMP (*9*, *10*). In the X-ray analysis of this crystal, the N^6 position of cAMP is exposed in AI, but blocked in site BI. On the other hand, the crystal structure indicated that position C8 of cAMP is freely accessible when bound to site BI, but blocked by Trp^{260}, Val^{182}, and Val^{192} when

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[1] Abbreviations: cAKI and cAKII, cAMP-dependent protein kinase isozymes I and II, respectively; RI and RII, regulatory subunits of cAKI and cAKII, respectively; C, catalytic subunit of cAK. The abbreviations used for the cyclic nucleotide analogues are given in Tables 1 and 2.

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bound to AI. This was taken to support the view that 8-substituted cAMP analogues are barred from site A, but bind well to site B of both RI and RII (*1*). In contrast, an early study reported that some 8-substituted cAMP analogues bind better to site AI than to site BI (*12*). This study aims to clarify this point. For this purpose, a number of new 8-substituted cAMP analogues were designed and synthesized. In addition, some previously studied cAMP analogues were resynthesized and isolated to higher purity than before. The analogues were designed to probe the importance of bulk, hydrophobicity, and hydrogen bonding potential close to position C8, and the effect of a positive charge at various distances and positions relative to C8. The cAMP affinity of each analogue was determined in absolute terms (equilibrium dissociation constants) for sites AI, BI, AII, and BII.

It will be shown that sites AI and AII, as well as BI and BII, possess distinct differences with respect to tolerance of bulk, positive charge, and preference for hydrogen bonding interaction potential of 8-substituted cAMP analogues. A consequence of the findings is that the subsite of AI facing position C8 of cAMP, which is buried in the crystal structure (*1*), must be able to expand and assume conformations quite different from that in the resolved crystal of cAMP-liganded RI. It will also be demonstrated that there are restrictions for binding of 8-substituted cAMP analogues to site BI, even if C8 of the cAMP analogue appears to be exposed at the surface of the crystal.

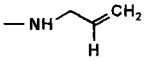
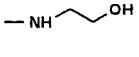
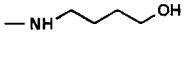
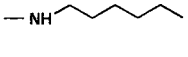
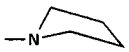
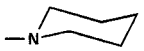
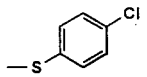
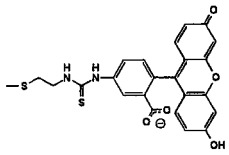
MATERIALS AND METHODS

Synthesis of Cyclic Nucleotide Analogues. All compounds were 8-substituted at position C8 of cAMP (see Figure 1 for the location of substituent R and Tables 1 and 2 for the formula of each substituent and compound numbering). 8-Aminoallyl-cAMP (**1c**, 8-AALL-cAMP), 8-aminoethylhydroxy-cAMP (**1d**, 8-AEOH-cAMP), 8-aminobutylhydroxy-cAMP (**1e**, 8-ABOH), 8-dimethylamino-cAMP (**1g**, 8-DMA-cAMP), 8-pyrrolidino-cAMP (**1h**, 8-PYRRO-cAMP), 8-piperidino-cAMP (**1i**, 8-PIP-cAMP), 8-aminoethylamino-cAMP (**3a**, 8-AEA-cAMP), 8-(−)-(*R*)-(1,2-diaminopropyl)-cAMP (**3b**, 8-R-APrA-cAMP), 8-(+)-(*S*)-(1,2-diaminopropyl)-cAMP (**3c**, 8-S-APrA-cAMP), 8-aminopropylamino-cAMP (**3d**, 8-APrA-cAMP), 8-aminobutylamino-cAMP (**3e**, 8-ABA-cAMP), and 8-piperazino-cAMP (**3g**, 8-PIAZ-cAMP) were synthesized as described by Long et al. with some modifications (*13*). The purity of all compounds was higher than 99.8%, and no starting material or cAMP could be detected by analytical HPLC on a 250 mm × 4.6 mm, 10 μm steel column packed with LiChrosorb RP 18 (MERCK, Darmstadt, Germany) and eluted with a mobile phase of water containing 0.01 M TEAF (pH 6.5), and from 10% (3g) to 45% methanol (**1i**) depending on the lipophilicity of the cyclic nucleotide. The structure of each cAMP analogue was confirmed by ^{1}H and ^{31}P NMR and FAB/MS analysis. ^{1}H and ^{31}P spectra (internal standard being Me_4Si and external standard 85% H_3PO_4) were recorded with a Bruker WH-360 spectrometer. The solvent was DMSO-d_6. Mass spectra were obtained with a Finnigan MAT spectrometer (model 8222) in FAB mode with glycerol as the matrix (*14*). 8-Aminohexyl-cAMP (**1f**, 8-AH-cAMP) was a kind gift from H.-G. Genieser (BIOLOG Life Science Institute, Bremen, Germany). 8-Amino-cAMP (**1a**, 8-NH_2-cAMP), 8-methylamino-cAMP (**1b**, 8-MA-cAMP), 8-bromo-cAMP (**2b**, 8-Br-cAMP), and 8-[[2-(fluoresceinylthioureido)amino]ethyl]thio]-cAMP (**2e**, 8-FLUO-cAMP) were purchased from BIOLOG Life Science Institute. 8-Aminohexylamino-cAMP (**3f**, 8-AHA-cAMP) and 8-*p*-chlorophenylthio-cAMP (**2c**, 8-CPT-cAMP) were obtained from Sigma Chemical Co. (St. Louis, MO). 8-Oxo-cAMP (**2a**, 8-OXO-cAMP) and 8-(2-hydroxypropyl)-cAMP [**2d**, 8-C(OH)$(CH_3)_2$-cAMP] were obtained as described previously (*15*).

