Chemistry and Mechanism of Vanadate-Promoted Photooxidative Cleavage of Myosin†

Jean C. Grammer,‡ Joseph A. Loo,§ Charles G. Edmonds,|| Christine R. Cremo,‡ and Ralph G. Yount*,‡

Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-4660, Pharmaceutical Research Division, Parke-Davis, Ann Arbor, Michigan 48105, and Environmental Molecular Science Laboratory, Pacific Northwest National Laboratory, Richland, Washington 99352

Received July 31, 1996⊗

ABSTRACT: Irradiation of the stable myosin subfragment 1(S1)·MgADP·orthovanadate (V_i) complex results in oxidation of an active site serine (Ser-180) to a serine aldehyde [Cremo, C. R., Grammer, J. C., & Yount, R. G. (1989) *J. Biol. Chem. 264*, 6608−6611]. This photomodified S1 will reform a new MgADP·V_i complex and upon a second irradiation, the S1 heavy chain is cleaved into 21 kDa NH_2-terminal and 74 kDa COOH-terminal fragments. When S1, in which the side chain of Ser-180 was tritiated, was photocleaved tritium was released from the protein suggesting that cleavage was occurring at Ser-180. The 21 kDa NH_2-terminal fragment was resistant to carboxypeptidase digestion, and the 74 kDa COOH-terminal fragment yielded no sequence by Edman degradation, indicating that parts of Ser-180 went to each fragment. To identify these parts, the two cleavage fragments were isolated and chemically (21 kDa) or enzymatically (74 kDa) cleaved, and the resulting peptides were separated by reversed phase HPLC. The peptides immediately down- and up-stream from Ser-180 were isolated and the blocking groups were identified by mass spectrometry. The 21 kDa fragment peptide was blocked with a carboxamide on Glu-179 (confirmed by HPLC and capillary electrophoresis in comparison with peptide standards), while the NH_2 group of Gly-181 of the 74 kDa fragment was blocked with an oxalyl group (verified by enzymatic analysis for oxalate). The side chain of Ser-180 was released as formate. O_2 is required for photocleavage. Cleavage experiments in the presence of $^{18}O_2$ showed one atom of ^{18}O labeled the oxalyl group. A mechanism in which O_2 adds to a free radical on the α-carbon of Ser-180 with a subsequent Criegee type rearrangement is proposed to explain both the kinetics and products of the photocleavage.

Monomeric vanadate ions (V_i) and the oligomers with which it is in equilibrium interact with many classes of proteins. Vanadate ion (VO_4^{3-}) is isostructural and isoelectronic with phosphate. Unlike phosphate, vanadate is photochemically active and irradiation of protein-bound vanadate species with UV light often results in specific oxidations of protein side chains and in polypeptide cleavage (Gibbons & Mocz, 1991). UV-induced oxidation or photocleavage mediated by vanadate appears to be a general phenomenon reported for at least 10 different enzymes [reviewed in Muhlrad and Ringel (1995)]. Dynein (Lee-Ford *et al*., 1986; Tang & Gibbons, 1987; Gibbons *et al*., 1987; Marchese-Ragona *et al*., 1989; Gibbons & Mocz, 1991) and myosin (Grammer *et al*., 1988; Cremo *et al*., 1988, 1989, 1990, 1991; Mocz, 1989; Mulhrad *et al*., 1991; Ringel *et al*., 1990) have been the most extensively studied. Other enzymes photomodified or photocleaved by irradiation of vanadate complexes are adenylate kinase (Cremo *et al*., 1992), tubulin (Correia *et al*., 1994), ribulosebisphosphate carboxylase/oxygenase (Mogel and McFadden, 1989), phosphogluconate dehydrogenase (Bergamini *et al*., 1995), aldolase (Crans *et al*., 1992), Ca^{2+} ATPase from the sarcoplasmic reticulum (Vegh *et al*., 1990; Molnar *et al*., 1991), phosphofructokinase (Signorini & Bergamini, 1990), and glycogen phosphorylase (Bergamini & Signorini, 1991). Isocitrate lyase is also specifically photomodified by vanadate at the active site (Ko *et al*., 1992) indicating that in certain cases vanadate (or vanadate polymers) will mimic polyanionic substrates which are not phosphates.

Although many polypeptide cleavages have been reported, little is known about the mechanism of the reactions. It is known that the +5 oxidation state of vanadium is involved in the oxidative reactions (Cremo *et al*., 1990) and that oxygen is required for photocleavage of dynein (Gibbons & Mocz, 1991). An important obstacle to mechanistic studies is that the cleaved polypeptide termini and other products of cleavage have been difficult to identify. The amino termini resulting from cleavage have in all reports proved resistant to sequencing by Edman degradation (Gibbons & Mocz, 1991; Correia *et al*., 1994; Cremo *et al*., 1991, 1992; Bergamini *et al*., 1995). This is unfortunate because identification of these termini could provide interesting structural information about phosphate and polyanionic binding sites for many enzymes. It is then of general interest to characterize further the photooxidative reaction mechanisms to extend the use of V_i[1] is a chemical probe.

† This work was supported by Grants DK-05195 (R.G.Y.), AR-40917 (C.R.C.), and GM-47423 (C.G.E.) from the National Institutes of Health and by a grant from the Muscular Dystrophy Association (R.G.Y.). The LKB Ultroscan XL laser densitometer used in these studies was purchased with funds provided by NSF Equipment Grant PCM 8400841.

* To whom correspondence should be addressed.
‡ Washington State University.
§ Parke-Davis.
|| Pacific Northwest Laboratories.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1996.

S0006-2960(96)01901-0 CCC: $12.00

In skeletal muscle myosin, the initial chemical steps in the cleavage of the peptide backbone at the active site have been outlined (Cremo *et al*., 1991). The photocleavage of myosin may be an unusual case in which the oxidative steps in a multistep mechanism can be performed sequentially and the stable intermediates characterized. Irradiation of the tightly bound myosin·MgADP·V_i complex covalently modifies the protein by promoting the oxidation of the hydroxymethyl side chain of Ser-180 to a "serine aldehyde" (Cremo *et al*., 1988, 1989). The active site of smooth muscle myosin can be similarly oxidized at Ser-181 (Cole & Yount, 1992). The skeletal myosin "serine-180 aldehyde"·MgADP·V_i complex can be further oxidized by UV irradiation. This step specifically cleaves the polypeptide chain at the active site (Cremo *et al*., 1989; Grammer *et al*., 1988) and concomitantly oxidizes Ser-243 to an "aldehyde" (Grammer & Yount, 1991). Ser-180 is located in the glycine-rich consensus sequence (phosphate binding loop) for ATP binding proteins (Saraste *et al*., 1990). The side chains of both Ser-181 and Ser-236 (analogous to Ser-180 and Ser-243 in rabbit skeletal myosin) have recently been shown to hydrogen bond with vanadate oxygens in the MgADP·V_i complex in the X-ray structure of the truncated heads of *Dictyostellium* myosin (Smith & Rayment, 1996). These results and those from earlier crystal structures of AlF_4·MgADP·DcS1, BeF_x·MgADP·DcS1 (Fisher *et al*., 1995), and $MgPP_i$·DcS1 structures (Smith & Rayment, 1995) are direct verification that vanadate photooxidation specifically modifies phosphate binding residues in the active site.

Here we further investigate the mechanism of the vanadate-promoted photocleavage reaction at the active site of skeletal muscle myosin subfragment 1. The approach was to isolate peptides from both the COOH and NH_2 termini of the photocleaved site. Characterization of these peptides showed that the COOH-terminal peptide was amidated and the NH_2-terminal peptide was blocked by an oxalyl group. From these data and other kinetic information we propose a mechanism which involves addition of molecular oxygen to a stabilized seryl radical generated by a light-catalyzed vanadate reduction to V^{IV} (vanadyl ion). A rearrangement followed by loss of formate and hydrolysis gives the amidated COOH terminus and the NH_2 terminus blocked with an oxalyl group. The mechanism of photocleavage of myosin likely has common elements with other photocleavage reactions. If so, this information should help in the development of new methods to identify the amino acid sequences at other photocleavage sites.

MATERIAL AND METHODS

The commercial compounds used (and their sources) were as follows: Li_3ADP (Pharmacia P-L Biochemicals); NaB^3H_4 (Dupont-NEN); chymotrypsin, TPCK-treated trypsin, oxalate oxidase, carboxypeptidases Y and P, CNBr, and eledoisin (Sigma); Aminoethyl-8, BCA protein assay reagents, succinic anhydride, and trinitrobenzene sulfonate (Pierce); ultrapure ammonium sulfate (ICN-Schwarz Mann); ultrapure guanidine hydrochloride, formate dehydrogenase, and urea (Boehringer Mannheim); sodium orthovanadate (Fischer). Vanadate solutions (100 mM) were prepared as described (Goodno, 1982).

Enzyme Preparations. Myosin was prepared according to Wagner and Yount (1975). Chymotryptic S1 was prepared by a modification of the method of Okamoto and Sekine (1985) as previously described (Grammer *et al*., 1988). Photomodified S1 and photocleaved S1 were prepared as described previously (Cremo *et al*., 1991; Grammer *et al*., 1988). Briefly, the S1·MgADP·V_i complex was formed by incubation of S1 with $MgCl_2$, ADP, and V_i, purified by centrifugal gel filtration, and irradiated with UV light to generate the photomodified S1 with the side chain of Ser-180 oxidized to an aldehyde. The MgADP·V_i complex with photomodified S1 was formed in a similar manner, purified, and irradiated, generating photocleaved S1. This procedure typically leads to about 50% cleavage of the heavy chain.

