Three New Potential cAMP Affinity Labels. Inactivation of Human Platelet Low K_m cAMP Phosphodiesterase by 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-Cyclic Monophosphate†

Paul G. Grant,*,‡ Dianne L. DeCamp,§ Jerome M. Bailey,§ Robert W. Colman,‡ and Roberta F. Colman§

Thrombosis Research Center, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, and Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

Received April 18, 1989; Revised Manuscript Received July 25, 1989

ABSTRACT: Three new analogues of cAMP have been synthesized and characterized: 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-cyclic monophosphate (2-BDB-TcAMP), 2-[(3-bromo-2-oxopropyl)thio]-adenosine 3′,5′-cyclic monophosphate (2-BOP-tcAMP), and 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-cyclic monophosphate (8-BDB-TcAMP). The bromoketo moiety has the ability to react with the nucleophilic side chains of several amino acids, while the dioxobutyl group can interact with arginine. These cAMP analogues were tested for their ability to inactivate the low K_m (high affinity) cAMP phosphodiesterase from human platelets. The 2-BDB-TcAMP and 2-BOP-TcAMP were competitive inhibitors of cAMP hydrolysis by the phosphodiesterase with K_i values of 0.96 ± 0.12 and 0.70 ± 0.12 μM, respectively. However, 2-BDB-TcAMP and 2-BOP-TcAMP did not irreversibly inactivate the phosphodiesterase at pH values from 6.0 to 7.5 and at concentrations up to 10 mM. These results indicate that although the 2-substituted TcAMP analogues bind to the enzyme, there are no reactive amino acids in the vicinity of the 2-position of the cAMP binding site. In contrast, incubation of the platelet low K_m cAMP phosphodiesterase with 8-BDB-TcAMP resulted in a time-dependent, irreversible inactivation of the enzyme with a second-order rate constant of 0.031 ± 0.009 min^{-1} mM^{-1}. Addition of the substrates, cAMP and cGMP, and the product, AMP, to the reaction mixture resulted in marked decreases in the inactivation rate, suggesting that the inactivation was due to reaction at the active site of the phosphodiesterase. These compounds should prove to be useful in studying the active sites of cAMP phosphodiesterases and cAMP binding sites of other proteins.

The catabolism of the important intracellular regulatory nucleotides cAMP and cGMP is mediated by cyclic nucleotide phosphodiesterases (EC 3.1.4.17). Multiple forms of this enzyme have been reported to exist in a wide variety of tissues and cell types (Appleman et al., 1982; Beavo et al., 1982; Manganiello et al., 1984). These forms of the enzyme differ in substrate specificity, kinetic characteristics, and physical characteristics and in the mode of regulation of their activities. The physiological significance of this multiplicity of cyclic nucleotide phosphodiesterases within cells is not well understood, nor have the exact metabolic roles of the individual forms been defined.

Minimal information is available on structure–function relationships for the cyclic nucleotide phosphodiesterases. Recently, some cyclic nucleotide phosphodiesterases have been sequenced in whole or in part (Charbonneau et al., 1986; Sass et al., 1986; Chen et al., 1986; Ovchinnikov et al., 1987). Each of the forms appears to be the product of a separate gene rather than the result of posttranscriptional or posttranslational modifications of a common precursor (Charbonneau et al., 1986). However, each of the forms analyzed contains a region of 200–270 amino acid residues that has considerable homology with the other phosphodiesterases. Charbonneau et al. (1986) have proposed that this conserved region may form the catalytic site of the cyclic nucleotide phosphodiesterases and the other nonhomologous regions may be the regulatory regions of the enzymes, but no direct evidence exists to support this hypothesis.

Affinity labeling has been extensively used to study ligand binding sites of enzymes. A number of cAMP analogues have been synthesized and utilized for affinity or photoaffinity labeling of cAMP binding sites of cAMP-dependent protein kinase, phosphofructokinase, histone kinase, and human erythrocyte membrane proteins (Cooperman & Brunswick, 1973; Haley, 1975; Pomerantz et al., 1975; Severin et al., 1975; Dreyfuss, 1978). We have previously found that 2′-*O*-(2-iodo-3-hydroxypropyl)adenosine 3′,5′-cyclic monophosphate inactivated human platelet low K_m (high affinity) cyclic AMP phosphodiesterase, although the inactivation was quite slow (Reimann et al., 1983).

In this paper we report the synthesis of three new cAMP analogues: 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-cyclic monophosphate (2-BDB-TcAMP),[1] 2-[(3-bromo-2-oxopropyl)thio]adenosine 3′,5′-cyclic monophosphate (2-BOP-TcAMP), and 8-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 3′,5′-cyclic monophosphate (8-BDB-TcAMP). These analogues are potentially useful as affinity labels for cAMP binding sites on proteins. The bromoketo group can

† This work was supported by grants from NIH (HL 36579) (R.W.C.), from NSF (DMB-88-04706) (R.F.C.), from the Southeastern Pennsylvania Chapter of the American Heart Association (P.G.G.), and from The Council for Tobacco Research (2340) (R.W.C.).

‡ Temple University School of Medicine.

§ University of Delaware.

[1] Abbreviations: 2-BDB-TcAMP, 2-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 3′,5′-cyclic monophosphate; 2-BOP-TcAMP, 2-[(3-bromo-2-oxopropyl)thio]adenosine 3′,5′-cyclic monophosphate; 8-BDB-TcAMP, 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-cyclic monophosphate; 2-TcAMP, 2-thioadenosine 3′,5′-cyclic monophosphate; 8-BrcAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; 8-TcAMP, 8-thioadenosine 3′,5′-cyclic monophosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)-amino methane; TLC, thin-layer chromatography.

0006-2960/90/0429-887$02.50/0

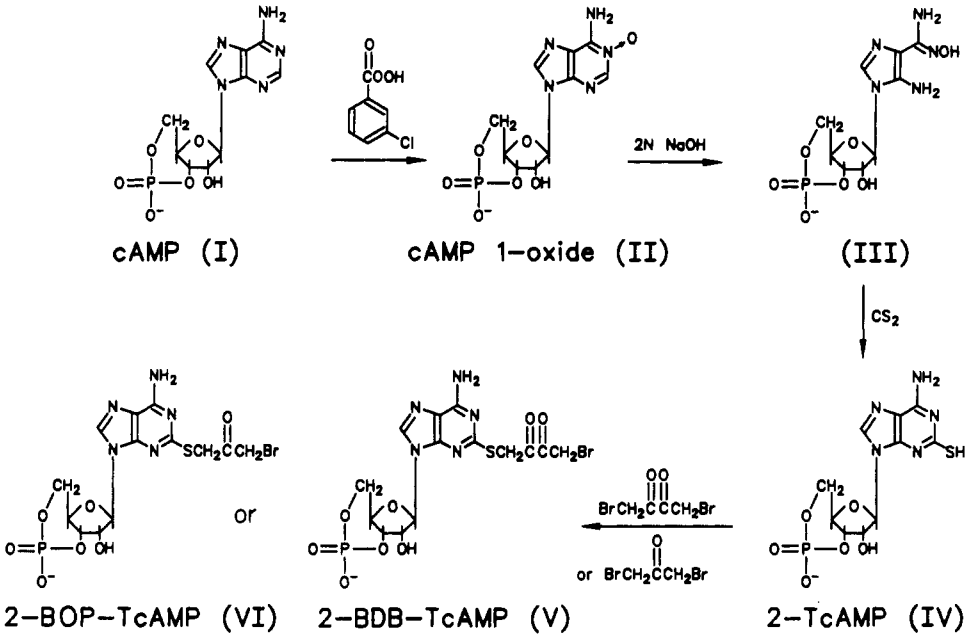

FIGURE 1: Synthesis of 2-BDB-TcAMP and 2-BOP-TcAMP.