Purification of R Subunits. cAKI and cAKII were prepared from rabbit skeletal muscle and bovine cardiac muscle, respectively (*15*). Free RI subunit was obtained by incubation of cAKI with 50 μM cAMP in 15 mM Hepes/NaOH (pH 7.2) containing 1 mM EDTA, 0.3 mM EGTA, and 0.5 mM DTE, and removal of free C subunit by passing the mixture three times through a phosphocellulose column equilibrated in the same buffer. The free RI was concentrated by absorption to a column of DEAE-Sepharose equlibrated in 10 mM potassium phosphate (pH 7.2) with 1 mM EDTA, 0.3 mM EGTA, and 0.5 mM DTE. After extensive washing in equilibration buffer with 50 mM KCl, RI was eluted in the same buffer with 200 mM KCl. It was then made 12 mM in cGMP, and left for 2 days at 4 °C to exchange endogenous cAMP with cGMP. Final purification was by size exclusion HPLC on two tandem columns [30 cm TSKW4000 and 60 cm TSKW3000, in 150 mM potassium phosphate buffer (pH 7.2)]. The fractions were analyzed by SDS−polyacrylamide gel electrophoresis, and only fractions with undegraded RI subunit were used. The purity of RI, based on Coomassie staining, was greater than 80%. The procedure for production of RII from cAKII was similar. The modifications were as follows: (a) the presence of 10 mM magnesium acetate and 1 mM ATP with cAMP during the first dissociation step, (b) elution from DEAE-Sepharose with 300 mM KCl, and (c) reduced exchange time (18 h) of cAMP with cGMP.

Determination of R Subunit Binding Site Affinity for [^{3}H]-cAMP and for cAMP Analogues. The RI and RII subunits were diluted to concentrations of 0.7 and 1.5 nM, respectively, with various concentrations of [^{3}H]cAMP in 10 mM Hepes/NaOH (pH 7.2), 2 mM EDTA, 0.3 mM EGTA, 130 mM KCl, 3 mM sodium phosphate, bovine serum albumin (1 mg/mL), the protease inhibitors apoprotinin (25 μg/mL), leupeptin (50 μM), antipain (5 μM), chymostatin (5 μM), pepstatin (10 μM), and soybean trypsin inhibitor (1 mg/mL), and the antioxidants (added after degassing) 2 mM DTE and 2 mM gluthatione. After preincubation at 0 °C for 5 h, the test tubes were transferred to a 37 °C bath. After 45 min, one aliquot (220 μL) was removed and mixed with 10 volumes of ice-cold 3.8 M $(NH_4)_2SO_4$ to precipitate the [^{3}H]-cAMP bound to R (both sites A and B). Another aliquot was mixed with 5 volumes of ice-cold Hepes buffer with 4 M NaCl and 0.1 mM unlabeled cAMP to achieve preferential exchange of [^{3}H]cAMP from site A before being mixed with 4 mL of ice-cold 3.8 M $(NH_4)_2SO_4$ after 4 h for RII and 16 h for RI. After 4 h in the 4 M NaCl buffer, >90% of the [^{3}H]cAMP bound to site AII was exchanged compared to <10% for site BII. After 16 h, >98% of the [^{3}H]cAMP bound to site AI was exchanged compared to <10% for site BI (see refs *12* and *15* for additional details). The amount of [^{3}H]cAMP bound to each cAMP binding site was plotted against the amount of bound and free [^{3}H]cAMP (free [^{3}H]-cAMP = total [^{3}H]cAMP − bound [^{3}H]cAMP), and the

Table 1: Affinities of Testkit 1 and 2 Analogues for the Binding Sites of RI and RII[a]

No.	Compound	Substituent	K_D AI [nM]	K_D AII [nM]	K_D BI [nM]	K_D BII [nM]
	cAMP	—H	3.00	8.00	2.00	6.00
1a	8-NH_2-cAMP		20.0	90.9	0.51	2.00
1b	8-MA-cAMP		38.5	308	0.61	3.75
1c	8-AALL-cAMP		17.3	67.2	1.00	12.2
1d	8-AEOH-cAMP		60.7	394	1.96	16.4
1e	8-ABOH-cAMP		106	1010	1.40	12.8
1f	8-AH-cAMP		90.9	930	1.11	6.90
1g	8-DMA-cAMP		11.9	10400	3.46	3.82
1h	8-PYRRO-cAMP		10.4	16000	15.6	5.17
1i	8-PIP-cAMP		1.43	170	33.3	2.23
2a	8-OXO-cAMP	=O	20.0	17.0	3.92	0.73
2b	8-Br-cAMP	—Br	2.31	72.7	2.00	0.88
2c	8-CPT-cAMP		0.769	148	1.18	0.316
2d	8-C(OH)$(CH_3)_2$-cAMP		47.0	5000	417	250
2e	8-FLUO-cAMP		4.50	167	2.90	1.62

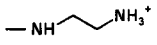
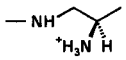
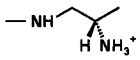
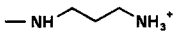
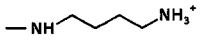
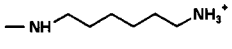
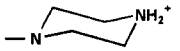
[a] The affinity of cAMP and selected analogues (**1a**−**i** and **2a**−**e**) for sites AI and BI of rabbit RIα and sites AII and BII of bovine RIIα is expressed as the equilibrium dissociation constant (K_D). The upper part of the table shows data for uncharged analogues with a nitrogen atom attached to the C8 position of cAMP (**1a**−**i**, testkit 1), and the lower part shows uncharged analogues with an oxygen (**2a**), bromine (**2b**), sulfur (**2c** and **2e**), or carbon (**2d**) attached to C8 (**2a**−**e**, testkit 2). The values represent the mean of five to seven experiments, and the standard deviation of the mean was less than 15%. Experimental details are described in Materials and Methods.

equilibrium binding dissociation constant (K_D^{cAMP}) was determined.

Unlabeled cAMP and the cAMP analogues were tested for their ability to compete with [^{3}H]cAMP for binding to sites AI, AII, BI, and BII, and the inhibition constants for the cAMP analogue ($K_i^{analogue}$) and cAMP (K_i^{cAMP}) were determined as described previously (*15*). Each analogue was tested at 10−15 concentrations covering a 500−16000-fold concentration range. Its affinity relative to cAMP is expressed as $K'_i{}^{analogue} = K_i{}^{cAMP}/K_i{}^{analogue}$. The absolute affinity of a cAMP analogue for a binding site is expressed as $K_D{}^{analogue} = K_D{}^{cAMP}/K'_i{}^{analogue}$. Each analogue was tested five to seven times, and the standard deviation of the mean was less than 15% of the values given in Table 1. It should be noted that the cAMP analogue mapping results were similar when partially purified cAKI replaced the purified RI as the binding reagent, indicating that the [^{3}H]cAMP binding was specific for RI in the semipurified preparation. The same held true when partially purified cAKII replaced RII, provided cAMP phosphodiesterase activity was counteracted by including 0.2 mM 3-isobutyl-1-methylxanthine in the incubation mixture.

