Differential Labeling of the Catalytic Subunit of cAMP-Dependent Protein Kinase with Acetic Anhydride: Substrate-Induced Conformational Changes†

Joseph A. Buechler, Thomas A. Vedvick, and Susan S. Taylor*

Division of Biochemistry, Department of Chemistry, University of California, San Diego, La Jolla, California 92093

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ABSTRACT: In order to identify regions that are sensitive to substrate-induced perturbations, the catalytic subunit of cAMP-dependent protein kinase was differentially labeled with [^{3}H]acetic anhydride. Treatment of the catalytic subunit with acetic anhydride in the absence of substrates led to the irreversible inhibition of activity, and MgATP protected against inactivation. After development of a purification protocol for the lysine-containing peptides, the reactivity of each lysine in the native enzyme was calculated. The reactivity profile of lysines in the apoenzyme revealed three distinct regions. In general, the lysines within the amino-terminal segment (residues 1–83) and the carboxy-terminal segment (192–345) were relatively reactive. In contrast, the five lysines in the middle of the protein (Lys-92, -105, -111, -168, and -189) were very unreactive, indicating that these groups are sequestered from the aqueous solvent. The reactivity of each lysine was then determined in the presence of MgATP and in the presence of MgATP and a 20-residue inhibitor peptide. Most of the substrate-induced changes in lysine reactivity were localized in the amino-terminal segment, while the reactivities of lysines in the carboxy-terminal region were not altered significantly by MgATP or inhibitor peptide. MgATP affords substantial protection to three residues in particular. Lys-72, predicted previously to be essential for nucleotide binding, was relatively reactive in the apoenzyme, whereas labeling was nearly abolished in the presence of MgATP. The reactivity of Lys-47 and Lys-76 also diminished significantly in the presence of MgATP and was abolished in the presence of MgATP and the inhibitor peptide. The protection of these residues, and the structural homologies that are shared between nucleotide binding proteins, enabled a model to be proposed for part of the ATP binding site in the catalytic subunit.

The protein kinases include a large number of related enzymes that share extensive sequence similarities throughout a conserved catalytic core (Hanks et al., 1988). cAMP-dependent protein kinase is perhaps the simplest protein kinase. The holoenzyme form of cAMP-dependent protein kinase is an inactive complex composed of two catalytic subunits and two regulatory subunits. The active monomeric catalytic subunits are released from the holoenzyme complex after cAMP binds with high affinity to the regulatory subunits [for a review, see Flockhart and Corbin (1982)]. The catalytic subunit phosphorylates Ser or Thr residues on specific protein substrates having a common recognition sequence. This recognition sequence typically is composed of two basic amino acids, usually arginines, preceding the phosphorylated residue, with one intervening amino acid [Kemp et al., 1977; for a review, see Bramson et al. (1984)].

Affinity labeling and group-specific labeling have identified several amino acids in the catalytic subunit that are most likely essential for catalysis. For example, Lys-72 was affinity labeled by an analogue of ATP, 5′-*p*-(fluorosulfonyl)benzoyl]-adenosine (FSBA) (Zoller et al., 1981). Recently, Asp-184, which may participate as a general base in catalysis or in binding Mg^{2+} of the MgATP complex, was covalently modified by a hydrophobic carbodiimide, dicyclohexylcarbodiimide (DCCD) (Buechler & Taylor, 1988). These two residues, Asp-184 and Lys-72, can be covalently cross-linked when the free catalytic subunit is treated with DCCD, thus positioning both residues in close proximity at the active site. The identification of this cross-link, in conjunction with kinetic evidence, led to a proposal for the enzyme mechanism, where conformational changes occur as a result of nucleotide and protein substrate binding (Buechler & Taylor, 1989).

Whereas affinity labeling can be an effective method for identifying specific amino acid residues that either contribute to catalysis or are near substrate binding sites, a more general approach has been used here to understand overall substrate-induced conformational changes in the catalytic subunit of cAMP-dependent protein kinase. The trace acetylation of lysine residues is a sensitive method for identifying amino acids that may interact directly with substrates or with other subunits in multisubunit complexes. This approach also can be used to characterize substrate-induced conformational changes (Schewale & Brew, 1982; Giedroc et al., 1985; Richardson & Brew, 1980). A modification of this technique was applied to the catalytic subunit in order to identify regions of the protein that are sensitive to MgATP binding and to the binding of an inhibitor peptide. General features of the enzyme have been mapped in terms of lysine reactivity, and overall regions that are perturbed or unaffected by substrate binding have been identified. In addition, several specific lysine side chains are protected by MgATP from modification by acetic anhydride. On this basis, a model for a portion of the nucleotide binding site is proposed.

Experimental Procedures

Materials. Reagents were obtained from the following sources: [^{3}H]acetic anhydride (100 mCi/mmol), ICN; acetic anhydride and acetonitrile (HPLC grade), Fisher Scientific; adenosine 5′-triphosphate (ATP), L-1-(tosylamino)-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, thermolysin (type x), and α-chymotrypsin, Sigma; trifluoroacetic acid (TFA) (HPLC grade), Pierce; glycine and tris-

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* To whom correspondence should be addressed.