react with most nucleophilic groups found in proteins (Hartman, 1977), and the dioxo group in 2-BDB-TcAMP and 8-BDB-TcAMP has the potential to react with arginine residues as well (Yankeelov, 1970; Riordan, 1973). We have evaluated these three compounds as affinity labels for the low K_m (high affinity) cAMP phosphodiesterase from human platelets. A preliminary account of this work has been presented (Grant et al., 1988a).

Experimental Procedures

Materials. cAMP, phosphorus standard solution, Malachite Green base, mercuric thiocyanate, and buffer salts were supplied by Sigma Chemical Co. 1,4-Dibromobutanedione was obtained from Aldrich Chemical Co. and was recrystallized from petroleum ether before use. 1,3-Dibromo-2-propanone was from Lancaster Synthesis. Bromine, sodium metabisulfate, ammonium molybdate, thiourea, and hydrogen peroxide were purchased from Fisher Scientific Co. 2-Methoxyethanol and *m*-chloroperoxybenzoic acid were from Aldrich Chemical Co. Ferric perchlorate was purchased from Fluka AG. [2,8-^{3}H]cAMP (28 Ci/mmol) was obtained from ICN Biomedicals, Inc.

NMR Procedures. NMR spectra were obtained with a Bruker WM 250-MHz spectrometer at room temperature. ^{1}H NMR samples were dissolved in D_2O containing dioxane (3.71 ppm) as an internal standard. H_3PO_4 (85%) was used as an external standard for ^{31}P NMR chemical shifts.

Thin-Layer Chromatography (TLC). TLC was performed on cellulose aluminum-backed sheets (EM Reagents, 0.1-mm thickness). Isobutyric acid/concentrated NH_4OH/H_2O (66:1:33) was used as the solvent system.

Bromide Analysis. Free bromide was measured by a modification of the procedure described by Zall et al. (1956), in which bromide displaces thiocyanate from mercuric thiocyanate. The liberated thiocyanate reacts with ferric ion to form a red complex, which is then measured spectrophotometrically. An aliquot (10–50 μL) containing 10–200 nmol of bromide was mixed with 100 μL of 60% perchloric acid. To this solution was added 240 μL of 0.17 M ferric perchlorate in 4 N perchloric acid. Methanol was added to bring the total volume to 1.0 mL. After 2 min, the absorbance at 463 nm was measured for standard bromide solutions and unknown samples. Samples containing covalently bound bromide were hydrolyzed with 0.2 M NaOH at room temperature for 1 h before addition of test reagents.

Phosphorus Analysis. The inorganic phosphorus determination was a modification of the procedures of Hess and Derr (1975) and Lanzetta et al. (1979). A 150–200-μL sample containing inorganic phosphate was mixed with 50 μL of 10 N H_2SO_4 and 800 μL of a fresh mixture (3:1) of Malachite Green (0.045% in 0.33 N HCl) and ammonium molybdate (4.2% in 3 N HCl). The 660-nm absorbance was measured after 10 min. Samples containing organic phosphorus were digested prior to analysis. A maximum of 10 nmol of sample in 200–400 μL of buffer was heated with 50 μL of 10 N H_2SO_4 at 190 °C for 2 h in a tube closed with aluminum foil. If the residue was colored at this point, 50 μL of 30% H_2O_2 was added and the sample was heated again at 190 °C for 1 h in an open tube.

Synthesis of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-Cyclic Monophosphate. The overall synthetic scheme is shown in Figure 1. cAMP 1-oxide (Figure 1, II) was synthesized by reaction of cAMP with *m*-chloroperoxybenzoic acid. Treatment of cAMP 1-oxide with NaOH followed by reaction with carbon disulfide yielded 2-thioadenosine 3′,5′-cyclic monophosphate (2-TcAMP) (Figure 1, IV). Condensation of 2-TcAMP with 1,4-dibromobutanedione gave 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-cyclic monophosphate (2-BDB-TcAMP) (Figure 1, V).

Preparation of Adenosine 3′,5′-Cyclic Monophosphate 1-Oxide. Adenosine 3′,5′-cyclic monophosphate 1-oxide (cAMP 1-oxide) was synthesized by the method described by Meyer et al. (1973) with slight modifications. A biphasic mixture containing 3 g of cAMP, 25 mL of 1 N sodium acetate, 25 mL of 1 N acetic acid, 45 mL of ethyl acetate, and 6 g of *m*-chloroperoxybenzoic acid was stirred for 48 h at room temperature. After this period, the aqueous (lower) layer was separated, chloroform (15 mL) and 25 mL of 1 N HCl were added to this layer, and the mixture was stirred at room temperature for 2 h. The aqueous (upper) layer was recovered, isopropyl alcohol (1200 mL) was added to it, and the cloudy white solution was maintained overnight at 4 °C. cAMP 1-oxide was collected by filtration and dried under vacuum. The ultraviolet absorption spectrum for cAMP 1-oxide ex-

hibited an A_{260nm}/A_{233nm} ratio of 0.23, similar to the value of 0.22 observed for AMP 1-oxide (Kapetanovic et al., 1985).

Preparation of 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamidoxime 3',5'-Cyclic Monophosphate. cAMP 1-oxide was treated with 2 N NaOH as described by Meyer et al. (1973). cAMP 1-oxide (3 g) was dissolved in 10 mL of 2 N NaOH and poured into a refluxing solution of 20 mL of 2 N NaOH. After refluxing for 10 min, the reaction was cooled on ice, and the pH was adjusted to 11 with AG50W-H4 (H^+ form, 100–200 mesh). The resin was filtered and washed with H_2O until the absorbance at 260 nm of the washings was less than 0.2. The filtrate and washings were applied to a 1.5 × 36 cm column of AG1-X2 (formate form, 100–200 mesh). The column was washed with water until the absorbance at 260 nm was less than 0.02. The column was then eluted with a linear gradient (300 mL of water and 300 mL of 0.5 N formic acid). Fractions (2.2 mL) were collected and were monitored at 260 nm. The product was located between fractions 105 and 145 and began crystallizing in the tubes. Fractions containing product were pooled and evaporated to a small volume, diluted with ethanol, and chilled on ice. The white powder was collected by filtration and washed with ethanol: yield 1.5 g.

