Vanadate Catalyzes Photocleavage of Adenylate Kinase at Proline-17 in the Phosphate-Binding Loop†

Christine R. Cremo,*,‡ Joseph A. Loo,§ Charles G. Edmonds,§ and Kristina M. Hatlelid∥,⊥

Biochemistry and Biophysics Department, Washington State University, Pullman, Washington 99164-4660, Chemical Sciences Department, Chemical Methods and Separations Group, Pacific Northwest Laboratories, Richland, Washington 99352, and Chemistry Department, The Colorado College, Colorado Springs, Colorado 80903

Received July 22, 1991; Revised Manuscript Received September 17, 1991

ABSTRACT: Irradiation of adenylate kinase (AK) from chicken muscle with 300–400-nm light in the presence of 0.25 mM vanadate ion first inactivated the enzyme and then cleaved the polypeptide chain near the NH_2 terminus. The addition of the multisubstrate analogue, P^1,P^5-bis(5′-adenosyl) pentaphosphate, prevented both effects. ATP, but not AMP, blocked both inactivation and cleavage in a saturable manner, suggesting that both effects were due to modification at the ATP-binding site. The polypeptide products of the photocleavage were isolated by HPLC and characterized by amino acid composition, peptide sequencing, and mass spectral analyses. The predominant (>90%) small peptide fragment contained the first 16 amino acids from the amino terminus of the enzyme. The amino terminus of this peptide contained an acetylated serine, and the "carboxy" terminus was modified by a cyclized γ-aminobutyric acid which originated from photooxidation and decarboxylation of proline-17 by vanadate. Edman sequencing indicated that the majority of the large peptide fragment ($M_r \sim 19\,500$) was amino-terminal blocked, but a small portion was sequenceable starting at either glycine-18 (7%) or serine-19 (2%). These studies indicate that in the ATP–AK complex proline-17 is close to the phosphate chain of ATP but not AMP, consistent with the latest evaluation of nucleotide-binding sites on mitochondrial matrix AK by X-ray crystallography [Diederichs, K., & Schulz, G. E. (1991) *J. Mol. Biol. 217*, 541–549]. Furthermore, this is the first report that an amino acid other than serine can be involved in vanadate-promoted photocleavage reactions.

Recent studies have shown that the phosphate analogue vanadate (V_i)[1] can catalyze highly specific photooxidations of proteins that often result in cleavage of polypeptide chains. The first examples of this phenomenon were discovered in the flagellar ATPase, dynein [see Gibbons and Mocz (1991), and references therein], and in the muscle ATPase myosin (Grammer et al., 1988; Mocz, 1989; Cremo et al., 1991). In myosin, the initial chemical steps in the cleavage of the peptide backbone have been outlined (Cremo et al., 1991). Irradiation of the tightly bound myosin–Mg^{2+}ADP–V_i complex covalently modifies the protein by oxidizing the hydroxymethyl side chain of Ser-180 to a "serine aldehyde" (Cremo et al., 1988, 1989). The serine aldehyde is then involved in a second V_i-dependent photooxidation step which specifically cleaves the polypeptide chain (Grammer et al., 1988; Cremo et al. 1989). Ser-180 is located in the glycine-rich consensus sequence (phosphate-binding loop) for ATP-binding proteins (Saraste, 1990). Recently, it has been reported that Ser-243 is also oxidized to an "aldehyde" in this second photooxidative step (Grammer & Yount, 1991). The exact role of serine in the mechanism of the sequential photooxidative reactions has not been determined.

Serine is the only amino acid to date which has been shown to be oxidized with V_i and UV light, suggesting that V_i-dependent photomodifications may be specific for serine. V_i is known to interact with many classes of proteins including ATPases, phosphatases, kinases, phosphorylases, nucleases, and enzymes of the citric acid cycle [for review, see Chasteen (1983)]. It is then of general interest to characterize further the photooxidative reactions to extend the use of V_i as a photoprobe for phosphate-binding sites.

In this study we have applied V_i-mediated photooxidation to a model protein, adenylate kinase (AK), which has been previously shown to interact with V_i (Pai et al., 1977; Boyd et al., 1985; Hatlelid & Cremo, 1990). The sequence of AK from chicken muscle has been determined (Kishi et al., 1986) and found to be homologous to other enzymes in the AK1 family (Schulz et al., 1986). AK contains a glycine-rich loop or phosphate-binding loop near its NH_2 terminus. This is a common motif found in most but not all ATP- and GTP-binding proteins (Saraste et al., 1990). The photooxidation and cleavage of myosin occurs in the analogous loop. In addition, AK is a relatively small protein in which the photooxidative products may be easily identified. For these reasons it was predicted that AK would be an ideal protein in which

† Supported by funds from The Colorado College (to K.M.H), the Howard Hughes Foundation, the NIH (AR 39937), the American Heart Association, the U.S. DOE Office of Health and Environmental Research (DE-AC06-76RLO 1830), the NSF Instrumentation and Instrument Development Program (DIR 8908096), and the NIH National Center for Human Genome Research (HG 00327). Pacific Northwest Laboratory is operated by Battelle Memorial Institute. The Varian 2200 spectrophotometer and LKB Ultroscan XL Laser densitometer used in these studies were purchased with funds provided by NSF Equipment Grant PCM 8400841.

‡ Washington State University.
§ Pacific Northwest Laboratories.
∥ The Colorado College.
⊥ Present Address: Graduate Program in Pharmacology and Toxicology, Arizona Health Sciences Center, Tucson, AZ 85724.

[1] Abbreviations: V_i, vanadate (which consists of varying amounts of monomeric tetrahedral vanadate and its oligomers, di-, tetra-, and pentavanadate depending upon total vanadate concentration); AK, adenylate kinase from chicken muscle; Ap_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate; TFA, trifluoroacetic acid; MS, mass spectrometry; ESI, electrospray ionization; ESI–MS, electrospray ionization mass spectrometry; AcN, acetonitrile; GABA, γ-aminobutyric acid.