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(hydroxymethyl)aminomethane (Tris), United States Biochemical Corp.; ultrapure urea, Schwarz/Mann Biotech; Cytoscint, Westchem; *Staphylococcus aureus* V8 protease, Miles Laboratories, Inc.

Sources of Proteins and Peptides. The catalytic subunit was prepared from porcine heart as described previously (Nelson & Taylor, 1981). The inhibitor peptide TTYADFIASGRTGRRNAIHD, which represents a fragment of the heat-stable inhibitor protein (Cheng et al., 1986), was synthesized on an Applied Biosystems Model 430A peptide synthesizer.

Assays. Enzymatic activity was assayed spectrophotometrically according to the method of Cook et al. (1982) using the synthetic peptide substrate LRRWSLG. This peptide was synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego.

Modification of Lysine Residues. Reactions were carried out in 0.1 M Tris·HCl (pH 8.0) and 5% glycerol. Catalytic subunit (25.6 μM, 2.7 mg total) was incubated on ice for 30 min with the following substrates: (a) 12 mM Mg^{2+}; (b) 12 mM Mg^{2+} and 2 mM ATP; (c) 12 mM Mg^{2+}, 2 mM ATP, and 35 μM inhibitor peptide. [^{3}H]Acetic anhydride (24.3 mCi/mmol, 100 mM in acetonitrile) was added to a final concentration of 3 mM. The reactions were quenched after 2 min by adding glycine or β-mercaptoethanol to a concentration of 30 mM. The samples were subsequently dialyzed against 0.1 M Tris·HCl (pH 8.0) to remove the glycine or β-mercaptoethanol and the acetate that formed from the hydrolysis of acetic anhydride. After denaturing the protein by adding solid urea to a final concentration of 8 M, the lysines were stoichiometrically labeled with four aliquots (100 μL each) of nonradioactive acetic anhydride (100 mM in acetonitrile).

In order to standardize the reactivity of each lysine residue, the maximum reactivity of each lysine residue was determined by denaturing the protein (25.6 μM, 1.5 mL) with 8 M urea and stoichiometrically labeling with four aliquots (80 μL each) of [^{3}H]acetic anhydride (24.3 mCi/mmol, 100 mM in acetonitrile). Prior to proteolysis, all samples were dialyzed extensively against 50 mM NH_4HCO_3 (pH 8.1).

Proteolysis. Tryptic digests were carried out at 37 °C with a 1:50 w/w ratio of TPCK-treated trypsin to catalytic subunit in 50 mM NH_4HCO_3 (pH 8.1). After 2 h, an equal aliquot of TPCK-treated trypsin was added, and incubation was continued for an additional 2 h. Chymotrypsin and thermolysin digests were carried out under the same conditions as those described above for trypsin. *S. aureus* V8 protease digests were done in 50 mM sodium phosphate (pH 8.0) under the same conditions as those described for trypsin.

High-Performance Liquid Chromatography (HPLC). The tryptic peptides were resolved by HPLC using an Altex 3200 system with a Vydac C4 column (0.46 × 25 cm). The buffers employed were (a) 10 mM sodium phosphate (pH 6.9) and (b) CH_3CN. Absorbance was monitored at 219 nm with a 100-30 Hitachi spectrophotometer equipped with a flow-through cell and at 280 nm with a SF 769 Kratos spectrophotometer equipped with a flow-through cell. Separation of chymotrypsin, thermolysin, and *S. aureus* V8 protease peptides was accomplished with the same system except a Vydac C18 column (0.46 × 25 cm) was used instead of the C4 column. Individual peptides were rechromatographed on a Vydac C18 column with a gradient of 0.1% TFA (pH 2.1) to CH_3CN prior to sequence analysis.

Sequencing. Gas-phase sequencing was carried out with an Applied Biosystems Model 470A protein sequencer. Phenylthiohydantoin (PTH)-amino acids were identified and quantitated by HPLC with an Applied Biosystems Model 120A analyzer. In addition, aliquots of each step were counted in Cytoscint for radioactivity.

Data Analysis. HPLC peak areas were determined by integration with Nelson analytical chromatography software, version 4.1. Radioactivity was measured by counting aliquots in Cytoscint. The maximum reactivity of each lysine was established by determining the radioactivity incorporated into each lysine when labeling was carried out on the denatured protein in the presence of 8 M urea. Labeling of the native enzyme, both in the presence and in the absence of various substrates, was then compared to the urea-denatured enzyme. The relative reactivity was determined by calculating the ratio of the modification in the native enzyme to the modification in the urea-treated enzyme. The ratio of the cpm to peak area of each lysine for the urea-denatured enzyme was determined, and these values represented 100% modification. The percent modification of lysines in peptides containing only one lysine residue was determined by rechromatographing each peptide, measuring the peak area directly from the HPLC profile, and quantitating the radioactivity associated with an aliquot of that peak. Peptides containing more than one lysine were sequenced, and the peak area of the PTH-acetyllysine was correlated with radioactivity. When peptides were too long to sequence in their entirety, the tryptic peptide was digested with a second protease, and the resulting peptides were analyzed as described above.