Preparation of 2-Thioadenosine 3',5'-Cyclic Monophosphate. Methanol (15 mL) was added to 0.35 g of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamidoxime 3',5'-cyclic monophosphate. Triethylamine was then added dropwise to form the soluble triethylammonium salt. Pyridine (10 mL) and carbon disulfide (4 mL) were added, after which the mixture was refluxed for 5 h. The yellowish brown mixture was allowed to cool to room temperature, and the solvents were removed under vacuum. The residue was dissolved in water (3 mL) and centrifuged to remove any insoluble material. The supernatant was applied to a 1 × 35 cm column of Sephadex G-10 and eluted with H_2O. Elution was monitored by absorbance at 290 nm. A broad yellow band was obtained and concentrated under vacuum to 5 mL and then applied to a 1.5 × 30 cm column of AG50W-H4 (H^+ form, 100–200 mesh), which was eluted with distilled water. Elution was monitored by absorbance at 290 nm. Fractions containing 2-thioadenosine 3',5'-cyclic monophosphate (2-TcAMP) were pooled and concentrated under vacuum to approximately 5 mL. The product was precipitated as a pale yellow powder by addition of 40 mL of a 1:1 mixture of isopropyl alcohol and methanol. The solvents were removed under vacuum: yield 74%. TLC exhibited a single ultraviolet absorbing spot at R_f = 0.36, whereas the corresponding 5'-monophosphate analogue had R_f = 0.24. NMR showed peaks at δ 8.06 (H_8) and 6.01 (H_1').

Preparation of 2-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 3',5'-Cyclic Monophosphate. 2-TcAMP was dissolved in methanol to give a solution of 26 mM by the addition of triethylamine dropwise to form the soluble triethylammonium salt. Sufficient triethylamine was added to adjust the pH to approximately 5.3 as estimated by pH paper. Recrystallized 1,4-dibromobutanedione was dissolved in 0.5 mL of methanol (820 mM). This solution was added (with rapid mixing) to 0.5 mL of the 2-TcAMP solution. Reaction occurred within 2 min as assessed spectrophotometrically from the decrease in absorbance at 290 nm. The reaction mixture was then placed on ice, and the product was precipitated by addition of 10 mL of diethyl ether. The precipitate was collected by centrifugation, redissolved in 0.5 mL of methanol, and again precipitated with diethyl ether. The resultant precipitate was washed with diethyl ether, dried under nitrogen to give a white powder, and stored at −80 °C. The yield of 2-BDB-TcAMP

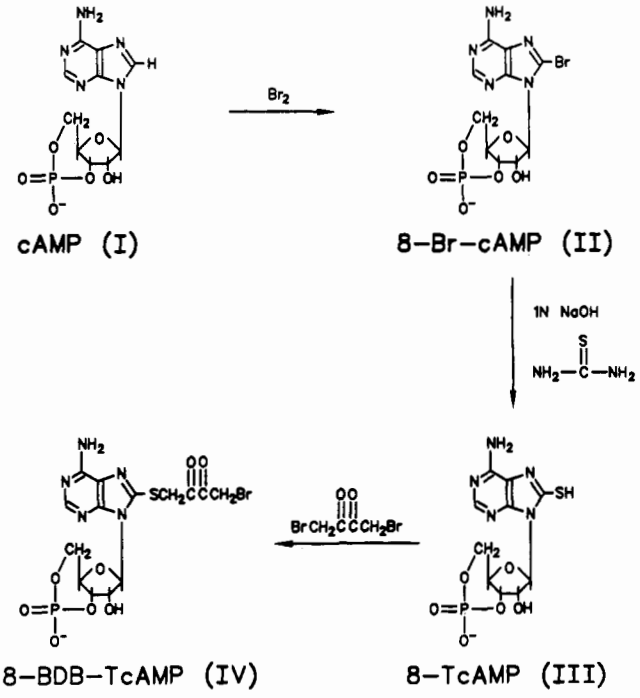

FIGURE 2: Synthesis of 8-BDB-TcAMP.

(Figure 1, V) from this step was 80%, and the product exhibited an R_f of 0.55 on TLC.

Preparation of 2-[(3-Bromo-2-oxopropyl)thio]adenosine 3',5'-Cyclic Monophosphate. 2-[(3-Bromo-2-oxopropyl)-thio]adenosine 3',5'-cyclic monophosphate (2-BOP-TcAMP) was synthesized by reaction of 2-TcAMP with 1,3-dibromo-2-propanone as follows. 2-TcAMP was dissolved in methanol (to yield 26 mM) by the addition of triethylamine to pH 5.3 as described above. 1,3-Dibromo-2-propanone (0.05 mL) was added to 0.45 mL of methanol (930 mM). This solution was added (with rapid mixing) to 0.5 mL of the 2-TcAMP solution. Reaction occurred immediately and could be assessed spectrophotometrically from the decrease in absorbance at 290 nm. The reaction mixture was placed on ice, and the product was precipitated by addition of 10 mL of diethyl ether. The precipitate was collected by centrifugation, redissolved in 0.5 mL of methanol, and again precipitated with diethyl ether. The resultant precipitate was washed with diethyl ether and dried under nitrogen to give a white powder. 2-BOP-TcAMP (Figure 1, VI) was stored dry and desiccated at −80 °C: yield, 80%; R_f = 0.65 on TLC.

Synthesis of 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 3',5'-Cyclic Monophosphate. The starting material, 8-bromocAMP (8-BrcAMP), was synthesized by the procedure of Ikehara and Uesugi (1969). 8-Thio-cAMP (8-TcAMP) was prepared by the method of Muneyama and co-workers with slight modifications (Muneyama et al., 1971). 1,4-Dibromobutanedione was coupled with the triethylamine salt of 8-TcAMP to yield the final product, as shown in Figure 2.

Preparation of 8-Bromoadenosine 3',5'-Cyclic Monophosphate. cAMP (400 mg, 1.1 mmol) was dissolved in 27.6 mL of H_2O and 1.2 mL of 1 N NaOH. To this solution was added 40 mL of 1 M sodium acetate, pH 4, followed by 11.1 mL of bromine water [10.96 mL of H_2O and 0.144 mL (5.5 mmol) of Br_2]. The solution was stirred overnight at room temperature, and then 70 mg of sodium metabisulfate was added to remove residual bromine. The solvent was removed under vacuum, and the residue was coevaporated three times with ethanol. Next, the residue was dissolved in 400 mL of H_2O and neutralized with concentrated NH_4OH. The solution

was applied to a 1 × 25 cm DEAE-Sephadex column equilibrated with 10 mM NH_4HCO_3. The column was eluted with a linear gradient (1.5 L of 10 mM NH_4HCO_3 and 1.5 L of 0.1 M NH_4HCO_3). Fractions (12 mL) were monitored for absorbance at 260 nm. Several small peaks eluting in fractions 110–120 were discarded. A broad peak containing 8-BrcAMP (Figure 2, II) eluted in fractions 125–182, which were pooled and lyophilized. The spectrum of 8-BrcAMP shows an absorption maximum at 263 nm when measured at pH 8.0. The sample was redissolved in water and lyophilized repeatedly until all the ammonium bicarbonate was removed. The yield was 88%.

