Amino Acid Specific ADP-ribosylation: Substrate Specificity of an ADP-ribosylarginine Hydrolase from Turkey Erythrocytes

Joel Moss,*,‡ Norman J. Oppenheimer,§ Robert E. West, Jr.,‡ and Sally J. Stanley‡

Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, and Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, San Francisco, California 94143

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ABSTRACT: An ADP-ribosylarginine hydrolase, which catalyzes the degradation of ADP-ribosyl[^{14}C]arginine to ADP-ribose plus arginine, was separated by ion exchange, hydrophobic, and gel permation chromatography from NAD:arginine ADP-ribosyltransferases, which are responsible for the stereospecific formation of α-ADP-ribosylarginine. As determined by NMR, the specific substrate for the hydrolase was α-ADP-ribosylarginine, the product of the transferase reaction. The ADP-ribose moiety was critical for substrate recognition; (phosphoribosyl)[^{14}C]arginine and ribosyl[^{14}C]arginine were poor substrates and did not significantly inhibit ADP-ribosyl[^{14}C]arginine degradation. In contrast, ADP-ribose was a potent inhibitor of the hydrolase and significantly more active than ADP > AMP > adenosine. In addition to ADP-ribosyl[^{14}C]arginine, both ADP-ribosyl[^{14}C]guanidine and (2′-phospho-ADP-ribosyl)[^{14}C]arginine were also substrates; at pH >7, ADP-ribosyl[^{14}C]guanidine was degraded more readily than the [^{14}C]arginine derivative. Neither arginine, guanidine, nor agmatine, an arginine analogue, was an effective hydrolase inhibitor. Thus, it appears that the ADP-ribosyl moiety but not the arginine group is critical for substrate recognition. Although the hydrolase requires thiol for activity, dithiothreitol accelerated loss of activity during incubation at 37 °C. Stability was enhanced by Mg^{2+}, which is also necessary for optimal enzymatic activity. The findings in this paper are consistent with the conclusion that different enzymes catalyze ADP-ribosylarginine synthesis and degradation. Furthermore, since the hydrolase and transferases possess a compatible stereospecificity and substrate specificity, it would appear that the two enzymatic activities may serve as opposing arms in an ADP-ribosylation cycle.

ADP-ribosylation is a covalent modification in which the ADP-ribose moiety of NAD is transferred to proteins (Hayaishi & Ueda, 1977; Vaughan & Moss, 1981). Two types of reactions, mono- and poly-ADP-ribosylation, have been distinguished on the basis of the attachment to a protein backbone of either a single ADP-ribose moiety or of a branching, polymeric structure, respectively (Hayaishi & Ueda, 1977; Vaughan & Moss, 1981; Miwa et al., 1979; Kawaichi et al., 1980; Okazaki et al., 1980; Pekala & Moss, 1983). Mono-ADP-ribosyltransferases have been described in viruses, bacteria, and animal tissues (Hayaishi & Ueda, 1977; Vaughan & Moss, 1981). Their function has been defined for bacterial toxins that appear to exert their effects on cells by ADP-ribosylation of regulatory proteins in critical metabolic pathways (Iglewski & Kabat, 1975; Pappenheimer, 1977; Moss et al., 1984). A nitrogenase in *Rhodospirillum rubrum* is inactivated by ADP-ribosylation (Pope et al., 1985). The role of mono-ADP-ribosyltransferases intrinsic to animal cells is unclear, although they may act in a manner similar to that of the toxins (Lee & Iglewski, 1984). One subset of these enzymes catalyzes the mono-ADP-ribosylation of arginine and protein (Moss & Vaughan, 1978; Moss et al., 1980; Yost & Moss, 1983; Tanigawa et al., 1984; Soman et al., 1984; Godeau et al., 1984). These NAD:arginine ADP-ribosyltransferases were identified in turkey erythrocytes (Moss & Vaughan, 1978; Moss et al., 1980; Yost & Moss, 1983), rabbit skeletal muscle (Soman et al., 1984), chicken liver (Tanigawa et al., 1984), and *Xenopus* tissues (Godeau et al., 1984).

Recently, an enzymatic activity was described in mouse fibroblasts that degraded the ADP-ribosyl protein linkage at the ribose–protein bond (Smith et al., 1985). Since the substrate ADP-ribosyl protein was formed by NAD:arginine ADP-ribosyltransferase, it was assumed that the ADP-moiety was attached to the protein at an arginine residue through the guanidino group (Moss & Vaughan, 1978). To define further the cleavage reaction, ADP-ribosylarginine was used as a model substrate, thus permitting identification of both products of the reaction, ADP-ribose and arginine (Moss et al., 1985a). An activity was purified from turkey erythrocytes that catalyzed the degradation of ADP-ribosylarginine resulting in release of the ADP-ribose moiety with regeneration of arginine. The arginine subsequently served as an ADP-ribose acceptor in the presence of NAD and an NAD:arginine ADP-ribosyltransferase, indicating that the cleavage reaction had not resulted in modifications in the guanidino group of arginine (Moss et al., 1985a). Thus, in animal tissues, it would appear that ADP-ribosylation of proteins is a reversible modification.

The family of NAD:arginine ADP-ribosyltransferases in turkey erythrocytes possesses different substrate specificities and physical, kinetic, and regulatory properties (Moss et al., 1980; Yost & Moss, 1983; Moss et al., 1985b). These transferases utilize both β-NAD and β-NADP as substrates, leading to the formation of ADP-ribosylarginine and (2′-phospho-ADP-ribosyl)arginine, respectively (Moss et al., 1979b). The transferase-catalyzed reaction is stereospecific and leads to formation of the α-anomer (Moss et al., 1979b). Since turkey erythrocytes contain NAD:arginine ADP-ribosyltransferases and ADP-ribosylarginine hydrolase activities (Moss et al., 1980, 1985a; Yost & Moss, 1983), it appeared that in this system ADP-ribosylation and 2′-phospho-ADP-ribosylation of arginine residues might be a reversible modification of proteins. For these two enzymatic activities

* Address correspondence to this author.
‡ National Institutes of Health.
§ University of California, San Francisco.

to constitute opposing arms in an ADP-ribosylation cycle, however, they must exhibit compatible product–substrate relationships. Highly purified NAD:arginine ADP-ribosyltransferase from erythrocytes was utilized to generate stereospecific substrates, which were tested with ADP-ribosylarginine hydrolase. It appears from these studies that the hydrolase is stereospecific and exhibits a substrate specificity compatible with the transferase activities.