Results

In order to determine the reactivities of lysine residues in the catalytic subunit toward acetic anhydride, conditions were established that were sufficient to inhibit enzymatic activity but not to denature or precipitate the enzyme. The procedure employed here was similar to earlier methods for the trace acetylation of lysine residues (Schewale & Brew, 1982; Giedroc et al., 1985). The major difference in the present study is that higher levels of total acetylation were achieved. Incubation of the catalytic subunit with 3 mM acetic anhydride resulted in the loss of 55% of the initial enzymatic activity within 2 min when $MgCl_2$ alone was present. After 2 min, the reaction was terminated by the addition of an excess of β-mercaptoethanol. Under these conditions, the enzyme remained completely soluble. Previous studies on this modified enzyme showed that it was still capable of binding MgATP (Buechler & Taylor, 1988). Although a K_d(MgATP) was not determined, having the capacity to bind MgATP indicated that a conformation similar to the native enzyme was retained. When the catalytic subunit was incubated with MgATP prior to being treated with acetic anhydride, only 15% inactivation was observed after 2 min. Thus, the modification of lysine residues in the apoenzyme by acetic anhydride inhibited catalytic activity, and MgATP was capable of protecting against this inactivation.

When the lysine side chains are stoichiometrically modified with acetic anhydride, the specificity of trypsin is limited to arginine residues. Consequently, 12 tryptic peptides containing lysine residues were predicted on the basis of the primary sequence of the catalytic subunit. However, since Arg-336 was extremely resistant to trypsin digestion, the 2 carboxy-terminal tryptic peptides eluted primarily as a single peptide, and only 11 peptides were observed, as indicated in Table I. The HPLC elution profile of these tryptic peptides is shown in Figure 1 (lower panel). This profile represents peptides derived from the catalytic subunit that was stoichiometrically labeled with [^{3}H]acetic anhydride in the presence of urea. The

Table I: Lysine-Containing Peptides Resulting from Tryptic Digestion of the Catalytic Subunit Modified by Acetic Anhydride

peptide	sequence
1	GNAAA^{5}AKKGS^{10}EQESV^{15}KEFLA^{20}KAKED^{25}FLKKW^{30}ENPAQ^{35}NTAHL^{40}DQFER^{45}
2	IKTLG^{50}TGSFG^{55}R
3	VMLV^{60}KHKET^{65}GNHFA^{70}MKILD^{75}KQKVV^{80}KLKQI^{85}EHTLN^{90}EKR
4	IL^{95}QAVNF^{100}PFLVK^{105}LEYSF^{110}KDNSN^{115}LYMVM^{120}EYVPG^{125}GEMFS^{130}HLR
5	DLKPE^{170}NLLID^{175}QQGYI^{180}QVTDF^{185}GFAKR^{190}
6	VK^{192}GR
7	T^{195}WTLCG^{200}TPEYL^{205}APEII^{210}LSKGY^{215}NKAVD^{220}WWALG^{225}VLIYE^{230}MAAGY^{235}PPFFA^{240}DQPIQ^{245}IYEKI^{250}VSGKV^{255}R
8	FPSH^{260}FSSDL^{265}KDLLR^{270}
9	NLLQV^{275}DLTKR^{280}
10	FGNLK^{285}NGVND^{290}IKNHK^{295}WFATT^{300}DWIAI^{305}YQR
11	KV^{310}EAPFI^{315}PKFKG^{320}PGDTS^{325}NFDDY^{330}EEEEI^{335}RVSIN^{340}EKCGK^{345}EFSEF^{350}

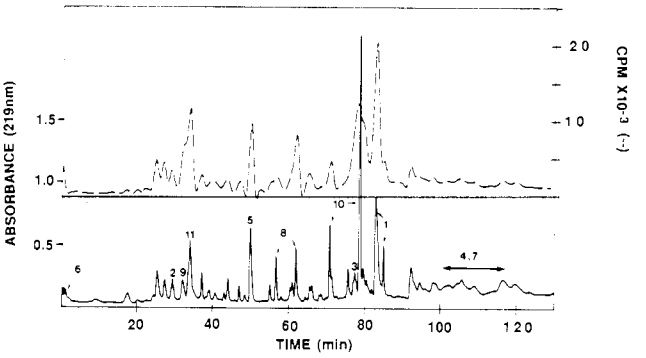

FIGURE 1: Separation of tryptic peptides labeled with [^{3}H]acetic anhydride. The catalytic subunit was treated with acetic anhydride as described under Experimental Procedures. After digestion with TPCK-treated trypsin, the resulting peptides were separated by HPLC as described. The tryptic peptides were eluted with a 120-min linear gradient from 10% to 40% CH_3CN followed by a 10-min linear gradient from 40% to 50% CH_3CN (bottom panel). The radioactivity was determined by counting 5% of each 1-mL fraction in Cytoscint (upper panel).

