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Identification of Peptides from the Adenine Binding Domains of ATP and AMP in Adenylate Kinase: Isolation of Photoaffinity-Labeled Peptides by Metal Chelate Chromatography†

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Received September 24, 1991; Revised Manuscript Received March 3, 1992

ABSTRACT: Photoaffinity labeling with azidoadenine nucleotides was used to identify peptides from the ATP and AMP binding domains on chicken muscle adenylate kinase. Competition binding studies and enzyme assays showed that the 8-azido analogues of Ap_4A and ATP modified only the $MgATP^{2-}$ site of adenylate kinase, whereas the 2-azido analogue of ADP modified the enzyme at both the ATP and AMP sites. The positions of the two nucleotide binding sites on the enzyme were deduced by isolating and sequencing the modified peptides. Photolabeled peptides were isolated by a new procedure that used metal chelate chromatography to affinity purify the photolabeled peptides prior to final purification by reverse-phase HPLC. The sequences of the peptides that were photolabeled with the 8-azido analogues corresponded to residues K28–L44, T153–K166, and T125–E135 of the chicken muscle enzyme. The residues that were present in both tryptic- and *Staphylococcus aureus* V-8 protease-generated versions of these peptides were assigned to the ATP binding domain on the basis of selective photoaffinity labeling with the 8-azidoadenine analogues. These peptides and an additional peptide corresponding to positions I110–K123 were photolabeled with 2-N_3ADP. Since I110–K123 was photolabeled by 2-N_3ADP but not by 8-N_3Ap_4A, it was assigned to the AMP binding domain.

Adenylate kinase (EC 2.7.4.3) catalyzes a phosphoryl exchange between $MgATP^{2-}$ and uncomplexed AMP. The enzyme contains two nucleotide binding sites that differ in their specificity (Noda, 1973) and possesses a glycine-rich region, thought to be a common motif for nucleotide phosphate binding (Fry et al., 1985). Because of these structural features and its relatively small size and ubiquitous nature, adenylate kinase is generally regarded as the prototype nucleotide binding protein. Consequently, a considerable amount of structural information is known about the enzyme from ligand binding studies (Hamada et al., 1979; Reinstein et al., 1990), comparative analysis of the primary structure (Fry et al., 1985; Schulz et al., 1986), X-ray crystallography (Schulz et al., 1974; Pai et al., 1977; Egner et al., 1987), and NMR (Fry et al., 1985, 1988). However, the assignment of the two nucleotide binding domains of adenylate kinase remains controversial [cf. Kim et al. (1989) and Tsai and Yan (1991)].

Photoaffinity labeling with azidonucleotide analogues provides a direct approach for elucidating nucleotide binding domains on proteins. Azidoadenine nucleotides bind in the adenine nucleotide binding domain of proteins, often serving as substrates for the enzymatic reaction. Photolysis with UV light activates the azido moiety which, if bound to the protein, will react within the binding domain. In theory, once a protein is photolabeled, the regions containing the nucleotide binding domain can be identified by isolating the modified peptide(s) (Potter & Haley, 1983). However, technical problems associated with recovery of modified peptides have hampered the broad application of photoaffinity labeling as a technique for identifying active-site peptides. The major hindrance is the lability of the labeled photoprobe during peptide separation by reverse-phase HPLC. The problem stems from inherent lability of both the N-glycosidic bond of the nucleotide and certain covalent bonds formed upon photoinsertion (Lewis et al., 1989; Haley, 1991). In fact, cases where photolabeled

† Supported in part by Research Grant GM-35766 from the National Institutes of Health.
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0006-2960/92/0431-4479$03.00/0

peptides have been isolated have usually involved photoinsertion into a tyrosine residue (Cross et al., 1987; Kim & Haley, 1991) which apparently forms a bond stable enough to preserve the radioactive tag through peptide purification. Other types of photoinsertions are probably less stable since the bulk of radioactive label is generally lost from the peptide during reverse-phase HPLC. Thus, before photoaffinity labeling with the azido nucleotide analogues can be used routinely to identify nucleotide binding sites, it is necessary to develop procedures for separating photoaffinity-labeled peptides from unmodified peptides that will preserve the label through purification.

In the present study, the 8-azido analogues of Ap_4A[1] and ATP were found to photoaffinity label the $MgATP^{2-}$ binding site of chicken muscle adenylate kinase. The 2-azido analogue of ADP modified both the $MgATP^{2-}$ and AMP sites. Photoaffinity-labeled peptides were isolated by metal chelate chromatography using Fe^{3+}–iminodiacetic acid (IDA)–Sepharose before final purification by reverse-phase HPLC and sequence analysis. The procedure made it possible to assign the adenine nucleotide binding domains for $MgATP^{2-}$ and AMP. The results provide new insights into the adenine binding domains of adenylate kinase and demonstrate the efficacy of metal chelate chromatography as a technique for selectively purifying peptides photolabeled with nucleotide photoaffinity probes.

Materials and Methods

Materials. Adenylate kinase from chicken muscle and iminodiacetic acid–Sepharose 6-B (IDA–Sepharose) were purchased from Sigma Chemical Co. (St. Louis, MO).[2] [γ-^{32}P]-8-Azidoadenosine 5′-triphosphate (8-N_3ATP), [γ-^{32}P]ATP, 2-N_3ATP, 8-N_3AMP, and 2-N_3AMP were synthesized as described previously (Potter & Haley, 1983; Kim & Haley, 1990).

Synthesis of 8-N_3Ap_4A and [β-^{32}P]8-N_3Ap_4A. 8-N_3ATP (13.5 μmol) was dissolved in 1 mL of 1 M MOPS, pH 6.8, and 250 mM $MgCl_2$. Excess 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and AMP were added, and the solution was stirred at 25 °C for 14 h in the dark. The reaction mixture was diluted with 1 mL of saturated EDTA and 2 mL of H_2O, and 8-N_3Ap_4A was isolated by anion-exchange chromatography on a 20- × 1.5-cm column of DEAE-cellulose using a 400-mL linear gradient from 0 to 300 mM triethylammonium bicarbonate. The product, which eluted immediately after 8-N_3ATP with a 45% yield, was characterized by thin-layer chromatography (R_f = 0.49 on silica gel in isobutyric acid/H_2O/concentrated ammonium hydroxide, 66:33:1, compared to R_f = 0.39 for 8-N_3ATP), UV spectra (λ_{max} = 264 nm in H_2O), UV photodecomposition, resistance to alkaline phosphatase, and digestion with snake venom phosphodiesterase. [β-^{32}P]8-N_3Ap_4A was synthesized by the same procedure using [γ-^{32}P]8-N_3ATP (sp act. = 10–25 mCi/μmol). The product yield was 35–40% with a sp act. of 5–15 mCi/μmol. The product was characterized as described above and by comigration with nonradioactive 8-N_3Ap_4A standards on TLC.