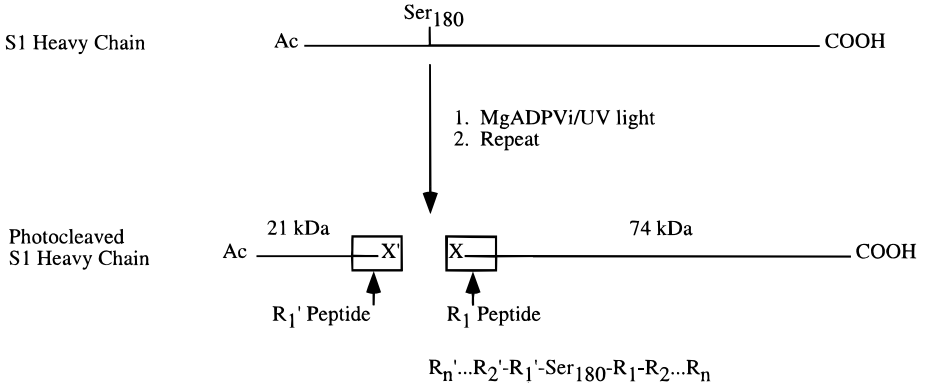
Analytical Methods. SDS gel electrophoresis was as described by Laemmli (1970) utilizing 12% acrylamide, 0.32% bisacrylamide, and 0.1% SDS. Gels were stained in 0.05% Coomassie Blue R in methanol/water/acetic acid (5:5:1, v/v/v) and destained in the same solvent. To quantitate the photocleavage, destained gels were soaked in water and scanned on an LKB Ultroscan XL laser densitometer. Modified S1 protein concentrations were determined either by a dye binding assay (Bradford, 1976) or by the BCA protein assay (Pierce) using unmodified S1 as the standard as previously described (Wells *et al*., 1979); $\epsilon_{280}^{1\%} = 7.5$ cm^{-1} (Wagner & Weeds, 1977). The protein concentrations of the isolated photocleavage fragments were determined using the BCA assay with bovine serum albumin as the standard.

Isolation and Cleavage of the 21 kDa Fragment. To isolate the 21 kDa photocleavage fragment (see Scheme 1), photocleaved S1 (35 mg) was separated on several 3 mm thick 12% SDS−polyacrylamide gels and cleavage fragments were visualized by precipitating non-protein-bound SDS as the K^+ salt by briefly soaking the gels in cold 1 M KCl. The protein bands remain clear on an opaque background. The 21 kDa NH_2-terminal fragment was excised, minced, and extracted with 25 mM Tris, 192 mM glycine, pH 8.3, 0.1% SDS, for 36 h at 45 °C and filtered. The filtrate was dialyzed against two changes of deionized water, lyophilized and precipitated with three volumes of cold ethanol to remove residual SDS. Prior to CNBr digestion, cysteines were reduced and aminoethylated as follows: a 10-fold molar excess of DTE was added to the 21 kDa fragment in 0.2 M Bicine, 8 M urea (pH 8.1). After 2 h under an argon atmosphere, the solution was adjusted to pH 8.5 and two aliquots of a 12.5-fold excess (over total thiol) of Aminoethyl-8 were added at 0 and 1 h while at 50 °C to block all free thiols. After an additional 1.5 h, unreacted Aminoethyl-8 was destroyed by addition of a 4-fold excess of β-mercaptoethanol. After 1 h, the solution was dialyzed against two changes of 5 mM acetic acid over 24 h and finally dialyzed into 70% formic acid overnight. The thiol-blocked 21 kDa fragment (3.3 mg, 158 nmol) was cleaved at methionines by addition of 1:1 (wt/wt) CNBr/21 kDa fragment and allowed to react for 24 h at room temperature.

Isolation and Cleavage of the 74 kDa Fragment. Initially, we attempted to extract the 74 kDa cleavage fragment (see

[1] Abbreviations: S1, skeletal myosin subfragment 1; TNBS, trinitrobenzene sulfonate; HPLC, high-pressure liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; pmS1, photomodified S1; V_i, orthovanadate; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecylsulfate; DTE, dithioerythritol; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; TCA, trichloroacetic acid; ACN, acetonitrile; TFA, trifluoroacetic acid; DcS1, truncated subfragment 1 from *Dictyostelium discoideum* myosin II.

Scheme 1: Summary of Photocleavage of S1 and Strategy for the Isolation of Peptides around the Cleavage Site at Ser-180[a]



[a] Photocleaved S1 was produced and the cleavage fragments were isolated as described under Materials and Methods. The peptide R_1', containing the blocked COOH terminus (X′), was isolated from the NH_2-terminal 21 kDa cleavage fragment. Similarly, the peptide R_1, containing the blocked NH_2 terminus (X), was isolated from the COOH-terminal 74 kDa cleavage fragment. The NH_2 terminus of the heavy chain of S1 is naturally acetylated (Ac).

Scheme 1) from SDS gels as was done for the 21 kDa fragment. While recovery of the 21 kDa was approximately 90%, the yield from the 74 kDa fragment was only 5−10%. Therefore, the 74 kDa fragment was separated by preparative SDS gel electrophoresis on a Bio-Rad model 491 Prep Cell apparatus using a 4% acrylamide stacking gel and a 7% acrylamide separating gel. The fragments were continuously eluted with 25 mM Tris, 192 mM glycine, pH 8.3, 0.05% SDS during the electrophoresis, and 5 mL fractions were collected. Aliquots of fractions were analyzed on 12% SDS−PAGE (Laemmli, 1970) 1.5 mm minigels (Hoeffer Mighty Small), and fractions containing the 74 kDa fragment were pooled, dialyzed against two changes of deionized water, lyophilized, resuspended in minimal water, and precipitated with three volumes of cold ethanol. The 74 kDa fragment was succinylated to block lysine ϵ-amino groups as follows: after resuspension of the pellet in 100 mM phosphate, pH 8.0, 6 M guanidine hydrochloride, a 50-fold excess of succinic anhydride (over lysines) was added slowly with vigorous stirring at room temperature, maintaining the pH at 8.0 with a pH stat. After the last addition, and upon pH stabilization, the solution was made 1 M in NH_2OH and the pH adjusted to 10 with NaOH to deblock succinylated tyrosyl, histidyl, cysteinyl, seryl, and threonyl side chains (Gounaris & Perlmann, 1967). After 1 h, the solution was dialyzed overnight against two changes of 50 mM ammonium bicarbonate. The solution was made 1 M in urea followed by three additions of 1/100 (wt/wt) of TPCK-treated trypsin at 0, 30, and 60 min, and digestion continued overnight at room temperature.

By use of the Bio-Rad Prep Cell, we were able to routinely recover >35% of the 74 kDa fragment. (As the recovery from the Prep Cell was determined just prior to succinylation, losses from other steps are included in this value, and therefore the recovery from the electrophoresis is likely greater.) We have also used the Prep Cell to isolate a [^{3}H]-labeled tryptic 23 kDa fragment of S1 with a 95% recovery of radioactivity as determined immediately after elution from the gel. In both cases, it was possible to load 50−75 mg of protein and still retain acceptable resolution, thus purifying large quantities of peptide fragments in a single run.

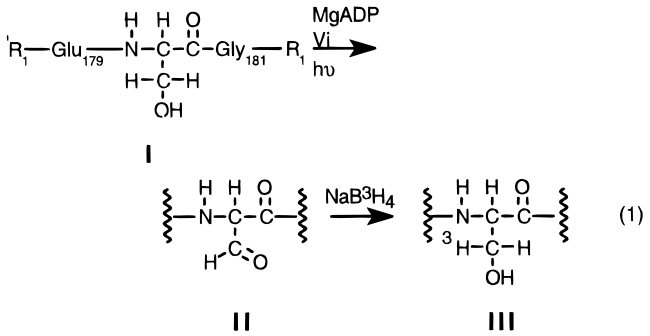
HPLC Separations. Prior to HPLC separations, the digests were clarified by filtration through 0.45 μ Nylon-66 filters (Rainin, Inc.). The peptides were separated by HPLC using Brownlee RP300 C8 semipreparative (7 mm × 25 cm) and analytical columns (4.6 mm × 13 cm) (Applied Biosystems) or a Vydac C4 analytical column (4.6 mm × 13 cm) (Separations Group). The HPLC system was either a microprocessor-controlled Altex/Beckman dual-pump setup connected to a Beckman model 165 dual-wavelength detector or a dual-pump Rainin system connected to a Waters 991 photodiode array detector.

Peptide Sequence and Amino Acid Analyses and Synthesis. Sequence analyses were performed on an ABI 475A pulse liquid sequencer (Applied Biosystems). Data were acquired and analyzed with the standard program Run 470-I (ABI). Amino acid content was analyzed with a Beckman model 121 MB amino acid analyzer with post-column ninhydrin detection. Peptide syntheses were performed on an ABI 431A synthesizer (Applied Biosystems) using standard Fmoc chemistry.