Materials and Methods

Materials. Arginine, agmatine, guanidine, ovalbumin, ADP-ribose, NAD, NADP, nicotinamide, ATP, histone, ADP, AMP, adenosine, alkaline phosphatase, and snake venom phosphodiesterase were purchased from Sigma; DE-52 and carboxylmethylcellulose were from Whatman; turkey blood was from Pel-Freez; glycine was from Schwarz/Mann; Sepharose CL-4B, concanavalin A–Sepharose, and phenyl-Sepharose were from Pharmacia; AG 1-X2, Affi-Gel 501, and Affi-Gel 601 (boronate resin) were from Bio-Rad; sodium acetate, NaCl, and $MgCl_2$ were from Fisher; dithiothreitol and MES[1] were from Boehringer-Mannheim; HEPES was from Calbiochem-Behring; [^{14}C]guanidine (30 Ci/mol), [*carbonyl*-^{14}C]NAD (53 Ci/mol), and [^{14}C]arginine (336 Ci/mol) were from Amersham.

Assays. (*a*) *NAD:Arginine ADP-ribosyltransferase.* NAD:arginine ADP-ribosyltransferase activity was assayed in a total volume of 0.3 mL containing 50 mM potassium phosphate (pH 7.0), 32.4 μM [*carbonyl*-^{14}C]NAD, 1 mg/mL ovalbumin, 200 mM NaCl, and 10 mM agmatine. Reaction was initiated with ADP-ribosyltransferase (0.16 milliunit). After incubation for 30 min at 30 °C, two 0.1-mL samples were transferred to columns (0.5 × 4 cm) of AG 1-X2; [*carbonyl*-^{14}C]nicotinamide was eluted with four 1.2-mL additions of water, as described previously (Moss et al., 1976; Moss & Vaughan, 1977). One unit of transferase activity is defined at 1 μmol of ADP-ribose transferred to agmatine per minute. All assays were run in duplicate.

(*b*) *ADP-ribosylarginine Hydrolase.* Hydrolase activity was assayed in a total volume of 0.1 mL containing 50 mM potassium phosphate, pH 7.5, 50 μM ADP-ribosyl[^{14}C]arginine (4000 cpm), 10 mM $MgCl_2$, and 5 mM dithiothreitol (Moss et al., 1985a). Reaction was initiated with cleavage enzyme (60 milliunits). After 30 min at 30 °C, 80 μL was applied to a phenyl boronate–polyacrylamide column (0.5 × 4 cm). [^{14}C]Arginine was eluted with four additions of 1.25 mL of 0.05 M sodium acetate, pH 5. Unless indicated otherwise, all assays were run in quadruplicate. One unit of hydrolase activity corresponds to 1 nmol of ADP-ribosylarginine hydrolyzed per minute at 30 °C. Activity of the organomercurial agarose-purified enzyme was linear with protein concentration and time (data not shown). Unless noted otherwise, experiments were performed with the organomercurial agarose-purified enzyme.

(*c*) *Protein.* Protein was determined by the method of Lowry et al. (1951) or by a dye-binding assay (Bio-Rad).

Preparation of ADP-ribosyl[^{14}C]arginine, (2′-Phospho-ADP-ribosyl)[^{14}C]arginine, (Phosphoribosyl)[^{14}C]arginine, Ribosyl[^{14}C]arginine, and ADP-ribosyl[^{14}C]guanidine. (*a*) *Preparation of ADP-ribosyl[^{14}C]arginine.* The substrate for the hydrolase-catalyzed reaction was synthesized in a mix containing 50 mM HEPES, pH 8.0, 5 mM potassium phosphate, 5% propylene glycol, 100 mM NaCl, 10 mM NAD, 20 mM [^{14}C]arginine (1.8×10^7 cpm), 1 mg/mL ovalbumin, and NAD:arginine ADP-ribosyltransferase (994 milliunits) (final volume 2 mL). After 90 min at 30 °C, the sample was applied to an Affi-Gel 601 column (bed volume 1.5 mL) that was washed with 30 mL of 0.1 M glycine, pH 9.0, 0.1 M NaCl, and 0.01 M $MgCl_2$ and eluted with 50 mM sodium acetate, pH 5.0. The pH 5.0 acetate fractions (2.5 mL) were brought to pH 7.0 with 1 M Na_2HPO_4 (0.15 mL). Radiolabeled product in the acetate eluate was characterized by thin-layer and high-performance liquid chromatography as described earlier (Yost & Moss, 1983; Moss et al., 1985a).

(*b*) *Preparation of (Phosphoribosyl)[^{14}C]arginine.* (Phosphoribosyl)[^{14}C]arginine was generated from ADP-ribosyl[^{14}C]arginine in a reaction mix containing 50 mM HEPES, pH 8.0, 10 mM $MgCl_2$, 778 μM ADP-ribosyl-[^{14}C]arginine (6.6×10^5 cpm), and 50 milliunits of snake venom phosphodiesterase (final volume 1 mL). After 1 h at 30 °C, the sample was applied to an Affi-Gel 601 column (bed volume 1.5 mL) that was washed and eluted as described above for the preparation of ADP-ribosyl[^{14}C]arginine. The extent of conversion of ADP-ribosyl[^{14}C]arginine to (phosphoribosyl)[^{14}C]arginine was determined by thin-layer chromatographic analysis on cellulose MN300–poly(ethylenimine) (Sybron/Brinkmann) developed in 0.9 M acetic acid–0.3 M LiCl.

(*c*) *Preparation of Ribosyl[^{14}C]arginine.* Ribosyl[^{14}C]arginine was generated from ADP-ribosyl[^{14}C]arginine under the same reaction conditions utilized above to prepare (phosphoribosyl)[^{14}C]arginine except for the addition of alkaline phosphatase (25 units). The formation of ribosyl-[^{14}C]arginine was monitored by thin-layer chromatography on cellulose MN300–poly(ethylenimine) plates developed in 1-butanol–acetic acid–water (12:3:5). In this system, ribosyl[^{14}C]arginine and (phosphoribosyl)[^{14}C]arginine are readily separated (Moss et al., 1985a).