Enzyme Assays. For determination of enzyme activity after inactivation by photolabeling, adenylate kinase was assayed spectrophotometrically as described (Tian et al., 1988). To determine substrate specificity, adenylate kinase was assayed by monitoring the production of ADP in 25-μL reactions containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20 μg of adenylate kinase, and the substrates indicated in the text. Reactions were terminated by the addition of methanol to 80% (v/v). After 60 min at −20 °C, precipitated protein was removed by centrifugation. The supernatant was dried in vacuo at 40 °C, which destroyed the azido compounds, causing loss of their absorption at 240–300 nm. Following resuspension in HPLC buffer, the reactions were analyzed for ADP by boronate chromatography on a 5- × 250-mm SelectiSpher-10 HPLC boronate column (Pierce, Rockford, IL) using 0.12 M Na_2HPO_4, pH 7.8, as the mobile phase. Isocratic flow at 1 mL/min separated ATP, ADP, and AMP with retention times of 8, 11, and 17.5 min, respectively. Nucleotides were detected by absorbance at 260 nm.

Photolabeling of Adenylate Kinase. Photoaffinity labeling was conducted at 4 °C in 25-μL reactions containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 115 pmol of adenylate kinase, and the indicated amount of photoprobe. Reactions were initiated by addition of photoprobe. After a 15-s incubation, the reactions were photolyzed for 1 min with 254-nm radiation from a hand-held ultraviolet lamp (3000 μW/cm^2). Reactions were terminated by addition of SDS and dithiothreitol, and ^{32}P incorporation into the adenylate kinase polypeptide was determined following SDS–PAGE and autoradiography (Salvucci & Haley, 1990). Autoradiograms were scanned with an image aquisition densitometer (BioImage, MilliGen/Biosearch, Ann Arbor, MI) to determine the relative ^{32}P incorporation, quantified on the basis of whole-band analysis.

For peptide isolation from the ATP binding site, 1 mg of adenylate kinase was photolabeled with 10 μM [^{32}P]8-N_3Ap_4A or [^{32}P]8-N_3ATP in a 4-mL reaction under the conditions described above. To increase the level of photolabeling, a second round of photolabeling was performed by adding fresh probe to the reaction. Unreacted azido probe was reduced to the amine by the addition of dithiothreitol to 50 mM, and the reaction was concentrated to 0.3 mL by centrifugal ultrafiltration in a Centricon-10 device (Amicon, Danvers, MA). Photolabeled protein was separated from most of the unincorporated probe by gel-filtration chromatography at 23 °C on a 1- × 20-cm column of Sephadex G-50-80 that was equilibrated with 1% ammonium acetate, pH 8.3 (buffer A). For peptide isolation from the AMP site, [β-^{32}P]2-N_3ADP was produced in situ by incubating 1 mg of adenylate kinase at 4 °C for 2 min in a 4-mL reaction containing 80 μM [γ-^{32}P]ATP and 80 μM unlabeled 2-N_3AMP in 20 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$. Reactions were photolyzed for 1 min, terminated by addition of DTT, and concentrated before gel filtration as described above.

Purification of Photolabeled Peptides by Fe^{3+}–Chelate Chromatography and Reverse-Phase HPLC. A 500-μL column of IDA–Sepharose was prepared for Fe^{3+}–chelate chromatography by washing with water and then equilibrating with 5 column volumes of 50 mM $FeCl_3$ (Muszynska et al., 1986). The column was washed to remove unbound Fe^{3+} by successive additions of H_2O, buffer A, buffer A containing 0.5 M NaCl, and buffer A. The washed resin was added to the desalted, photolabeled adenylate kinase together with TPCK-treated trypsin [10% (w/w)] or 10 units of *Staphylococcus*

[1] Abbreviations: Ap_4A, adenosine 5′,5‴-tetraphosphate adenosine; Ap_5A, adenosine 5′,5‴-pentaphosphate adenosine; N_3ADP, azidoadenosine 5′-diphosphate; N_3Ap_4A, azidoadenosine 5′,5‴-tetraphosphate adenosine; N_3ATP, azidoadenosine 5′-triphosphate; PVDF, poly(vinylidene difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

aureus V-8 protease, and the mixture was incubated overnight at room temperature with gentle rocking. The resin was transferred to a plastic column and eluted with successive 15-mL additions of buffer A, buffer A containing 0.5 M NaCl, buffer A, buffer A containing 4 M urea, buffer A, and buffer A containing 10 mM KH_2PO_4, pH 8. The first 2 mL of the phosphate elution was further fractionated by reverse-phase HPLC on a 4.6- × 250-mm C8 column (ISCO, Lincoln, NE). The starting mobile phase (0.1% TFA) was delivered at 0.2 mL/min for the first 25 min and then increased to 1 mL/min upon initiation of an acetonitrile gradient. Photolabeled peptides were detected by their absorbance at 215 nm and ^{32}P cpm using an on-line detector (Flo-one, Packard Instruments) and by determining Cerenkov radiation in 1-mL column fractions. Fractions containing radioactivity were dried in vacuo and subjected to sequence analysis at the University of Kentucky Macromolecular Facility using an Applied Biosystems 477A pulse liquid protein sequencer with on-line 120A PTH identification.

Isolation of Photolabeled Peptides from PVDF Membranes. Photolabeled adenylate kinase was bound to Immobilon-P, PVDF membranes (Millipore, Bedford, MA), either by filtration of the photolabeling reaction through a 23-mm disk or after SDS–PAGE and electrophoretic transfer overnight at 0.1 A in 50 mM Tris–borate. After rinsing in H_2O, the PVDF membrane containing bound photolabeled protein was cut into 0.2-cm strips and the strips were blocked by incubation with 0.1% (w/w) poly(vinylpyrrolidone)-40 in methanol (Bauw et al., 1989). The membrane strips were rinsed sequentially in H_2O and buffer A and then incubated overnight with trypsin and Fe^{3+}–IDA–Sepharose as described above. Photolabeled peptides were eluted from the Fe^{3+}–IDA–Sepharose column as described above.

Results

Synthesis of 8-Azidoadenosine 5′,5‴-Tetraphosphate Adenosine (8-N_3Ap_4A). The synthesis of 8-N_3Ap_4A and [β-^{32}P]8-N_3Ap_4A was based on the procedure of Ng and Orgel (1987). 8-N_3ATP was activated by a water-soluble carbodiimide (CDI) and simultaneously reacted with a molar excess of AMP at 25 °C for 14 h. This simple procedure produced 8-N_3Ap_4A in relatively good yield. Following purification, the identity of the product was confirmed by TLC and the following other methods. The compound was resistant to alkaline phosphatase, indicating the absence of unblocked phosphates. However, it could be digested with snake venom phosphodiesterase showing the presence of phosphodiester linkages. Additional evidence for a linkage between AMP and 8-N_3ATP was obtained when [γ-^{32}P]8-N_3ATP was used in place of unlabeled 8-N_3ATP. The product formed in this reaction possessed the ^{32}P label. The UV spectra of the compound showed a λ_{max} of 264 nm, which is the same as that of an equimolar mixture of AMP and 8-N_3ATP. Irradiation of the compound with UV light caused photodecomposition, evident by a change in a λ_{max} from 264 to 258 nm. This result indicates that a non-azido adenine ring is present in the compound and is intact after photolysis. The purity of the compound was established by isosbestic points determined by monitoring the UV spectra after photolysis of the sample for various lengths of time.