Mass Spectral Analysis. Electrospray ionization mass spectrometry (ESI-MS) was performed using a TAGA 6000E tandem quadrupole instrument (Sciex, Thronhill, Ontario, Canada) equipped with an ESI source and atmosphere/vacuum interface previously described (Smith *et al.*, 1990, 1991). Solutions of the analyte at approximately 24 pmol/μL in either 5% aqueous acetic acid or 1:2 acetonitrile:water containing 3.3% acetic acid (v/v) were delivered at 0.5 μL/min in combination with a flowing sheath electrode flow of 3 μL/min of methanol with the ion source and interface operating under standard conditions. Data were acquired at 0.2 *m*/*z* intervals with the mass spectrometer scanning from *m*/*z* 50 to 1400. Typically 20 spectra were averaged, the centroid of each ion manually selected and the relative molecular masses calculated according to procedures as described (Mann *et al.*, 1988; Edmonds & Smith, 1990). Tandem mass spectrometry was performed with a laboratory frame collision energy of 75 volts and an argon collision gas thickness of 1.5×10^{14} atoms/cm^2. The mass axis of both analyzing spectrometers were calibrated using fragment ions produced by nozzle−skimmer dissociation (voltage difference between the nozzle and skimmer elements set at 215 volts) of the undecapeptide, substance P. ESI mass spectrometry of the peptides isolated from the $^{16}O_2/^{18}O_2$-generated 74 kDa fragment was performed with a hybrid magnetic sector mass spectrometer (Finnigan MAT 900a, Bremen, Germany) (Loo *et al.*, 1993).

RESULTS

Evidence that Ser-180 Is the Site of Photocleavage. We have previously shown that Ser-180 in skeletal myosin S1 (eq 1, I) is oxidized to an aldehyde (II) upon irradiation of



(1)

the S1·MgADP·V_i complex. Reduction of the Ser-180 "aldehyde" form (II; referred to as photomodified S1) by NaB^3H_4 regenerates essentially native S1 (III; Cremo *et al*., 1988) in which Ser-180 is radiolabeled on the β carbon. The [^{3}H]S1 (III) was then used to prepare the [^{3}H]S1·MgADP·V_i complex which upon irradiation gives photomodified [^{3}H]-S1. Typically, the specific activity of the [^{3}H]S1 is decreased by only 30−40% upon subsequent photomodification indicating the tritium isotope effect favored loss of ^{1}H over ^{3}H from the side chain. The photomodified [^{3}H]S1·MgADP·V_i complex was prepared and irradiated and samples were removed for quantification of protein-bound radioactivity and photocleavage at the times indicated (Figure 1; see also footnote 2). The photomodified [^{3}H]S1 complex had an initial specific activity of 3400 cpm/nmol. As the irradiation progressed the specific activity of S1 decreased to 1200 cpm/nmol while the percent of heavy chain cleaved rose to 36%. In four experiments, the $t_{1/2}$ for photocleavage was 1.5 ± 0.1 min while the $t_{1/2}$ for ^{3}H release was 0.6 ± 0.1 min. The $t_{1/2}$ for ADP and V_i release was intermediate to these values (∼1.0 min). These results indicate that the photocleavage is a multistep reaction.

General Strategy. To show unequivocally that Ser-180 was the site of photocleavage, and to further deduce the cleavage mechanism, information about the newly generated termini was needed (see Scheme 1). In preliminary experiments, the COOH terminus of the 21 kDa fragment was found to be resistant to carboxypeptidase Y digestion and the 74 kDa fragment was resistant to repeated Edman degradation attempts. Therefore the 21 and 74 kDa fragments were isolated, and peptide cleavage methods based on the known sequence of S1 (Tong & Elzinga, 1983) were chosen to allow the identification and purification of the peptides containing the blocking groups, i.e., R_1' from the COOH terminus of the 21 kDa fragment and R_1 from the NH_2 terminus of the 74 kDa fragment (Scheme 1).

Isolation and Characterization of the R_1' Peptide. The 21 kDa cleavage fragment was purified as described under Materials and Methods and fragmented with CNBr. Only seven methionines are in the 21 kDa fragment (Tong & Elzinga, 1983) assuming that the cleavage occurs at Ser-180. Seven peptides plus homoserine would be expected from CNBr cleavage, as two of the methionines (Met-91 and Met-92) are adjacent to each other. Of these seven peptides,

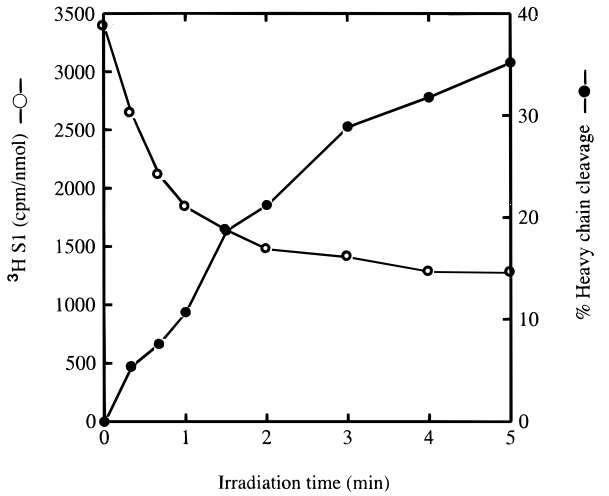


FIGURE 1: Photocleavage and loss of [^{3}H] from [^{3}H]S1 as a function of irradiation time. The S1·MgADP·V_i complex was prepared, purified, and irradiated as described under Materials and Methods. To form [^{3}H]S1, the photomodified S1 was reduced with a 4-fold excess of NaB^3H_4 for 45 min on ice, quenched with a 40-fold excess of arabinose,[2] and purified by centrifugation through a Sephadex G50 gel filtration column (Penefsky, 1977). [^{3}H]S1 was used to form the [^{3}H]S1·MgADP·V_i complex and to generate photomodified [^{3}H]S1 in a manner analogous to native S1. The [^{3}H]-photomodified S1·MgADP·V_i complex was formed, purified and irradiated as described under Materials and Methods and samples removed at the indicated times for analysis of protein-bound radioactivity (○) and photocleavage (●). Radioactivity was determined after precipitation of the [^{3}H]S1 with an equal volume of cold 10% TCA, rinsing the [^{3}H]S1 pellet with 5% TCA, solubilization in 1 M Tris (free base), 1% SDS, and counting in a Beckman LS9000 scintillation counter using BCS (Amersham) as the scintillant. The extent of photocleavage was determined by applying 15 μg of each sample onto an SDS−PAGE gel as described under Materials and Methods. Gels were stained, destained and scanned as described under Materials and Methods to determine the areas under the 95, 74, and 21 kDa fragments.

two were expected to be large (>20 amino acids) and one was a dipeptide. Of the remaining four peptides, two lacked aromatic amino acids, one of which, Leu_{166}-"Glu"$_{179}$, was the peptide of interest. The other peptide, *N*-Ac-Ser_1-Asp_5, was a small hydrophilic peptide which was not expected to be retained by the reversed phase HPLC separation chosen (see below). These two peptides would absorb at 215 nm but little at 280 nm, allowing the R_1' peptide to be identified. The HPLC separation of the CNBr digest is shown in Figure 2. The chromatogram between 50 and 70 min is uncomplicated, and we were able to eliminate several peaks from consideration. The triplet peak between 50 and 52 min was eliminated due to the presence of 280 nm absorption, while the peaks at 54 and 61 min were attributed to components in the solvent system. Therefore, the likely R_1' peptide was the peak at 57 min which had no detectable 280 absorbance (arrow). This peptide was further purified by HPLC using a shallow gradient (Figure 2, inset), and the peak between 101 and 108 min was collected and submitted to sequence and amino acid analysis (Table 1). Both the amino acid analysis and the sequence yield data shown in Table 1 identify the peptide as the expected 14 amino acid R_1' peptide starting at Leu_{166}. To our surprise, the peptide sequenced through to Glu_{179} with no unusual amino acids in evidence. This was an unexpected result as previous attempts to digest the 21 kDa fragment with carboxypeptidase Y were unsuccessful, indicating that the COOH terminal amino acid was modified. Amino acid analysis (Table 1) gave the predicted

[2] In subsequent studies, we have found that 40-fold glyceraldehyde is a more effective quench for NaB^3H_4.