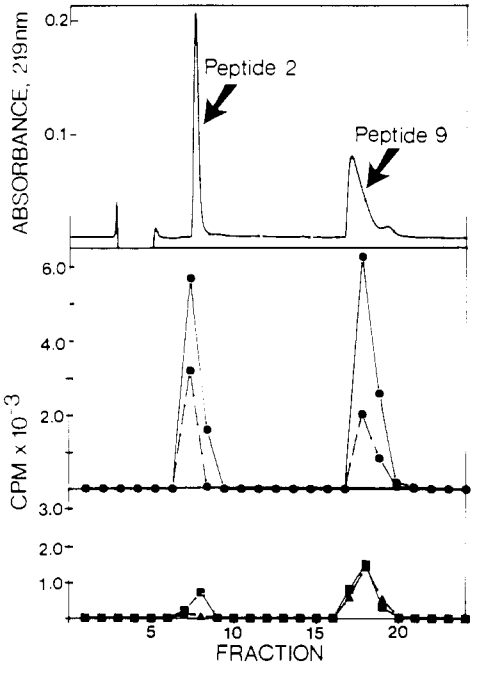

FIGURE 2: Purification of peptides 2 and 9. The fractions from Figure 1 containing peptides 2 and 9 were pooled and rechromatographed as described under Experimental Procedures. The peptides were eluted with a 60-min linear gradient from 10% to 30% CH_3CN (top panel). Radioactivity was determined by counting 10% of each 1-mL sample in Cytoscint. The radioactivity profiles of the peptides derived from the catalytic subunit that was modified in the presence of urea (—) and $MgCl_2$ (– –) are shown in the middle panel. The incorporation of radioactivity when acetylation was carried out in the presence of MgATP (■) and MgATP plus inhibitor peptide (▲) is indicated in the lower panel.

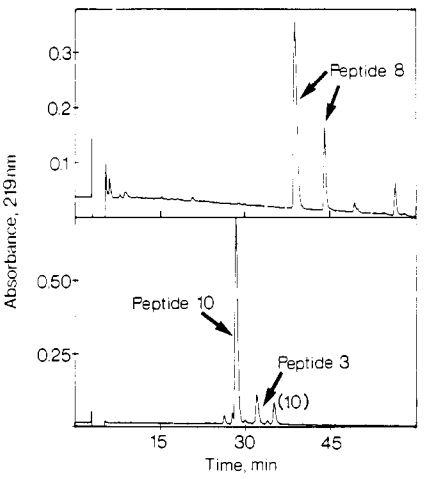

FIGURE 3: Purification of tryptic peptides labeled with [^{3}H]acetic anhydride. Fractions corresponding to peptide 8 and peptides 3 and 10 were pooled and rechromatographed as described under Experimental Procedures. Peptide 8 was eluted with a 60-min linear gradient from 20% to 32% CH_3CN (top panel). Peptides 3 and 10 were eluted with a 60-min linear gradient from 25% to 38% CH_3CN (bottom panel).

accompanying radioactivity profile is shown in the upper panel of Figure 1. In order to calculate the relative reactivity of each lysine residue in the catalytic subunit, each of the peptides shown in Table I had to be located in the HPLC profile of tryptic peptides.

Each of the peaks of radioactivity (Figure 1, upper panel) eluting before 90 min, with the exception of peptide 11, was purified by rechromatographing with a different elution gradient. The region containing peptides 2 and 9 in Figure 1 was pooled and rechromatographed as shown in Figure 2, upper panel. Purification of peptide 8 and of peptides 3 and 10 is shown in Figure 3, upper and lower panels, respectively. The peptide that eluted at 34–36 min, which corresponded to peptide 11, did not elute with a TFA gradient presumably because of the large number of acidic and hydrophobic residues. The purified peptides were subsequently identified by sequencing, and the major peaks are marked in Figure 1 (lower panel). Peptides 4 and 7 eluted as broad peaks after 90 min. It should be noted that the retention times of several peptides varied slightly between HPLC runs. For example, peptide 3 also eluted with peptides 4 and 7, and peptide 8 eluted in varying amounts in at least three different retention times, the two shown in Figure 1 and a third at approximately 64 min. The minor peaks that are not marked in Figure 1 were identical with the major peptides that are indicated. The reason for their elution at slightly different positions from the major peptides was not clear, since the reactivities of the lysines in the minor peaks were, within experimental error, the same as the major peaks. Because the reactivity of each lysine was calculated as a function of the peak area relative to cpm, the

Table II: Lysine-Containing Peptides Resulting from Further Proteolytic Digestion of the Indicated Tryptic Peptides

peptide	proteolytic enzyme	resultant peptides	sequence
1	thermolysin	1A	AKKGSEQESVKE
		1B	LAK
		1C	AKED
		1D	LKKWENPAQNTAH
4	chymotrypsin	4A	LVKLEY
		4B	SFKDNSNLY
5	chymotrypsin	5A	DLKPENLLIDQQGY
		5B	GFAKR
7	chymotrypsin	7A	SKGY
		7B	NKAVDW
		7C	EKIVSGKVR
11	V8 protease	11A	KVEAPFIPKFKGPGDTSNFDDYE
		11B	KCGKE

absolute yield of any given peptide was not a factor.