Photoaffinity Labeling of Adenylate Kinase. Preliminary experiments established that 8-N_3Ap_4A was a better photoaffinity probe for adenylate kinase than 8-N_3ATP, labeling the enzyme at lower concentrations and with greater efficiency of photoinsertion. With 8-N_3Ap_4A, ^{32}P incorporation into the 26-kDa adenylate kinase polypeptide was dependent upon photolysis with ultraviolet radiation (Figure 1). The substrates, ATP and ADP, and an active-site-directed inhibitor, Ap_4A, afforded almost complete protection against photolabeling, but AMP had little effect. These protection studies indicate that 8-N_3Ap_4A interacts primarily at the ATP site of adenylate kinase. Photoaffinity labeling of adenylate kinase with 8-N_3Ap_4A exhibited saturation kinetics; 10 μM 8-

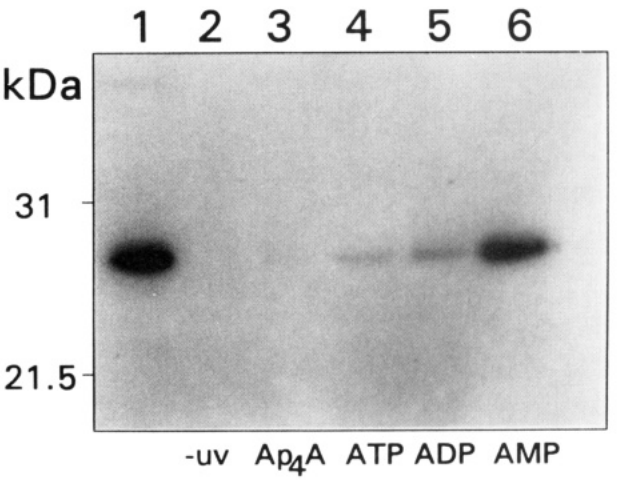

FIGURE 1: Photoaffinity labeling of chicken muscle adenylate kinase with [β-^{32}P]8-N_3Ap_4A. Autoradiogram of adenylate kinase polypeptides, separated by SDS–PAGE, after incubation with 2 μM [β-^{32}P]8-N_3Ap_4A either alone (lanes 1 and 2) or in the presence of 50 μM Ap_4A (lane 3), ATP (lane 4), ADP (lane 5), or AMP (lane 6). In lane 2, adenylate kinase was incubated with [β-^{32}P]8-N_3Ap_4A but not photolyzed.

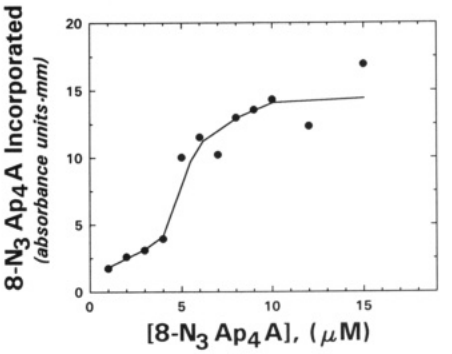

FIGURE 2: Effect of [β-^{32}P]8-N_3Ap_4A concentration on photoincorporation of [β-^{32}P]8-N_3Ap_4A into adenylate kinase. Adenylate kinase was photolyzed in the presence of the indicated concentrations of [β-^{32}P]8-N_3Ap_4A and separated by SDS–PAGE. Incorporation of [β-^{32}P]8-N_3Ap_4A was determined from densitometer scans of the autoradiograms.

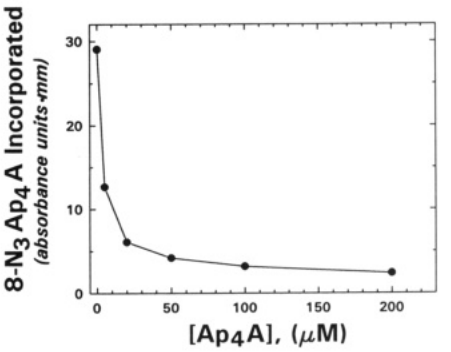

FIGURE 3: Effect of Ap_4A concentration on photoincorporation of [β-^{32}P]8-N_3Ap_4A. Adenylate kinase was photolyzed in the presence of 2 μM [β-^{32}P]8-N_3Ap_4A and the indicated concentrations of Ap_4A and was separated by SDS–PAGE. Incorporation of [β-^{32}P]8-N_3Ap_4A was determined from densitometer scans of the autoradiograms.

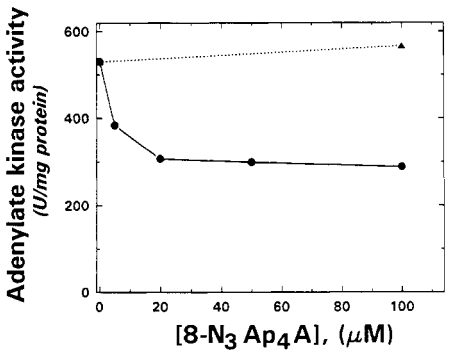

FIGURE 4: Inactivation of adenylate kinase activity by photoaffinity labeling with 8-N_3Ap_4A. Adenylate kinase (3 μg) was either photolyzed (●) or incubated in the dark (▲) in the presence of the indicated concentrations of 8-N_3Ap_4A in 25 μL. Unincorporated photoprobe was reduced by the addition of 2.5 μL of 100 mM dithiothreitol. Twenty-microliter aliquots were assayed for adenylate kinase activity at 30 °C by transfer to a 0.5-mL reaction containing 2 mM Mg^{2+}ATP and 2 mM ADP.

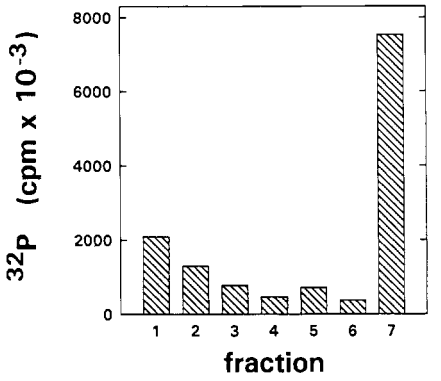

FIGURE 5: Isolation of photolabeled peptides by Fe^{3+}–chelate chromatography. Elution profile of radioactivity from a 0.5- × 2-cm Fe^{3+}–IDA–Sepharose column. The resin was incubated overnight with 1 mg of desalted, photolabeled adenylate kinase and 100 μg of trypsin and then eluted successively with 15 mL of 1% ammonium acetate, pH 8.3 (buffer A) (1 and 2), buffer A containing 0.5 M NaCl (3), buffer A (4), buffer A containing 4 M urea (5), buffer A (6), and buffer A containing 10 mM KH_2PO_4 (7).