(*d*) *Preparation of ADP-ribosyl[^{14}C]guanidine.* ADP-ribosyl[^{14}C]guanidine was synthesized in a reaction mix identical with that used in the preparation of ADP-ribosyl-[^{14}C]arginine except for the substitution of [^{14}C]guanidine for [^{14}C]arginine.

(*e*) *Preparation of (2′-Phospho-ADP-ribosyl)arginine.* (2′-Phospho-ADP-ribosyl)[^{14}C]arginine was synthesized enzymatically with erythrocyte NAD:arginine ADP-ribosyltransferase A, which was shown previously to use NAD and NADP as donors of ADP-ribose and 2′-phospho-ADP-ribose, respectively. The reaction mix contained 994 milliunits of NAD:arginine ADP-ribosyltransferase A, 50 mM HEPES, pH 8.0, 10 mM NAD, 1 mg/mL ovalbumin, 0.1 M NaCl, and 20 mM [^{14}C]arginine (4×10^7 cpm) in a total volume of 2 mL. (2′-Phospho-ADP-ribosyl)[^{14}C]arginine was isolated by affinity chromatography on a phenyl boronate–acrylamide resin (1.5 mL); the compound was eluted with 50 mM sodium acetate, pH 5.0, as noted above. The phenyl boronate eluate was applied to an AG 1-X2 resin (1.5 mL) (Bio-Rad), which was washed with 1 N formic acid; the procedure effectively resolved ADP-ribosyl[^{14}C]arginine from (2′-phospho-ADP-ribosyl)[^{14}C]arginine. The product was lyophilized, solubilized in 20 mM potassium phosphate, pH 7.5, and stored at −20 °C. The compound was characterized by high-performance liquid chromatographic analysis (as noted in Figure 2) and thin-layer chromatography.

Protein Purification. (*a*) *NAD:Arginine ADP-ribosyltransferase.* Transferase was purified from turkey erythrocytes as described previously (Moss et al., 1980). The enzyme was stored in 50 mM potassium phosphate, pH 7.0–50% propylene glycol at 4 °C.

(*b*) *Purification of ADP-ribosylarginine Hydrolase.* Packed and washed red blood cells (144 mL) from 300 mL of turkey blood were homogenized in 20 mM potassium phosphate, pH

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

Table I: Purification of ADP-ribosyl[^{14}C]arginine Hydrolase from Turkey Erythrocytes[a]

step	ADP-ribosylarginine hydrolase				NAD:arginine ADP-ribosyltransferase	
	protein (mg)	units (nmol·min^{-1})	sp act. (units/mg)	yield (%)	units (μmol·min^{-1})	yield (%)
supernatant	41 600	2000	0.048	100	8.37	100
DE-52 eluate	823	1420	1.73	70.9	4.76	56.9
phenyl-Sepharose A	200	803	4.02	40.1	0.24	2.9
phenyl-Sepharose B					3.24	39.7
carboxymethylcellulose	202	787	3.90	39.3	0.148	1.8
organomercurial agarose	161	736	4.60	36.8	0.052	0.6
Ultrogel AcA-54	11	424	38.6	21.2		

[a] Details of the purification procedure are given in the text. ADP-ribosylarginine hydrolase and NAD:arginine ADP-ribosyltransferase activities were determined in standard assays (see Materials and Methods).

7.5 (600 mL), in a Waring Blendor; the homogenate was centrifuged at 27000g for 30 min and the resulting supernatant fraction (680 mL) mixed for 18 h with DE-52 (1500 mL) diluted with 1820 mL of 20 mM potassium phosphate, pH 7.5. The DE-52 was then washed extensively in a funnel with a total of 8000 mL of 20 mM potassium phosphate, pH 7.5. The hydrolase was eluted with 20 mM potassium phosphate, pH 7.5–0.5 M NaCl (1000 mL); this DE-52 eluate was applied to phenyl-Sepharose (142 mL) that was then washed with 20 mM potassium phosphate, pH 7.5–0.5 M NaCl (1000 mL). The enzyme was eluted first with 205 mL of 50 mM potassium phosphate, pH 7.5–50% propylene glycol (Table I, phenyl-Sepharose A) and then with 560 mL of 60% propylene glycol–100 mM potassium phosphate, pH 7.5 (Table I, phenyl-Sepharose B). As noted in Table I, phenyl-Sepharose A contained the ADP-ribosylarginine hydrolase activity, while phenyl-Sepharose B contained NAD:arginine ADP-ribosyltransferase activity. After 2/3 (v/v) dilution with 20 mM potassium phosphate, pH 7.5, phenyl-Sepharose A was applied to carboxymethylcellulose (150 mL). The nonadherent fraction was then applied to an organomercurial agarose column (1.5 × 8 cm), which was washed with 20 mM potassium phosphate, pH 7.5–20% propylene glycol. The hydrolase activity was eluted with 20 mM potassium phosphate, pH 7.5–20 mM dithiothreitol. The hydrolase appears to be unstable if stored at these concentrations of dithiothreitol (see below). Dithiothreitol was removed by batchwise chromatography on phenyl-Sepharose; the elution procedure is as noted above. The hydrolase was further purified by gel permeation chromatography on Ultrogel AcA-54 equilibrated with 20 mM dithiothreitol, 20 mM potassium phosphate, and 100 mM NaCl. The approximate molecular weight of the hydrolase on the basis of its elution from the gel permeation column was 40 000. This column effectively separated the hydrolase from any residual NAD:arginine ADP-ribosyltransferase activity. These data futher support the proposal that different enzymes mediate ADP-ribosylarginine synthesis and degradation. The purification procedure, summarized in Table I, gives an 800-fold purification of the hydrolase from the supernatant fraction and clearly separates it from NAD:arginine ADP-ribosyltransferases, which bind more tightly to phenyl-Sepharose and elute after the hydrolase from the gel permeation column. The recovery of NAD:arginine ADP-ribosyltransferase activity during hydrolase purification is shown in Table I. Clearly, the synthetic and degradative enzymes can be resolved.

