Influence of Phallotoxins and Metal Ions on the Rate of Proteolysis of Actin†

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ABSTRACT: The rate of proteolytic degradation of rabbit skeletal muscle actin by trypsin, α-chymotrypsin, and, mainly, subtilisin was followed by dual wavelength spectroscopy at 285 nm by reference at 320 nm. Phalloidin and phallacidin, two toxic bicyclic heptapeptides from the mushroom *Amanita phalloides,* protect F-actin against degradation by the proteolytic enzymes. G-actin, which does not combine with phalloidin when maintained in the monomeric state by working at low ionic strength, and bovine serum albumin, which also has no affinity to the toxin, are hydrolyzed at the same rates in the presence or absence of phalloidin. The proteolysis of F-actin is distinctly retarded by KCl alone, i.e., without phalloidin, whereas Mg^{2+} or Ca^{2+} as sole cations permit a rather high rate of hydrolysis. An even faster degradation of F-actin by subtilisin is observed in the presence of Mg^{2+} plus cytochalasin B. Adenosine diphosphate and triphosphate have no influence on the rate of the enzymatic degradation. The *S* sulfoxide of phalloidin, the nontoxic diastereomer of the toxic *R* form, exerts only a limited inhibitory effect on the enzymatic hydrolysis; secophalloidin, another nontoxic derivative, which does not bind to F-actin has practically no effect.

The phallotoxins, bicyclic heptapeptides from toxic *Amanita* mushrooms (Wieland & Wieland, 1972), bind strongly to F-actin, thus stabilizing the microfilamentous protein against various depolymerizing or denaturing agents (Wieland, 1977a,b). Since enzymatic degradation of proteins is strongly enhanced (Rupley, 1967) by a preceding denaturation, a strengthening of the structure of F-actin could be expected to cause also resistance against the attack of proteases. We, therefore, studied the hydrolytic cleavage of actin by subtilisin, trypsin, and α-chymotrypsin in the presence of some stabilizing agents like K^+ ions, ADP, and ATP, but mainly phalloidin.

As a simple means of following the rate of hydrolysis, we used the difference ultraviolet spectra of proteins and their products of hydrolysis. During changes of the tertiary, secondary, or primary structure, the ultraviolet maxima of proteins undergo shifts to shorter wavelengths and slight decreases in intensity (for a detailed discussion, see Yanari & Bovey, 1960). This is due to the environmental change of the aromatic amino acid side chains which are more or less hydrophobically bound in the native protein and on hydrolysis are exposed to a predominantly aqueous milieu of lower polarizability.

The difference spectra of some proteins and their products of hydrolysis by subtilisin are shown in Figure 1. The minima (or maxima, depending on the arrangement of the cuvettes) appear at 280–285 nm and 290–295 nm corresponding to the chromophores of tyrosine and tryptophan, respectively. The intensities of the (negative) peaks at both wavelengths roughly parallel the relative tyrosine to tryptophan ratios in the respective proteins. In bovine serum albumin this ratio is 19:2 (Peters & Hawn, 1967), in actin from rabbit skeletal muscle 16:4 (Collins et al., 1975), in γ-immunoglobulin 42:20 (Edelman, 1970), and in lactate dehydrogenase M_4 from pig skeletal muscle 22:20 to 20:24 (Pesce et al., 1967).

The rate of enzymatic hydrolysis was measured by following the time course of the decrease of the absorption at 285 nm as related to the (constant) absorption at 320 nm by dual wavelength spectroscopy (Shibata, 1976a,b).

Experimental Section

Materials and Methods. Subtilisins (subtilopeptidase A, EC 3.4.21.14, 5 U/mg), trypsin (from bovine pancreas, EC 3.4.21.4, 33 U/mg), and lactate dehydrogenase (from pig muscle, EC 1.1.1.27) were purchased from Boehringer-Mannheim GmbH; α-chymotrypsin (bovine pancreas, EC 3.4.21.1, 45 U/mg) was from Serva, Heidelberg, W. Germany; bovine serum albumin was obtained from Behringwerke AG, Marburg/Lahn; and γ-globulin (from bovine serum, Cohn-fraction II) was from Biomol, Ilvesheim. Pellet F-actin was a gift from Dr. P. Dancker (Dancker & Hoffmann, 1973). G-actin was prepared from pellet F-actin by depolymerization with 0.1 mM ATP or 0.1 mM ADP in 1 mM Tris-HCl, pH 7.4, at 0 °C for 1 h. F-actin was obtained by homogenizing the pellet in 0.1 M KCl, or by polymerization of G-actin in the presence of 0.1 M KCl and 0.7 mM $MgCl_2$ or 0.7 mM $CaCl_2$. The phallotoxins were samples from our laboratory. Cytochalasin B was from Aldrich Chem. Co. It was added as 10 μL of a 2.1×10^{-2} M solution in *N,N'*-dimethylformamide to 1 mL of the buffer. The solvent alone had no effect in control experiments. The concentrations of actin and of the phallotoxins were determined spectroscopically using $\epsilon_{300} = 1.18 \times 10^4$ M^{-1} cm^{-1} for the phallotoxins. Ultraviolet spectra, difference UV spectra (Wieland et al., 1975), and dual wavelength spectra were obtained with an Aminco DW-1 spectrophotometer. Viscosities were determined with a Cannon-Manning 150 semimicro viscosimeter.