Preparation of 8-Thioadenosine 3′,5′-Cyclic Monophosphate. A solution of 1 N NaOH (1.23 mL), thiourea (133 mg, 1.7 mmol), and 8-BrcAMP (350 mg, 0.79 mmol) was evaporated to a solid residue. The residue was dissolved in 2-methoxyethanol (17.5 mL) and refluxed for 2 h. The reaction mixture was evaporated to dryness, redissolved in 5 mL of H_2O, and centrifuged for 10 min at 13000 rpm. The brown supernatant was applied to a 1 × 25 cm DEAE-Sephadex column equilibrated with 10 mM NH_4HCO_3; 12-mL fractions were collected and monitored for absorbance at 290 nm. The column was washed with the starting buffer and then eluted with a linear gradient (2 L of 10 mM NH_4HCO_3 and 2 L of 0.6 M NH_4HCO_3). Four peaks appeared during the gradient elution. The third peak (fractions 150–190) had a spectrum identical with that of 8-BrcAMP. The fourth peak, identified as 8-TcAMP (Figure 2, III) by its absorption maximum at 291 nm (pH 8), eluted in fractions 201–220. The pooled fractions were evaporated and desalted by lyophilization, with a yield of 39%. The R_f was 0.38 on TLC.

Preparation of 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 3′,5′-Cyclic Monophosphate. Conversion of III to IV (Figure 2) was carried out in the same manner as described for the synthesis of 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate by deCamp et al. (1988). 8-TcAMP (20 mg) was dissolved in 1 mL of H_2O and applied to a 1.8 × 50 cm column of AG50W-X4 (pyridinium). The column was eluted with 0.05 M pyridinium acetate, pH 7.0, at room temperature. Fractions (10 mL) were collected and monitored for absorbance at 291 nm. Fractions 4–10 were pooled, and 7 μL of triethylamine was added. The pool was dried under vacuum and coevaporated three times with methanol. The residue was dissolved in methanol to give a concentration of 26 mM, and the pH was adjusted to 5 (estimated by pH paper) by the addition of triethylamine. 1,4-Dibromobutanedione (0.01 g, recrystallized) was dissolved in 0.5 mL of methanol (820 mM) and rapidly mixed with 0.5 mL of the 8-TcAMP solution. Reaction occurred within 25 min and could be assessed spectrophotometrically by the decrease in absorbance at 306 nm and the increase in absorbance at 278 nm when the reaction mixture was diluted into 0.05 M MES buffer, pH 6. The product was precipitated by the addition of 10 mL of diethyl ether. The precipitate was collected by centrifugation, redissolved in 0.5 mL of methanol, and precipitated again with diethyl ether. The resultant precipitate was washed with diethyl ether, dried under nitrogen, and stored desiccated at −80 °C. 8-BDB-TcAMP (Figure 2, IV) was obtained as a powder in approximately 40% yield for this step. The overall yield was 17%, and the R_f was 0.61 on TLC.

Purification of Low K_m cAMP Phophodiesterase from Human Platelets. Low K_m (high affinity) cAMP phosphodiesterase was purified from the cytosolic fraction of outdated human platelets obtained from the American Red Cross by a combination of DEAE-cellulose chromatography followed by chromatography on Blue Dextran–Sepharose as previously described (Grant & Colman, 1984). The enzyme was purified approximately 2500-fold. The purified phosphodiesterase had a molecular weight of 61000 by $NaDodSO_4$–polyacrylamide gel electrophoresis and represents an enzymatically active proteolytic portion of the native molecule with a molecular weight of 110000 (Macphee et al., 1986; Grant et al., 1988b).

Measurement of cAMP Phosphodiesterase Activity. Cyclic AMP phosphodiesterase activity was measured by using the column procedure as described by Thompson et al. (1979) except that all assays were performed in 0.1 mL rather than 0.4 mL and the reaction buffer was 125 mM Tris-HCl, pH 7.5, containing 20 mM $MgCl_2$ and 1 mg/mL bovine serum albumin. [^{3}H]cAMP was purified prior to use as described by Van Lookeren Campagne and Van Haastert (1983). Inhibitory constants (K_i) for inhibitors of cAMP hydrolysis were calculated from Lineweaver–Burk plots of velocity of cAMP hydrolysis versus cAMP concentration. Assays were performed at cAMP concentrations from 0.1 to 2 μM. Hydrolysis of cAMP was 10% or less in all assays.

Reactions of 2-BDB-TcAMP and 2-BOP-TcAMP with Purified Low K_m cAMP Phosphodiesterase. Samples of purified low K_m cAMP phosphodiesterase were dialyzed against 100 mM MES, pH 6.0, 100 mM MES, pH 6.5, 100 mM PIPES, pH 7.0, or 100 mM HEPES, pH 7.5, containing 2–20 mM $MgCl_2$ and 20% glycerol. The reagents 2-BDB-TcAMP and 2-BOP-TcAMP were dissolved in 50 mM MES, pH 5.0, and small volumes ($^1/_{10}$ final reaction volume) were added to the dialyzed enzyme to start the reaction. The actual pHs of reaction mixtures were 5.98, 6.47, 6.93, and 7.45, respectively. Incubations were conducted at 24 or 30 °C. At timed intervals, aliquots were withdrawn and diluted 50-fold in 125 mM Tris-HCl, pH 7.5, containing 20 mM $MgCl_2$, 1 mg/mL bovine serum albumin, and 2 mM dithiothreitol. The diluted samples were then assayed for phosphodiesterase activity as described above. Control samples were incubated under identical conditions but in the absence of the cAMP analogues.

Reaction of 8-BDB-TcAMP with Low K_m cAMP Phosphodiesterase. Prior to the reaction, 1.5-mL polypropylene microcentrifuge tubes were filled with a 1 mg/mL solution of bovine serum albumin and incubated for 1 h at 24 °C. The tubes were then drained, rinsed extensively with water, and dried.

The purified low K_m cAMP phosphodiesterase was dialyzed overnight versus 50 mM HEPES, pH 7.0, containing 2 mM $MgCl_2$ and 20% glycerol (dialysis buffer). The dialyzed phosphodiesterase (5–10 μg/mL) was incubated with varying concentrations of 8-BDB-TcAMP at 24 °C. The 8-BDB-TcAMP was dissolved in 50 mM MES, pH 5.0, and small volumes (one-fifth the final reaction volume) were added to the dialyzed enzyme to start the reaction. Protecting ligands were added to the reaction mixture in dialysis buffer and were preincubated with the phosphodiesterase for 2–5 min prior to the addition of the 8-BDB-TcAMP. Control samples were incubated under identical conditions in the absence of the 8-BDB-TcAMP.

At timed intervals, aliquots (2 μL) were withdrawn and diluted 50-fold in 50 mM Tris-HCl, pH 7.5, containing 20 mM $MgCl_2$, 0.5 M NaCl, and 10% glycerol (elution buffer) with 1 mg/mL bovine serum albumin (100 μL total volume). The diluted samples were applied to 1-mL Sephadex G-50 columns prepared in elution buffer by centrifugation as described by Tuszynski et al. (1983) to remove unbound 8-BDB-TcAMP and any protecting ligands. After addition of the diluted samples, the columns were centrifuged, a 50-μL aliquot of

elution buffer without BSA was added to each column, and the columns were recentrifuged. The eluate and wash from each column were pooled, and the phosphodiesterase activity in each sample was measured. Protein determinations were performed by using the BCA assay (Pierce) in microtiter plates as described by Smith et al. (1985).