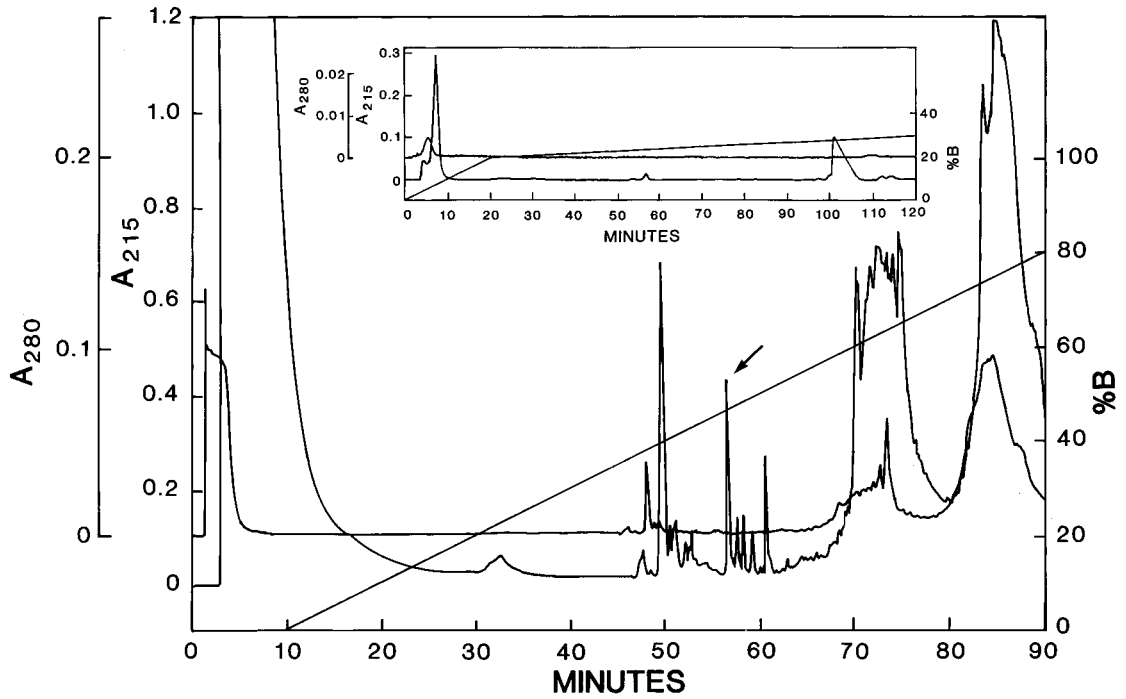


FIGURE 2: Reversed phase HPLC separation of the R_1' peptide. The NH_2-terminal 21 kDa peptide was isolated and cleaved with CNBr as described under Materials and Methods. The lyophilized CNBr digest was redissolved in formic acid, diluted 3-fold with water, filtered, and injected onto a Vydac C4 column (220 × 4.6 mm). The column was developed at 1 mL/min with a 1%/min linear gradient of 0.11% TFA (solvent A) to 0.1% TFA in 60% ACN (solvent B) and one mL fractions were collected. Inset. The peptide eluting at 57 min (arrow) was collected and further purified on a Vydac C4 column using a 1%/min gradient to 20 min followed by a 0.1%/min gradient using solvents A and B. In both chromatograms, the elution time corresponds to the 215 nm absorbance trace (lower trace) while the 280 nm trace (upper) is offset by 2 min in the negative direction.

Table 1: Sequence of Amino Acids of R_1' Peptide and Amino Acid Composition of R_1' and R_1 Peptides

	sequence yield			amino acid composition			
	R_1' peptide			R_1' peptide		R_1 peptide	
cycle	amino acid	yield (pmol × 10^{-2})	amino acid	nmol[a]	theory	nmol[a]	theory
1	Leu	7.4	Asx	2.3	2	1.1	1
2	Thr	2.5	Thr	2.0	2	2.0	2
3	Asp	2.7	Ser	0.9	1	nil	0
4	Arg	0.8	Glx	3.3	3	nil	0
5	Glu	3.2	Gly	1.1	1	2.2	2
6	Asn	2.1	Ala	nil	0	1.1	1
7	Gln	2.4	Val	nil	0	1.1	1
8	Ser	0.9	Ile	2.2	2	nil	0
9	Ile	2.1	Leu	2.1	2	nil	0
10	Leu	1.9	Lys	nil	0	2.1	2
11	Ile	2.1	Arg	1.0	1	1.0	1
12	Thr	0.7					
13	Gly	1.1					
14	Glu	0.6					
15	nil	nil					

[a] Amino acid composition values were normalized to Arg.

amino acids at the expected levels, including three Glu, and no unusual amino acid was detected.

To verify our results with the 21 kDa fragment, the R_1' peptide was also tested for its susceptibility to carboxypeptidase Y digestion. There was no difference in retention time of the peptide on reversed phase HPLC after extensive digestion (data not shown). These data confirm that the COOH terminus of the 21 kDa cleavage fragment was blocked with a group derived from Ser-180 that was unstable to the conditions of the Edman degradation and amino acid analysis.

The analysis of the purified R_1' peptide by ESI-MS is shown in Figure 3. Ions at mass-to-charge ratios (m/z) of 530.2 and 794.9 correspond to the $[M + 3H]^{3+}$ and $[M + 2H]^{2+}$ molecular ions of the peptide, respectively. With an internal standard peptide ([D-Trp^{2,7,9}]-substance P, M_r 1604.94) in the solution, a relative molecular mass of 1587.72 ± 0.11 was measured for the isolated peptide. Tandem mass spectrometry of the $[M + 2H]^{2+}$ (Figure 3, lower) yielded singly and doubly charged product ions consistent with the sequence. Nomenclature for the product ions have been previously described (Loo *et al.*, 1990), where the positive charge resides on the NH_2 terminal fragment for a- and b-type products and charge retention on the COOH terminal fragment for y-type product ions. Similar sequence information was obtained (data not shown) by inducing fragmentation in the atmospheric pressure/vacuum interface (Smith *et al.*, 1990, 1991). The ESI-MS and ESI-MS/MS data were consistent with an amidated COOH terminus.

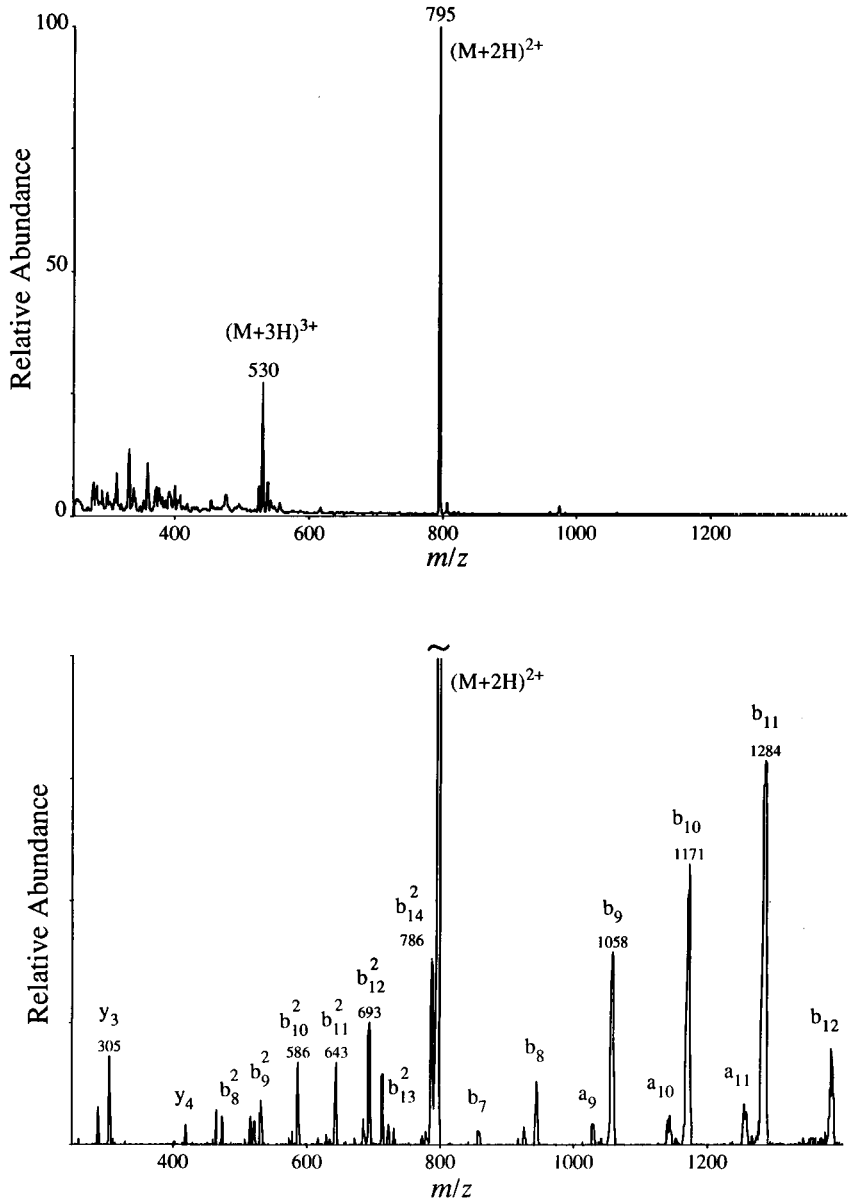


FIGURE 3: Mass spectral analysis of the R_1' peptide. Upper: ESI spectrum of the R_1' peptide. The mass/charge ratio values are shown below the assigned ions. Lower: Tandem MS of the $[M + 2H]^{2+}$ ion. Nomenclature for the product ions has been previously described (Loo *et al.*, 1990).

To confirm the presence of a COOH terminal carboxamide, two synthetic peptides of Glu_{170}-Glu_{179} were prepared: one contained a free carboxyl on the COOH terminus whereas the other contained a carboxamide (Table 2). The retention times for the two synthetic peptides on reversed phase HPLC and capillary zone electrophoresis were compared with the retention time of a truncated R_1' peptide (ENQSILITGX′; Table 2). In both methods, the truncated tryptic R_1' peptide had the same retention times as the synthetic peptide with the amidated COOH terminus. Co-chromatography of the amidated synthetic peptide and tryptic R_1' peptide revealed only one peak with each method, while co-chromatography of the free COOH-terminus synthetic peptide and tryptic R_1' gave rise to two peaks. These data confirm that the COOH-terminus blocking group of the R_1' peptide is an amide.