Determination of Rates of Proteolysis. Enzymatic proteolysis was run in cuvettes of 0.438 cm width containing a total of 1.00 mL of solution of the protein and the additional reagents. The rate of hydrolysis was measured, partly in parallel, by: (a) change of absorbance at 285 nm relative to the absorbance at 320 nm using the dual wavelength mode in a 0.438-cm wide cuvette and a slit of 3-nm band pass; (b) viscosimetry (the results are given in η_{spec} (specific viscosity = [outflow time of protein solution/outflow time of solvent] − 1); (c) precipitation of hydrolysis samples at different times

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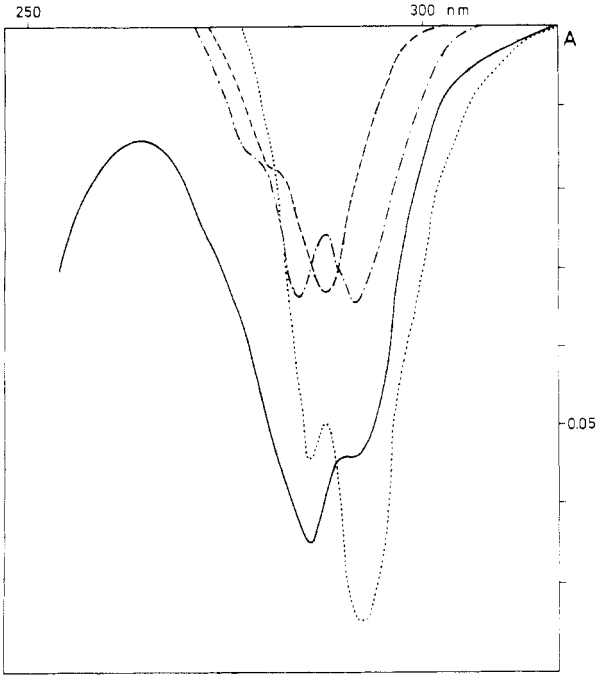

FIGURE 1: Ultraviolet difference spectra of the products of hydrolysis by subtilisin and the native proteins. Subtilisin (0.73×10^{-5} M) was mixed with the protein solutions in 0.1 M KCl, 1 mM Tris-HCl (pH 7.4) in the sample cuvette, incubated at 30 °C for 120 min, and measured against the separated components. (—) F-actin (2.2×10^{-5} M); (- - - -) bovine serum albumin (1.5×10^{-5} M); (·····) γ-globulin (0.67×10^{-5} M); (·····) lactate dehydrogenase from pig muscle (0.7×10^{-5} M).

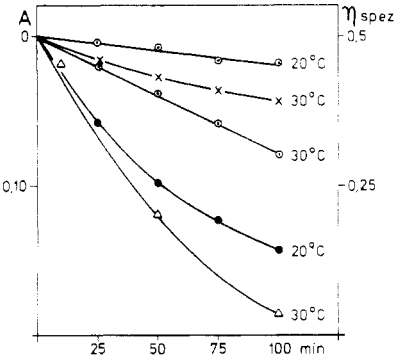

FIGURE 2: Time course of proteolytic degradation of rabbit muscle actins measured by dual wavelength spectroscopy at 285 nm (reference 320 nm) and by viscosimetry. Final concentrations of subtilisin 7.3×10^{-6} M, of trypsin 8.3×10^{-6} M, of F-actin 1.98×10^{-5} M in 0.1 M KCl and 0.1 mM ADP containing 1 mM Tris-HCl of pH 7.4. Final concentration of G-actin was 2×10^{-5} M in 0.1 mM ADP containing 1 mM Tris-HCl of pH 7.4. The experiments were run at 20 and 30 °C as indicated at the curves. (⊙—⊙) F-actin; (●—●) G-actin with subtilisin (in the absence or presence of phalloidin); (X–X) F-actin with trypsin or α-chymotrypsin, all measured spectroscopically; (△—△) F-actin with subtilisin measured by viscosimetry.

with trichloroacetic acid, centrifuging and measuring the absorption of the supernatant at 275 nm (Rick, 1974). The experimental conditions are indicated in the legends of the respective figures.