Structural Analysis and Quantum Mechanical Calculations of cAMP Analogues; Relation to the Coordinates of Binding Sites A and B of RI. The hydrophobicity of the cAMP analogues was estimated on the basis of K_W values, as validated previously (*16*, *17*). The relative lipophilicity (L_r) is defined as $K_W{}^{analogue}/K_W{}^{cAMP}$. p$K_a$ values of the analogues

Table 2: Affinities of Testkit 3 Analogues for the Binding Sites of RI and RII[a]

No.	Compound	Substituent	K_D AI [nM]	K_D AII [nM]	K_D BI [nM]	K_D BII [nM]
	cAMP	—H	3.00	8.00	2.00	6.00
3a	8-AEA-cAMP		546	4570	30.2	531
3b	8-R-APrA-cAMP		455	5714	28.6	429
3c	8-S-APrA-cAMP		1071	5000	25.2	261
3d	8-APrA-cAMP		211	800	1.41	37.9
3e	8-ABA-cAMP		92.9	513	0.59	17.4
3f	8-AHA-cAMP		54.5	777	0.49	15.5
3g	8-PIAZ-cAMP		28.3	20000	435	173

[a] The table shows $K_D^{analogue}$ values for binding of seven cAMP analogues (**3a**−**g**, testkit 3) with a C8 substituent carrying a positively charged amino group, For details, see the footnote of Table 1.

were calculated with the ACD/pK_a DB software (Advanced Chemistry Development Inc., Toronto, ON). The coordinates of the X-ray structure of the deletion mutant (Δ1−91)rRIa of the regulatory subunit of cAKI were kindly provided by W. R. G. Dostmann (University of Vermont, Burlington, VT). The program HyperChem5.0 (Hypercube, Waterloo, ON) was used for structural analysis of the X-ray structure and for molecular modeling of selected cAMP analogues. The conformation of cAMP bound to sites AI and BI of (Δ1−91)rRIα was identified by deletion of all atoms from the protein. For molecular modeling of cAMP analogues, the torsion angle χ_{CN} [χ_{CN} being O4′−C1′−N9−C4 (*18*)] of the glycosidic bond was constrained to the value found for cAMP in site AI ($\chi_{CN} = 53.2°$) or BI ($\chi_{CN} = 69.3°$). For the calculations, the analogues were considered to be ionized carrying a negative charge at the cyclic phosphate moiety and a positive charge at the aliphatic amino group in position 8 (testkit 3). The extracted cAMP from the X-ray structure was used as the starting geometry, and preoptimized substituents (by MMplus force field) were attached to C8 of the adenine. The resulting cAMP analogues were preoptimized with the MMplus force field and then treated quantum mechanically with the pm3 Hamiltonian. The dipole moments were calculated only for the adenine moieties of the cAMP analogues because stacking interactions between cyclic AMP binding sites and cAMP are expected only for the adenine base and Trp^{260} and Tyr^{371} of sites AI and BI, respectively (*1*). The charged terminal amino group of the testkit 3 substituents, the conformational flexibility of the aliphatic hydrocarbon spacers of some 8-substituents, and the negative charge of the cyclic phosphate are unlikely to be relevant for the stacking interaction between binding site and adenine moiety (*1*), but affected the calculated dipole moments (data not shown).

The dipole moments of the base moiety were calculated on the CNDO level of theory by selecting the aromatic systems of the optimized cyclic phosphates (using the "extend to sp^3" command in HyperChem). Starting with the N1 atom, the current selection was extended throughout the purine system until single-bonded atoms or sp^3−sp^3 single bonds were reached. This selection included the C1′ atom of the ribose, the hydrogen atom at C1′, and the entire amino group at position 6. The smallest substituents attached to position 8, like amino (**1a**), oxo (**2a**), and bromo (**2b**), were completely included, while only the C8 nitrogen was included in tertiary substituted amines (**1g**−**i** and **3g**), and only the C8−N−H fragment in the secondary substituted 8-amino derivatives (like **3a**−**f**). These activated subsystems were treated quantum mechanically (CNDO) to determine the dipole moments.

RESULTS

Rationales for Selection of cAMP Analogues and Their Chemical Characterization. To systematically study the influence of chemical modifications at position 8 of the cAMP molecule, the compounds were selected and arranged into three groups.

*Testkit 1 (**1a**−**i**).* Each of these compounds (see row 1 of Table 1) has a nitrogen directly attached to the C8 position of cAMP. They were chosen to investigate the effect of different amino group substitutions, and the effect of flexible or rigid bulk. These derivatives do not carry a positive charge under physiological conditions since a primary (**1a**), secondary (**1b**−**f**), or tertiary (**1g**−**i**) amino group directly attached to the aromatic purine base exhibits a pK_a of 3−4 (*18*, *19*). Also, the N7 and N1 positions are uncharged at pH 7.2. In compounds **1g**−**i**, the base moiety is considered to be restricted to the syn conformation of the glycosidic bond, as a result of the bulkiness of the substituents (*20*, *21*). Compounds **1a**−**e** have hydrogen bonding donor potential close to C8. **1a** can form two hydrogen bonds by the primary amine at position 8, and **1b**−**f** are able to form a single

hydrogen bond by the secondary substituted amino group. The compounds with most steric extension directly adjacent to the C8 position are 8-PYRRO-cAMP (**1h**; 3.69 Å distance between the Cα hydrogen atoms), 8-DMA-cAMP (**1g**; 3.77 Å maximal distance between the Cα hydrogen atoms), and 8-PIP-cAMP (**1i**; 4.26 Å). 8-PYRRO-cAMP (**1h**) is less bulky close to the C8 position than 8-PIP-cAMP (**1i**) because its N atom joins the pyrrolidino ring under a lower angle (117.5°) than the corresponding N atom in 8-PIP-cAMP (121.9°), as was verified by molecular modeling. Although the Cα atoms meet the N atom in 8-DMA-cAMP (**1g**) under a greater angle (124.8°) than in **1i**, the maximal distance between their hydrogen atoms (3.77 Å) is shorter. This is because the optimal chair conformations of **1i** require that each Cα has one equatorial hydrogen atom, which are more distant from each other than the corresponding H atoms of the optimized staggered methyl groups in 8-DMA-cAMP.