The earlier sequence data (Table 1) was consistent with the presence of a Glu_{179}-amide, as the amide would be cleaved at the last cycle of the Edman degradation, to yield Glu as the last amino acid. With this knowledge, the R_1' peptide was shown to be degraded by the less specific carboxypeptidase P which is known to cleave ammonia or

Table 2: Capillary Zone Electrophoresis (CZE) and HPLC Retention Times of Various Peptide Analogs and the Truncated R_1' Peptide

peptide[a]	retention time (min)	
	CZE[b]	RP HPLC[c]
ENQSILITGE (COOH)	14.4	46
ENQSILITGE ($CONH_2$)	12.4	43
truncated R_1' peptide	12.4	43

[a] Two variations of the peptide ENQSILITGE which corresponds to E_{170}-E_{179} in the rabbit myosin heavy chain sequence (Tong & Elzinga, 1983) were synthesized on an ABI 431A protein synthesizer. One of the peptides contained an amide blocked COOH terminus while the other had a free COOH terminus. To prepare the truncated R_1' peptide, the 14 amino acid R_1' peptide was cleaved at R_{169} with trypsin and the E_{170}-E_{179} peptide was purified by reversed phase HPLC as described in Figure 2. [b] Capillary zone electrophoresis was performed on a Bio-Rad HPE 100 using 100 mM phosphate, pH 2.5, as electrolyte. [c] Reversed phase HPLC separations were performed on a Brownlee RP300 C8 analytical column using 0.11% TFA with a 1%/min gradient of 0.1% TFA in acetonitrile.

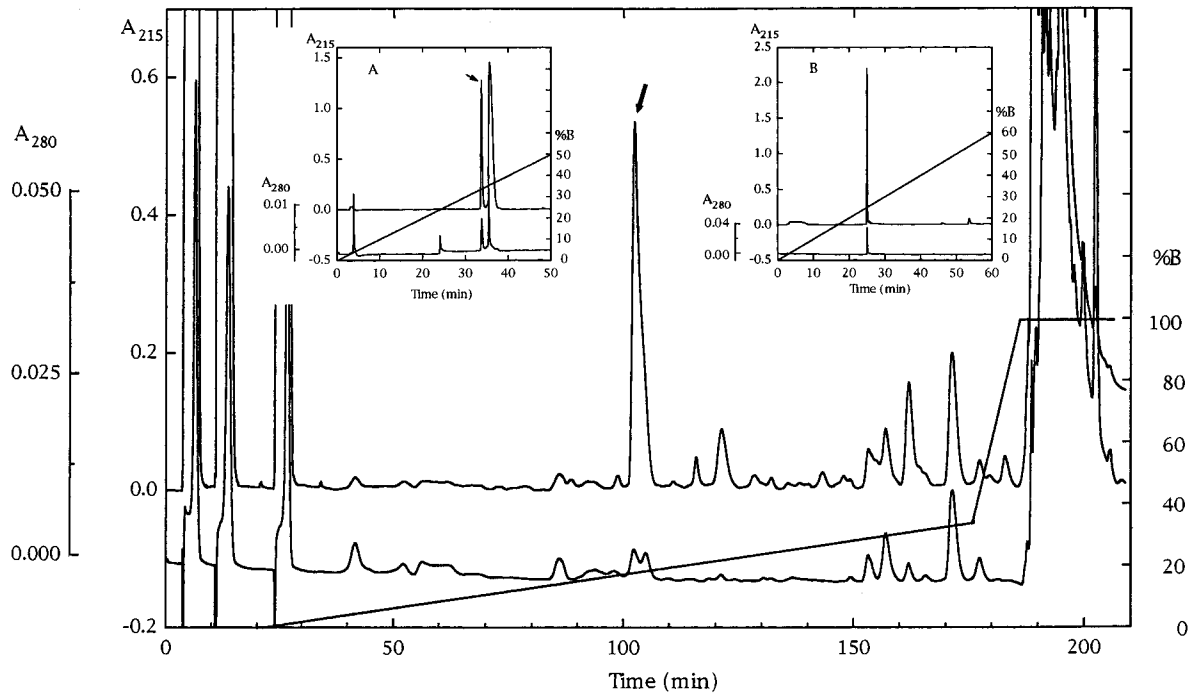


FIGURE 4: HPLC purification of the R_1 peptide. The COOH-terminal 74 kDa photocleavage fragment was isolated, succinylated, and trypsinized as described under Materials and Methods. The tryptic peptides generated were separated by reversed phase HPLC on a Brownlee C8 semipreparative column equilibrated in 0.11% TFA. The digest was applied by multiple injections (5.5 mL) using a 7.5 mL loop, and upon the last injection, the column was developed by a 0.2%/min gradient of 0.11% TFA to 0.1% TFA in 60% acetonitrile. The elution was monitored at 215 nm (upper trace) and 280 nm (lower trace). Inset A. The peptide pool eluting at 102 min (arrow) was collected and re-chromatographed on a Brownlee C8 analytical column at 1 mL/min with a 1%/min gradient using the TFA system as above. To test for the presence of a blocked NH_2 terminus, aliquots from the two peptide pools (34 and 36 min) were neutralized with 500 mM sodium borate, pH 9.0, and reacted with 50−100 μL of 7.2 mg/mL trinitrobenzenesulfonate (TNBS). After 30 min, the reaction mixture was rechromatographed on the analytical column exactly as described above and the retention times compared. The 36 min peptide(s) was modified by TNBS and eluted now at 56 and 60 min. Inset B. The peptide eluting at 34 min (arrow, panel A), which did not react with TNBS, was collected and further purified on the C8 analytical column using a 1%/min gradient of 5 mM potassium phosphate, pH 6.9, to 65% acetonitrile at 1 mL/min. 1-min fractions were collected. The peptide eluting at 25 min was desalted on the C8 analytical column using the TFA system as above and was analyzed as the "R_1 peptide".

amidated amino acids from peptides with COOH-terminal carboxamides (Remington & Breddam, 1994).

Isolation and Characterization of the R_1 Peptide. The 74 kDa fragment was isolated and the ϵ-amino groups of all lysines were succinylated as described under Materials and Methods. Succinylation was performed for two reasons. First, after tryptic cleavage of the 74 kDa fragment, all peptides except the blocked R_1 peptide (see Scheme 1) would have a new, free amino group at their NH_2 terminus. It was therefore possible to check for the presence of free amino groups by the reaction of TNBS with each HPLC purified peptide to detect the non-reactive R_1 peptide. Secondly tryptic digestion of a succinylated 74 kDa fragment would cleave only at Arg residues, thereby decreasing the potential number of tryptic peptides.

The tryptic digest of the succinylated 74 kDa cleavage fragment was separated by HPLC as described under Materials and Methods (Figure 4). From preliminary studies utilizing a similarly digested 50 kDa fragment derived from the 74 kDa fragment (in which only 11 peptides should be generated), the approximate retention time of the expected R_1 peptide, X-Gly_{181}-Arg_{190}, was determined to be ~100 min, and consequently, after 32% B (160 min after the third injection), the gradient was ramped to 100%. As can be seen, the chromatogram had few peaks during the shallow gradient with only one major peak (upper trace) at 102 min. Re-chromatography of this major peak on analytical reversed phase HPLC gave two peaks (figure 4, inset A). The peaks were pooled separately, treated with TNBS, and re-chromatographed using the same conditions as in Figure 4, inset A. The peptide at 34 min did not react with the TNBS (HPLC retention time unchanged), while the peak at 36 min gave two new peaks at 56 and 60 min with concomitant loss of the 36 min peak (data not shown). The putative R_1 peptide (34 min) was pooled and re-chromatographed at pH 6.9 as shown in Figure 4, inset B, resulting in a single peptide. The amino acid analysis of the HPLC purified peptide (Table 1) was consistent with the composition of the 10 amino acid NH_2-terminal tryptic peptide expected (Gly_{181}-Arg_{190}). An attempt to sequence this peptide was unsuccessful, confirming that the NH_2 terminus was blocked.

The electrospray ionization mass spectrum of the purified succinylated R_1 peptide is shown in Figure 5, upper. The data are consistent with a peptide of measured molecular mass of 1303.1 daltons. An oxalyl (residue mass 73 daltons) NH_2-terminal moiety (X shown below), would bring the derivatized peptide to a calculated mass of 1303.6 daltons which is in close correspondence with the measured value. A second unassigned peptide of mass 1074.6 daltons is also present $[M + 2H]^{2+}$ at the asterisk in Figure 5, upper.

The result of tandem mass spectrometry of the $[M + 2H]^{2+}$ ion of this component appearing at *m/z* 652.6 is shown in Figure 5, lower. The majority of product ions may be assigned as b- and y-type shown in the inset in figure 5, lower. The masses of the assigned fragmentations are consistent with the mass and sequence of the NH_2-terminal peptide containing two succinylated lysine residues and an oxalyl group on the NH_2 terminus.