0006-2960/92/0431-491$03.00/0

to study the exact chemical steps in the specific oxidation and cleavage at nucleotide-binding sites by V_i.

AK was of particular interest to us because of the considerable differences between two models [see Tsai and Yan (1991) for review] describing the positions of the substrates ATP and AMP within the crystal structure of the protein. For this reason, it has been the subject of many recent mutagenesis experiments [see Tsai and Yan (1991) for review]. The substrate-binding models have been derived either from ^1H NMR methods (Fry et al, 1985; Mildvan & Fry, 1987) or from X-ray diffraction studies of protein crystals (Pai et al., 1977). A more recent crystal structure (Egner et al., 1987) has provided indirect evidence for yet a third model which reverses the positions of the substrates [reversed X-ray model; see Tsai and Yan (1991)] originally proposed from the X-ray data (Pai et al., 1977). Recently, Diederichs and Schulz (1991) have analyzed a crystalline AMP–AK complex from beef heart mitochondrial matrix that confirms the indirect evidence of Egner et al. (1987). The purpose of our study was to use V_i-mediated photooxidation as a method to identify amino acids that bind the phosphoryl moieties of either ATP or AMP, providing direct chemical evidence for the modeling of substrate-binding positions in this controversial protein.

We show that AK was inactivated and subsequently cleaved into two fragments after irradiation in the presence of V_i. Both inactivation and cleavage were inhibited specifically by ATP but not AMP, suggesting that the modifications occurred at the polyphosphate region of the ATP-binding site. Structural characterization of purified cleavage fragments by ESI–MS, amino acid analysis, and sequence analysis indicated that the cleavage involved photooxidative decarboxylation of Pro-17 to a novel diimide structure. Pro-17 of AK and Ser-180 of myosin are in equivalent positions within their respective glycine-rich consensus sequences. These data provide direct chemical evidence that Pro-17 is close to the polyphosphates of ATP in the binary ATP–AK complex. This result supports recent structural information (Diederichs & Schulz, 1991) and mutagenesis studies (Yan et al., 1990b; Kim et al., 1990; Tsai & Yan, 1991).

Materials and Methods

Stock solutions of V_i (sodium orthovanadate, Aldrich) were prepared as described by Goodno (1982) and stored at −20 °C. $A_{p5}A$ (disodium salt), Tris, chicken muscle adenylate kinase (lyophilized and essentially salt free), and triethanolamine were purchased from Sigma and used without further purification. AMP and ATP were from Pharmacia. For a typical experiment, the AK was dissolved in the indicated buffer and centrifuged at 4 °C in a microfuge (Beckman) for 5 min to clarify the solution. Dissolved enzyme samples were used immediately. The protein concentration of the supernatant was determined spectrophotometrically using an $\epsilon_{277}^{1\%} = 5.38\ cm^{-1}$.

Enzymatic activity was determined at 25 °C by measuring ADP formation by the pyruvate kinase/lactate dehydrogenase coupled enzyme assay (Rhoads & Lowenstein, 1968). Assays (3.3 mL) were carried out at 25 °C in 75 mM triethanolamine (pH adjusted to 7.6 with NaOH at room temperature), 1.2 mM AMP, 1.0 mM ATP, 10 mM $MgCl_2$, 120 mM KCl, 1 mM phosphoenolpyruvate, 0.21 mM NADH, 5 units of pyruvate kinase (Boehringer Mannheim), and 20 units of lactate dehydrogenase (Boehringer Mannheim). The reaction mixture was preincubated at 25 °C for 5 min prior to the addition of the appropriate amount of AK to give an absorbance change per min at 365 nm below 0.03. One unit of enzyme activity is defined as the amount that produces 1 μmol of ADP/min.

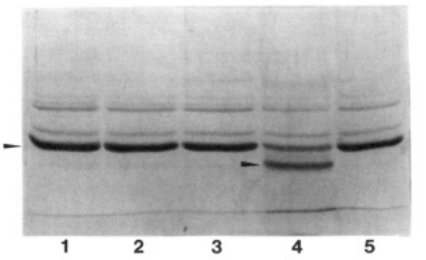


FIGURE 1: Cleavage of AK by UV light and V_i. AK (30 μM) in 2.5 mM Tris-HCl (pH 8.0 at 4 °C)/0.1 M KCl was treated as follows: Lane 1, untreated protein; Lane 2, 20-min irradiation in the absence of V_i; Lane 3, 0.25 mM V_i without irradiation; Lane 4, 20-min irradiation in the presence of 0.25 mM V_i; Lane 5, same as for lane 4 with the addition of 0.10 mM Ap_5A and 5 mM $MgCl_2$. Irradiation and gel electrophoresis were as described under Materials and Methods.

The specific activity was between 1100 and 1200 units/mg, which is very similar to that reported by Tagaya et al. (1989) for the chicken muscle enzyme expressed in *Escherichia coli*.

Protein samples were irradiated with a Hanovia 450-W medium-pressure Hg lamp (Ace Glass) at an average distance of 9 cm in a small Pyrex Petri dish, 10-mL Pyrex beaker, or 1.5-mL plastic microcentrifuge tube, cooled on an ice bath lined with aluminum foil to reflect radiation. A Pyrex filter (Petri dish cover) was used to remove radiation below 300 nm. The amount of cleavage varied slightly between experiments depending upon the distance of the sample from the lamp, absorbance and path length of the sample, and the vessel in which the sample was irradiated.

SDS gel electrophoresis was performed as described by Laemmli (1970) with 12% acrylamide, 0.32% bis(acrylamide), and 0.1% SDS, on a 0.75-mm-thick mold (Hoeffer Mighty Small). Gels were stained with 0.05% Coomassie Blue R in methanol/water/acetic acid (5:5:1 v/v/v), destained in the same solvent, and soaked in water prior to scanning and photographing. An LKB Ultroscan XL laser densitometer was used to determine the integrated intensity of each stained band as an estimate of the amount of protein cleavage.