N_3Ap_4A was sufficient to saturate photolabeling and the apparent K_d(8-N_3Ap_4A) extropolated from the response curve was 4.5 μM (Figure 2). However, the response of photoincorporation to 8-N_3Ap_4A concentration was not hyperbolic.

Protection of 8-N_3Ap_4A photolabeling of adenylate kinase by increasing concentrations of Ap_4A is shown in Figure 3. Half-maximal protection against photoincorporation of 2 μM [β-^{32}P]8-N_3Ap_4A occurred with 5 μM Ap_4A, and 200 μM Ap_4A afforded almost complete protection. Covalent modification of adenylate kinase by 8-N_3Ap_4A reduced the catalytic activity of the enzyme (Figure 4). A single photolabeling event reduced adenylate kinase activity by nearly 50%, indicating an initial photoinsertion efficiency of this magnitude. Multiple photolysis events caused additional reduction in enzyme activity (data not shown). A maximum inactivation of adenylate kinase activity by a single photolysis step with 8-N_3Ap_4A was obtained at 25 μM 8-N_3Ap_4A.

Azido Nucleotide Analogues as Substrates for the Adenylate Kinase Reaction. The ability of 2- and 8-azidoadenine nucleotides to act as substrates in the adenylate kinase reaction was assessed by determining ADP formation using various combinations of nucleotides and nucleotide analogues. ADP (plus N_3ADP) was formed by adenylate kinase with substrate combinations: (a) 8- or 2-N_3ATP and AMP and (b) ATP and 2-N_3AMP (data not shown). However, no ADP (or 8-N_3ADP) was formed when 8-N_3AMP and ATP were presented to adenylate kinase. The inability of 8-N_3AMP to serve as a substrate for the adenylate kinase reaction was reported in a previous study (Owens, 1983).

Isolation of Photolabeled Peptides from the ATP Binding Domain of Adenylate Kinase. Photoaffinity labeling with 8-N_3Ap_4A was used to covalently modify adenylate kinase at the ATP binding domain. Following two rounds of photolabeling with 10 μM [β-^{32}P]8-N_3Ap_4A and desalting, adenylate kinase was digested overnight with either trypsin or *S. aureus* V-8 protease in the presence of Fe^{3+}–IDA–Sepharose, a metal ion chromatography resin that has been used for affinity purification of phosphoproteins and peptides (Andersson & Porath, 1986; Murakami et al., 1990). Preliminary studies with photolabeled peptides from adenylate kinase established that several hours were required for binding of the photolabeled peptides to the resin. For this reason, proteolysis and binding were conducted simultaneously by incubating photolabeled adenylate kinase with both Fe^{3+}–IDA–Sepharose and protease.

Preliminary experiments with adenylate kinase also showed that, unlike phosphopeptides (Murakami et al., 1990), peptides photolabeled with azidonucleotides form a coordination complex with Fe^{3+}–IDA–Sepharose that is very tight above pH 8. Therefore, after incubation of the photolabeled adenylate kinase with protease and Fe^{3+}–IDA–Sepharose, the resin was eluted routinely with buffer, 0.5 M NaCl, and 4 M urea to disrupt electrostatic, hydrophobic, and ionic interactions that might otherwise retain unmodified peptides. Also, the use of a relatively high pH of 8.3 for chromatography served as an additional precaution against nonspecific adsorption.

A typical elution profile of radioactivity from the Fe^{3+}–IDA–Sepharose column is shown in Figure 5. Similar results were obtained for photolabeled peptides produced by digestion with V-8 protease. In general, greater than 70% of the radioactivity was adsorbed by the Fe^{3+}–IDA–Sepharose column during incubation and remained bound until elution with 10 mM P_i. Reverse-phase HPLC of the various fractions showed that most of the peptide material did not bind to the column (Figure 6A). Consequently, the A_{215} profile of the unretained fraction was characterized by a high degree of complexity, similar to the A_{215} profile of proteolytically digested adenylate kinase prior to passage through the column (data not shown). Reverse-phase HPLC revealed that fractions corresponding to the NaCl, urea, and P_i elutions were of lower complexity, particularly the P_i fraction, which contained most of the radioactivity but only a small number of peptide peaks (Figure 6B). No peptides were detected in the P_i fraction in control experiments in which unmodified adenylate kinase was digested with trypsin and subjected to Fe^{3+}–chelate chromatography (Figure 6C).

Reverse-phase HPLC resolved the P_i fraction into two to four peaks of radioactivity (Figure 7). An identical ^{32}P profile was obtained with photolabeled peptides that were chromatographed directly on the reverse-phase column without prior separation by Fe^{3+}–chelate chromatography, and similar results were obtained with photolabeled peptides produced by digestion with V-8 protease. In all cases, the major peak of radioactivity eluted with the unretained material, probably representing unincorporated ^{32}P carried through the isolation procedure or generated by breakdown of the photoinserted nucleotide (see below). Sequence analysis showed that this peak did not contain peptides. One or two smaller peaks eluted after the start of the gradient, and these contained peptides. When these peptides were rechromatographed on the reverse-phase column, most of the label was lost from the peptides and eluted with the unretained fraction (data not shown).

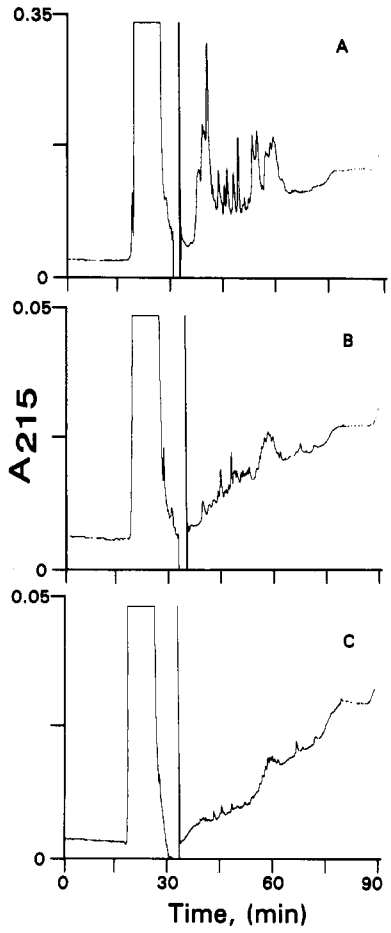

FIGURE 6: Reverse-phase HPLC separation of peptides in the unretained and P_i fractions from the Fe–IDA–Sepharose column monitored at A_{215}. Adenylate kinase, either photolabeled with [β-^{32}P]8-N_3Ap_4A (A and B) or unmodified (C), was digested with trypsin in the presence of Fe^{3+}–IDA–Sepharose. The column was eluted as described in Figure 5, and the unretained fraction (i.e., fraction 1 in Figure 5) (A) and the P_i elution fraction (i.e., fraction 7 in Figure 5) (B and C) were chromatographed by reverse-phase HPLC on a C8 column. The mobile phase, 0.1% TFA (starting solvent) and 70% acetonitrile in 0.1% TFA, was delivered at 0.2 mL/min HPLC for the first 25 min and then changed to 1 mL/min upon initiation of a linear gradient to 42% acetonitrile over the next 40 min and to 70% acetonitrile over the following 45 min.