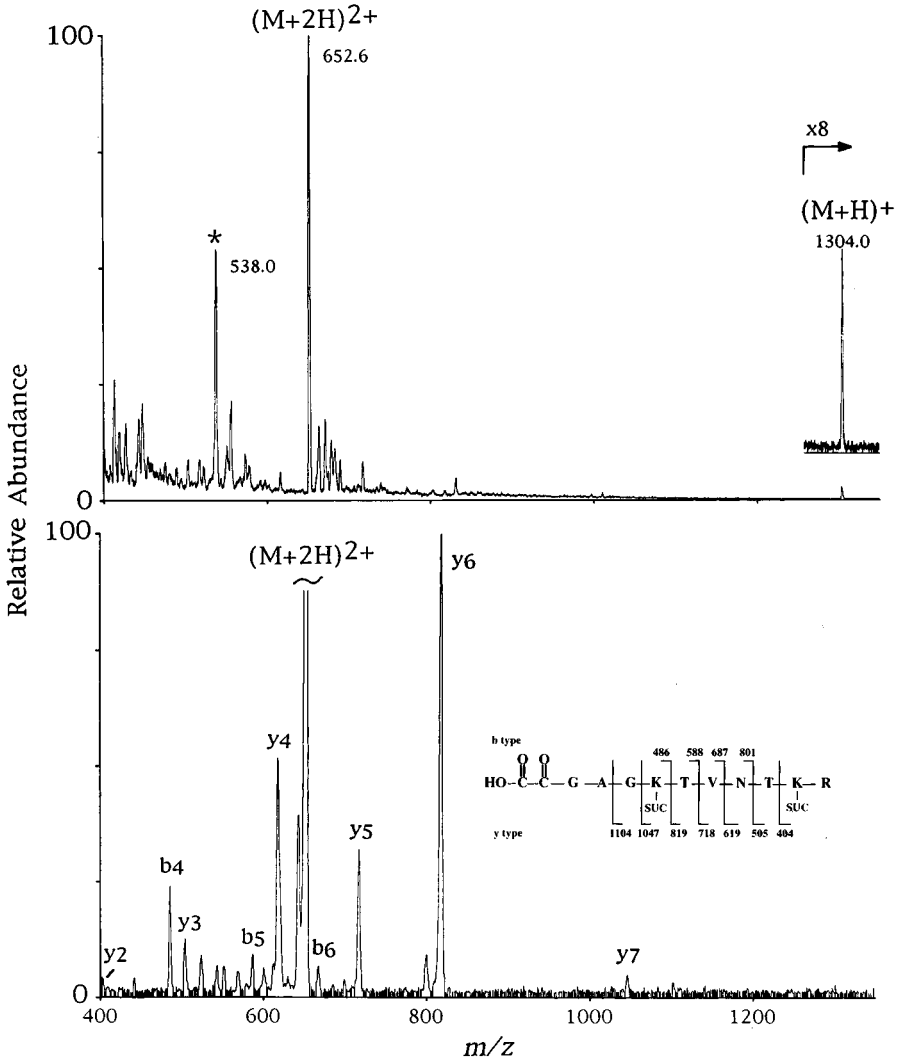


FIGURE 5: Mass spectral analysis of the R_1 peptide. ESI mass spectrum (upper) and partial tandem mass spectrum (lower) of the $[M + 2H]^{2+}$ parent ion. Upper: Mass/charge ratio (m/z) values are shown below the assigned ions. The $[M + H]^+$ response is shown at 8 times the scale of the abscissa. Lower: Conventional notation for the sequence specific fragmentation of the polypeptide is shown according to the scheme given in the inset.

Table 3: Oxalate Analysis of the R_1 Peptide[a]

peptide	nmol of oxalate	
	expected	found
eledoisin (no added oxalate)	0.0	0.0
eledoisin (+ 12.5 nmol oxalate)	12.5	12.1
R_1 peptide	15.0	14.1

[a] Oxalate was analyzed enzymatically using oxalate oxidase in a Sigma 591-C oxalate diagnostic kit (Sigma, Inc.). 15 nmol of peptide (either eledoisin or R_1) was hydrolyzed with 5 μL of 6 N HCl in a sealed, evacuated vial for 22 h at 110 °C. After hydrolysis, 20 μL of the Sigma sample diluent (prepared twice as concentrated as directed) was added to each vial and the contents transferred to a microfuge tube followed by a 10 μL rinse with sample diluent. The samples were neutralized to pH 7.0−7.5 with 4 N NaOH and assayed as described by Sigma except the charcoal extraction step was not performed. Known quantities of oxalate were also hydrolyzed as described above and used as standards to correct for oxalate losses (<50%) during hydrolysis.

To confirm the presence of an oxalyl group on the NH_2 terminus, oxalate oxidase was used to determine the amount of oxalate from acid hydrolyzed R_1 peptide (Table 3). To ensure that hydrolysis would not interfere with the enzyme assay, an undecapeptide, eledoisin, was used as a control. As seen in Table 3, no oxalate was found in the eledoisin hydrolyzate whereas the expected level of oxalate was found in the eledoisin sample to which oxalate was added prior to the hydrolysis. Upon hydrolysis and subsequent analysis of 15 nmol of the R_1 peptide, the amount of oxalate found (14.1 nmol) was consistent with a single oxalyl group on the NH_2-terminal amino acid. As the R_1 peptide contains two succinyl groups, controls were performed in which succinate was added to the assay at levels up to four times those expected in the sample. As was expected from the known specificity of oxalate oxidase, the added succinate did not affect the assay (data not shown).

$^{18}O_2$ Experiments. Photocleavage could not be detected upon irradiation of the photomodified S1·MgADP·V_i complex in the absence of O_2. To probe the role of O_2, photomodified S1·MgADP·V_i complex was irradiated in the presence of 98% $^{18}O_2$. The R_1 peptide from the resulting 74 kDa fragment was isolated as described above and analyzed by ESI-MS. As shown in Table 4, the R_1 peptide isolated from the $^{18}O_2$-generated 74 kDa fragment had a mass of two higher than the original R_1 peptide, indicating that one of the two oxalyl carboxyl oxygens came from O_2 (see also Figure 5). Tandem mass spectrometry resulted in a fragment which placed the ^{18}O within the first three residues as expected (data not shown). The mass spectrum of the peptide isolated from the $^{18}O_2$-generated 74 kDa fragment also contained a mass corresponding to the unlabeled (i.e.,

Table 4: ESI-MS Data for R_1 Peptides Isolated Following Cleavage in the Presence of $^{16}O_2$ or $^{18}O_2$

	^{16}O labeled R_1 peptide		^{18}O labeled R_1 peptide[a]	
ion	*m/z*	*m/z* (theoretical)	*m/z*	*m/z* (theoretical)
$[M + H]^+$	1303.58	1303.61	1305.62	1305.62
$[M + 2H]^{2+}$	652.32	652.31	653.32	653.31

[a] Following formation of the photomodified S1·MgADP·V_i complex as described under Materials and Methods, sucrose was added (3 mg/mg of S1) and the sample was lyophilized to dryness in a 15 × 85 mm culture tube. The tube was sealed with a septum and the dried sample was deoxygenated by repeated argon and vacuum purges through a gated syringe. Deoxygenated S1 buffer (prepared by multiple freeze/thaw cycles with alternating argon/vacuum purges) was transferred to the evacuated, deoxygenated photomodified S1·MgADP·V_i complex (~60 mg of pmS1) via a double-headed transfer needle by argon pressure. The dissolved sample was repeatedly purged with argon and quickly evacuated (to avoid frothing) several more times. $^{18}O_2$ (Isotech Inc., 98.1 atom %) was introduced into the evacuated sample, and the solution was irradiated for 4 min with gentle agitation while the test tube was lying on its side on ice. The R_1 peptide (72 nmol) was isolated from 7.8 mg of 74 kDa polypeptide as described under Materials and Methods.

Table 5: Formate Determination after UV Irradiation

sample	nmol of formate[a]	nmol expected[b]
control S1	0.5	0
pmS1 CoADP-V_i complex	4.7	1.6
pmS1 MgADP-V_i complex	23.1	21.5

[a] Formate was analyzed by the formate dehydrogenase assay (Bergmeyer *et al*., 1983) in which the generation of NADH upon oxidation of formate was measured. The photomodified S1·Mg (or Co) ADP·V_i complexes were prepared and irradiated as described in Materials and Methods except that the final buffer used in the purification was 50 mM potassium phosphate, pH 7.0 (no azide was added as this inhibits formate dehydrogenase). Approximately 5 mg of S1 was used per assay. This amount of S1 (43 nmol) at 50% photocleavage should yield about 21 nmol of formate. Control S1 was treated exactly as the other samples except no V_i was added at either step where the V_i complex was formed. All samples were irradiated for 4 min and deproteinized by centrifugation through a Centricon 50 filter (Amicon). The filtrate was then assayed for formate. [b] The amount expected was determined by measuring the amount of photocleavage seen for each sample as described under Material and Methods.

^{16}O-containing) R_1 peptide. In two independent preparations, the ratio of ^{18}O-labeled to ^{16}O-labeled R_1 peptide was approximately 60:40. It is unlikely this was due to contaminating $^{16}O_2$ as the method used for deoxygenation (see Table 4 legend) of the photomodified S1·MgADP·V_i complex was shown to prevent cleavage therefore implying complete O_2 removal. Rather, we think ^{16}O is introduced by exchange of ^{18}O with $H_2{}^{16}O$ in a step just prior to hydrolysis of the peptide chain (see Discussion).