Some of the peptides shown in Table I contained only one lysine and did not require further analysis. Peptides with multiple lysines were either sequenced directly after purification or required further proteolysis. The peptides requiring further proteolysis and the lysine-containing peptides that were obtained from treatment with the indicated proteolytic enzyme are shown in Table II. In most cases (peptides 4, 5, and 7), proteolysis was carried out to obtain smaller peptides containing only one or two lysines. Peptide 1 had to be digested with thermolysin because the α-NH_2 group is blocked with myristic acid (Carr et al., 1982). Because it could not be purified with a TFA gradient, peptide 11 was digested directly with *S. aureus* V8 protease. The HPLC elution profiles of the proteolytic digests of peptide 1 (upper panel), peptide 5 (middle panel), and peptide 11 (lower panel) are shown in Figure 4. Each of the peaks of radioactivity from Figure 4 (not shown) was rechromatographed with a TFA gradient, and the marked peptides were identified by sequencing.

Modification of the native catalytic subunit with 3 mM [^{3}H]acetic anhydride was carried out under 3 different conditions: (1) in the presence of Mg^{2+} alone, (2) in the presence of MgATP, and (3) in the presence of MgATP plus a 20-residue inhibitor peptide. In each case, the reaction was terminated after 2 min by quenching excess reagent with β-mercaptoethanol. The partially modified protein was then denatured, and lysine residues were stoichiometrically modified with unlabeled acetic anhydride, as described under Experimental Procedures. After digestion with trypsin, the peptides were separated by HPLC and purified as described above.

Using the above protocol, it was possible to quantitate the reactivities of all the lysines in the catalytic subunit after the peptides were purified. The reactivities of single lysine residues in a peptide (1B, 1C, 2, 4A, 4B, 5A, 5B, 6, 7A, 7B, 8, and 9) were quantitated by rechromatographing the peptides. The calculation of the percent modification of Lys-47 and Lys-279, found in peptides 2 and 9, respectively, will be described here as an example. The rechromatograph of fractions 32–34, which contain peptides 2 and 9, is shown in Figure 2. An equal aliquot of each fraction containing the peptides was counted for radioactivity (Figure 2, middle and lower panels). In addition, the peak area for each peptide, based on the absorbance at 219 nm, was determined by integration. The numerical values of the peak area and the total amount of radioactivity associated with each peak are shown in Table III. The relative reactivity was then calculated as described under Experimental Procedures.

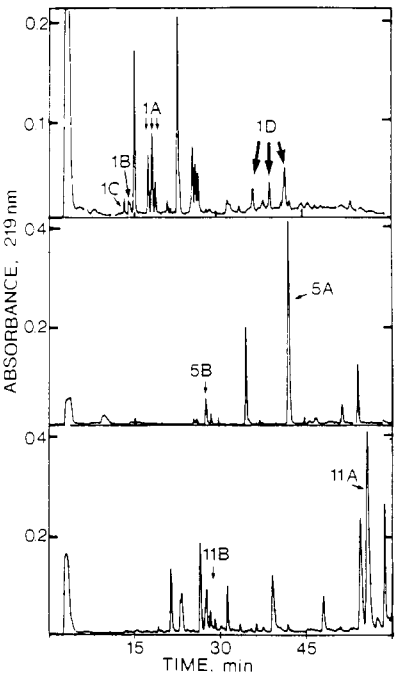

FIGURE 4: Separation of proteolytic digests of tryptic peptides. Peptides 1, 5, and 11 were digested with the proteolytic enzymes listed in Table II as described under Experimental Procedures. The resulting peptides were separated by HPLC as described. The thermolysin peptides derived from peptide 1 were separated with a 60-min linear gradient from 0% to 22.5% CH_3CN (top panel). The chymotrypsin peptides derived from peptide 5 were separated with a 60-min linear gradient from 0% to 25% CH_3CN (middle panel). The *Staph. aureus* V8 protease peptides derived from peptide 11 were separated with a 60-min linear gradient from 0% to 22.5% CH_3CN (bottom panel).