Results

Characterization of 2-[(4-Bromo-2,3-dioxobutyl)thio]-adenosine 3',5'-Cyclic Monophosphate and 2-[(3-Bromo-2-oxopropyl)thio]adenosine 3',5'-Cyclic Monophosphate. The purity of these new bromodioxobutyl and bromooxopropyl cAMP analogues was assessed by thin-layer chromatography as described under Experimental Procedures. In each case a single ultraviolet-absorbing spot was observed which exhibited an R_f distinguishable from that of 2-thioadenosine 3',5'-cyclic monophosphate.

The ultraviolet absorption spectra of 2-BDB-TcAMP has a maximum at 246 ($\epsilon = 25.1 \times 10^3$ M^{-1} cm^{-1}) with a shoulder at 270 nm ($\epsilon = 11.1 \times 10^3$ M^{-1} cm^{-1}). This property allows the reaction between 2-TcAMP and 1,4-dibromobutanedione to be followed spectrophotometrically, as the 2-TcAMP exhibits absorption maxima at 290 (15×10^3 M^{-1} cm^{-1}) and 256 nm (21×10^3 M^{-1} cm^{-1}) when measured in 0.10 M MES buffer, pH 6.0. Reaction with 1,4-dibromobutanedione causes a decrease in the extinction coefficient at 290 nm (5.8×10^3 M^{-1} cm^{-1} for the BDB analogue). The ultraviolet absorption spectrum of 2-BOP-TcAMP is identical with that observed for 2-BDB-TcAMP. Therefore, the reaction of 2-TcAMP with 1,3-dibromopropanone may also be followed spectrophotometrically from the decrease in absorbance at 290 nm.

The bromide and phosphorus contents of these new nucleotide analogues were measured as described under Experimental Procedures. In each case, the organic phosphorus and hydrolyzable bromide contents have been expressed as a ratio to the spectrophotometrically determined cAMP analogue. For 2-BDB-TcAMP, 1.03 and 1.00, respectively, were measured as the ratios of bromide:nucleotide and phosphorus:nucleotide. For 2-BOP-TcAMP, 0.97 and 1.02, respectively, were the ratios of bromide:nucleotide and phosphorus:nucleotide.

The proton NMR spectrum of 2-BDB-TcAMP in D_2O had peaks centered at δ 8.17 (H_8), 6.15 (H_1'), 4.65 (H_3'), 4.51 (H_2'), 4.42 (H_5'), 4.26 (H_4'), and 3.74–3.85 (CH_2Br); the CH_2S was obscured by the HDO peak. For comparison, 2-thioadenosine 3',5'-cyclic monophosphate had peaks centered at δ 8.06 (H_8), 6.01 (H_1'), 4.67 (H_3'), 4.55 (H_2'), 4.45 (H_5'), and 4.28 (H_4'); and cAMP (Blackburn et al., 1973) exhibited peaks at δ 8.21 (H_8), 8.14 (H_2'), 6.09 (H_1'), 4.75 (H_3'), 4.71 (H_2'), 4.60 (H_5'), and 4.37 (H_4'). The 2-bromooxopropyl-TcAMP had resonances at δ 8.16 (H_8), 6.05 (H_1'), 4.73 (H_3'), 4.55 (H_2'), 4.45 (H_5'), 4.32 (H_4'), 4.25 (CH_2Br), and 4.61 (CH_2S). Assignments of the ribose protons were made by comparison with the proton NMR spectrum of cAMP (Blackburn et al., 1973). Assignments of the CH_2Br and CH_2S protons were made by comparison to the proton NMR spectrum of 1,3-dibromo-2-propanone and 1,4-dibromobutanedione as described previously (Bailey & Colman, 1987).

The proton-decoupled ^{31}P NMR spectrum of 2-BDB-TcAMP was performed at 25 °C in a 2-mL sample volume, pH 8.0, containing 25% D_2O as an internal field frequency lock and EDTA to eliminate any line broadening due to paramagnetic impurities. A single resonance at −1.08 ppm was observed for 2-BDB-TcAMP confirming this phosphate as a 3',5'-cyclic phosphate (Cozzone & Jardetzky, 1976).

Characterization of 8-[(4-Bromo-2,3-dioxobutyl)thio]-adenosine 3',5'-Cyclic Monophosphate. 8-BDB-TcAMP was characterized by TLC, NMR, bromide assay, and phosphorus assay. On TLC, a single fluorescent, UV-absorbing spot was observed with an R_f of 0.61, which was clearly distinguishable from 8-TcAMP (0.38) and 1,4-dibromobutanedione (R_f = 0.78).

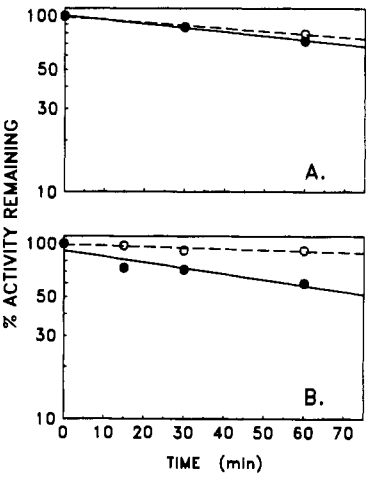

FIGURE 3: Reaction of 2-BDB-TcAMP (A) and 2-BOP-TcAMP (B) with low K_m cAMP phosphodiesterase from human platelets. (A) Incubation was performed at pH 7.0 and 24 °C in the presence of 5 mM 2-BDB-TcAMP. Control (●); 2-BDB-TcAMP (○). (B) Incubation was done at pH 6.5 and 24 °C in the presence of 5 mM 2-BOP-TcAMP. Control (●); 2-BOP-TcAMP (○).

The proton-decoupled ^{31}P NMR of 8-TcAMP showed a singlet at −0.80 ppm, indicative of a 3',5'-cyclic monophosphate. For comparison, the value obtained by Cozzone and Jardetzky (1976) for cAMP at pH 7 was −1.58 ppm. The proton spectrum had peaks centered at 6.20 ppm (d, H_1') and 8.30 ppm (s, H_2).

The extinction coefficient of 8-BDB-TcAMP is 1.64×10^4 M^{-1} cm^{-1} when measured at 278 nm in 0.1 N HCl. A stoichiometric amount of organic phosphorus (1.00) and hydrolyzable bromide (1.05) relative to spectrophotometrically determined 8-BDB-TcAMP was found.

Reaction of 2-BOP-TcAMP and 2-BDB-TcAMP with Platelet Low K_m cAMP Phosphodiesterase. Incubation of 2-BOP-TcAMP or 2-BDB-TcAMP (at concentrations up to 10 mM) with purified low K_m cAMP phosphodiesterase from human platelets at pH values from 6.0 to 7.5 and at 24 or 30 °C did not cause irreversible inactivation for periods as long as 2 h (Figure 3). Alterations in the $MgCl_2$ concentration of the buffers (0–20 mM) did not result in any inactivation (data not shown). At higher concentrations, the nucleotide analogues often appeared to stabilize the enzyme against denaturation (Figure 3B) as does cAMP.