The five-membered pyrrolidino ring system of **1h**, with its envelope conformation, is the most rigid of the N-containing substituents. The six-membered piperidino ring of **1i** can adopt two distinct chair conformations, a boat conformation and a twist conformation, and is therefore more flexible. Compounds **1a** and **1b** have relative lipophilicities (L_r = 0.52 and 1.02, respectively) lower than or similar to that of cAMP. The other members of testkit 1 have enhanced lipohilicity (up to an L_r of 12.0 for **1i**). The dipole moments were found to be similar in value [range from 1.81 (**1h**) to 4.16 D (**1a**)] and direction in space (range from 18° to 46°) to those for cAMP (3.04 D and 34.5°).

*Testkit 2 (**2a**−**e**).* Compounds **2a**−**e** (Table 1) were included to serve as comparison to the 8-amino-substituted analogues of testkit 1. The compounds were uncharged at positions N1 and N7, except for 8-OXO-cAMP (**2a**), which according to pK_a calculation had a pK_a value of 7.66, implying the existence of some negatively charged **2a** species at pH 7.2. The compounds differed considerably in the bulk of the substituent. **2a** and **2b** were the least bulky of all the analogues tested, whereas **2d** had the most bulk close to C8 of all the analogues that were tested (distance between the Cα hydrogens of 4.37 Å). Compound **2e** (8-FLUO-cAMP) had the overall bulkiest substituent ever mapped (fluorescein coupled to C8 via a six-atom spacer).

Unlike compounds **1a**−**f** and **3a**−**f**, compounds **2a**−**c** and **2e** cannot readily participate as donors in hydrogen bonding close to C8. On the other hand, they can readily participate in hydrogen bonding as acceptors. The predicted efficiency rank of the analogues as hydrogen bonding acceptors is as follows: **2a** > **2c**, **2e** > **2b**.

Compound **2a** is less lipophilic (L_r = 0.74) than cAMP, whereas the others are more lipophilic (L_r^{2b} = 1.82; L_r^{2c} = 36.3). The value of the dipole moments and their orientation in space differ significantly from those (3.04 D and 34.5°) of cAMP, being 3.71 D and 129° for **2a**, 0.69 D and 7° for **2b**, 0.61 D and 79° for **2c**, and 0.82 D and 138° for **2d**.

*Testkit 3 (**3a**−**g**).* Compounds **3a**−**f** (Table 2) are aminoalkylamino analogues of cAMP. They are able to form a hydrogen bond by the secondary substituted amino group at position 8. They have no charge at N1 or N7 at physiological pH, but their distal amino groups are positively charged at physiological pH. In compounds **3a**−**c**, the charged amino group is about the same distance from C8 as the OH group of compound **1d**. The diastereomeric analogues (*R*)- and (*S*)-APrA-cAMP (**3b,c**) were synthesized to probe the chirality of the binding sites and their sensitivity for bulk attached to the second carbon in the spacer. In compounds **3a** and **3d**−**f**, the distance between the positive charge attached at the 8-position via a terminal primary amino group and the adenine moiety was altered by a stepwise increase in the alkyl spacer length. The resulting aminoalkylamino analogues exhibit increasing conformational flexibility as a function of increasing chain length. As a counterpart to this series of sterically mobile substituents, we designed 8-PIAZ-cAMP (**3g**) with a more rigid cyclic spacer comparable to **1i**, carrying a positive charge. Compound **3g** has as much bulk directly adjacent to the C8 position (4.25 Å interatomic distance between the Cα hydrogens) as **1i** (4.26 Å). The lipophilicity of the testkit 3 analogues ranged from 0.31 for compound **3a** to 1.78 for compound **3f**. The dipole moments of the purine ring systems (range from 1.52 to 3.15 D, angles from 22° to 66°) are similar to those of the testkit 1 compounds, i.e., similar to those of cAMP (3.03 D and 34.5°). Only compound **3g** has a significantly reduced dipole moment of 0.56 D with an angle of 59°. Thermodynamically optimized structures of compounds **3a**, **3d**, and **3e** derived from the cAMP molecule in site BI (χ_{CN} = 53.16°) are depicted in Figure 2. The analysis suggested, surprisingly, that the energetically most optimal conformation of 8-ABA-cAMP (**3e**) had an intramolecular salt bridge from the terminal amino group to the equatorial oxygen of the cyclic phosphate. In contrast, compounds **3a**−**d** and **3g** were not able to adopt this type of interaction, predominantly as a result of the short spacers (data not shown). It should be noted that the binding experiments were conducted at physiological ionic strength, which would render such intramolecular ionic interactions less favorable than in the computer simulations. The possibility of salt bridge formation by 8-ABA-cAMP should nevertheless not be excluded.

Determination of Absolute Affinities of cAMP Analogues for Sites A and B of the R Subunits of cAKI and cAKII. So far, results of cyclic nucleotide analogue mapping studies have been given in relative units. Since the affinity of cAMP itself has rarely been determined in mapping studies, the absolute analogue affinities have not been published. It was decided to determine the equilibrium dissociation binding constant (K_D) for the binding of [^{3}H]cAMP to the binding sites (AI and BI) of rabbit RIα and the binding sites (AII and BII) of bovine RIIα. On the basis of the determined K_D^{cAMP} values and analogue affinity relative to cAMP, the $K_D^{analogue}$ values of twenty-one 8-modified cAMP analogues were determined for site AI, AII, BI, and BII (Tables 1 and 2). Site AII was distinct in having the lowest affinity for all the compounds, except 8-OXO-cAMP (**2a**) whose affinity for site AII (K_D = 17 nM) was marginally higher than for site AI (K_D = 20 nM). The highest affinity for site AI (K_D = 0.77 nM) was shown by 8-CPT-cAMP (**2c**). The highest site AI/AII affinity ratio (about 1600) was shown by 8-PYRRO-cAMP (**1h**). 8-AHA-cAMP (**3f**) had the highest affinity for site BI (K_D = 0.49 nM) and the highest site BI/BII ratio (32). 8-CPT-cAMP (**2c**) had the highest affinity (K_D = 0.32 nM) for binding to BII. The highest BII/BI ratio (16) was shown by 8-PIP-cAMP (**1i**).