Mass spectral analyses were performed at the Pacific Northwest Laboratories in Richland, WA, by ESI–MS. The ESI source and the interface to the triple-quadrupole mass spectrometer (TAGA 6000E; Sciex, Thornhill, Ontario, Canada) have been previously described (Smith et al., 1990; Loo et al., 1990). Peptides were continuously infused through the ESI source capillary at a rate of 8 pmol min^{-1}, with the methanol sheath flow rate controlled at 3 μL min^{-1}. Argon was used as the target gas for the collisionally activated dissociation (CAD) tandem MS experiments.

Results

The products of photocleavage of AK (21.5 kDa, 193 amino acids) by V_i were analyzed by gel electrophoresis (Figure 1). Treatment of AK (lane 1) with V_i in the absence of UV light (lane 3) or irradiation without V_i (lane 2) had no effect, whereas irradiation in the presence of V_i (lane 4) generated a peptide of approximately 19.5 kDa and a small cleavage peptide (approximately 20 amino acids) comigrating with the dye front on the gel. These results indicate that both V_i and light were required to cleave the enzyme, and this cleavage was near either the NH_2 or COOH terminus. Under the conditions of Figure 1, 63% of the protein was cleaved. The presence of the potent multisubstrate inhibitor Ap_5A, which is thought to bind to both the ATP- and AMP-binding sites (Schulz et al., 1990; equilibrium dissociation constant = 10 nM; VanDerLijn et al., 1979) completely prevented cleavage

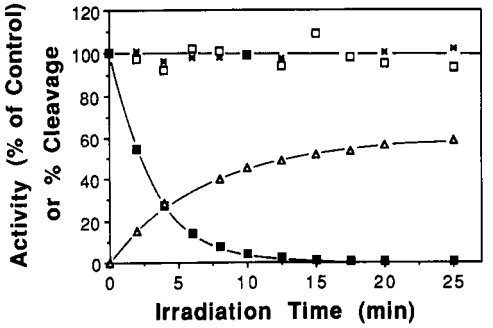


FIGURE 2: Activity and cleavage versus irradiation time. AK (16.6 μM) in 2.5 mM Tris (pH 8.0 at 4 °C)/0.1 M KCl was irradiated for the indicated times (□, activity) in the absence of V_i; (×, activity) with 0.25 mM V_i and 25 μM Ap5A; or (■, activity and Δ, cleavage) with 0.25 mM V_i. Aliquots were then assayed for activity and applied to gels to determine the extent of cleavage. Activity is expressed as a percentage of an untreated control. Cleavage is expressed as a simple percentage. Control experiments indicated that the samples did not further inactivate or cleave after the lamp was turned off (data not shown). Irradiation, gel electrophoresis, and enzyme assay procedures are described under Materials and Methods.

of the protein (lane 5). In general, the amount of cleavage could be increased to 100% with increasing V_i concentrations (to 1.5 mM) and longer irradiations (data not shown). These 100% cleavage conditions were not used, thus avoiding potential nonspecific V_i interactions with the protein. The amount of cleavage did not depend upon the $MgCl_2$ concentration, but it decreased from 65 to 30% when the concentration of the buffer Tris was increased from 2.5 to 50 mM (data not shown). This inhibition of cleavage by Tris has also been found in studies of V_i-catalyzed cleavage of myosin (Cremo et al., 1990).

To determine the effect of photocleavage on the activity of the enzyme, the amount of cleavage and activity were measured versus irradiation time (Figure 2). During the irradiation, the protein was progressively inactivated and cleaved but these two effects were not directly correlated. After a 25-min irradiation, the enzyme had <5% of the activity of the control which was irradiated in the absence of V_i. However only 55–60% (not 95%) was cleaved, suggesting that photocleavage was preceded by a chemical modification that inactivated the protein. Addition of the potent multisubstrate inhibitor Ap_5A (Leinhard & Secemski, 1973) prevented both inactivation (Figure 2) and cleavage (Figure 1) of the protein, indicating that both effects were due to modifications at the active site.

To examine further the site specificity of the V_i-dependent reaction, we analyzed the effects of the substrates ATP and AMP on the inactivation (Figure 3A) and cleavage (Figure 3B). The protection against both inactivation and cleavage by ATP was saturable, providing half-maximal protection at approximately 50 μM. In contrast, the protection against cleavage or inactivation by AMP did not appear to saturate within the range of AMP concentrations tested. For AMP the apparent half-maximal protection was approximately 1.5 mM for inactivation and 1.0 mM for cleavage. These data suggest that the V_i-mediated cleavage occurred predominantly at the ATP-binding site.

The correlation between enzyme activity and percentage of cleavage (Figure 3C) as derived from the data in Figure 3A,B illustrates two points. First, the enzyme was fully inactive at only ~50% cleavage, consistent with the data in Figure 2. Second, the line drawn through the data intersects the y-axis at ~90%, indicating that ~10% of the enzyme was inactivated before detectable cleavage (detection limit ~ 2%). Therefore,