However, when tested as reversible inhibitors, both 2-BDB-TcAMP and 2-BOP-TcAMP (at 2.5 and 5 μM) were observed to be competitive inhibitors of cAMP hydrolysis by the low K_m cAMP phosphodiesterase, with K_i values of 0.96 ± 0.12 and 0.70 ± 0.12 μM, respectively (data not shown), indicating similar affinity to the substrate cAMP (K_m = 0.2 μM) (Grant & Colman, 1984). These results suggest that 2-BDB-TcAMP and 2-BOP-TcAMP bound to the phosphodiesterase so that the lack of irreversible inactivation was not due to a failure of the cAMP analogues to bind to the enzyme. As determined by the thin-layer chromatography, neither analogue was hydrolyzed by the low K_m cAMP phosphodiesterase in 60 min, while cAMP was fully hydrolyzed in 15 min at 24 °C.

Reaction of 8-BDB-TcAMP with Platelet Low K_m cAMP Phosphodiesterase. When tested as a reversible inhibitor,

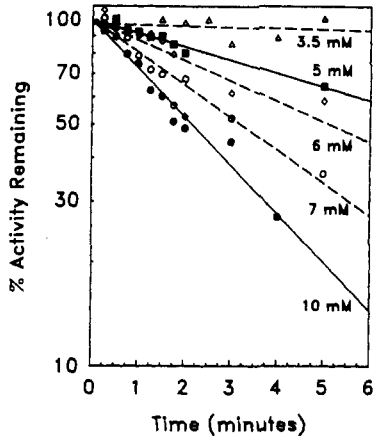

FIGURE 4: Inactivation of low K_m cAMP phosphodiesterase by 8-BDB-TcAMP. The incubation was conducted at pH 7.0 and 24 °C as described under Experimental Procedures. The results shown are representative results from one experiment. The results were corrected for loss of activity in control samples (maximum of 2–3% over the course of the experiment) and normalized for the protein concentration of the samples. Concentrations of 8-BDB-TcAMP used are indicated on graph.

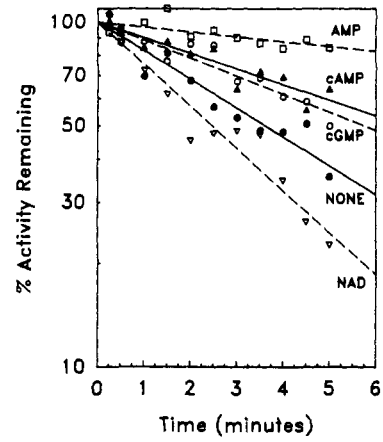

FIGURE 5: Effect of various nucleotides on inactivation of low K_m cAMP phosphodiesterase by 8-BDB-TcAMP. Enzyme (8 μg/mL) was incubated at pH 7.0 and 23 °C with 7 mM 8-BDB-TcAMP as described under Experimental Procedures. All additional nucleotides were added to a final concentration of 20 mM. The results were corrected for loss of activity in control samples and normalized for the protein content of the samples.

8-BDB-TcAMP (at 10 and 50 μM) was observed to be a competitive inhibitor (K_i = 9.4 ± 2.0 μM) of cAMP hydrolysis by the low K_m cAMP phosphodiesterase, indicating weaker affinity than cAMP or the 2-substituted analogues. In contrast to the results with 2-BOP-TcAMP and 2-BDB-TcAMP, incubation of the purified phosphodiesterase with 8-BDB-TcAMP at concentrations from 1 to 10 mM resulted in a rapid, time-dependent loss of phosphodiesterase activity (Figure 4). The reaction rate, measured over a period of 5 min, obeyed pseudo-first-order kinetics and was dependent on reagent concentration (Figure 4). Calculation of the second-order rate constant from a plot of k_{obs} versus concentration of 8-BDB-TcAMP yielded a value of 0.031 ± 0.009 min^{-1} mM^{-1} (N = 19). Dilution and gel filtration of enzyme samples did not reverse the inactivation caused by 8-BDB-TcAMP, indicating that the inactivation is due to an irreversible reaction.

If the 8-BDB-TcAMP is inactivating the phosphodiesterase by a reaction at its active site, the normal substrates and/or products should protect the phosphodiesterase against inactivation. As demonstrated in Figure 5, addition of cAMP, cGMP, and AMP to the reaction mixture resulted in a decrease in the rate of inactivation, indicating protection of the active site. In contrast, NAD^+, which is not a normal ligand for this enzyme, did not protect against inactivation. Further evidence that 8-BDB-TcAMP is reacting at the active site of the low K_m cAMP phosphodiesterase is provided by the fact that it is hydrolyzed completely in 15 min by a concentration of the enzyme which also hydrolyzes cAMP completely during the same period. These results suggest that the inactivation of the phosphodiesterase by 8-BDB-TcAMP is occurring by reaction at the active site of the enzyme.

The K_is for inhibition of cAMP hydrolysis by cGMP, AMP, and NAD^+ are 0.04 μM (Grant & Colman, 1984), 4.7 ± 0.4 mM, and >70 mM, respectively (data not shown). The degree of protection by AMP appears to be greater than that observed with cAMP or cGMP, although the K_m and K_i for the cyclic phosphate compounds indicate that these bind more tightly to the phosphodiesterase than does AMP. The amount of cAMP and cGMP added (20 mM) should have saturated the substrate binding site. This result may suggest that 8-BDB-TcAMP is reacting more at a "product binding" site, and as a result AMP gives a greater degree of protection.

Discussion

Inhibition of the cyclic nucleotide phosphodiesterase can produce an increase in the cAMP levels within cells, which in turn can affect many cellular processes including stimulation of glycogenolysis, lipolysis, steroidogenesis, and inhibition of platelet activation. The potential medical usefulness of phosphodiesterase inhibitors has led to the synthesis of many cyclic nucleotide analogues for evaluation as cyclic nucleotide phosphodiesterase inhibitors (Michal et al., 1974; Miller et al., 1984; Ogreid et al., 1985).

Miller and co-workers (Christensen et al., 1975; Khwaja et al., 1975; Meyer et al., 1975; Miller et al., 1980, 1984) have studied a series of 2- and 8-substituted cAMP as well as cGMP analogues as inhibitors of partially purified phosphodiesterases from a number of sources including bovine heart and rabbit kidney and lung. They found that the 2-substituted cAMP analogues were substrates for a phosphodiesterase preparation from rabbit kidney undergoing hydrolysis to the corresponding AMP analogue (Meyer et al., 1975; Miller et al., 1984). However, cAMP analogues with substituents at either the 2- or 8-position could act as inhibitors of cAMP hydrolysis (Khwaja et al., 1975; Meyer et al., 1975; Miller et al., 1980, 1984). The inhibitory potency of these analogues varied markedly depending on the source of the cAMP phosphodiesterase. Substitutions at the 2-position had a much greater effect on the bovine heart phosphodiesterase than on the rabbit lung phosphodiesterase: short-chain alkyl groups decreased the inhibitory potency of these compounds for the bovine heart phosphodiesterase but had little effect on their potency for inhibiting the rabbit lung phosphodiesterase. In contrast, both cAMP phosphodiesterases were reasonably tolerant of straight-chain alkylthio substituents at the 8-position (Miller et al., 1984). These results suggest that the bovine heart phosphodiesterase may have more rigid steric requirements in the region of the cAMP binding site adjacent to the 2-position than does the phosphodiesterase from rabbit lung.