Table I: Sequence Analysis of Photolabeled Tryptic Peptides from the Radioactive Peaks at 36 and 46 min in the HPLC Chromatogram

	PTH-amino acids (pmol)[a]				
cycle	36 min			46 min	
1	I (75)	A (115)	R (24)	Y (11)	K (4)
2	V (64)	T (67)	L (110)	G (25)	I (24)
3	H (21)	E (95)	E (95)	Y (14)	V (28)
4	K (31)	P (59)	T (28)	T (19)	H (5)
5	Y (30)	V (83)	Y (30)	H (1)	
6		I (44)	Y (75)	L (17)	
7		A (33)	K (17)	S (6)	
8		F (27)		T (11)	
9		Y (24)		G (10)	
10		K (13)		D (8)	
11				L (7)	
12				L (13)	

[a] Picomole yield of PTH-amino acid residues.

A fourth peak of radioactivity was sometimes present late in the gradient (~63 min). This peak, which corresponded to a broad A_{215} peak, probably contained partially digested protein since both absorbance and radioactivity increased with shorter digestion times (data not shown).

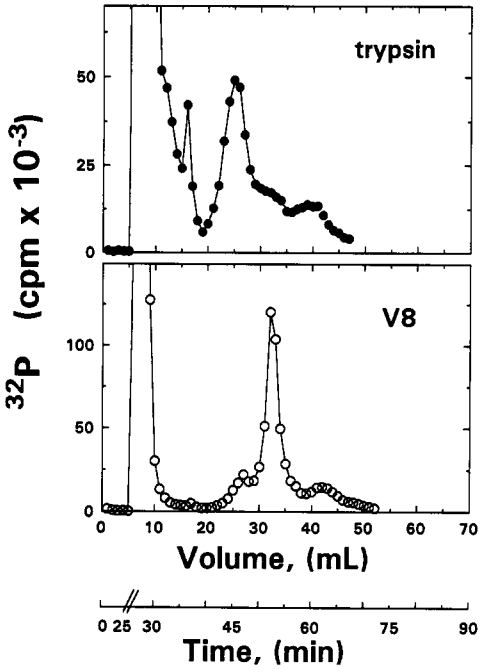

FIGURE 7: ^{32}P profile of reverse-phase HPLC separation of photolabeled peptides in the P_i fractions from the Fe^{3+}–IDA–Sepharose column. Adenylate kinase was photolabeled with [β-^{32}P]8-N_3Ap_4A and digested with trypsin (●) or *S. aureus* V-8 protease (○) in the presence of Fe^{3+}–IDA–Sepharose. Photolabeled peptides were eluted with P_i and chromatographed by reverse-phase HPLC as described in Figure 6. An abscissa showing the corresponding times of the various fractions in the profile is included for reference.

Table II: Sequence Analysis of Photolabeled V8 Protease Peptides from the Radioactive Peaks at 46 and 53 min in the HPLC Chromatogram

	PTH-amino acids (pmol)[a]			
cycle	46 min			53 min
1	T (24)	K (17)	T (24)	T (66)
2	Y (6)	I (6)	M (15)	Y (15)
3	Y (7)	V (20)	V (20)	Y (31)
4	K (9)	H (1)	K (9)	K (22)
5	A (6)	K (4)	R (7)	A (27)
6	T (4)	Y (3)	L (14)	T (19)
7	E (4)	G (4)	L (11)	E (15)
8	P (3)	Y (3)	K (3)	P (9)
9	V (2)	T (2)	R (5)	V (9)
10	I (2)		G (9)	I (9)
11	A (1)		E (3)	A (9)
12	F (2)		T (4)	F (8)
13	Y (2)		S (3)	Y (7)
14	K (1)		G (5)	K (3)
15			R (2)	G (7)
16			V (2)	R (5)
17			D (2)	G (8)
18			D (3)	I (4)
19			N (1)	V (5)
20			E (2)	R (2)
21			E (2)	Q (3)
22				L (NQ)[b]
23				N (2)
24				A (2)
25				E (1)

[a] Picomole yield of PTH-amino acid residues. [b] NQ denotes residue assignments that were made but not quantitated due to insufficient chromatographic resolution.

ATP Binding Site Peptides. Sequence analysis of the radioactive fractions from the reverse-phase column identified five tryptic peptides corresponding to two separate regions of the adenylate kinase sequence: K28–L44 and R150–K165 (Table I). Peptides from these same regions of adenylate kinase and also the region T125–E145 were isolated from

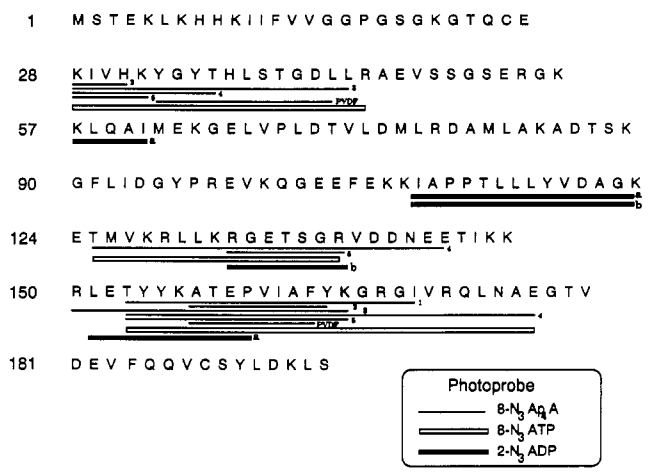

FIGURE 8: Corresponding locations of photolabeled peptides on the primary sequence of chicken muscle adenylate kinase (Kishi et al., 1986). Peptides photolabeled with 8-N_3Ap_4A (—), 8-N_3ATP (□), and 2-N_3ADP (■) were digested with either V-8 protease or trypsin and separated by Fe^{3+}–chelate chromatography and reverse-phase HPLC. The numbers and letters next to the peptides refer to separate photolabeling experiments with 8-N_3Ap_4A (1–5) and 2-N_3ADP (a and b). Peptides labeled "PVDF" were recovered from PVDF membranes.

adenylate kinase digested with V-8 protease (Table II).