Total hydrolase activity appeared to increase during the initial purification steps; it seems that accurate determination of hydrolase activity in impure erythrocyte cytosol preparations is complicated by the presence of coexisting enzymatic activities such as phosphodiesterases that degrade the substrate (Hayaishi & Ueda, 1977; Futai & Mizuno, 1967; Futai et al., 1968). Addition of Mg^{2+}-dependent phosphodiesterases to the assay resulted in an apparent loss of hydrolase activity (Table II). The heat-treated phosphodiesterase was inactive (Table II). Addition of alternative phosphodiesterase substrates, which resulted in preservation of ADP-ribosyl[^{14}C]arginine, prevented the apparent loss of hydrolase activity (Table II).

Table II: Effect of Phosphodiesterase on ADP-ribosylarginine Hydrolase Activity[a]

additions to incubation I	ADP-ribosylarginine hydrolysis (pmol·min^{-1})	
	control	+PDE inhibitors
none	47.6	37.2
phosphodiesterase (PDE)	0	32.6
heat-treated phosphodiesterase	49.2	38.4

[a] Assays contained 62.5 mM potassium phosphate, pH 7.5, 6.25 mM dithiothreitol, 12.5 $MgCl_2$, and 62.5 μM ADP-ribosyl[^{14}C]arginine and, where indicated, phosphodiesterase (5 milliunits), heat-treated (10 min at 95 °C) phosphodiesterase, and/or phosphodiesterase inhibitors (24 μM ADP-ribose, 2.4 mM pyrophosphate, 5 mM GTP) in a total volume of 80 mL. After 10 min at 30 °C, ADP-ribosylarginine hydrolase (58 milliunits) was added in 0.02 mL. After ~30 min at 30 °C, samples (80 μL) were withdrawn for [^{14}C]arginine determination.

Results

Stereochemistry of Turkey Erythrocyte ADP-ribosylarginine Hydrolase Reaction. The NAD:arginine ADP-ribosyltransferase from turkey erythrocytes, in the presence of β-NAD and arginine, catalyzed the stereospecific formation of α-ADP-ribosylarginine; the enzymatic product anomerized with time to yield an α,β mixture. The stereospecificity of the reaction catalyzed by the ADP-ribosylarginine hydrolase from turkey erythrocytes was determined by ^{1}H NMR spectroscopy to monitor the reaction. As can be seen, the initial ADP-ribosylarginine substrate is present as a 60:40 ratio of α- to β-anomers by the resonance assignments reported previously (Oppenheimer, 1984) (Figure 1). Stereospecificity for the hydrolase was examined with a 500 μM solution of ADP-ribosylarginine and with standard assay conditions for the hydrolase; representative spectra showing the time course of the reaction are presented in Figure 1. One hour after addition of the hydrolase, the resonance of the α-ribosyl anomeric proton of ADP-ribosylarginine at 5.337 ppm was decreased to less than 25% of its original value with the concomitant appearance of resonances for the α- and β-anomeric protons of ADP-ribose at 5.322 and 5.213 ppm, respectively. After 2.5 h, the resonance for the α-ribosyl proton of ADP-ribosylarginine was no longer detected, whereas the resonance for the β-ribosyl proton at 5.148 ppm had decreased by less than 20%. These spectra clearly show the selective loss of the α-anomer of ADP-ribosylarginine and thus establish that the turkey enzyme is an α-specific hydrolase.

Substrate Specificity. The organomercurial agarose-purified enzyme catalyzed the degradation of ADP-ribosyl[^{14}C]argi-

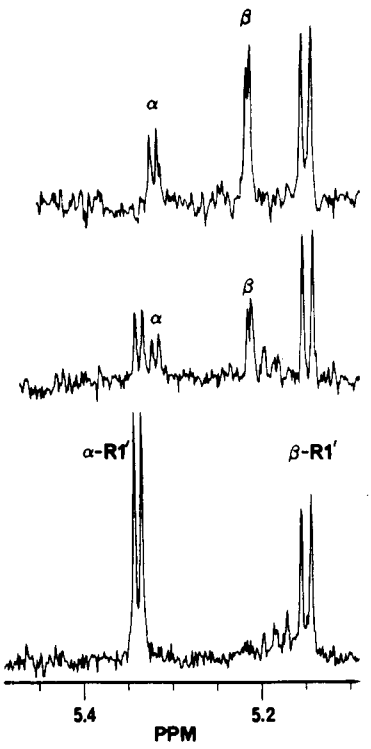

FIGURE 1: Stereospecificity of turkey erythrocyte ADP-ribosylarginine hydrolase. ADP-ribosylarginine was prepared and purified as outlined previously (Oppenheimer, 1984) except that an ADP-ribosyltransferase from erythrocytes was used instead of cholera toxin. Although only the α-anomer is synthesized enzymatically (Oppenheimer, 1978; Moss et al., 1979a,b), in the course of purification ADP-ribosylarginine anomerizes extensively to give the initial 60:40 mixture of α- to β-anomers that was used directly. Samples to study the enzyme-catalyzed hydrolysis of ADP-ribosylarginine were prepared in D_2O in a 5-mm NMR tube and had the following final concentrations: 50 mM sodium phosphate (pD 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.5 mM ADP-ribosylarginine in a total initial volume of 360 μL. A total of 40 μL of an aqueous solution of the ADP-ribosylarginine hydrolase was added to the sample, and spectra were recorded every 20 min. The reaction was conducted at 25 °C. Spectra were obtained on a GN 500-MHz Fourier-transform NMR spectrometer employing quadrature phase detection. The spectral width was 5200 Hz, and 256 free induction decays were accumulated with 16K data points for each spectrum. The pulse width and delay times were adjusted to give equilibrium intensities for the anomeric protons, and a 1-s presaturation pulse was applied in order to suppress the residual HDO resonance. Total spectral accumulation time was 8 min for each spectrum. The spectra were referenced to the internal chemical shift standard sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]-propionate (TSP). The figure gives a portion of the 500-MHz ^1H NMR spectrum showing the time-dependent changes caused by incubation of ADP-ribosylarginine with the turkey ADP-ribosylarginine hydrolase. The spectrum at the bottom shows the anomeric protons of ADP-ribosylarginine and was obtained prior to addition of the turkey hydrolase. The spectrum after incubation for 1 h (middle) shows that the resonance of the α-ribosyl anomeric proton of ADP-ribosylarginine at 5.337 ppm has decreased to less than 25% of the original amount. In addition, new resonances corresponding to the α- and β-anomeric protons of ADP-ribose are observed at 5.322 and 5.213 ppm, respectively. After 2.5 h, the spectrum shows no detectable resonance for the α-ribosyl proton of ADP-ribosylarginine (top), and only the resonances for the β-ribosyl proton (5.148 ppm) and the anomeric protons of ADP-ribose are present.