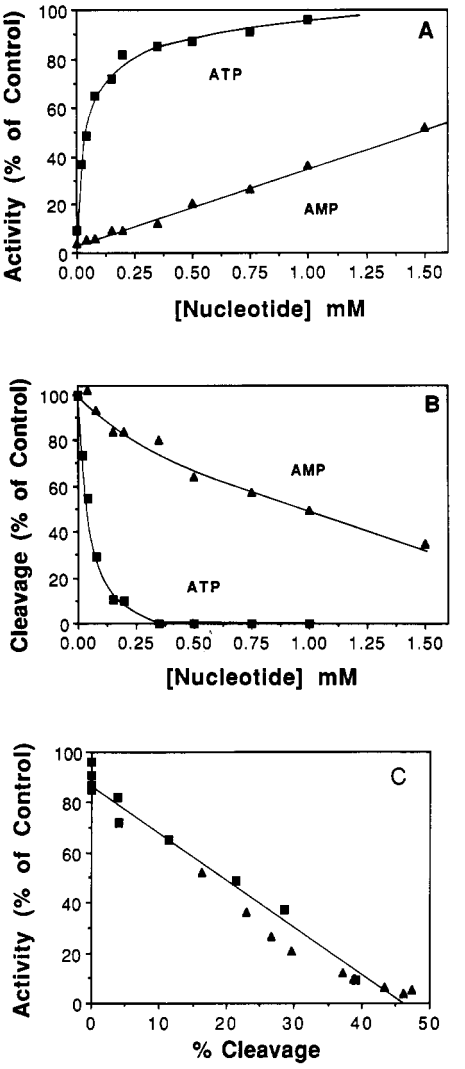


FIGURE 3: Protection by nucleotides against inactivation (A) and photocleavage (B) of AK and correlation between activity and cleavage (C). For (A) and (B), AK (29 μM) in 2.5 mM Tris (pH 8.0 at 4 °C)/0.1 M KCl/5 mM $MgCl_2$/0.25 mM V_i was irradiated for 10 min in the presence of the indicated concentrations of ATP. The AMP protection experiment was identical except that $MgCl_2$ was omitted. Stock solutions of nucleotides were adjusted to pH 8.0 and diluted into the above buffer prior to addition to the protein to avoid pH and Tris concentration differences in the samples. Samples were assayed for activity and analyzed by gel electrophoresis as described under Materials and Methods. Activity values are expressed as a percentage of identically treated controls (minus irradiation). Cleavage values are expressed as a percentage of a control sample that was irradiated in the absence of nucleotide. For (C), the data in (A) and (B) were replotted (■, ATP points; ▲, AMP points) except that the cleavage was expressed as a simple percentage, not as a percentage of a control. The line was fit by eye through the data.

the data indicate that an intermediate inactive form of the enzyme was generated prior to cleavage.

Knowledge of which species of V_i interacts at the active site of AK would help account for the specificity of the photocleavage for the ATP-binding site. V_i may bind to AK in a polymeric form, as V_i ions are known to rapidly equilibrate between the monomeric tetrahedral form and the di-, tetra-, and pentameric oligomers depending upon the total V_i concentration (Baes & Mesmer, 1976; Habayeb & Hileman, 1980; Heath & Howarth, 1981). ^{51}V NMR analysis indicated that under our conditions (0.25 mM total V_i, pH 8.0) approximately 95% of the vanadium was in the monomeric form (data not shown). A small amount of the dimer was present, but no tetra-, penta-, or decavanadate could be detected [the

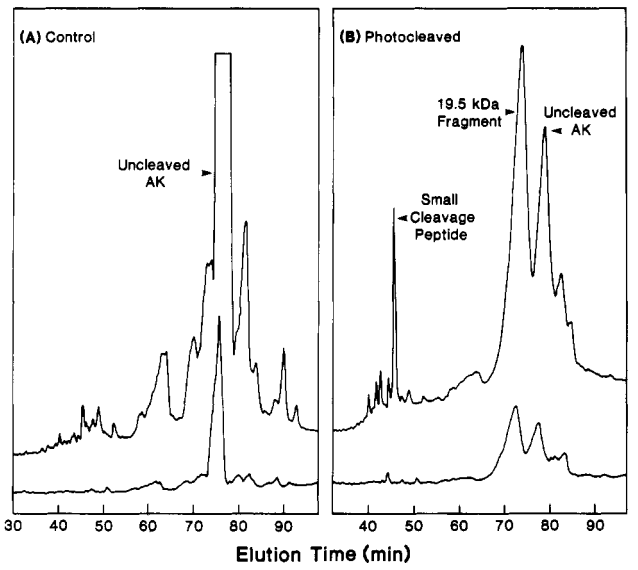


FIGURE 4: HPLC purification of small photocleavage peptide. AK (39 μM) in 2.5 mM Tris (pH 8.0 at 4 °C)/0.1 M KCl was irradiated for 20 min in the absence (A) or presence (B) of 0.25 mM V_i. Cleavage was estimated by gel electrophoresis to be about 60% for (B), and no cleavage could be detected for (A). Samples (350 μg) were acidified with TFA, filtered, and applied to a Brownlee Aquapore C8 column (220 × 4.6 mm) equilibrated at room temperature in 0.115% TFA/H_2O. A 1%/min gradient to 0.100% TFA/60% AcN, starting at 0 min, was applied at a flow rate of 1.0 mL/min. The chromatogram between 0 and 30 min was not shown because no peaks were detected in this time span. Upper trace: 215 nm, 0.2 OD full scale, corresponds to elution time. Lower trace: 260 nm, 0.05 OD full scale, with a 2-min lag from elution time.