The 4-bromo-2,3-dioxobutyl and 3-bromo-2-oxopropyl groups are favorable reactive side chains for potential affinity-labeling reagents. The bromoketo group can react with the nucleophilic side chains of many of the common amino acids found in proteins including cysteine (Batra & Colman, 1986), aspartate (Huang & Colman, 1988), histidine (Batra et al., 1989), tyrosine (DeCamp & Colman, 1989), and lysine and glutamate (Hartman, 1977). In addition, the dioxo group

has the added potential of being able to react with arginine residues (Yankeelov, 1970; Riordan, 1973). Nucleotide analogues incorporating these side chains have been used as affinity labels for several enzymes including glutamate dehydrogenase (Batra & Colman, 1984, 1986), NAD-specific isocitrate dehydrogenase (Huang & Colman, 1984, 1988), pyruvate kinase (DeCamp et al., 1988), and acetyl-CoA binding proteins (Owens & Barden, 1978; Clements et al., 1979; Katiyar et al., 1982). In the cases of NAD^+- and $NADP^+$-dependent isocitrate dehydrogenases, glutamate dehydrogenase, and pyruvate kinase, specific peptides containing amino acid residues modified by bromodioxobutyl nucleotide analogues have been isolated and characterized (Batra & Colman, 1986; Bailey & Colman, 1987; Huang & Colman, 1988; Batra et al., 1989; DeCamp & Colman, 1989).

The inactivation of the low K_m cAMP phosphodiesterase from human platelets by 8-BDB-TcAMP appears to be a specific affinity-labeling reaction occurring in proximity to the active site of the enzyme. The enzyme is irreversibly inactivated by 8-BDB-TcAMP since neither dilution nor gel filtration reverses the inactivation. The specificity of the reaction of 8-BDB-TcAMP with the phosphodiesterase is indicated by the protective effect of the substrates cAMP and cGMP and the product AMP in decreasing the rate of inactivation. In contrast, NAD^+, which is not a ligand for this enzyme, did not protect the enzyme from inactivation.

The lack of inactivation of the phosphodiesterase by 2-BOP-TcAMP and 2-BDB-TcAMP contrasted with the rapid reaction with 8-BDB-TcAMP suggests that the 2-substituted analogues interact with the phosphodiesterase quite differently from 8-BDB-TcAMP. The fact that 2-BDB-TcAMP and 2-BOP-TcAMP function as reversible inhibitors of cAMP hydrolysis indicates that the 2-substituted analogues can bind to the enzyme. In view of this result, the failure of the 2-substituted analogues to cause inactivation suggests that the enzyme lacks appropriately reactive groups accessible from the 2-position of the cAMP binding site.

The results of Miller and co-workers (Christensen et al., 1975; Khwaja et al., 1975; Meyer et al., 1975; Miller et al., 1980, 1984) as well as our results with the 2- and 8-substituted analogues indicate that the nature of the cAMP binding site may vary considerably among the different forms of cAMP phosphodiesterase. These observations, in turn, suggest that the results of affinity-labeling studies utilizing the cAMP analogues here described might differ when performed on different cAMP phosphodiesterases. These cAMP affinity analogues may prove very useful in probing the structure of the active site of the various forms of cAMP phosphodiesterase as well as cAMP binding sites of other proteins.

References

Appleman, M. M., Ariano, M., Takemoto, D. J., & Whitson, R. H. (1982) *Handb. Exp. Pharmacol. 58*, 261–300.

Bailey, J. M., & Colman, R. F. (1987a) *Biochemistry 26*, 6858–6869.

Bailey, J. M. & Colman, R. F. (1987b) *J. Biol. Chem. 262*, 12620–12626.

Batra, S. P., & Colman, R. F. (1984) *Biochemistry 23*, 4940–4946.

Batra, S. P., & Colman, R. F. (1986) *Biochemistry 25*, 3508–3515.

Batra, S. P., Lark, R. H., & Colman, R. F. (1989) *Arch. Biochem. Biophys. 270*, 277–285.

Beavo, J. A., Hansen, R. S., Harrison, S. A., Hurwitz, R. L., Martins, T. J., & Mumby, M. C. (1982) *Mol. Cell. Endocrinol. 28*, 387–410.

Blackburn, B. J., Lapper, R. D., & Smith, I. C. P. (1973) *J. Am. Chem. Soc. 95*, 2873–2878.

Charbonneau, H., Beier, N., Walsh, K. A., & Beavo, J. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9308–9312.

Chen, C.-N., Denome, S., & Davis, R. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9313–9317.

Christensen, L. F., Meyer, R. B., Jr., Miller, J. P., Simon, L. N., & Robins, R. K. (1975) *Biochemistry 14*, 1490–1496.

Clements, P. R., Barden, R. E., Ahmad, P. M., & Ahmad, F. (1979) *Biochem. Biophys. Res. Commun. 86*, 278–284.

Cooperman, B. S., & Brunswick, D. J. (1973) *Biochemistry 12*, 4079–4084.

Cozzone, P. J., & Jardetzky, P. (1976) *Biochemistry 15*, 4853–4859.

DeCamp, D. L., & Colman, R. F. (1989) *J. Biol. Chem. 264*, 8430–8441.

DeCamp, D. L., Lim, S., & Colman, R. F. (1988) *Biochemistry 27*, 7651–7658.

Dreyfuss, G., Schwartz, K., Blout, E. R., Barrio, J. R., Liu, F.-T., & Leonard, N. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1199–1203.

Grant, P. G., & Colman, R. W. (1984) *Biochemistry 23*, 1801–1807.

Grant, P. G., DeCamp, D. L., Bailey, J. M., Colman, R. W., & Colman, R. F. (1988a) *FASEB J. 2*, A1042.

Grant, P. G., Mannarino, A. F., & Colman, R. W. (1988b) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9071–9075.

Haley, B. E. (1975) *Biochemistry 14*, 3852–3857.

Hartman, F. C. (1977) *Methods Enzymol. 46*, 130–153.

Hess, H. H., & Derr, J. E. (1975) *Anal. Biochem. 63*, 607–613.

Huang, Y.-C., & Colman, R. F. (1984) *J. Biol. Chem. 259*, 12481–12488.

Huang, Y.-C., & Colman, R. F. (1988) *J. Cell Biol. 107*, 854a.

Ikehara, M., & Uesugi, S. (1969) *Chem. Pharm. Bull. 17*, 348–354.

Kapetanovic, E., Bailey, J. M., & Colman, R. F. (1985) *Biochemistry 24*, 7586–7593.