nine. The K_m for ADP-ribosyl[^{14}C]arginine under standard assay conditions was 65.2 ± 8.0 μM (n = 5, ±SE). (2′-Phospho-ADP-ribosyl)arginine was also cleaved by the hydrolase with a K_m of 47.2 ± 5.5 μM (n = 2, ± half the range); V_{max} was 39.4% that of ADP-ribosylarginine. Degradation of (2′-phospho-ADP-ribosyl)arginine was not secondary to the formation of ADP-ribosylarginine by contaminating phosphatases (Figure 2). At similar concentrations, (phosphoribosyl)[^{14}C]arginine and ribosyl[^{14}C]arginine were not hydrolyzed (Table IIIA). Addition of either (phosphoribosyl)arginine or ribosylarginine to an assay containing ADP-ribosyl[^{14}C]arginine did not significantly affect the rate of [^{14}C]arginine formation (Table IIIB). Thus, the ADP-ribose moiety appeared to be important for substrate recognition. In agreement with the findings was the observation that hydrolase activity was inhibited competitively by ADP-ribose with inhibition by ADP-ribose > ADP > AMP (Figure 3). ADP-ribose was also more inhibitory than α-NAD > β-NAD; β-NAD was not hydrolyzed under hydrolase assay conditions. Although the ADP-ribose moiety was essential

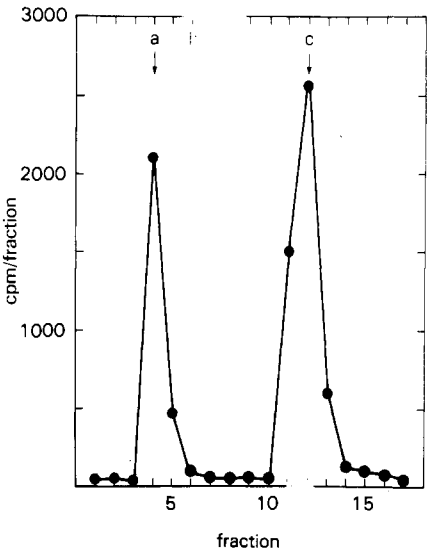

FIGURE 2: Hydrolysis of (2′-phospho-ADP-ribosyl)arginine by erythrocyte hydrolase. A total of 50 μM (phospho-ADP-ribosyl)[^{14}C]arginine (12000 cpm) was incubated with hydrolase (34 μg) in a total volume of 150 μL containing 50 mM potassium phosphate, pH 7.5, 10 mM $MgCl_2$, and 5 mM dithiothreitol at 30 °C for 30 min. One hundred microliters of reaction mix (containing 7700 cpm) was injected onto a Du Pont Zorbax SAX column (0.46 × 25 cm) with Upchurch guard column. Chromatography conditions were as follows: mobile phase, 200 mM sodium phosphate, pH 4.5; temperature, 35 °C; flow rate, 1 mL/min. One-minute samples were collected for scintillation counting. Standards are (a) [^{14}C]arginine, (b) ADP-ribosyl[^{14}C]arginine, and (c) (2′-phospho-ADP-ribosyl)[^{14}C]arginine. Without enzyme in the reaction mix, only a single peak corresponding to (2′-phospho-ADP-ribosyl)[^{14}C]arginine appeared.

Table III: Substrate Specificity of ADP-ribosylarginine Hydrolase

	ADP-ribosyl-arginine hydrolysis (pmol·min^{-1})
(A) substrate[a]	
ADP-ribosyl[^{14}C]arginine (48 μM)	30.4
(phosphoribosyl)[^{14}C]arginine (44 μM)	0
ribosyl[^{14}C]arginine (42 μM)	0
(B) additions[b]	
none	31.8
(phosphoribosyl)arginine (44 μM)	29.1
ribosylarginine (42 μM)	30.9

[a] ADP-ribosylarginine hydrolase (17.2 μg) initiated the reaction in a standard assay mix (0.1 mL) containing 50 mM potassium phosphate, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, and the indicated substrate. After 30 min at 30 °C, assays were terminated as noted under Materials and Methods. [b] The standard assay mix containing 48 μM ADP-ribosyl[^{14}C]arginine was supplemented with the indicated additions. Reaction was initiated with hydrolase (17.2 μg) (final volume 0.1 mL). After 30 min at 30 °C, 0.08 mL was placed over the phenyl boronate resin to isolate released [^{14}C]arginine. All assays were run in a standard mix.

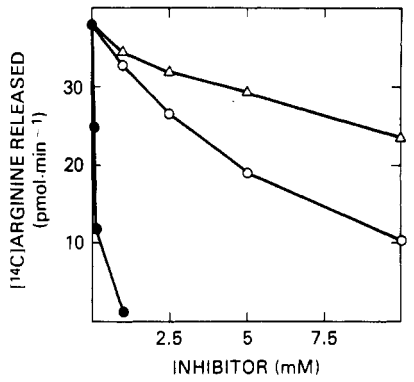

FIGURE 3: Effect of ADP-ribose and related compounds on activity of ADP-ribosylarginine hydrolase. ADP-ribosylarginine hydrolase (17.2 μg) was assayed in a standard mix (final volume 0.1 mL) containing as indicated ADP-ribose (●), ADP (○), and 5′-AMP (△). Incubation conditions were identical with those given in the legend to Table III.