Some of the analogues (notably, **1b**, **1i**, **2c**, and **3f**) were also tested as inhibitors of [^{3}H]cAMP binding to sites AI and BI of recombinant human RIα and bovine RIα, and

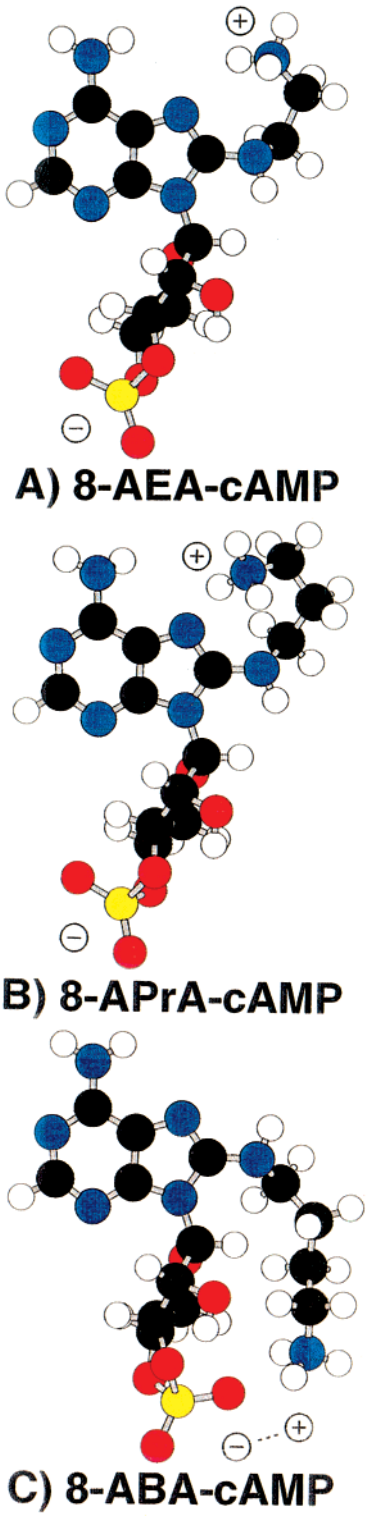


FIGURE 2: Computed predictions of conformations of maximal stability of 8-AEA-cAMP (A), 8-APrA-cAMP (B), and 8-ABA-cAMP (C). Note the predicted salt bridge formation between the cyclic phosphate and the terminal amino charge in 8-ABA-cAMP. Details of the computing and of the structure of substituents are found in Materials and Methods and in Table 2, respectively. The molecules are projected in the plane of the adenine ring.

found to bind to these sites like they do to the corresponding sites of rabbit RI (data not shown).

Importance for Binding Site Affinity of 8-Substituted cAMP Analogues of Hydrogen Bonding Donor and Acceptor Potential Close to C8. Compound **1a** can form two hydrogen bonds by the primary amine at position 8. Compounds **1b**−**f** and **3a**−**f** are able to form a hydrogen bond by the secondary substituted amino group at position 8. All 12 of these compounds with hydrogen bonding donor potential had site BI preference (affinity for site BI from 4- to 32-fold higher than for any other site). None of the other compounds had similar site BI preference (Tables 1 and 2). This suggests that hydrogen bond donation is particularly important for site BI binding. Compounds **2a**−**c** and **2e** have hydrogen bonding acceptor potential. These compounds exhibited site BII preference, suggesting that site BII differs from site BI by preferring hydrogen bond acceptor potential rather than donor potential. Site AI selected against either hydrogen donor bond potential or the strongest hydrogen bonding acceptor potential, suggesting a nonpolar environment close to the docking of C8. For site AII, eventual effects of hydrogen bonding were overwhelmed by the effects of bulk in the substituents (see below), making interpretation of hydrogen bonding effects difficult.

Importance for Binding Site Affinity of 8-Substituted cAMP Analogues of Dipole Moment, Lipophilicity, Charge Transfer Potential, and Syn/Anti Conformation. The dipole moment of the adenine ring is similar in direction for cAMP (34.5°) and all analogues of testkits 1 and 3 (18−66°), while analogues of testkit 2 exhibit significant alterations (7−138°). The strength of the dipole moment is increased for compounds **1a** and **2a**, and decreased for compounds **2b**, **2c**, and **3g**. The site BI preference of 8-AMINO-cAMP (**1a**) and site BII preference of 8-OXO-cAMP **(2a)** could not be ascribed to different dipole strengths (4.16 D for **1a** vs 3.71 D for **2a**). Compound **2a** differed from **1a** and all other testkit 1 compounds in dipole direction (129° vs 18−46° for the other compounds). It is unlikely that this difference was important for the BII versus BI preference of 8-OXO-cAMP, since 8-Br-cAMP (**2b**) and 8-CPT-cAMP (**2c**), which were similar to 8-OXO-cAMP (**2a**) with respect to site BII versus BI selectivity (Table 1), had very different dipole directions (7° and 79°, respectively). This reinforces the interpretation made above that the observed variation of BI versus BII selectivity of the compounds is related to differences in hydrogen bonding acceptor and donor potential. There was no overall correlation between dipole moments and affinity, suggesting that the differences in dipole moment are of minor importance in explaining the distinct binding behaviors of the 8-substituted compounds.

Site AI discriminated against cAMP with polar 8-substituents; all analogues with an oxo group (**2a**), primary amine (**1a**), or secondary amine (**1b**−**i** and **3a**−**f**) had decreased AI affinity. The compounds with highest affinity for site AI (**1i** and **2c**) were strongly hydrophobic. Nevertheless, there was no direct correlation between overall analogue lipophilicity and analogue affinity for any binding site, indicating that enhanced analogue lipophilicity may be more important for penetration of cell membranes than for interaction with the cAMP kinase. Neither of the testkit modifications added to or detracted from the aromatic character of the mother compound cAMP, so all compounds would be expected to retain the potential to form stacking interactions with the protein binding sites and therefore had similar charge transfer potentials of the adenine base.