Katiyar, S. S., Pan, D., & Porter, J. W. (1982) *Biochem. Biophys. Res. Commun. 104*, 517–552.

Khwaja, T. A., Boswell, K. H., Robins, R. K., & Miller, J. P. (1975) *Biochemistry 14*, 4238–4244.

Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95–97.

Macphee, C. H., Harrison, S. A., & Beavo, J. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6660–6663.

Manganiello, V. C., Yamamoto, T., Elks, M., Lin, M. C., & Vaughn, M. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 16*, 291–301.

Meyer, R. B., Shuman, D. A., Robins, R. K., Miller, J. P., & Simon, L. N. (1973) *J. Med. Chem. 16*, 1319–1323.

Meyer, R. B., Jr., Uno, H., Robins, R. K., Simon, L. N., & Miller, J. P. (1975) *Biochemistry 14*, 3315–3321.

Michal, G., Mühlegger, K., Nelboeck, M., Thiessen, C., & Weiman, G. (1974) *Pharmacol. Res. Commun. 6*, 203–252.

Miller, J. P., Boswell, K. H., Meyer, R. B., Jr., Christensen, L. F., & Robins, R. K. (1980) *J. Med. Chem. 23*, 242–251.

Miller, J. P., Sigman, C. C., Johnson, H. L., Novinson, T., Springer, R. H., Senga, K., O'Brien, D. E., & Robins, R. K. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 16*, 277–290.

Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., & Simon, L. N. (1971) *Biochemistry 10*, 2390–2395.

Ogreid, D., Ekanger, R., Suva, R., Miller, J. P., Sturm, P., Corbin, J. D., & Doskeland, S. O. (1985) *Eur. J. Biochem. 150*, 219-227.
Ovchinnikov, Y. A., Gubanov, V. V., Khramtsov, N. V., Ischenko, K. A., Zagranichny, V. E., Muradov, K. G., Shuvaena, T. M., & Lipkin, V. M. (1987) *FEBS Lett. 223*, 169-173.
Owens, M. S., & Barden, R. E. (1978) *Arch. Biochem. Biophys. 187*, 299-306.
Pomerantz, A. H., Rudolph, S. A., Haley, B. E., & Greengard, P. (1975) *Biochemistry 14*, 3858-3862.
Reimann, J. E., Grant, P. G., Colman, R. W., & Colman, R. F. (1983) *J. Protein Chem. 2*, 113-129.
Riordan, J. F. (1973) *Biochemistry 12*, 3915-3923.
Sass, P., Field, J., Nikawa, J., Toda, T., & Wigler, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9303-9307.
Severin, E. A., Nesterova, M. V., Sashchenko, L. P., Rasumova, V. V., Tunitiskaya, V. L., Kochetkov, S. N., & Gulyaev, N. N. (1975) *Biochim. Biophys. Acta 384*, 413-422.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76-85.
Thompson, W. J., Terasaki, W. L., Epstein, P. M., & Strada, S. J. (1979) *Adv. Cyclic Nucleotide Res. 10*, 69-92.
Tuszynski, G. P., Knight, L. C., Kornecki, E., & Srivastava, S. (1983) *Anal. Biochem. 130*, 166-170.
Van Lookeren Campagne, M. M., & Van Haastert, P. J. M. (1983) *Anal. Biochem. 135*, 146-150.
Yankeelov, J. A., Jr. (1970) *Biochemistry 9*, 2433-2439.
Zall, D. M., Fisher, D., & Garner, M. Q. (1956) *Anal. Chem. 28*, 1665-1668.

Positional Independence and Additivity of Amino Acid Replacements on Helix Stability in Monomeric Peptides†

Gene Merutka and Earle Stellwagen*
Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242
Received July 11, 1989; Revised Manuscript Received September 7, 1989

ABSTRACT: The 17-residue peptide acetylAEAAAKEAAAKEAAAKAamide, described as an autonomous folding unit (Marqusee & Baldwin, 1987), has been used to examine the effect of amino acid replacements on helix stability. Alanine residues(s) at positions 4, 9, and 14 in the peptide sequence were replaced either singly or multiply by either serine or methionine residues with solid-phase peptide synthesis. The thermal dependence of the helix/coil transition of each peptide was observed by far-ultraviolet circular dichroism. Within experimental variation, all three single replacements exhibit a common thermal transition, and all three double replacements exhibit a different common thermal transition. These results suggest that replacement of the central alanine residue in the repeat EAAAK located in the N-terminus, in the middle, or in the C-terminus of the peptide helix has the same effect on helix stability. The melting temperature of each thermal transition was estimated by assuming a linear van't Hoff plot and a change in molar ellipticity of 33 500 deg cm^2 dmol^{-1}. Such analysis indicates that each replacement of an alanine residue by a serine residue diminishes the melting temperature by 11 ± 1 °C and that each replacement of an alanine residue by a methionine residue diminishes the melting temperature by 6 ± 1 °C. These results suggest that the effect of these replacements on helix stability is additive.

During the last several years, it has been demonstrated that analogues of the N-terminal peptide of pancreatic ribonuclease exhibit the spectral features of a monomeric helix/coil transition in aqueous solution (Shoemaker et al., 1985, 1987). It is observed that the helical content of the peptide can be enhanced significantly by optimization of the distribution of charged groups relative to the helix dipole. Recently, it has been reported that the monomeric helical form of the 17-residue peptide acetylAEAAAKEAAAKEAAAKAamide can be a major component in aqueous solution (Marqusee & Baldwin, 1987). This peptide provides a model system to examine the inherent propensity of amino acids to stabilize helicity unfettered by the packing considerations that occur in protein structures. We plan to generate systematic analogues of this parent peptide to examine the relationship between their helical content and the amino acid residue helix probabilities obtained from analysis of crystallographic structures. Since 20^{17} sequence analogues of this peptide exist,

Table I: Peptide Sequences

sequence	notation
acetylAEAAAKEAAAKEAAAKAamide	AAA
acetylAEASAKEAAAKEAAAKAamide	SAA
acetylAEAAAKEASAKEAAAKAamide	ASA
acetylAEAAAKEAAAKEASAKAamide	AAS
acetylAEASAKEASAKEAAAKAamide	SSA
acetylAEASAKEAAAKEASAKAamide	SAS
acetylAEAAAKEASAKEASAKAamide	ASS
acetylAEASAKEASAKEASAKAamide	SSS
acetylAEAMAKEAAAKEAAAKAamide	MAA
acetylAEAAAKEAMAKEAAAKAamide	AMA
acetylAEAAAKEAAAKEAMAKAamide	AAM
acetylAEAMAKEAMAKEAAAKAamide	MMA
acetylAEAMAKEAAAKEAMAKAamide	MAM
acetylAEAAAKEAMAKEAMAKAamide	AMM
acetylAEAMAKEAMAKEAMAKAamide	MMM

† This investigation was supported by Public Health Service Program Project Grant HE 14388 from the National Heart, Lung and Blood Institute and by National Science Foundation Biological Instrumentation Program Grant DMB 8413658.
* Corresponding author.

0006-2960/90/0429-894$02.50/0