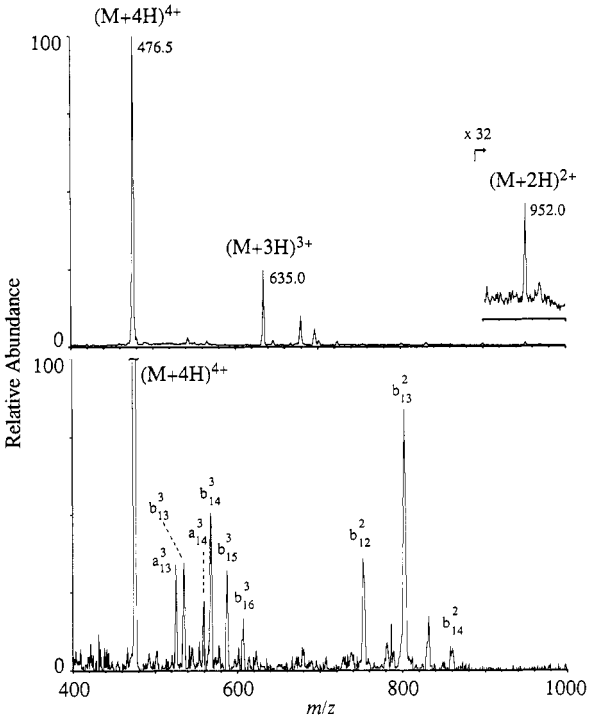


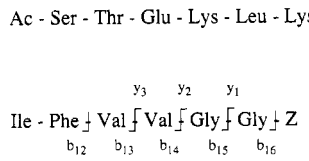
FIGURE 5: MS analysis of the NH_2-terminal cleavage peptide. ESI mass spectrum (top) and partial CAD tandem MS spectrum (bottom) of the $(M + 4H)^{4+}$ parent ion. The peptide (lyophilized from TFA/H_2O/AcN, see Figure 4 legend) was dissolved in a solution of 71:25:4 H_2O/AcN/acetic acid. Approximately 33 pmol of peptide was consumed in the experiments. Top: relative molecular masses are shown below the indicated ions. The $(M + 2H)^{2+}$ peak is shown at 32 times the scale of the abscissa. Bottom: conventional notation (Biemann, 1988) for sequence-specific fragmentation of polypeptides is used (see Scheme I), augmented with a superscript indicating the charge state. Absence of a superscript indicates a singly charged ion.

metastable decamer which has been shown to inhibit AK activity (Boyd et al., 1985) is purposely destroyed in the preparation of the V_i solutions used here (Goodno, 1982)]. It has been previously shown by ^{51}V NMR that the tetramer interacts specifically at the active site of AK (Hatlelid & Cremo, 1990). However, these experiments were performed at much higher V_i concentrations (i.e., more tetravanadate) than the cleavage experiments reported here. In a cleavage experiment the irradiation time is long (10–20 min; Figure 2), so it is possible that cleavage occurs by utilizing catalytic amounts of tetramer which may not be detected by ^{51}V NMR. One method to determine which species affects cleavage is to correlate the rates of cleavage to the concentrations of the various species present in the V_i solution (as measured by ^{51}V NMR). This experiment was not attempted because the concentration of V_i in the +5 oxidation state progressively decreases by reduction to the +4 state as the irradiation proceeds (Cremo et al., 1990). We have attempted to determine which species of V_i inhibits the activity of AK by a similar correlation, even though the inhibiting species may not be the species which catalyzes the photocleavage reaction. We found that salt and pH effects interfered at the high concentrations of V_i required to observe appreciable inhibition of activity (2–3 mM V_i for apparent 50% inhibition). Even without conclusive information about which V_i species binds to AK, the specificity of the reaction for the ATP-binding site convinced us to pursue the structural characterization of the V_i cleavage fragments.

To purify and characterize the cleavage fragments, control (uncleaved) and photocleaved enzyme were both separated on a reversed-phase HPLC column (Figure 4A,B). The uncleaved protein consisted of minor contaminants and a major peak corresponding to unmodified AK (eluting at 75–78 min). The cleaved enzyme sample consisted of residual uncleaved protein, a 19.5-kDa fragment eluting at 72–74 min, and a conspicuous small peptide eluting at 46 min (small cleavage peptide). We surmised that the small cleavage peptide was the NH_2- or COOH-terminal peptide that was predicted from the data in Figure 1. The peptide was purified further on the C8 column with a 1%/min gradient from 5 mM potassium phosphate, pH 6.9, to 50% AcN/H_2O, followed by a desalting step in the TFA system (data not shown) to give a single peak. The two fractions of this peak were analyzed for purity by capillary zone electrophoresis (Bio-Rad) in 50 mM potassium phosphate buffer, pH 2.5. The first fraction contained a predominant peptide (the small cleavage peptide; >90%) and a single minor contaminant peptide, and the second fraction was enriched in the minor contaminant.

The analysis of the HPLC-purified small cleavage peptide (first fraction from above) by ESI–MS is shown in Figure 5. Ions at mass-to-charge ratio (m/z) of 476.5, 635.0, and 952.0 correspond respectively to the $(M + 4H)^{4+}$, $(M + 3H)^{3+}$, and $(M + 2H)^{2+}$ molecular ions of the peptide with a measured relative molecular mass of 1902.0 ± 0.1. The relatively low intensity ions at m/z 680 and 700 and ions below m/z 400 were assigned as contaminants. This assigment was based upon MS analysis (data not shown) of the second fraction (see above) from the HPLC purification. Tandem MS of the 4+ (Figure 5, bottom) and the 3+ (data not shown) multiply protonated molecules yield multiply charged product ions due to cleavage from regions near residues 12–16 (see Scheme I). The majority of the fragment ions observed were due to cleavage of peptide bonds. The positive charge resides on the NH_2-terminal fragment for a and b ions, whereas charge retention occurs at the COOH terminus for y ions. Similar mass spectra were obtained (data not shown) by increasing

Scheme I: Observed Tandem MS Fragmentation Pattern of the NH_2-Terminal Cleavage Peptide[a]



[a] The b ions are acylium ions which lose CO to form a ions [see Biemann (1988)].

the voltage difference between the nozzle and skimmer ion sampling elements of the atmospheric pressure/vacuum interface, thereby increasing the collision energy to induce dissociation of all ions (Smith et al., 1990; Loo et al., 1990). All observed a_n and b_n product ions were consistent with the sequence S_1TEKLKHHKIIFVVGG$_{16}$ with an acetylated NH_2 terminus and a modified COOH-terminal residue (shown as Z on Scheme I; see below). For example, the $b_{16}{}^3$ fragment ion at m/z 606.8 indicated that the mass of the peptide minus the COOH-terminal modification was 1817.4, in good agreement with an acetylated peptide. Furthermore, the mass of the COOH-terminal-modified residue was determined by difference from the relative molecular mass, to be approximately 84 Da. In addition, ions in the tandem mass spectrum at m/z 143, 200, and 299 (data not shown) were consistent with y_1, y_2, and y_3 sequence ions and a COOH-terminal group of mass 84.