In compounds **1g**−**i**, **2d**, and **3g**, the glycosidic bond is believed to be forced into the syn conformation. The fact

that **1i** has a high affinity for site AI and BII and **1g** a high affinity for site BI supports the contention that cAMP and cAMP analogues are bound to these sites in the syn conformation (*1*, *9*, *10*). Compounds **1g**, **1h**, **2d**, and **3g** have very low affinity for site AII, but also other analogues (**1e** and **3d**), whose 8-substituents appear to be less bulky and therefore should allow the anti conformation, exhibited a low affinity for site AII. In comparison, **1i** showed a smaller decrease of site AII affinity (Tables 1 and 2). The study presented here therefore did not allow any firm conclusion about whether cAMP analogues may bind to site AII preferentially in the anti conformation.

Importance for Binding Site Affinity of 8-Substituted cAMP Analogues of the Bulk and Rigidity of a C8 Substituent. Site AII had decreased affinity for all 8-substituted compounds. The affinity was less decreased when the 8-substituents were small, like 8-oxo (**2a**), 8-bromo (**2b**), and 8-amino (**1a**). Compounds that combine rigidity with high proximal bulk (**2d**, **1g**, and **1h**) had about 1000-fold decreased affinity for site AII, indicating that this site is extremely sensitive to bulk close to C8. Only site AII discriminated appreciably against bulk more distant from C8, like in compounds **2c** and **2e**. It is concluded that site AII differs from all the other binding sites in its high sensitivity to both proximal and distal bulk of 8-substituents. Site AI tolerated bulk far better than site AII, and better than site BI. Site AI had the highest affinity of all sites for both 8-C(OH)$(CH_3)_2$-cAMP (**2d**) and 8-PIP-cAMP (**1i**). With the exception of 8-DMA-cAMP (**1g**), site BI had less tolerance for proximal bulk (see the data for **1h**, **1i**, **3g**, and **2e**) than either site AI or site BII.

The fluorescent and extremely bulky 8-FLUO-cAMP (**2e**) had affinity for sites AI, BI, and BII similar to those of cAMP. This indicates that this compound and other similarly large compounds can be used as high-affinity probes of either cAMP binding site (AI and BI) of RI and site BII of RII.

Importance for Binding Site Affinity of 8-Substituted cAMP Analogues of Positive Charge of a C8 Substituent. Compounds **3a**−**c** and **3g** have a charged amino group close to C8. They have affinities for all binding sites 1−2 orders of magnitude lower than those of similar analogues (**1c**, **1d**, and **1i**) without a charge. The decrease in affinity resulting from introducing a charge in 8-PIP-cAMP (**1i**) to form 8-PIAZ-cAMP (**3g**) was more severe for RII than for RI: 22-fold for site AI, 115-fold for site AII, 14-fold for site BI, and 92-fold for site BII. The exact spatial position of the charge seemed to be less important than its overall distance from C8, since the stereoisomers **2b** and **2c** had binding affinities similar to that of 8-AEA-cAMP (**2a**). Replacing the amino group of 8-AEA-cAMP (**2a**) with a hydroxyl group [8-AEOH-cAMP (**1d**)] resulted in a more than 20-fold increased affinity for all binding sites.

A gradual relief of the negative effect of a positive charge was noted for all binding sites as the chain length between C8 and the charge increased to three (**3d**) and four (**3e**) carbons. It appeared that a terminal amino group actually enhanced site BI binding. Replacing the amino group of 8-ABA-cAMP (**3e**) with a hydroxyl group (**1e**) led to decreased site BI affinity and slightly enhanced site BII affinity. Comparison of the mapping data for the six-carbon chain 8-AH-cAMP (**1f**) and its amino-substituted counterpart 8-AHA-cAMP (**3f**) revealed that the terminal amino group contributed positively to site BI binding, had little effect for site AI and AII binding, and slightly decreased the affinity for site BII.

It is concluded that whereas a positive charge close to C8 was detrimental for binding to all binding sites, it was accommodated when separated from C8 by a chain of four or more carbon atoms. A terminal amino group distant from C8 enhanced site BI versus BII selectivity. In fact, 8-AHA-cAMP had the highest site BI versus BII selectivity (32-fold) on record.

The relevance of the computer prediction that the energetically most optimal conformation of 8-ABA-cAMP had an intramolecular salt bridge from the terminal amino group to the equatorial oxygen of the cyclic phosphate (Figure 2) could be tested by comparing the binding behavior of 8-ABA-cAMP and 8-APrA-cAMP (**3d**), which is unable to form a salt bridge. Since 8-APrA-cAMP had a higher affinity than cAMP for site BI, internal salt bridge formation may be unimportant in explaining the high site BI affinity for 8-aminoalkylamino-substituted cAMP analogues. The reason may be that the binding experiments were conducted at physiological ionic strength. This renders ionic interactions less favorable than in the computer simulations in a vacuum based on the atoms of the analogues alone.

DISCUSSION

The current knowledge of the geometry of the cAMP binding sites of cAK is based primarily on the X-ray-resolved structure of truncated bovine RIα cocrystallized with cAMP bound to site AI and site BI (*1*). The results of this study serve to (1) provide a fuller picture of how cyclic nucleotides interact with the subdomains of AI and BI facing the C8 position of cAMP, (2) challenge the uniqueness of the conformation of the sites, particularly site AI, in the crystal by showing high site AI affinity for analogues which cannot be accommodated in site AI as depicted in the crystal structure, and (3) point to important differences between sites AII and AI and between sites BI and BII. The results also give clearer guidelines about how to construct novel cAMP analogues with site BI and site BII preference.

The results will first be discussed for each type of binding site of RI, using the same residue numbering as for bovine RIα in ref *1*. Thereafter, the results for sites AII and BII of RII will be discussed, sometimes incurring residues of bovine RIIα aligned in homologous positions (*3*) to critical residues in RIα.