The reactivities of lysine residues in peptides containing more than one lysine (1A, 1D, 3, 7C, 10, 11A, and 11B) were quantitated by sequencing the peptides. The PTH derivative for acetyllysine eluted as a distinct peak slightly before PTH-alanine. Consequently, the peak area of each PTH-acetyllysine could be calculated by integration. An equal aliquot of each PTH-amino acid was collected and counted for radioactivity. The relative reactivity was then calculated as described under Experimental Procedures, with an average value of 380 constituting the ratio of peak area to cpm for lysines in the protein that was modified by [^{3}H]acetic anhydride in the presence of urea. Table IV lists the cpm collected from several sequence steps and the peak areas for each of the PTH-acetyllysine steps for peptide 3.

The calculated values for the relative reactivity of each lysine in the native enzyme are shown in Figure 5. The lysine reactivities in the presence of MgATP, and in the presence of MgATP and the inhibitor peptide, are also indicated. The total amount of radioactivity incorporated into the protein was

Table III: Numerical Values of Peak Area and cpm for Peptides 2 and 9[a]

	A		B		C		D	
peptide	peak area	cpm	peak area	cpm	peak area	cpm	peak area	cpm
2	2 507 965	7 239	2 711 505	3 267	2 604 789	913	2 812 624	163
9	2 044 244	8 851	2 334 217	2 912	2 542 065	2 571	2 475 796	2 509

[a] Tryptic peptides 2 and 9 were isolated following modification by acetic anhydride in the presence of (A) urea, (B) $MgCl_2$, (C) MgATP, and (D) MgATP plus inhibitor peptide.

Table IV: Numerical Values of Peak Area and cpm for Peptide 3[a]

		A		B		C		D	
step	amino acid	cpm	peak area	cpm	peak area	cpm	peak area	cpm	peak area
1	Val	60		39		41		30	
2	Met	48		58		51		32	
3	Leu	48		86		34		36	
4	Val	56		306		33		25	
5	Lys	3989	1607887	1611	1503758	738	877548	534	565237
6	His	250		268		110		66	
7	Lys	3947	1495126	832	1334026	487	782501	343	470257
8	Glu	274		116		91		65	
16	Lys	2116	726925	907	673664	105	390658	41	221086
17	Ile	354		369		51		43	
18	Leu	214		194		58		34	
19	Asp	190		165		58		39	
20	Lys	1802	650073	172	564438	60	335555	38	174643
21	Gln	378		202		73		66	
22	Lys	1537	597328	907	501445	348	283013	171	167871
23	Val	364		230		116		72	
24	Val	210		119		63		46	
25	Lys	1397	516032	1106	411749	533	247085	289	139935
26	Leu	324		277		151		82	
27	Lys	1339	456701	526	379321	307	224070	123	129457
28	Gln			188		101		69	
35	Glu			67		51		38	
36	Lys			76	128788	50	65715	39	40987
37	Arg			70		51		35	

[a] Peptide 3 was purified and sequenced as described under Experimental Procedures. The numerical values of peak area (arbitrary units) are only for the PTH-acetyllysine contained in the indicated step. Peptide 3 was derived from enzyme modified in the presence of (A) urea, (B) $MgCl_2$, (C) MgATP, and (D) MgATP plus inhibitor peptide.

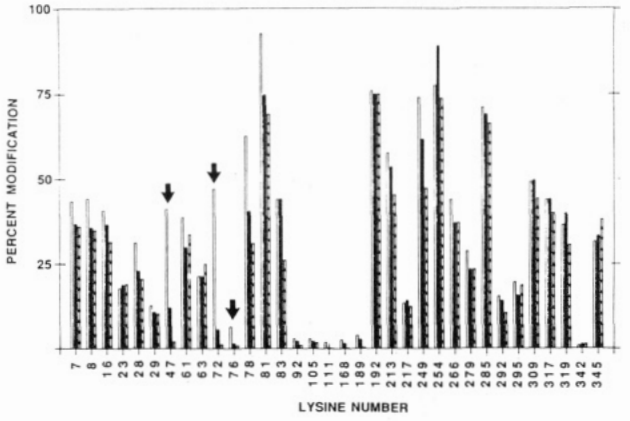

FIGURE 5: Relative reactivity of lysine residues toward acetic anhydride in the presence of (a) (open bars) 12 mM $MgCl_2$, (b) (solid bars) 12 mM $MgCl_2$, and 2 mM ATP, (c) (open bars with inserted "x") 12 mM $MgCl_2$, 2 mM ATP, and 35 μM inhibitor peptide.

reduced by approximately 65% when the native enzyme was compared to the denatured protein. The addition of MgATP reduced the incorporation by 69.9%, while the total incorporation of radioactivity was reduced 73.3% in the presence of the inhibitor peptide and MgATP.

Discussion

The catalytic subunit of cAMP-dependent protein kinase was differentially labeled with [^{3}H]acetic anhydride to determine the reactivities of lysine residues and to identify regions of the catalytic subunit that are perturbed in response to substrate binding. The apoenzyme can be separated readily into distinct regions based on the reactivities of lysine residues (Figure 5). The amino-terminal region, including Lys-7 through Lys-83, contains lysine side chains that are relatively reactive with the exception of Lys-76. The lysines in the next region, Lys-92 through Lys-189, are very unreactive. Although there are five lysines contained within this region, none of them react to any extent with acetic anhydride, either in the presence or in the absence of substrates. The remaining carboxy-terminal region, which constitutes close to half of the protein, contains 14 lysines that also are relatively reactive. Only Lys-342 at the carboxy terminus shows no appreciable modification.