To examine the reproducibility of the procedure for isolating photolabeled peptides, adenylate kinase was photolabeled with [β-^{32}P]8-N_3Ap_4A in five separate experiments and the photolabeled peptides were isolated. When aligned with the primary sequence of adenylate kinase, the peptides from all 5 experiments corresponded to one of three distinct regions of adenylate kinase and clustered around specific residues in each of the three regions (i.e., V30–Y35, R133–G138, A157–Y165; Figure 8). Peptides from each of the three regions were also recovered by Fe^{3+}–chelate chromatography and reverse-phase HPLC of adenylate kinase that was photoaffinity labeled with [γ-^{32}P]8-N_3ATP and digested with V-8 (Figure 8).

Isolation of Photolabeled Peptides from PVDF Membranes. Peptides were also recovered from adenylate kinase that was photolabeled and then bound to PVDF membrane. In two separate experiments, adenylate kinase was photolabeled with [β-^{32}P]8-N_3Ap_4A, bound to PVDF membrane, and digested with trypsin in the presence of Fe^{3+}–IDA–Sepharose. In one experiment, photolabeled adenylate kinase was simply filtered through a disk of PVDF membrane. In the other experiment, the adenylate kinase polypeptide was transferred electrophoretically to PVDF membrane after separation by SDS–PAGE. In both cases, only about 20% of the label was released from the membrane after digestion with trypsin. Nearly 100% of the released radioactivity was bound to the Fe^{3+}–IDA–Sepharose and required P_i for elution (data not shown). The profile of radioactivity obtained upon separation of this fraction by reverse-phase HPLC was similar to the chromatogram presented in Figure 7 in the positions of the radioactive peptide peaks. Sequence analysis showed that in both experiments the radioactive peptide peak contained only a single peptide: Y33–L43 in one experiment and A157–F164 in the other (Figure 8).

Peptides from the AMP Binding Site. The observation that adenylate kinase could catalyze phosphoryl exchange using 2-N_3AMP as a substrate indicated that the AMP site could be covalently modified by photoaffinity labeling with 2-N_3AMP or 2-N_3ADP. For these experiments, [^{32}P]2-N_3ADP was produced in situ by supplying adenylate kinase with Mg[γ-^{32}P]ATP^{2-} and unlabeled 2-N_3AMP. The product of this reaction, [β-^{32}P]2-N_3ADP, should label both nucleotide sites, an expectation confirmed by preliminary experiments which showed that 1 mM ATP, which provided complete protection against photolabeling with 8-N_3ATP, afforded only partial protection against photolabeling with [β-^{32}P]2-N_3ADP (data not shown). The ^{32}P profile of tryptic peptides from adenylate kinase photolabeled with 2-N_3ADP after separation by Fe^{3+}–chelate chromatography was similar to that shown in Figure 5. Radioactivity in the P_i fraction was resolved into several peaks by reverse-phase HPLC. The sequences of the isolated peptides corresponding to these peaks are shown in Figure 8. In addition to peptides photolabeled with 8-N_3Ap_4A, thought to be from the ATP site, a unique peptide corresponding to positions I110–K123 was isolated in two separate experiments.

Discussion

The ATP and AMP Sites of Adenylate Kinase. The $MgATP^{2-}$ and AMP binding domains of adenylate kinase have been assigned to various locations on the enzyme on the basis of sometimes conflicting evidence from NMR (Fry et al., 1985; Mildvan & Fry, 1987), X-ray crystallography (Pai et al., 1977), and, more recently, site-directed mutagenesis (Yan et al., 1990; Kim et al., 1990). The difficulty in making definitive assignments for the two nucleotide binding sites is caused, in part, by the high degree of structural similarity of the substrates which limits the extent of selectivity possible for the two binding sites. For example, in addition to $MgATP^{2-}$ and MgADP, the ATP binding site of adenylate kinase will bind uncomplexed ATP, ADP, and probably AMP (Vetter et al., 1990). The AMP site is more specific, binding uncomplexed ADP and AMP, but not ATP (Shyy et al., 1987). An attempt was made to produce adenylate kinase with bound ATP for definitive assignment of the ATP site by crystallography, but this attempt was not successful (Pai et al., 1977). Adenylate kinase has been crystallized with AMP (Diederichs & Schulz, 1991), but, because of possible binding of AMP to the ATP site, the assignment of the AMP site can only be regarded as tentative. Similarly, the adenine nucleotide binding sites identified in the X-ray crystal structure of the adenylate kinase–Ap_5A complex cannot be distinguished (Egner et al., 1987).

In the present study, chicken muscle adenylate kinase was photolabeled specifically at the ATP site with 8-N_3Ap_4A. AMP was ineffective in protecting against photolabeling with 8-N_3Ap_4A, indicating that only the ATP site was modified by the diadenosine photoprobe. The ability of adenylate kinase to bind 8-azidoadenine nucleotides and to catalyze phosphoryl transfer from the 8- and 2-azido analogues of ATP is consistent with broad selectivity characteristic of the ATP site. In contrast, the AMP site of adenylate kinase is known to exhibit much greater specificity. Of the AMP analogues tested, only 8,5′-cAMP, 2′-dAMP (Fry et al., 1987), and 2-N_3AMP (this study) can serve as substrates in the reaction. Fry et al. (1987) have attributed the reactivity of 8,5′-cAMP and 2′-dAMP to the conformation that these compounds assume in solution. From NMR studies with Cr^{3+}AMPPCP, these authors suggest that AMP is bound to adenylate kinase in an anti conformation, involving multiple enzyme–ligand interactions. A requirement for the anti conformation for binding to the AMP site may explain the preference of adenylate kinase for the 2-azido analogue of AMP, which also assumes primarily an anti conformation in solution (Czarnecki, 1984). In contrast, the 8-azidoadenine nucleotide analogues do not appear to bind to the AMP site (Figure 1). The lack of binding may be

because the 8-azidoadenine analogues are primarily in the syn conformation as expected for 8-substituted purine nucleotides (Tavale & Sobell, 1970).