The amino acid analysis of the HPLC-purified small cleavage peptide (see Figure 4B) is shown in Table I. The analysis was consistent with the composition of the 16 NH_2-terminal residues of AK (Kishi et al., 1986), except that 1 mol of GABA/mol of peptide was found. This unusual component was most likely a hydrolyzed product of a COOH-terminal modification due to the V_i cleavage. An attempt was made to sequence this peptide by Edman degradation, but no phenylthiohydantoin derivatives of any amino acids were detected, suggesting that the serine at the NH_2 terminus was blocked. This blocking group was consistent with the MS data which indicated the presence of an acetylated NH_2-terminal serine.

The four additional minor peptides eluting between 40 and 45 min on the HPLC chromatogram (Figure 4B) were also characterized. These four sequences (data not shown) did not correspond to the AK sequence. They appeared to arise from cleavage of the minor contaminant protein(s) eluting at 63–64 min (Figure 4A), on the basis of data from a time course of cleavage (data not shown).

Chromatographic fractions (nos. 71 and 72, Figure 4B) containing the 19.5-kDa fragment were submitted for sequence analysis (data not shown). After seven cycles, the following two sequences were found: G_{18}SGKGTQ and S_{19}GKGTQX in a molar ratio of 2.7:1.0. It was calculated from the sequencing yield and the amount of protein submitted for analysis [on the basis of a bicinchoninic assay (Pierce)] that the two sequences represented only 7 and 2%, respectively, of the total protein in the sample. These data suggest that the majority of the 19.5-kDa fragment was not sequenceable, and a minor portion contained cleavage sites NH_2-terminal to Gly-18 and Ser-19.

Discussion

Irradiation with UV light in the 300–400-nm range in the presence of V_i cleaved the polypeptide chain of AK into two predominant fragments (Figure 1). The cleavage was preceded by a modification that inactivated the enzyme (Figures 2 and 3C). The nature of this precleavage modification is currently under investigation in our laboratory. To localize the V_i modifications within the active site, the two substrates AMP and MgATP were tested for their ability to protect against V_i inactivation (Figure 3A) and cleavage (Figure 3B). The differences between the AMP and ATP concentrations which provided half-maximal protection of inactivation and cleavage (for AMP, 1.0–1.5 mM; for MgATP, 0.05 mM) cannot be accounted for by differences in the equilibrium dissociation constants of the nucleotides (160 μM for MgATP and 370 μM for AMP; Tsai & Yan, 1991). The weak protection found for AMP may reflect the weak cross-binding of AMP to the MgATP site (4.3 mM; Sanders et al., 1989). These data support the conclusion that V_i is interacting preferentially with the ATP-binding site.

Analysis of the V_i cleavage fragments (Figures 1 and 4) indicated that the cleavage was at a single site near either the NH_2 or COOH terminus of the enzyme. The amino acid composition (Table I) of the purified small cleavage peptide (Figure 4) matched the composition of the NH_2-terminal 16 amino acid peptide of AK. The significant difference was the presence of 1 mol of GABA/mol of peptide. These data suggest that the COOH terminus of the peptide contained a part of Pro-17. MS analysis indicated the molecular mass of the peptide was 1902.0 Da (Figure 5). After an acetylated NH_2-terminal serine evident from the tandem MS data (Figure 5, bottom) plus the 16 amino acids was accounted for, the molecular mass measured by MS indicated an additional modified residue of 83.6 Da, presumably on the new COOH terminus of the peptide. This is almost identical to the mass of GABA linked to the COOH-terminal amino acid as a lactam.

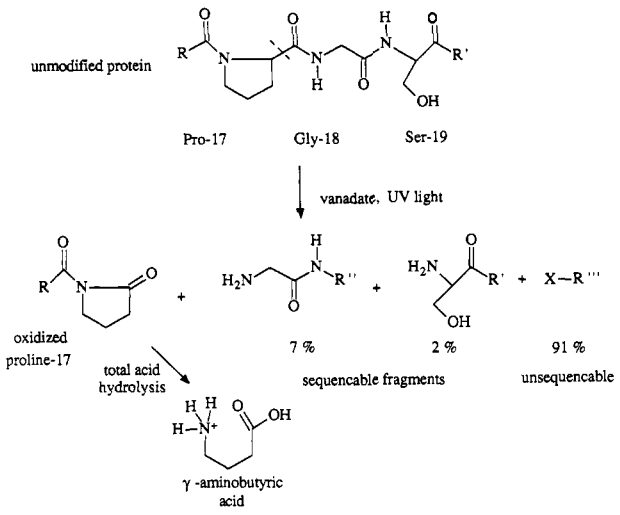
Scheme II shows a chemical reaction sequence that is consistent with both the amino acid analysis and the MS analysis of the small cleavage peptide. The enzyme was

Table I: Amino Acid Analysis of Small Cleavage Peptide[a]

amino acid	relative number of residues: analysis of cleavage peptide[b]	theoretical[c]
Asp	0.3	0
Thr	0.9	1
Ser	0.8	1
Glu	1.1	1
Pro	0.2	0
Gly	2.1	2
Ala	0	0
Val	2.1	2
Met	0	0
Ile	1.7	2
Leu	1.1	1
Tyr	0	0
Phe	1.0	1
Lys	3.1	3
His	1.9	2
Arg	0	0
γ-aminobutyric acid (GABA)	1.0	0

[a] Amino acids were determined with a Beckman Model 121 MB amino acid analyzer, after hydrolysis in double-distilled 6 N HCl (Burdick and Jackson) for 22 h at 110 °C. Data were analyzed by comparing the peak area for each amino acid in the sample to the peak area for the same amino acid in a standard mixture containing 1 nmol of each amino acid. GABA eluted at 85.2 min between phenylalanine (56.1 min) and ammonia (101.3 min). [b] Values are expressed relative to phenylalanine. [c] Values are for the 16 amino acid NH_2-terminal peptide of chicken muscle AK which contains the sequence STEKLKHHKIIFVVGG. The sequence does not contain the NH_2-terminal methionine residue suggested from the cDNA sequence (Kishi et al., 1986). This residue is also absent in chicken AK expressed in *E. coli* (Tanizawa et al., 1987).