Although MgATP protected the enzyme from inactivation by acetic anhydride, the overall modification of lysine residues was still substantial. This suggests that some residues, such as Lys-81 and Lys-295, are not essential for function or for structurally maintaining the active conformational state of the enzyme. MgATP did significantly alter the reactivity of a few residues and slightly changed many others, even though the overall modification of lysine side chains was still high. The amino-terminal region was the most sensitive to MgATP-induced changes. Three lysines, Lys-47, Lys-72, and Lys-76, were protected by MgATP from modification with acetic anhydride. One of these lysines, Lys-72, is invariant in all protein kinases and was shown to be an essential amino acid by affinity labeling with FSBA (Zoller et al., 1981). Subsequent nucleotide displacement studies with the FSBA-modified catalytic subunit, using *lin*-benzoadenosine derivatives, confirmed that the side chain of Lys-72 lies in close proximity to the β- and/or γ-phosphate of MgATP (Bhatnagar et al., 1984). On this basis, the MgATP protection of Lys-72 against modification by acetic anhydride was expected. The protection of Lys-47 and Lys-76, on the other hand, was not predicted. It is unknown if Lys-47 and Lys-76, which are not conserved in other protein kinases, contribute directly to the binding of MgATP.

The fact that MgATP protects Lys-47 and Lys-72 from modification by acetic anhydride confirms that the ATP binding site is located in the amino-terminal portion of the catalytic subunit. In addition, comparison with other adenine nucleotide binding proteins allows one to propose a more precise model for a portion of this nucleotide binding region. Rossmann et al. (1974), by comparing the crystal structure of lactate dehydrogenase with glyceraldehyde-3-phosphate dehydrogenase, found many general features of secondary and

tertiary structure were conserved, even though a comparison of amino acid sequences revealed no striking similarities. They predicted this nucleotide-fold motif would be conserved in all adenine nucleotide binding proteins. The motif consists, in general, of a sheet of β-strands with two α-helices above the plane of the sheet and two below. In the case of lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, there are six parallel β-strands. The carboxy-terminal end of the β-sheet and a crevice between two of the strands constitute a major part of the adenine nucleotide binding site. Although there are variations in the connectivity of the β-strands and on the size of the α-helices, this overall structure has been reproduced in nearly every adenine and guanine nucleotide binding protein where crystallographic data are available. The primary sequence is generally not conserved with the exception of a triad of glycines, Gly-X-Gly-X-X-Gly, that lie between the first β-strand and the subsequent α-helix (Brändén, 1980). The bound nucleotide, particularly the phosphate region, frequently is close to the triad of glycines, although the orientation of the nucleotide varies somewhat in each structure (Egner et al., 1987; deVos et al., 1988; Wierenga et al., 1985).

On the basis of these criteria, a model can be developed for a portion of the nucleotide fold for the catalytic subunit. The affinity labeling of Lys-72 with FSBA first localized the ATP binding site in the amino-terminal region of the polypeptide chain (Zoller et al., 1981). Differential labeling with acetic anhydride now has confirmed that the amino-terminal domain is most affected by ATP binding. Since the sequence similarities with other kinases in the catalytic domain begin at about residue 45, it is reasonable that residues 45–50 constitute the first β-strand. The placement of a triad of glycines, Gly-50, Gly-52, and Gly-55, at the end of β-strand 1 is consistent with all known nucleotide-fold motifs (Brändén, 1980). These glycines are the only common feature of primary sequence shared by nucleotide binding proteins, and they are also highly conserved throughout the family of protein kinases. The region extending from Gly-55 to Lys-72 would be an α-helix followed by a second β-strand if the catalytic subunit is similar to most other adenine nucleotide binding proteins. A model of this region, shown in Figure 6, displays the α-carbon backbone of lactate dehydrogenase containing the sequence of the catalytic subunit, with the three highly invariant glycines of the catalytic subunit being superimposed with those found in lactate dehydrogenase. A similar model was first described by Wierenga et al. (1986) for lactate dehydrogenase when they established an amino acid sequence fingerprint for this region of the nucleotide fold. Wierenga et al. also indicated that residues 45–75 of the catalytic subunit possibly folded into a $\beta\alpha\beta$ structure similar to lactate dehydrogenase.