Fate of the Serine-180 Side Chain. The oxalyl and the amide groups on the termini of the two photocleavage fragments account for all the backbone atoms of Ser-180. However, the side chain atoms of Ser-180 remained unaccounted for. Tritium from the β-hydroxymethyl group of Ser-180 of ^3H labeled S1 was released from the protein in some form early in the photocleavage reaction (Figure 1). The released compound was not volatile under reduced pressure, ruling out [^3H]formaldehyde or the complete oxidation to ^3H_2O and CO_2 as cleavage products. This suggested formate as the most logical cleavage product. Table 5 shows that irradiation of the photomodified S1·MgADP·V_i complex generated formate at levels corresponding to the amount of photocleavage. Irradiation of the photomodified S1·CoADP·V_i complex which is known to be photostable (Grammer et al., 1988) generated only minor amounts of formate. Formate was also identified by HPLC on a SynChropak AX100 anion exchange column (250 × 2.1 mm, SynChrom, Inc., Lafayette, IN) using 0.1 M potassium phosphate, pH 4.0, as the solvent as recommended by the manufacturer and monitoring the absorbance at 210 nm. This method separated formate from acetic and lactic acids. Furthermore, if the photomodified [^3H]S1·MgADP·V_i complex was irradiated, the released ^3H was shown to co-elute on HPLC with a ^{14}C formate standard (data not shown).

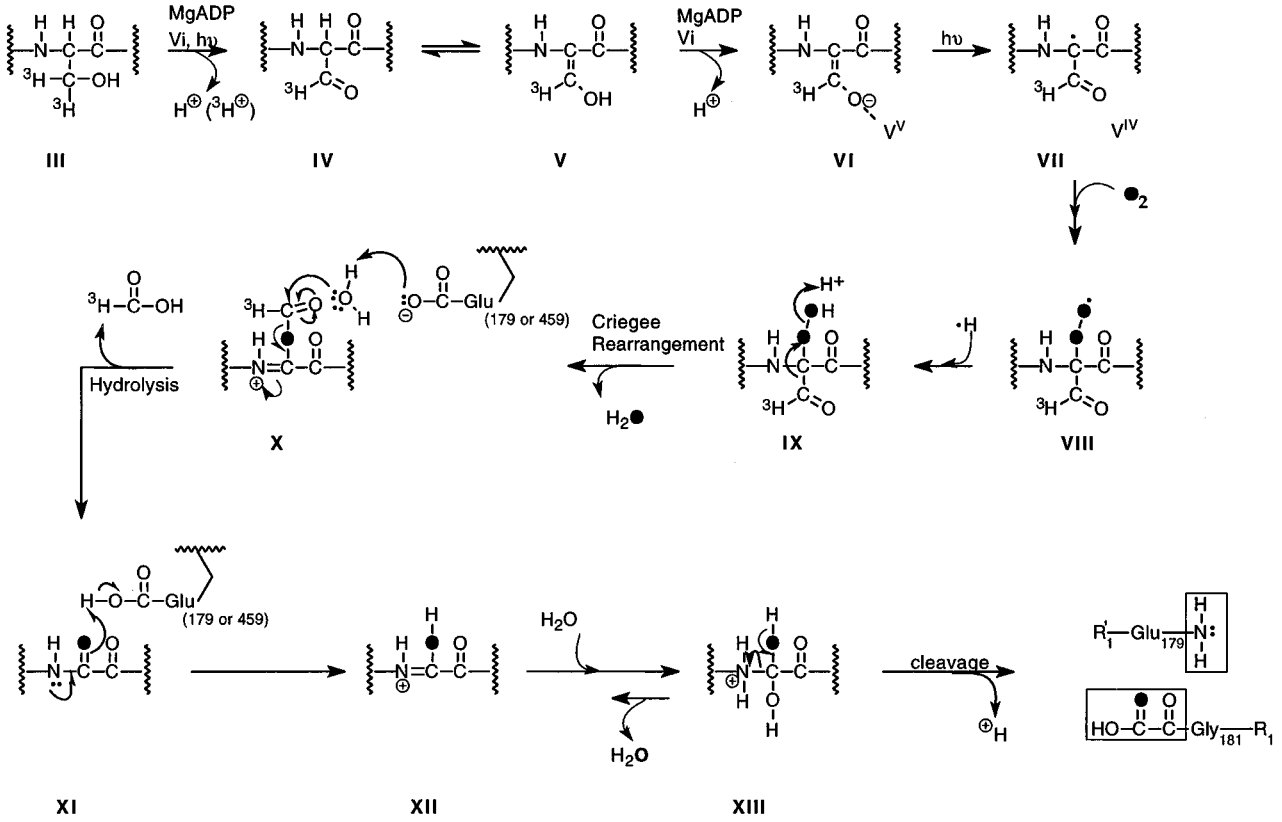
DISCUSSION

The purpose of this paper was to characterize the chemistry and mechanism of vanadate-promoted photocleavage of the myosin heavy chain elicited by irradiation of the S1·MgADP·V_i complex. This reaction is known to occur in at least two steps. The first step leads to oxidation of the side chain of Ser-180 to an aldehyde (see eq 1; Grammer *et al*., 1988; Cremo *et al*., 1988). If MgADP and V_i were retrapped at the active site of this photomodified S1 and irradiated, the 95 kDa heavy chain fragment was cleaved to an NH_2-terminal 21 kDa and a COOH-terminal 74 kDa fragments. Neither of these fragments were sequencable by standard techniques suggesting that parts of Ser-180 were blocking the new COOH and NH_2 termini. New strategies were devised to characterize these blocking groups. The approach was to first isolate the 21 and 74 kDa fragments by large scale gel electrophoresis and then to cleave these fragments into smaller peptides with CNBr and trypsin, respectively. Knowledge of the amino acid sequence and the site of photocleavage was essential in devising analytical schemes which would allow the blocked peptides to be identified after HPLC separation. Thus, the blocked CNBr peptide (called R_1') from the 21 kDa fragment was known from the sequence not to contain any aromatic amino acids and to be of moderate size. Other CNBr peptides were either very large, very small, or would contain aromatic amino acids. Thus by estimating the time of elution and by the absence of 280 nm absorption it was possible to identify and purify the R_1' peptide from HPLC separations.

A different approach was taken to identify the blocked peptide (R_1) from the 74 kDa fragment. Here, all free ϵ-amino groups of lysyl residues in the 74 kDa polypeptide were succinylated before trypsin treatment. This decreased the number of tryptic peptides and meant all tryptic peptides except the desired blocked peptide from the cleavage site would have a new free NH_2 group. The peptides with free NH_2 groups were identified by reaction with the chromophoric trinitrobenzenesulfonate reagent which also drastically increased their time of elution on reversed phase HPLC. The blocked R_1 peptide was thus readily identified and characterized by mass spectrometry, amino acid analysis, and by assay for oxalate which was found to block the amino group of Gly-181.

The above experiments showed the polypeptide chain was cleaved between the α-amino group and the α-carbon of Ser-180. Two isotopic tracers, ^3H and $^{18}O_2$, were used to determine the fate of the β-carbon side chain of Ser-180. First [^3H]S1 was prepared by reducing the aldehyde group at Ser-180 in photomodified S1 with NaB^3H_4. This placed a ^3H specifically on the β-carbon of Ser-180. No other amino acids were significantly labeled (Cremo *et al*., 1989). This [^3H]S1 then was used to follow the kinetics of the photo-

Scheme 2



cleavage step vs loss of ^3H. The results showed ^3H was lost before photocleavage occurred (Figure 1). ADP and V_i were lost at intermediate times (data not shown). The ^3H was shown to be on formate which was released ($t_{1/2} \approx 0.6$ min) from the protein before the polypeptide backbone was cleaved ($t_{1/2} \approx 1.5$ min). These differing reaction times indicated that a multistep oxidation was occurring before cleavage occurred.

To further examine these steps the cleavage reaction was carried out in the presence of 98% $^{18}O_2$. In control experiments molecular O_2 was shown to be essential for photocleavage. Here, one atom of ^{18}O was found in the oxalyl group. The second atom is believed to be lost as $H_2{}^{18}O$. A mechanism which satisfies all the experimental findings is shown in Scheme 2. A key step involves the generation of a free radical at the α-carbon of the serine aldehyde (Ser-180) (structure VII, Scheme 2) by photoreduction of vanadate (V^V) to vanadyl (V^{IV}). Molecular O_2 adds to the free radical. The β-carbon aldehyde then migrates to the carbon bound oxygen with loss of the second oxygen as H_2O (structure X). Reactions of this type are called Criegee rearrangements and are known to be weakly acid catalyzed (Plesničar, 1983). It is possible that either Glu-179 or Glu-459 known from the crystal structure to be nearby (Rayment *et al*., 1993) could act as acid catalysts in this reaction. The subsequent hydrolysis of the formyl group may in turn be facilitated by Glu-179 or Glu-459 acting as a base to activate H_2O to attack the formyl carbon. Formate is released from the protein and the oxalyl carbonyl next to the amino group is reversibly hydrated many times (structures XII and XIII) before hydrolytic cleavage occurs. It is this step which we believe allows the loss of ^{18}O from the distal carboxyl group of oxalate and which explains the 40/60 $^{16}O/^{18}O$ ratio found in the R_1 peptide generated in the presence of 98% O_2 (Table 4). Again this hydration may be acid catalyzed by Glu-179 or Glu-459 although adjacent carbonyls are known to be highly hydrated naturally because of their strong polarity.