Scheme II: Summary of Photocleavage Chemistry[a]



[a] The sequence of three residues in the glycine-rich loop of AK are shown. R = the NH_2-terminal 16 amino acids. R′ or R″ = the remainder of the 21.5-kDa protein attached to either serine or glycine, which are sequenceable. The dotted line indicates the predominant position of cleavage by V_i and UV light. The α-carbon of Pro-17 is oxidized to a carbonyl to form a diimide structure which gives γ-aminobutyric acid upon total acid hydrolysis of the peptide. A total of 91% of the protein from the other side of the cleavage site did not sequence. X = unknown terminal structure attached to R‴, the remaining fragment.

Table II: Consensus Sequences Containing Amino Acids Implicated in Vanadate-Mediated Photocleavage

enzyme	sequence no.	sequence[a]
adenylate kinase (chicken skeletal muscle)	15–23[b]	GGPGSGKGT
myosin ATPase[c] (rabbit skeletal muscle)	178–186[d]	GESGAGKTV

[a] Oxidized amino acids are shown in boldface type. [b] Numbering as in Schultz et al. (1986). [c] Cremo et al. (1989). [d] Numbering exclusive to rabbit protein.

specifically cleaved within Pro-17 with concomitant oxidation of the α-carbon of Pro-17 to a carbonyl. After a total acid hydrolysis of the peptide for amino acid analysis, GABA would be expected to form as was found in the amino acid analysis (Table I).

The structural analysis of the NH_2 terminus of the 19.5-kDa fragment has not yet been completed. A total of 91% of the protein was not sequenceable (see Scheme II), suggesting that its NH_2 terminus was blocked. It is possible that the small amounts of sequenceable fragments were breakdown products of the unsequenceable 19.5-kDa fragment, as they were exposed to acidic conditions during HPLC. The equivalent termini in the cleavage of myosin (Mocz, 1989; Cremo et al., 1991) and dynein (Gibbons & Mocz, 1991) have also been shown to resist sequence analysis. The exact chemical structure of this modified terminus of AK is currently under investigation. With this data it may be possible to deduce a mechanism for the photocleavage reaction.

This is the first report of a V_i-mediated photooxidation involving a proline residue. Previously, only serine residues have been shown to be involved (Cremo et al., 1988, 1989, 1990; Mogel & McFadden (1989). Table II is a comparison of sequences from AK and skeletal muscle myosin, which are known to contain V_i cleavage sites. In both instances the oxidized amino acids occur in the same position of the glycine-rich consensus sequence for the phosphate-binding loop. The oxidized Ser-180 in myosin has been placed at the γ-phosphoryl-binding site of ATP (Cremo et al., 1989) because during the cleavage reaction the V_i is bound exclusively at the active site by the formation of a stable highly purified myosin–MgADP–V_i complex. No evidence for a similar complex of V_i with either ADP or ATP could be found in AK (C. Cremo, unpublished observation). At this time it is not known whether the V_i is cleaving AK at the α-, β-, or γ-phosphoryl position of ATP. Since the cleavage sites in both AK and myosin occur in the same position in the glycine-rich consensus sequence, regardless of the residue, this position may be important to the coordination of the phosphoryl groups of ATP during catalysis. Mutations of Pro-17 [to Leu, Reinstein et al. (1988); to Gly and Val, Tagaya et al. (1989)] decrease the K_ms for both substrates but do not change the V_{max}s appreciably, suggesting that Pro-17 is more important to substrate binding than to transition-state stabilization.

The protection of V_i photocleavage by ATP at Pro-17, which is at the tip of the phosphate-binding loop, is consistent with the recent reversed assignments for substrate binding proposed from indirect results of the crystal structure of the AK–Ap_5A complex (Egner et al., 1987). It is also consistent with the findings from the recent crystal structure of the AK–AMP complex from beef heart mitochondrial matrix (Diederichs & Schulz, 1991), which place all of the amino acids in the glycine-rich loop including Pro-17 greater than 4.5 Å from the AMP molecule. The phosphate of AMP contacts the guanidinium groups of Arg-44 and Arg 97 (Diederichs & Schulz, 1991) which are distant from Pro-17. Recent mutagenesis experiments have confirmed this position of AMP by showing that mutations of these two arginines [Arg-44, Yan et al. (1990a,b) and Kim et al. (1990); Arg-97, Tsai and Yan (1991)] drastically alter interactions with AMP but not with ATP. The photooxidation by V_i of Pro-17 in AK provides direct chemical evidence which refutes the original substrate positions proposed by Pai et al. (1977) within the crystal structure of AK (Schultz et al., 1974; Dreusicke et al., 1988).

In conclusion, we have shown that V_i alone in the absence of nucleotides was a highly specific reagent to photooxidize Pro-17 in the glycine-rich loop of AK. On the basis of substrate protection studies, Pro-17 appeared to interact with the phosphoryl groups of ATP but not AMP. These results provide evidence in support of the reversed X-ray model (Tsai & Yan, 1991) of substrate binding in AK and establish V_i as a generally useful photoactivatable probe for phosphate-binding sites in proteins.