In general, assignment of the ATP and AMP sites of adenylate kinase have either supported the model proposed by Pai et al. (1977) from the crystal structure of the porcine muscle enzyme or favored a reversal of the $MgATP^{2-}$ and AMP sites that were proposed in this model. In the present study, the ability to modify adenylate kinase exclusively at the ATP binding domain by photoaffinity labeling with 8-N_3Ap_4A and 8-N_3ATP facilitated identification of peptides that are in close proximity to the adenine ring of ATP. That binding and labeling occurred specifically at the $MgATP^{2-}$ site was indicated by (1) the ability of 8-N_3ATP to serve as a substrate for the reaction, (2) the complete protection afforded by ATP against photolabeling with 8-N_3Ap_4A, and (3) the lack of protection afforded by AMP. Due to the lability of the photoinserted bond to sequencing conditions, we were unable to identify the specific residues that were modified in the photolabeled peptides. However, photolabeled peptides clustered around certain residues, and the regions of overlap between tryptic and V8 peptides were limited. These data suggest a position for the adenine ring of ATP near residues V30–H36 and A157–Y160. Residues in the tryptic peptide K134–R140 may also be close in proximity, but this assignment is more tentative, since K134–R140 was photolabeled in only some of the experiments. It should be noted that residues K134–R140 are adjacent to a helical region that moves upon induced fit of the substrates (Pai et al., 1977). Site-directed mutation of arginine residues in this region suggested a role for these residues in catalysis, but not substrate binding (Tsai & Yan, 1991). Thus, conformational changes during catalysis appear to include movement of this region of the protein with the possible effect of bringing it to a position close to the adenine domain for ATP.

Assignment of residues in tryptic peptide K28–R45 to the ATP site is consistent with (1) the proposal of Hamada et al. (1979) for placement of the adenine ring between Y32 and Y34 (i.e., 33 and 35 of the chicken muscle enzyme) on a synthetic peptide corresponding to residues 1–44 of muscle adenylate kinase; (2) the NMR studies of Fry et al. (1985) which position the adenine ribose moiety near residues that correspond to I29, V30, H37, and L38 of the chicken enzyme; and (3) the identification of residues corresponding to T39 and G40 of the chicken muscle enzyme in the adenine "B" site of the crystal structure of the yeast adenylate kinase–Ap_5A complex (Egner et al., 1987). Peptide A157–K166 has not been assigned previously to the ATP site. However, two tyrosine residues adjacent to this peptide (i.e., Y153 and 154) have been implicated in nucleotide binding on the basis of NMR studies of the porcine muscle enzyme (McDonald & Cohen, 1975). In the crystal structure, residues A157–Y165 of this peptide transverse a helical region that could overlap with residues K28–R45 (Pai et al., 1977). In the present study, peptide A157–K166 was photolabeled with 8-N_3ATP and 2-N_3ADP and in all 5 experiments with 8-N_3Ap_4A. Thus, on the basis of photolabeling, we believe that the adenine binding domain for the $MgATP^{2-}$ site is flanked by residues near positions A157–Y165 and V30–Y35.

Competition binding studies showed that the 8-azidoadenine analogues do not bind to the AMP site of adenylate kinase. Therefore, it was necessary to use the 2-azido analogue of ADP to modify peptides in the AMP binding domain. 2-N_3ADP should photolabel peptides in both the $MgATP^{2-}$ and AMP domains since the 2-azidoadenine analogues of both ATP and AMP could serve as substrates in the adenylate kinase reaction. The results confirmed this prediction, for in addition to peptides that were assigned to the $MgATP^{2-}$ site on the basis of photolabeling with 8-N_3ATP and 8-N_3Ap_4A, a unique peptide, I110–K123, was photolabeled with 2-N_3ADP. This peptide is probably within the binding domain for AMP.

Precedents exist for assignment of residues in I110–K123 to the AMP site. For example, NMR studies with Cr^{3+}-AMPPCP indicate an involvement of L116 in AMP binding (Fry et al., 1987). Also, crystallographic studies with the yeast adenylate kinase–Ap_5A complex have shown that several residues in peptide I110–K123 comprise part of the adenine "A" site (Egner et al., 1987). As noted above, residues T39 and G40 that appear to occupy the ATP binding domain on the basis of photolabeling were included in the adenine "B" site of Egner et al. (1987). Peptide I110–L123 appears to be somewhat removed from these residues in the crystal structure of the porcine muscle enzyme. It should be noted that the assignment of residues in peptide I110–K123 to the AMP domain does not preclude the involvement of the C-terminal helix in binding AMP as proposed by Hamada et al. (1979), since together these two peptides form one of two hydrophobic pockets on the enzyme (Schulz et al., 1974). Alternatively, the AMP could interact with peptide I110–K123 and the helical region containing R149 (R150 in the chicken muscle enzyme), a residue that appears to exert a marked influence over binding affinity and catalytic activity (Tsai & Yan, 1991; see below). Thus, final positioning of ATP and AMP based on photoaffinity labeling would place the adenine ring of ATP in a pocket between peptides K28–R45 and A157–K166, with the phosphates interacting with the highly conserved glycine-rich flexible loop region and AMP in close proximity to peptide I110–K123 interacting with either the C-terminal peptide or residues near R150.

In a recent review, Tsai and Yan (1991) provide a comprehensive discussion of the assignment of the nucleotide binding domains of adenylate kinase. Largely on the basis of evidence from site-directed mutagenesis, they proposed a new model for ATP and AMP binding domains of adenylate kinase. Many of the photoaffinity labeling results do not agree with assignments in the model, particularly the data showing that the AMP binding domain includes I110–K123. The reason for the discrepancy is unknown, but may be related to the fact that single amino acid changes can have unpredictable effects on enzyme function (Wente & Schachman, 1991), sometimes involving interactions quite distinct from the point of modification. For example, in the case of adenylate kinase, the mutation of R149M has a considerable effect on both substrate binding and catalysis, decreasing the k_{CAT} by almost 4 orders of magnitude and increasing the K_m for *both* $MgATP^{2-}$ and AMP by 130-fold (Tsai & Yan, 1991). In contrast, photoaffinity labeling can be a much more targeted approach, relying on interactions that occur exclusively within the binding domain. In the present study, the specificity of photolabeling and protection (Figures 1–4) indicated that adenylate kinase was modified exclusively within the adenine binding domains. Thus, the peptides that were photolabeled must occur within the binding domain. The directness of this approach and the selectivity of the AMP site for the 2-azidoadenine nucleotides made it possible to assign the adenylate kinase binding domains for ATP and AMP. Further refinement of the binding domains beyond the present level will require synthesis of a tighter binding transition-state photoprobe that has less freedom of movement within the binding domain. The results of the present study indicate that an asymmetric diadenosine

probe containing 2- and 8-azidoadenosine bridged by 4 or 5 phosphates would effectively cross-link peptides from the ATP and AMP binding domains, thus providing a very specific reagent for resolving the fine structure of the adenine bindng domains of adenylate kinase.