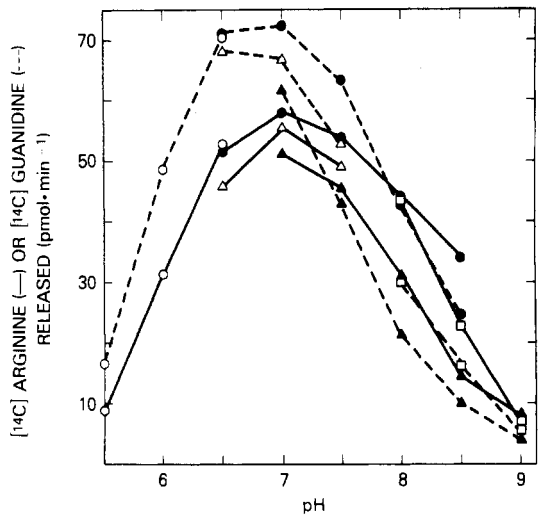

FIGURE 4: Effect of pH on activity of ADP-ribosylarginine hydrolase with ADP-ribosyl[^{14}C]arginine and ADP-ribosyl[^{14}C]guanidine as substrates. ADP-ribosylarginine hydrolase (10.2 μg) initiated the reaction in assays containing 50 mM of the indicated buffers, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 50 μM of either ADP-ribosyl-[^{14}C]arginine (—) or ADP-ribosyl[^{14}C]guanidine (- -). MES (○); potassium phosphate (●); imidazole (△); Tris (▲); glycine (□).

for substrate degradation, the arginine group was not. At 1 mM, arginine, guanidine, and agmatine, an arginine analogue, did not significantly inhibit the hydrolase reaction (data not shown). ADP-ribosyl[^{14}C]guanidine, synthesized by the erythrocyte NAD:arginine ADP-ribosyltransferase, was a substrate for the hydrolase; the K_m under standard assay conditions was 27.1 ± 3.6 μM (n = 5, ±SE). At pH <7, the enzyme exhibited more activity toward ADP-ribosyl[^{14}C]-guanidine than toward ADP-ribosyl[^{14}C]arginine (Figure 4). The ratio of activity in assays containing ADP-ribosyl[^{14}C]-arginine to those with ADP-ribosyl[^{14}C]guanidine increased with pH (Figure 4). Degradation of ADP-ribosyl[^{14}C]-guanidine, like that of ADP-ribosyl[^{14}C]arginine, was dependent on Mg^{2+} and thiol and sensitive to inhibition by ADP-ribose (data not shown).

Enzyme Stability. The purified enzyme was unstable, as noted under Materials and Methods, if stored in thiol. Incubation of the enzyme at 37 °C in dithiothreitol led to rapid loss of activity (Figure 5); Mg^{2+} stabilized both control and thiol-treated enzyme (Figure 5).

Effectors of ADP-ribosylarginine Synthesis and Degradation. The ADP-ribosylarginine hydrolase was inhibited significantly (>80%) by 5 mM NaF and 200 mM NaCl; histone

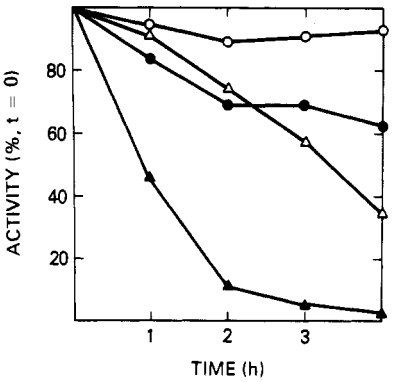

FIGURE 5: Effect of dithiothreitol and Mg^{2+} on stability of ADP-ribosylarginine hydrolase. ADP-ribosylarginine hydrolase (191 μg) was incubated at 37 °C in the presence of 3.33 mM potassium phosphate, pH 7.5, 3.33% propylene glycol, and, as noted, 10 mM $MgCl_2$ (○), 20 mM dithiothreitol (▲), $MgCl_2$ + dithiothreitol (△), or no additions (●) (final volume 1.5 mL). At the indicated times, samples (60 μL) were assayed for 30 min at 30 °C.

did not affect activity (data not shown). An NAD:arginine ADP-ribosyltransferase from erythrocytes was not affected by NaF (data not shown); it was previously shown to be activated by NaCl and histone (Moss & Stanley, 1981; Moss et al., 1981a,b). Mg^{2+} and dithiothreitol, activators of the hydrolase (Moss et al., 1985), did not affect ADP-ribosyltransferase activity. The fact that agents such as NaF, histone, Mg^{2+}, and thiol have different effects on the hydrolase and ADP-ribosyltransferase is consistent with the conclusion that different enzymes catalyze the synthetic and degradative reactions.

Discussion

The present studies demonstrate that the enzymes involved in ADP-ribosylarginine cleavage are clearly different from those responsible for ADP-ribosylarginine synthesis (Moss et al., 1980; Yost & Moss, 1983). The purification procedure clearly separates the hydrolase from two NAD:arginine ADP-ribosyltransferases present in the soluble fraction of turkey erythrocytes (Moss et al., 1980; Yost & Moss, 1983). The hydrolase and the ADP-ribosyltransferases appear to be regulated by different effectors. The presence of different enzymes and effectors controlling ADP-ribosylarginine synthesis and degradation is reminiscent of the protein kinase–phosphatase system (Krebs & Beavo, 1979).

The ADP-ribosyltransferases and ADP-ribosylarginine hydrolase could conceivably operate as opposing arms in an ADP-ribosylation cycle. The erythrocyte transferases catalyze the stereospecific ADP-ribosylation of a guanidino compound such as arginine, yielding an α-anomeric product; all transferases analyzed thus far including the poly(ADP-ribose) polymerase and the bacterial toxins chloleragen and *Escherichia coli* heat-labile enterotoxin appear to possess the same stereospecificity (Oppenheimer, 1978, 1984; Moss et al., 1979a,b; Ferro & Oppenheimer, 1978). The ADP-ribosylarginine hydrolase preferentially cleaves the α-anomer, consistent with the stereospecific coupling of the transferase–hydrolase reactions. α-ADP-ribosylarginine formed in vitro undergoes nonenzymatic anomerization. α,β-Anomerization may, however, be an in vitro phenomenon observed only following ADP-ribosylation of a model acceptor such as arginine. It is possible that α,β-anomerization does not occur in vivo; ADP-ribosylation of a physiological protein acceptor may result in an α-anomeric linkage that is stabilized by physical constraints imposed by the protein; i.e., the ADP-ribosylation site may not tolerate a β-anomeric linkage. Under these