ACKNOWLEDGMENTS

We thank J. Grammer, R. Yount, K. Facemyer, and M. Gresser for helpful discussions. Gerhard Munske performed the sequence analyses at the WSU Laboratory for Bioanalysis and Biotechnology. Don Appel (WSU) helped with the ^{51}V NMR experiments, and T. J. Zamborelli and D. Collins (The Colorado College) provided technical assistance in the earlier stages of this work.

Registry No. V_i, 14333-18-7; AK, 9013-02-9; ATP, 56-65-5; G_{18}-SGKGTQ, 137695-58-0; Ac-Ser-Thr-Glu-Lys-Leu-Lys-His-His-Lys-Ile-Ile-Phe-Val-Val-Gly-Gly-Z, 137718-25-3.

REFERENCES

Baes, C. F., & Mesmer, R. E. (1976) in *The Hydrolysis of Cations*, pp 197–210, Wiley-Interscience, New York.

Bieman, K. (1988) *Biomed. Environ. Mass Spectrom. 16*, 99–111.

Boyd, D. W., Kustin, K., & Niwa, M. (1985) *Biochem. Biophys. Acta 827*, 472–475.
Chasteen, N. D. (1983) *Struct. Bonding (Berlin) 53*, 105–138.
Cremo, C. R., Grammer, J. C., & Yount, R. G. (1988) *Biochemistry 27*, 8415–8420.
Cremo, C. R., Grammer, J. C., & Yount, R. G. (1989) *J. Biol. Chem. 264*, 6608–6611.
Cremo, C. R., Long, G. T., & Grammer, J. C. (1990) *Biochemistry 29*, 7982–7990.
Cremo, C. R., Grammer, J. C., & Yount, R. G. (1991) *Methods Enzymol. 196*, 442–449.
Diederichs, K., & Schulz, G. E. (1991) *J. Mol. Biol. 217*, 541–549.
Dreusicke, D., Karplus, A., & Schulz, G. E. (1988) *J. Mol. Biol. 199*, 359–371.
Egner, U., Tomasselli, A. G., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 649–658.
Fry, D. C., Kuby, S. A., & Mildvan, A. S. (1985) *Biochemistry 24*, 4680–4694.
Gibbons, I. R., & Mocz, G. (1991) *Methods Enzymol. 196*, 428–442.
Goodno, C. C. (1982) *Methods Enzymol. 85*, 116–123.
Grammer, J. C., & Yount, R. G. (1991) *Biophys. J. 59*, 226a.
Grammer, J. C., Cremo, C. R., & Yount, R. G. (1988) *Biochemistry 27*, 8408–8415.
Habayeb, M. A., & Hileman, O. E., Jr. (1980) *Can. J. Chem. 58*, 2255–2261.
Hatlelid, K. M., & Cremo, C. R. (1990) *Biophys. J. 57*, 423a.
Heath, E., & Howarth, O. W. (1981) *J. Chem. Soc., Dalton Trans.*, 1105–1110.
Kim, H. J., Nishikawa, S., Tokutomi, Y., Takenaka, H., Hamada, M., Kuby, S. A., & Uesugi, S. (1990) *Biochemistry 29*, 1107–1111.
Kishi, F., Maruyama, M., Tanizawa, Y., & Nakazawa, A. (1986) *J. Biol. Chem. 261*, 2942–2945.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lienhard, G. E., & Secemski, I. I. (1973) *J. Biol. Chem. 248*, 1121–1123.
Loo, J. A., Edmonds, C. G., & Smith, R. D. (1990) *Science 248*, 201–204.
Mildvan, A. S., & Fry, D. C. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol. 58*, 241–313.
Mocz, G. (1989) *Eur. J. Biochem. 179*, 373–378.
Mogel, S. N., & McFadden, B. A. (1989) *Biochemistry 28*, 5428–5431.
Pai, E. F., Sachsenheimer, W., Schirmer, R. H., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 37–45.
Reinstein, J., Brune, M., & Wittinghofer, A. (1988) *Biochemistry 27*, 4712–4720.
Rhoads, D. G., & Lowenstein, J. M. (1968) *J. Biol. Chem. 243*, 3963–3972.
Sanders, C. R., II, Tian, G., & Tsai, M.-D. (1989) *Biochemistry 28*, 9028–9043.
Saraste, M., Sibbald, P. R., & Wittinghofer, A. (1990) *Trends Biochem. Sci. 15*, 430–434.
Schulz, G. E., Biedermann, K., Kabasch, W., & Schirmer R. H. (1974) *J. Mol. Biol. 80*, 857–864.
Schulz, G. E., Schiltz, E., Tomasselli, A. G., Frank, R., Brune, M., Wittinghofer, A., & Schirmer, R. H. (1986) *Eur. J. Biochem. 161*, 127–132.
Schulz, G. E., Müller, C. W., & Diederichs, K. (1990) *J. Mol. Biol. 213*, 627–630.
Smith, R. D., Loo, J. A., Edmonds, C. G., Barinaga, C. J., & Udseth, H. R. (1990) *Anal. Chem. 62*, 882–899.
Tagaya, M., Yagami, T., Noumi, T., Futai, M., Kishi, F. Nakazawa, A., & Fukui, T. (1989) *J. Biol. Chem. 264*, 990–994.
Tanizawa, Y., Kishi, F., Kaneko, T., & Nakazawa, A. (1987) *J. Biochem. 101*, 1289–1296.
Tsai, M.-D., & Yan H. (1991) *Biochemistry 30*, 6806–6818.
VanDerLijn, P., Barrio, J. R., & Leonard, N. J. (1979) *Biochemistry 18*, 5557–5561.
Yan, H., Shi, Z., & Tsai, M.-D. (1990a) *Biochemistry 29*, 6385–6392.
Yan, H., Dahnke, T., Zhou, B., Nakazawa, A., & Tsai, M.-D. (1990b) *Biochemistry 29*, 10956–10964.