The mechanism in Scheme 2 explains the kinetics of product release and cleavage as well as the fate of ^3H and ^{18}O labels and the final chemical character of the products. The above mechanism may also explain how the side chain of Ser-243 is oxidized to an aldehyde during the photocleavage step (Grammer & Yount, 1991). The peroxo radical at Ser-180 (structure VIII) must be reduced by a hydrogen atom. We propose this H• may come from a β-carbon C−H from Ser-243. The loss of a second electron from the β-carbon atom via another O_2 molecule would convert the radical alcohol of Ser-243 into an aldehyde. Alternatively, the Ser-243 radical is close enough to the active site that vanadium in the +4 state could act as a reductant to complete the oxidation and return to vanadate in the +5 state. This suggestion is consistent with the close location of Ser-243 to vanadate trapped at the active site with MgADP (Smith & Rayment, 1996) in the crystal structure of truncated dictyostelium myosin subfragment 1.

One importance of the above findings is that they may serve as a paradigm for identification of other vanadate-promoted photocleavage sites. In particular, as found here it may be possible to use carboxypeptidase P to deblock the COOH-terminal side of the cleavage site so that subsequent chemical sequencing can be used to identify the site where vanadate binds. Serine carboxypeptidases, unlike the more familiar metallocarboxypeptidases from the pancreas, do not require a free COOH for activity [reviewed in Remington and Breddam (1994)]. They will act to cleave ammonia or amidated amino acids from the COOH terminus of peptides. One reason carboxypeptidase Y was ineffective here may be because it acts very slowly on peptides whose COOH-terminal residues contain charged sidechains. The fact

carboxypeptidase P was active means that a variety of carboxypeptidases should be tried if the COOH terminus of cleavage sites appears to be blocked. Treatment of peptides and small proteins with a mixture of carboxypeptidases Y and P together with MALDI-MS analysis has been used successfully for COOH-terminal sequencing (Thiede *et al*., 1995). Using this approach it may be possible to characterize the second and third vanadate photocleavage sites in S1 (Cremo *et al*., 1990; Mocz, 1989) which occur with no added nucleotide. One of these sites is believed to be near the actin binding site (Okamoto & Cremo, 1993) and the other (observed only at pH 6.0) generates a 31 kDa NH_2-terminal peptide (Muhlrad *et al*., 1991) which may represent cleavage at Ser-243.

Not all cleavage reactions will necessarily be between the α-amino and α-carbon atoms as seen here. For example, adenylate kinase is known to be photocleaved with vanadate at Pro-17 (Cremo *et al*., 1992). One of the photocleavage products from Pro-17, γ-aminobutyric acid, indicates cleavage occurred between carbons 1 and 2 of the prolyl residue. The cleavage mechanism here clearly does not involve a Criegee rearrangement but likely does require molecular O_2 to oxidize the α-carbon atom of Pro-17 to a carbonyl group. The current work offers a model system to show how the site of vanadate photocleavage can be identified by a combination of chemical, enzymatic, chromatographic, and physical techniques.

ACKNOWLEDGMENT

We thank Dr. Gerhard Munske at the WSU Laboratory for Biotechnology and Bioanalysis for the peptide sequence analysis and peptide synthesis and Dr. Gurusiddiah for the amino acid analyses. We are indebted to Dr. Paul Hopkins (University of Washington) and Dr. Rob Ronald (Washington State University) for discussions of the Criegee rearrangement and the chemistry of the photocleavage reaction.

REFERENCES

Bergamini, C. M., & Signorini, M. (1991) *Biochem. Int. 25*, 143−149.
Bergamini, C. M., Signorini, M., Hanau, S., Rippa, M., Polverino de Laureto, P., & Cremonini, M. A. (1995) *Arch. Biochem. Biophys. 321*, 1−5.
Biemann, K. (1988) *Biomed. Environ. Mass Spectrom. 16*, 99−111.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Cole, D. G., & Yount, R. G. (1992) *Biochemistry 31*, 6186−6192.
Correia, J. J., Lipscomb, L. D., Dabrowiak, J. C., Isern, N., & Zubieta, J. (1994) *Arch. Biochem. Biophys. 309*, 94−104.
Crans, D. C., Sudhakar, K., & Zamborelli, T. J. (1992) *Biochemistry 31*, 6812−6821.
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.
Cremo, C. R., Loo, J. A., Edmonds, C. G., & Hatlelid, K. M. (1992) *Biochemistry 31*, 491−497.
Edmonds, C. G., & Smith, R. D. (1990) *Methods Enzymol. 193*, 412−431.
Fisher, A. J., Smith, C. A., Thoden, J., Smith, R., Sutoh, K., Holden, H. M., & Rayment, I. (1995) *Biochemistry 34*, 8960−8972.
Gibbons, I. R., & Mocz, G. (1991) *Methods Enzymol. 196*, 428−442.
Gibbons, I. R., Lee-Eiford, A., Mocz, G., Phillipson, C. A., Tang, W. I., & Gibbons, B. H. (1987) *J. Biol. Chem. 262*, 2780−2786.
Goodno, C. C. (1982) *Methods Enzymol. 85*, 116−123.
Gounaris, A. D., & Perlmann, G. E. (1967) *J. Biol. Chem. 242*, 2739−2745.
Grammer, J., & Yount, R. G. (1991) *Biophys. J. 59*, 226a.
Grammer, J. C., Cremo, C. R., & Yount, R. G. (1988) *Biochemistry 27*, 8408−8415.
Ko, Y. H., Cremo, C. R., & McFadden, B. A. (1992) *J. Biol. Chem. 267*, 91−95.
Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680−685.
Lee-Eiford, A., Ow, R. A., & Gibbons, I. R. (1986) *J. Biol. Chem. 261*, 2337−2342.
Loo, J. A., Edmonds, C. G., & Smith, R. D. (1990) *Science 248*, 201−204.
Loo, J. A., Ogorzalek Loo, R. R., & Andrews, P. C. (1993) *Org. Mass Spectrom. 28*, 1640−1649.
Mann, M., Meng, C. K., & Fenn, J. B. (1988) *Anal. Chem. 61*, 1702−1710.
Marchese-Ragona, S. P., Facemeyer, K. C., & Johnson, K. A. (1989) *J. Biol. Chem. 264*, 21361−21368.
Mocz, G. (1989) *Eur. J. Biochem. 179*, 373−378.
Mogel, S. N., & McFadden, B. A. (1989) *Biochemistry 28*, 5428−5431.
Molnar, E., Varga, S., & Martonosi, A. (1991) *Biochim. Biophys. Acta 1068*, 17−26.
Muhlrad, A., & Ringel, I. (1995) *Metal Ions Biol. Syst. 31*, 211−230.
Muhlrad, A., Peyser, Y. M., & Ringel, I. (1991) *Biochemistry 30*, 958−965.
Okamoto, Y., & Sekine, T. (1985) *J. Biochem. (Tokyo) 98*, 1143−1145.
Okamoto, Y., & Cremo, C. (1993) in *Mechanism of Myofilament Sliding in Muscle Contraction* (Sugi, H., & Pollack, G. H., Eds.) pp 247−254, Plenum Press, New York.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.
Plesničar, B. (1983) in *The Chemistry of Peroxides* (Patai, S., Ed.) pp 551−554, J. Wiley & Sons, New York.
Rayment, I., Rypniewski, W. R., Schmidt-Bäse, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G., & Holden, H. M. (1993) *Science 261*, 50−58.
Remington, S. J., & Breddam, K. (1994) *Methods Enzymol. 244*, 231−248.
Ringel, I., Peyser, Y. M., & Muhlrad, A. (1990) *Biochemistry 29*, 9091−9096.
Saraste, M., Sibbald, P. R., & Wittinghofer, A. (1990) *Trends Biochem. Sci. 15*, 430−434.
Signorini, M., & Bergamini, C. M. (1990) *Biochem. Biophys. Res. Comm. 172*, 919−924.
Smith, C., & Rayment, I. (1995) *Biochemistry 34*, 8973−8981.
Smith, C., & Rayment, I. (1996) *Biochemistry 35*, 5404−5417.
Smith, R. D., Loo, J. A., Edmonds, C. G., Barinaga, C. J., & Udseth, H. R. (1990) *Anal. Chem. 62*, 882−899.
Smith, R. D., Loo, J. A., Ogorzalek Loo, R. R., Bushman, M., & Udseth, H. R. (1991) *Mass Spectrom. Rev. 10*, 359−451.
Thiede, B., Wittmann-Liebold, B., Bienert, M., & Krause, E. (1995) *FEBS Lett. 357*, 65−69.
Tong, S. W., & Elzinga, M. (1983) *J. Biol. Chem. 21*, 13100−13110.
Vegh, M., Molnar, E., & Martonosi, A. (1990) *Biochim. Biophys. Acta 1023*, 168−183.
Wagner, P. D., & Yount, R. G. (1975) *Biochemistry 14*, 1900−1907.
Wagner, P. D., & Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455−473.
Wells, J. A., Werber, M. M., Legg, J. I., & Yount, R. G. (1979) *Biochemistry 18*, 4793−4799.

BI961901G