circumstances, $\beta \rightarrow \alpha$ anomerization would not be a rate-determining factor in the release of the ADP-ribose moiety hydrolase, and tighter regulatory control could result. Two types of NAD:arginine ADP-ribosyltransferases have been described on the basis of the their ability to utilize either NAD or NADP and NAD as substrates. In erythrocytes, NAD:arginine ADP-ribosyltransferase A in the presence of NAD or NADP synthesizes ADP-ribosylarginine or (2′-phospho-ADP-ribosyl)arginine, respectively (Moss et al., 1979b). In contrast, NAD:arginine ADP-ribosyltransferase B like cholera toxin (Moss et al., 1977) clearly prefers NAD as substrate and displays little activity with NADP. If the in vitro reactions mimic in vivo activity, then transferases may generate both ADP-ribosyl and 2′-phospho-ADP-ribosyl protein. As noted in this paper, the erythrocyte ADP-ribosylarginine hydrolase cleaved in the ribosyl–arginine linkage in both ADP-ribosylarginine and (2′-phospho-ADP-ribosyl)arginine, although the V_{max} with the phosphorylated compound was significantly less. The hydrolase thus appears able to cleave potential products of the transferase reaction.

The ADP-ribose moiety plays a critical role in substrate recognition by the cleavage enzyme. ADP-ribosylarginine was clearly a better substrate than (phosphoribosyl)arginine and ribosylarginine; these latter two compounds were poor inhibitors of ADP-ribosylarginine cleavage. As expected, ADP-ribose was a potent inhibitor of activity and clearly better in this regard than ADP or AMP. Both ADP-ribosyl[^{14}C]arginine and ADP-ribosyl[^{14}C]guanidine were degraded by the hydrolase; an intact arginine moiety was therefore not essential for ribosyl–guanidine bond cleavage. In agreement, arginine, guanidine, and agmatine, an arginine analogue, did not significantly inhibit enzymatic activity. Thus, the primary recognition site for the hydrolase appears to be the ADP-ribose moiety.

Animal tissues contain several enzymes that may participate in the degradation of ADP-ribosylarginine (Hayaishi & Ueda, 1977; Futai & Mizuno, 1967; Futai et al., 1968; Knowles, 1980). Phosphodiesterases act on the pyrophosphate moiety to generate (phosphoribosyl)arginine and 5′-AMP (Hayaishi & Ueda, 1977; Futai & Mizuno, 1967; Futai et al., 1968); phosphatases degrade (phosphoribosyl)arginine to ribosylarginine (Knowles, 1980). Both of these enzymes are fairly common in animal tissues (Hayaishi & Ueda, 1977; Futai & Mizuno, 1967; Futai et al., 1968; Knowles, 1980). The hydrolase clearly prefers ADP-ribosylarginine as a substrate; (phosphoribosyl)arginine, a product of phosphodiesterase action, and ribosylarginine, which resulted from sequential phosphodiesterase–phosphatase degradation of ADP-ribosylarginine, were clearly less readily cleaved by the hydrolase. These findings are consistent with the hypothesis that action of phosphodiesterases on ADP-ribosyl (arginine) protein may prevent cleavage of the ribosyl–(arginine) protein linkage and, thus regeneration of the (arginine) protein acceptor. The fate of ribosyl (arginine) protein is currently unclear. Conceivably, there may be other enzymes with different substrate specificity that can cleave the phosphodiesterase and the phosphodiesterase–phosphatase products.

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Registry No. ADP, 58-64-0; AMP, 61-19-8; ADP-ribose, 20762-30-5; ADP-ribosyl[^{14}C]arginine, 103960-51-6; (2′-phospho-ADP-ribosyl)[^{14}C]arginine, 103960-52-7; ADP-ribosyl[^{14}C]guanidine, 103960-53-8; (phosphoribosyl)[^{14}C]arginine, 103960-54-9; ribosyl-[^{14}C]arginine, 103960-55-0; ADP-ribosylarginine hydrolase, 98668-52-1; α-ADP-ribosylarginine, 103960-56-1; adenosine, 58-61-7.

References

Ferro, A. M., & Oppenheimer, N. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 809–813.

Futai, M., & Mizuno, D. (1967) *J. Biol. Chem. 242*, 5301–5307.

Futai, M., Mizuno, D., & Sugimura, T. (1968) *J. Biol. Chem. 243*, 6325–6329.

Godeau, F., Belin, D., & Koide, S. S. (1984) *Anal. Biochem. 137*, 287–296.

Hayaishi, O., & Ueda, K. (1977) *Annu. Rev. Biochem. 46*, 95–116.

Iglewski, B. H., & Kabat, D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2284–2288.

Kawaichi, M., Ueda, K., & Hayaishi, O. (1980) *J. Biol. Chem. 255*, 816–819.

Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877–919.

Krebs, E. G., & Beavo, J. A. (1979) *Annu. Rev. Biochem. 48*, 923–959.

Lee, H., & Iglewski, W. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2703–2707.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Miwa, M., Saikawa, N., Yamizumi, A., Nishimura, S., & Sugimura, T. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 595–599.

Moss, J., & Vaughan, M. (1977) *J. Biol. Chem. 252*, 2455–2457.

Moss, J., & Vaughan, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3621–3624.

Moss, J., & Stanley, S. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4809–4812.

Moss, J., Manganiello, V. C., & Vaughan, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4424–4429.

Moss, J., Osborne, J. C., Jr., Fishman, P. H., Brewer, H. B., Jr., Vaughan, M., & Brady, R. O. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 74–78.

Moss, J., Garrison, S., Oppenheimer, N. J., & Richardson, S. H. (1979a) *J. Biol. Chem. 254*, 6270–6272.

Moss, J., Stanley, S. J., & Oppenheimer, N. J. (1979b) *J. Biol. Chem. 254*, 8891–8894.

Moss, J., Stanley, S. J., & Watkins, P. A. (1980) *J. Biol. Chem. 255*, 5838–5840.

Moss, J., Stanley, S. J., & Osborne, J. C., Jr. (1981a) *J. Biol. Chem. 256*, 11452–11456.

Moss, J., Stanley, S. J., & Osborne, J. C., Jr. (1981b) *J. Biol. Chem. 257*, 1660–1663.

Moss, J., Burns, D. L., Hsia, J. A., Hewlett, E. L., Guerrant, R. L., & Vaughan, M. (1984) *Ann. Intern. Med. 101*, 653–666.