Regulatory Subunit Type I Site A. The fact that the accessibility of the C8 position of cAMP in the cAMP-liganded crystal structure of site AI is blocked by Trp^{260}, Val^{182}, and Val^{192} suggested that C8-substituted cAMP analogues are not well tolerated by site A (*1*). This study shows clearly that site AI can accommodate even bulky C8 substituents, like in compounds **1g**−**i**, **2d**, and **2e**. In fact, 8-PIP-cAMP (**1i**) and the proximally extremely bulky 8-C(OH)$(CH_3)_2$-cAMP (**2d**) bind to site AI with higher affinity than to any other site (Table 1). This is surprising since computer visualization of the crystal coordinates indicates that C8 points to Ile^{175}, which forms the bottom of a narrow hydrophobic pocket, whose walls are formed by Trp^{260}, Val^{182}, and Val^{192}. Considerable rearrangements of this hydrophobic pocket must occur relative to the crystal to accommodate the bulky substituents of compounds **1h**,

1i, and **2d**. With the exception of Trp^{260}, which stacks with the adenine ring of cAMP, none of the residues mentioned above have been defined as essential for cAMP binding or R−C interaction, and we suggest that the analogues can expand this part of the AI pocket without disrupting the cAMP binding affinity or its ability to modulate R−C interaction. Such inherent flexibility of the site AI binding pocket can also explain the observation that 8-azido-cAMP bound to site AI can label Tyr^{244} of C-terminally truncated RI (*3*). In the crystal structure of RI, Tyr^{244} is far from the C8 position of bound cAMP (*1*). The induced fit hypothesis for ligand binding has several precedents (*22*). One example is the matrix metalloproteinase human stromelysin (MMP-3), whose crystals have been resolved with two structurally different inhibitors as ligands. In that enzyme, a loop region of 10 amino acids undergoes an unexpected conformational shift when one ligand is replaced by another one carrying a hydrophobic phenyl ring substituent (*23*).

The hydrophobic nature of the region of site AI facing C8 explains the negative influence of polar and charged 8-substituents on its cAMP analogue affinity (Tables 1 and 2). The potential hydrogen bonding donor substituents in 8-NH_2-cAMP (**1a**) and 8-MA-cAMP (**1b**) decreased the cAMP analogue affinity 40−60-fold compared to that for site BI, as expected from the fact that the stacking interaction of site AI is to Trp^{260}, which, unlike Tyr^{371} of BI, lacks a free hydroxyl group.

Regulatory Subunit Type I Site B. The C8 position of cAMP bound to site BI is accessible from the surface of RI in the crystal structure, which has reinforced the belief that all analogues modified in position 8 have excellent binding affinities for site BI (*1*). This general notion needs to be modified. In fact, site BI was more sensitive than site AI to substituents with flexible bulk (compound **1i**), rigid bulk (**1h**), and extreme bulk (**2d**) close to the C8 position (Table 1). This sensitivity to proximal bulk of substituents may be explained by 8-substituent interference with the Tyr^{371}−Glu^{324} hydrogen bonding interaction. In the cAMP-liganded state of BI, the Tyr^{371} residue is believed to be involved in hydrogen bonding to the highly conserved Glu^{324}, which is thought to be an important anchoring for adjusting an optimal orientation for charge transfer interaction between Tyr^{371} and the purine component of cyclic AMP (*1*). Otherwise, the BI subsite surrounding the C8 position is mainly hydrophobic, explaining why even a long hydrophobic chain like in 8-AH-cAMP (**1f**) and the fluorescein group of compound **2e** are well accommodated.

The hydrophobic nature of the BI subsite facing C8 does not explain the enhancement of cAMP affinity by polar substituents close to C8 like in 8-NH_2-cAMP (**1a**), 8-MA-cAMP (**1b**), 8-AAll-cAMP (**1c**), 8-AEOH-cAMP (**1d**), 8-ABOH-cAMP (**1e**), 8-AH-cAMP (**1f**), 8-APrA-cAMP (**3d**), 8-ABA-cAMP (**3e**), and 8-AHA-cAMP (**3f**). Consideration of the crystal data indicates that these compounds have the potential to serve as donors for hydrogen bonding interaction (on the basis of their secondary amino group attached to C8) with the hydroxyl group of Tyr^{371}.

Whereas a polar group can be accommodated in an overall hydrophobic environment due to hydrogen bonding, this is not possible when an additional charge is introduced close to C8 as in compounds **3a**−**c** and **3g**. In fact, 8-PIAZ-cAMP (**3g**), whose substituent combines a charge and bulk close to C8, has an affinity for site BI more than 200-fold lower than that of cAMP. The detrimental effect of the charged amino group was abolished when it was separated from the proximal secondary amine by a spacer of three or more linear carbon atoms (**3d**−**f**). This beneficial effect of the spacer was probably due to a combination of three factors. One is the increased distance between the charge and the C8 position; the second is a positive contribution to binding by the hydrophobic spacer itself, and the third is the fact that a positive anchoring for the free charge is available only an extended distance from C8. The last possibility received support from the finding that 8-AHA-cAMP (**3f**) had more than twice as high an affinity as 8-AH-cAMP (**1f**), and 8-ABA-cAMP (**3e**) more than twice as high an affinity as 8-ABOH-cAMP (**1e**). To explain the site BI-specific affinity enhancement by the terminal amino group, a survey was undertaken of the location of acidic residues relative to position C8 of the cAMP molecule in site BI of the published crystal structure of RI. Only two such residues (Asp^{320} and Glu^{324}) were close enough to C8 to interact with the terminal amino group of 8-APrA-cAMP (**3d**). Glu^{324} does not form ionic bonds in the cAMP-liganded state, and thereby differs from the corresponding Glu^{200} in site A, which forms a structurally important ionic bond with Arg^{241} (*1*). Glu^{324} could therefore in principle interact with the terminal amino group of 8-aminoalkylamino-cAMP analogues. It is unlikely, however, that the long spacers of 8-ABA-cAMP and 8-AHA-cAMP can be accommodated in the more buried region surrounding Glu^{324} without disrupting the local structure, which contains or contacts several residues (Tyr^{321}, Gly^{323}, Ala^{326}, Ala^{334}, Ala^{335}, and Tyr^{371}) known to be essential for high-affinity cyclic nucleotide binding to site BI (*1*). The carboxyl group of Asp^{320}, on the other hand, appeared to be exposed on the surface and surrounded by hydrophobic residues which would provide a bed for even the longest hydrophobic spacer [8-AHA-cAMP (**3f**)] that was tested. The most probable anchor of the terminal amino group of 8-aminoalkylamino analogues is therefore Asp^{320}.