Improved Procedure for Isolating Photolabeled Peptides. Photoaffinity labeling with ^{32}P-labeled azido nucleotide analogues has been widely used as a method for identifying nucleotide binding proteins even in very crude homogenates [for review see Potter and Haley (1983)]. However, isolation of photolabeled peptides using this technique has been less successful. The lack of success is due to two major technical problems: (a) the sometimes low efficiency of photolabeling and (b) the lability of the photoinserted probe to reverse-phase HPLC techniques. These problems greatly hinder recovery of the modified peptide. To obviate these problems, a general method was sought that would efficiently separate photolabeled peptides from unmodified peptides without substantial loss of the label. The results with adenylate kinase show that Fe^{3+}–chelate chromatography provides a one-step method for rapidly and efficiently separating photolabeled peptides from non-photolabeled peptides. The procedure described here also has been used to identify the photolabeled peptides from other nucleotide binding proteins including recombinant murine granulocyte macrophage colony stimulating factor (rMGM-CSF) and human interleukin-1 (hIL-1B) (in preparation). In addition, Al^{3+}–chelate chromatography has also been used to isolate photolabeled peptides (M. Olcott and B. E. Haley, in preparation). In experiments with adenylate kinase and other proteins, Fe^{3+}–IDA–Sepharose generally bound at least 70% of the photolabeled peptides, forming a complex that could be washed extensively with buffer, salt, and urea to remove virtually all of the nonspecifically bound peptides. Alternatively, the tryptic digest containing 4 M urea could be passed through the resin to avoid nonspecific protein–protein interactions from the onset. In the case of adenylate kinase, several hours were required for complete binding of the photolabeled peptides to Fe^{3+}–IDA–Sepharose. However, experiments with other proteins have shown that, depending on the protein, the time required for binding of photolabeled peptides to Fe^{3+}–IDA–Sepharose can vary from a few minutes to several hours (unpublished observation). Thus, for some proteins, photolabeled peptides can be isolated under gentle conditions within 3–4 h of the photolabeling event.

The ability to purify photolabeled peptides rapidly in a one-column procedure minimizes the extent of breakdown of the photoprobe. Our investigations with adenylate kinase and other proteins (unpublished data) have shown that, for a given protein, more than one peptide from the binding domain may be modified in a single photoaffinity labeling experiment. Also, different versions of the same peptide may be produced if the probe modifies more than one residue in the peptide, inserts into the peptide backbone, or provides steric hindrance to proteolytic digestion. For example, photoaffinity labeling of adenylate kinase with 8-N_3Ap_4A produced both truncated and extended versions of the same peptides (Tables I and II). Thus, even after affinity purification, peptides still must be separated by reverse-phase HPLC or some other chromatographic technique before they can be sequenced. Since most of the label is lost from the photolabeled peptides upon separation by reverse-phase HPLC, the most important consideration is to minimize the extent of breakdown of the probe prior to this step so that a detectable level of radioactivity will still be associated with the peptide after reverse-phase HPLC. Additionally, we have found that most photolabeled peptides retain radiolabel better if injected onto the reverse-phase column at a slow (less than 0.5 mL/min) flow rate. The results with adenylate kinase and a number of other proteins indicate that metal ion chelate chromatography provides a general method for selectively purifying photolabeled peptides. Non-photolabeled peptides and proteins are not retained by the resin at pH 8.3 or after elution with NaCl and urea (Figure 6); therefore, large amounts of proteolytic enzyme can be used with proteins that are recalcitrant to digestion. Still, the likelihood of success in isolating photolabeled peptides from a certain amount of a given protein ultimately depends upon the efficiency of photoinsertion and the stability of the bond formed. In the experiments with adenylate kinase, sequenceable amounts of photolabeled peptides were recovered routinely after photolabeling 1 mg (4.5 nmol) of adenylate kinase. Sequenceable amounts of photolabeled peptides were also recovered in the experiments with PVDF membrane even though only 200 μg of protein was photolabeled and recovery of radioactivity from the membrane after digestion with trypsin was only 20%. These results indicate that Fe^{3+}–chelate chromatography can be used to selectively purify photolabeled peptides starting with relatively low amounts (i.e., <50 μg) of photolabeled proteins. The ability to identify photolabeled peptides after SDS–PAGE and transfer of photolabeled adenylate kinase to PVDF membrane indicates that it may be possible to use photoaffinity labeling followed by peptide isolation via Fe^{3+}–chelate chromatography to identifying nucleotide binding domains on proteins in relatively complex mixtures.

Acknowledgments

We acknowledge Mr. Chifu Huang for providing valuable technical assistance to the project. We thank Dr. R. Chollet (University of Nebraska) for information on the application of metal ion chromatography for phosphopeptide isolation.

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Highly Efficient Photoaffinity Labeling of the Hormone Binding Domain of Atrial Natriuretic Factor Receptor†

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Received October 4, 1991; Revised Manuscript Received February 18, 1992

ABSTRACT: A high-efficiency photoaffinity derivative of atrial natriuretic factor (ANF) was developed for studying the peptide binding domain of the receptor protein and for better characterization of this receptor in tissues with a low density of binding sites. The position of the photosensitive residue was chosen on the basis of a molecular conformational model and on structure–activity relationship studies which both indicate that the carboxy-terminal end of the peptide is part of a hydrophobic pole likely to interact deeply within the ANF binding pocket of the receptor. Selection of the photoreactive residue *p*-benzoylphenylalanine (BPA) as a substitute for arginine in position 125 of the peptide sequence led to a photoaffinity derivative with a high (63%) efficiency of covalent incorporation to the receptor protein. This derivative (BPA-ANF) has a 10-fold lower affinity when compared with ANF, but it is a full agonist in stimulating cGMP production and inhibiting aldosterone secretion in bovine adrenal zona glomerulosa. Photoaffinity labeling with BPA-ANF specifically identifies ANF-R_1 and ANF-R_2 receptor proteins with a 10-fold higher efficiency than with azido derivatives of ANF or with cross-linking agents. This new ANF derivative therefore appears to be useful for studying ANF receptors in tissues with low levels of expression, for locating receptor following cellular internalization, and for tagging proteolytic fragments of the receptor amenable to amino acid microsequencing.

The discovery of the expanding family of cardiac natriuretic peptides and of their specific receptors has led to the identification of a unique type of transmembrane signaling mechanism for hormones and neuropeptides. These peptides, which include atrial natriuretic factor (ANF)[1] first identified in atrial extracts (Thibault et al., 1984; De Bold, 1985), brain natriuretic peptide (BNP) initially isolated from brain (Sudoh et al., 1988), and adrenal (Nguyen et al., 1989) and C-type

† This work was supported by grants from the Medical Research Council of Canada to the Research Program on Molecular Pharmacology and Genetics of Cardiac Natriuretic Peptides and for maintenance of the Molecular Modelling Resources (MT-10131) and from the Heart and Stroke Foundation of Quebec.

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[1] Abbreviations: ANF, atrial natriuretic factor; SDS, sodium dodecyl sulfate; IBMX, isobutylmethylxanthine; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid, sodium salt; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; BNP, brain natriuretic peptide; BPA-ANF, [Tyr[116],*p*-benzoyl-Phe[125]]ANF(99–125); C-ANF, [Cys[116]-ANF(102–116)-NH_2; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

0006-2960/92/0431-4487$03.00/0