The model in Figure 6 is consistent with the results of the differential labeling of lysine residues. The protection of Lys-72 from modification by acetic anhydride is accounted for since it lies at the carboxy-terminal end of β-strand 2, and MgATP typically binds at the carboxy-terminal end of the β-sheet (Brändén, 1980). Lys-72 also is located near the triad of glycines, which most likely interact with the phosphates of ATP. The model also places Lys-47 near the carboxy end of a β-sheet and close to the bound nucleotide. The reactivities of Lys-61 and Lys-63 are relatively unaffected by MgATP, indicating that they are located away from the carboxy end of the structure. Finally, the reactivities of Lys-78, Lys-81, and Lys-83 are consistent with their being directed away from the ATP binding site, although this is not shown in Figure 6. This combined evidence makes it likely that the catalytic

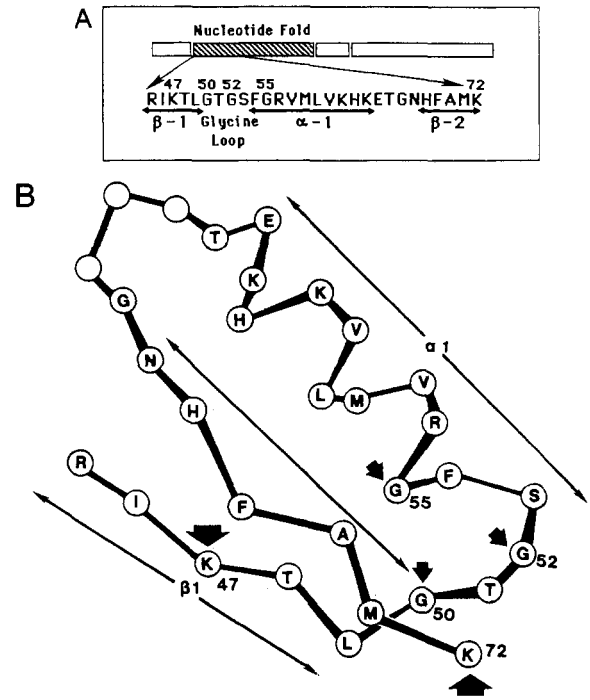

FIGURE 6: Proposed model of the N-terminal region of the nucleotide fold in the catalytic subunit. Panel A shows the location of this portion of the nucleotide binding site in the overall polypeptide chain. The predicted nucleotide fold in its entirety is indicated by hatching. The region that is predicted to correspond to β-strands 1 and 2 and α-helix 1 is shown below. (B) The α-carbon backbone is based on the coordinates of lactate dehydrogenase (Adams et al., 1970; Wierenga et al., 1985). The triad of glycines is indicated by small arrows. Lysines that are protected by MgATP are denoted by large arrows.

subunit contains the general features that are common to the nucleotide-fold motifs of adenine nucleotide binding proteins.

The striking unreactivity of the lysines in the central portion of the protein is unusual. These lysines could be involved in salt bridges or simply buried and thus sequestered from the aqueous solvent. If these lysines were contained within a hydrophobic pocket, they also would be inaccessible to acetic anhydride, although this would constitute a rather extensive hydrophobic site in order to account for all five lysines. The adenine nucleotide is generally considered to bind in a hydrophobic pocket; therefore, it is possible that at least one of these lysines is located there. In addition, it has been suggested that the region surrounding the α-phosphate of ATP is hydrophobic (Bhatnagar et al., 1983). Finally, two residues contained within this region, Glu-91 and Asp-184, are predicted to lie in a hydrophobic region based on their reactivity with the hydrophobic carbodiimide dicyclohexylcarbodiimide (Buechler & Taylor, 1988).

Since the overall reactivity of the lysine residues decreases from 35.3% in the apoenzyme to 30.1% with MgATP bound, and 26.7% with the inhibitor peptide and MgATP, it is apparent that the lysine residues are becoming less accessible to the solvent as substrates bind. Although the protection of Lys-47, -72, and -76 is most apparent, in fact nearly all of the lysines in the amino-terminal region show partially reduced reactivity in the presence of MgATP. Even some residues in the carboxy-terminal region, such as Lys-249, are partially protected by MgATP. In most cases, these changes are further enhanced by inhibitor peptide. This suggests that conformational changes are occurring throughout large regions of the catalytic subunit as the substrates bind, and specific regions can be identified that are sensitive to the binding of substrates. The amino-terminal half of the catalytic subunit, as discussed

before, is clearly sensitive to MgATP, while the carboxy-terminal half is generally unaffected. Although there are not any lysine residues that appear to contribute directly toward binding the inhibitor peptide, there are several residues whose reactivities change when both the inhibitor peptide and MgATP are present. The changes in lysine reactivity seen here may reflect movements similar to other kinases that act on small molecules. Hexokinase, for example, consists of two lobes that exist in an open configuration in the apoenzyme. Substrate binding leads to conformational changes that bring the two lobes closer together so that both lobes contribute directly to the active site (Anderson et al., 1979).

Structural and functional information can be deduced indirectly from affinity labeling and group specific labeling; however, eventually it is essential to have a high-resolution crystal structure of the apoenzyme and of a complex containing bound substrates before substrate-induced conformational changes are to be fully interpreted.

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