Regulatory Subunit Type II Site A. Site AII shares with site AI and BI the ability to discriminate against a charge close to the C8 position. AII has in addition an extreme ability to discriminate against bulk close to C8, compounds **1g**−**i**, **2c**, **2d**, and **3g** having affinities for site AII from 2−3 orders of magnitude lower than for site AI. It appears that site AII has much less freedom than any other site to adjust to 8-substituents. No modification of the 8-position of cAMP has yet been made that enhances the binding to site AII. It can be concluded that site AII differs fundamentally from sites AI, BI, and BII with respect to the subsite facing position C8 of cAMP. The amino acid residues close to C8 of sites AI and BI were either the same or only conservatively changed both relative to each other and relative to those in a similar place in the sequence alignment of domain BII. It is therefore noteworthy that two striking differences become apparent when the amino acid sequence is compared between site AII and AI. One is that Val^{192} of AI is replaced by the bulkier Tyr^{196} in AII. Tyr^{196} is labeled by 8-azido-cAMP bound to site AII of N-terminally truncated (Δ1−94)RII (*24*). Another difference is that Trp^{260}, which stacks with cAMP in site AI, is replaced by Ser^{264} in site AII.

Regulatory Subunit Type II Site B. The amino acid residues known from mutagenesis or affinity labeling to be important

for cAMP binding to site BI, like Ile[293], Tyr[321], Gly[323], Glu[324], Ala[326], Arg[333], Ala[334], Ala[335], and Tyr[371] (*1*), are all conserved in the B domain of RII (*25*). Tyr[379] of bovine RII is believed to form stacking interactions with the adenine ring of cAMP in a way similar to that of Tyr[371] in RI (*3*). The position of the free hydroxyl of Tyr[379] close to the C8 position of bound cAMP is supported not only by homology with RI but also by direct demonstration of labeling by 8-azido-cAMP (*3*). Three residues (Val[300], Leu[316], and Val[337]) in site BI, located in the vicinity of the C8 position of cAMP, are different in the homologous positions of BII (Ile[310], Cys[326], and Ala[347]). Asp[320] of BI, proposed to be an anchor for the terminal amino group of 8-ABA-cAMP and 8-AHA-cAMP (see the preceding paragraph), is replaced by Gln[330] in the presumed homologous position of RII.

Site BII differs from site BI in lacking a consistent preference for N-substituents with hydrogen bonding donor potential close to C8. All 12 of compounds (**1a**−**f** and **3a**−**f**) with such potential had a higher site BI versus BII preference than cAMP itself. This can be explained if the hydroxyl group of Tyr[379] of site BII differs from the homologous Tyr[371] of BI in preferentially supporting hydrogen bonding acceptors rather than hydrogen bonding donors. This is strongly supported by the clear site BII versus BI preference of compounds **2a**−**c** and **2e**, which all exhibit hydrogen bonding acceptor interaction potential.

Site BII had a better tolerance than site BI for hydrophobic bulk next to C8, as shown by the site BII versus BI preference of compounds **1h**, **1i**, and **2d**. The hydrophobicity would normally indicate that the subsite of BII facing C8 of cAMP in not exposed to solvent and the protein surface. This is not the case since we have found that 8-azido-cAMP tightly bound to site BII reacts much faster with β-mercaptoethanol supplied in the solvent than with the OH group of Tyr[379] (*26*). The unusual and energetically unfavorable surface exposure of hydrophobic residues in the cAMP binding site might be a consequence of conformational changes induced by cAMP binding. One possibility is displacement by cAMP of buried hydrophobic residues that were associated with Tyr[379] in the cAMP-free RII subunit. The fact that the surface outside the C8 subsite of BII can accommodate hydrophobic bulk is demonstrated also by the more than 3-fold enhanced affinity of 8-FLUO-cAMP (**2e**) relative to that of cAMP.

A general finding was that site BII, contrary to common belief (*1*, *3*, *10*), does not easily accept all C8-substituted cAMP analogues. In fact, cAMP analogues with charged C8 substituents such as 8-AEA-cAMP (**3a**) and PIAZ-cAMP (**3g**) have higher affinity for site AI than for site BII (Table 1). This may be explained by the hydrophobicity of the BII subsite facing C8 postulated above.

Concluding Remarks. It is obvious from the above discussion that site AII not only has the lowest cAMP affinity of mammalian cAK-associated binding sites under physiological binding conditions (Table 1) but also is very different from sites AI, BI, and BII in the region facing C8 of bound cAMP. Site AII tolerates poorly all 8-substituents. Site AI has a high affinity for some hydrophobic, bulky 8-substituents (like **1i**). Site BI has particularly low affinities for cAMP analogues with substituents carrying bulk and charge close to C8 (like **3g**). Site BII has a low affinity for cAMP with 8-substituents carrying charge close to C8 (like **3a**−**c** and **3g**). The most provoking finding is that, even in site AI in which the C8 position of the bound cAMP faces an internal narrow cavity (*1*), rather bulky 8-substituents can be accommodated, indicating considerable flexibility in the region facing the adenine portion of cAMP. It is probable that the published crystal structure of RI with bound cAMP represents only one of many conformations of cyclic nucleotide-liganded RI. The study presented here shows that site BI selectivity can be achieved by supplying hydrogen bonding donor potential close to C8 and a positive charge a distance of at least three carbons from C8. Whether double bonds, like in 8-AALL-cAMP (**1c**), can be introduced in 8-AHA-cAMP to enhance further its site BI selectivity should be explored. Site BII selectivity is achieved through supply of hydrogen acceptor potential close to C8. Since sites AI and AII have similar preference for N^6-modified compounds which are barred from the B sites of RI and RII (*15*), it should be tested whether site AI versus AII selective 8-substituted analogues such as **1g**−**i**, **2c**, and **2d** retain the AI versus AII selectivity and gain AI versus BI and AI versus BII selectivity when modified also in the N^6-position of the adenine ring.

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