Moss, J., Jacobson, M. K., & Stanley, S. J. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5603–5607.

Moss, J., West, R. E., Jr., Osborne, J. C., Jr., & Levine, R. L. (1985b) in *ADP-Ribosylation of Proteins* (Althaus, F. R., Hilz, H., & Shall, S., Ed.) pp 512–517, Springer-Verlag, Berlin and Heidelberg.

Okazaki, H., Niedergang, C., & Mandel, P. (1980) *Biochimie 62*, 147–157.

Oppenheimer, N. J. (1978) *J. Biol. Chem. 253*, 4907–4910.

Oppenheimer, N. J. (1984) *Methods Enzymol. 106*, 399–403.

Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem. 46*, 69–94.

Pekala, P. H., & Moss, J. (1983) *Curr. Top. Cell. Regul. 22*, 1–49.

Pope, M. R., Murrell, S. A., & Ludden, P. W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3173–3177.
Smith, K. P., Benjamin, R. C., Moss, J., & Jacobson, M. K. (1985) *Biochem. Biophys. Res. Commun. 126*, 136–142.
Soman, G., Mickelson, J. R., Louis, C. F., & Graves, D. J. (1984) *Biochem. Biophys. Res. Commun. 120*, 973–980.
Tanigawa, Y., Tsuchiya, M., Imai, Y., & Shimoyama, M. (1984) *J. Biol. Chem. 259*, 2022–2029.
Vaughan, M., & Moss, J. (1981) *Curr. Top. Cell. Regul. 20*, 205–246.
Yost, D. A., & Moss, J. (1983) *J. Biol. Chem. 258*, 4926–4929.

Mechanism of Inactivation of Human Leukocyte Elastase by a Chloromethyl Ketone: Kinetic and Solvent Isotope Effect Studies

Ross L. Stein* and D. Amy Trainor

Department of Biomedical Research, Stuart Pharmaceuticals, A Division of ICI Americas Inc., Wilmington, Delaware 19897

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ABSTRACT: The mechanism of inactivation of human leukocyte elastase (HLE) by the chloromethyl ketone MeOSuc-Ala-Ala-Pro-Val-CH_2Cl was investigated. The dependence of the first-order rate constant for inactivation on concentration of chloromethyl ketone is hyperbolic and suggests formation of a reversible "Michaelis complex" prior to covalent interaction between the enzyme and inhibitor. However, the observed K_i value is 10 μM, at least 10-fold lower than dissociation constants for complexes formed from interaction of HLE with structurally related substrates or reversible inhibitors, and suggests that K_i is a complex kinetic constant, reflecting the formation and accumulation of both the Michaelis complex and a second complex. It is proposed that this second complex is a hemiketal formed from attack of the active site serine on the carbonyl carbon of the inhibitor. The accumulation of this intermediate may be a general feature of reactions of serine proteases and chloromethyl ketones derived from specific peptides and accounts for the very low K_i values observed for these reactions. The solvent deuterium isotope effect (SIE) on the inactivation step (k_i) is 1.58 ± 0.07 and is consistent with rate-limiting, general-catalyzed attack of the active site His on the methylene carbon of the inhibitor with displacement of chloride anion. The general catalyst is thought to be the active site Asp. In contrast, the SIE on the second-order rate constant for HLE inactivation, k_i/K_i, is inverse and equals 0.64 ± 0.05. The proton inventory (rate measurements in mixtures of H_2O and D_2O) for this reaction is "bowed down" from a straight line connecting the points in pure H_2O and D_2O and indicates multi-proton reorganization. These results are consistent with a mechanism for k_i/K_i in which (i) initial formation of the Michaelis complex is accompanied by solvent reorganization and (ii) the ketone moiety of the inhibitor exists as a fully formed hemiketal in the rate-limiting transition state.

Peptide-derived chloromethyl ketones are irreversible inhibitors of serine proteases (Powers, 1977). The stable complexes formed from interaction of CMKs[1] with several proteases have been examined by X-ray crystallographic methods and reveal two important structural features (James et al., 1980; Poulos et al., 1976): (i) a covalent bond between the methylene carbon of the ketone and $N^{\epsilon 2}$ of the active-site histidine imidazole and (ii) a covalent bond between the ketone carbonyl carbon and O^{γ} of the active site serine. These results support earlier studies demonstrating the presence of alkylated histidine residues in acid hydrolysates of CMK-inactivated proteases (Schoellmann & Shaw, 1963; Shaw & Ruscica, 1971) and the inability of anhydrochymotrypsin to interact with *N*-tosyl-PheCH_2Cl (Weiner et al., 1966).

Kinetic studies of the interaction of serine proteases with CMKs indicate the reversible formation of an enzyme–inhibitor complex prior to alkylation and thus adherence of these reactions to the minimal mechanism of Scheme I (Collen et al., 1980; Kurachi et al., 1973; Powers, 1977; Walker et al., 1985).

Scheme I

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_i} E\text{–}I$$

Kinetic investigations also reveal a correlation between inhibitor structure and inactivation rates. In general, peptide structural features known to enhance catalytic efficiency during substrate hydrolysis also tend to increase rates of inactivation by CMKs (Collen et al., 1980; Kurachi et al., 1973; Powers, 1977).

Despite this level of understanding, the mechanistic picture for the inactivation of serine proteases by CMKs is still incomplete. Important unanswered questions include the following: What is the structure of E·I? Is E·I the initial Michaelis complex or the hemiketal formed from interaction of the active site serine with the carbonyl carbon of the inhibitor? What elementary reaction steps rate limit the processes governed by k_i and k_i/K_i? What are the structures of the rate-limiting transition states for these steps? Are these transition states stabilized by protolytic catalysis? Finally, are these mechanistic features sensitive to the structure of the inhibitor? In this paper we will try to answer these questions for the inhibition of human leukocyte elastase by the chloromethyl ketone MeOSuc-Ala-Ala-Pro-Val-CH_2Cl.

[1] Abbreviations: CMK, chloromethyl ketone; Tos, *N*-tosyl; MeOSuc, *N*-(methoxysuccinyl); pNA, *p*-nitroanilide; HLE, human leukocyte elastase; CT, bovine chymotrypsin.

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