Results

As shown in Figure 2 the rate of hydrolysis by subtilisin is much higher for G-actin than for F-actin. At 20 °C during the first 20 min G-actin is degraded about 10 times faster than F-actin, which apparently offers much less points for attack due to its compact structure. At 30 °C F-actin is hydrolyzed by subtilisin two to three times faster (Figure 2) than at 20 °C. The degradation of F-actin by an equivalent amount of trypsin or α-chymotrypsin is about 30% slower than by subtilisin. Also shown in Figure 2 is the time course of decreasing viscosity of a solution of F-actin in the presence of subtilisin. One notes that the specific viscosity, η_{spec}, arrives at very low values after 100 min, whereas the difference of absorbance at 285 nm is still increasing after that time interval. Apparently the viscosity of the solvent ($\eta_{spec} = 0$) is nearly attained when the solution still contains relatively large fragments whose further degradation is evident by the spectrophotometric analysis in the dual wavelength spectroscopy. Rates of hydrolysis assessed by spectrophotometric determination of the fragments not precipitated by trifluoroacetic acid roughly parallel that of the results obtained by viscosimetry (data not shown).

The influence of phallotoxins on the rate of proteolytic degradation of F-actin and bovine serum albumin is shown in Figure 3. Phalloidin in a molar ratio of 1:1 prevents the hydrolysis of F-actin almost completely. A small decay during the first 10 min is most probably due to a contamination of the F-actin samples by denatured protein which does not bind phalloidin and therefore is accessible to the enzyme. Gradual lowering of the toxin concentration leads to an increased sensitivity of the protein. In order to relate the protecting effectivity of phallotoxins against proteolysis with that against other influences and with the toxicity of different derivatives, we also examined phallacidin, an equally toxic compound and secophalloidin, which is completely untoxic due to the opening of one peptide ring (Wieland, 1967). We found that phallacidin exerts a protective effectivity comparable with that of phalloidin. Secophalloidin proved completely ineffective. In addition also the *S* sulfoxide of phalloidin was checked in our experiments. This compound, which is formed together with the *R* diastereomer on oxidation of phalloidin by peroxyacetic acid (Faulstich et al., 1968), is nontoxic in the white mouse, in contrast to the *R* compound, which has the same toxicity as phalloidin (LD_{50} = 2.5 mg per kg body weight). The *S* sulfoxide exhibited a small, but significant, inhibitory effect on the hydrolysis of F-actin by subtilisin. The rate of degradation of F-actin by trypsin or by α-chymotrypsin is likewise reduced by an equimolecular concentration of phalloidin. Bovine serum albumin, a protein which does not combine with the toxin (Wieland et al., 1975), is hydrolyzed by subtilisin, trypsin, and α-chymotrypsin at the same rate in the presence or in the absence of phalloidin.

We have also examined whether phalloidin also protects G-actin from enzymatic degradation. Actin is maintained in the monomeric form at low ionic strength and in the presence of adenosine 5-diphosphate. We found that, in 1 mM Tris-HCl, containing 0.1 mM ADP the degradation of actin by subtilisin runs equally fast without and with phalloidin present (Figure 2).

Effect of Metal Ions and of Cytochalasin B on the Rate of Enzymatic Hydrolysis. The stabilizing effect of K^+ ions on the structure of F-actin, well established by several authors (e.g., Löw et al., 1975), could also be proved by the delaying effect of K^+ ions on the proteolytic degradation. As is evident from Figure 4, 0.1 M KCl reduces the rate of actin hydrolysis by subtilisin by a factor of about 4 as compared with the reaction in the absence of K^+, but in the presence of 0.7 mM Mg^{2+} or 0.7 mM Ca^{2+}. Addition of cytochalasin B (CB) to the experiments with K^+ or Ca^{2+} produced no significant accelerating effects. In the presence of Mg^{2+}, however, CB led to a considerably faster hydrolysis of F-actin than that with Mg^{2+} alone (Figure 4).

The stabilizing effect of phalloidin is also dependent on the kind of metal ions; high protection of F-actin from attack by

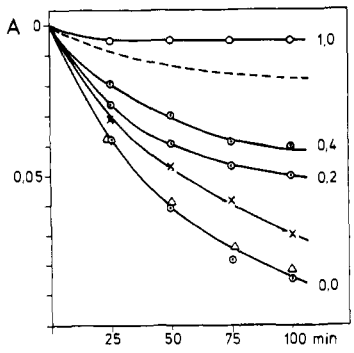

FIGURE 3: Dual wavelength measuring of proteolysis at 30 °C of F-actin (solid lines) and bovine serum albumin (- - - -) by subtilisin in the presence of various concentrations of phalloidin (O—O), of phalloidin sulfoxide A (X—X), and of secophalloidin (Δ—Δ). Concentrations of F-actin and subtilisin and composition of reaction medium as in Figure 2. Molar ratios of phalloidin/actin are indicated at the curves; ratios of phalloidin sulfoxide and of secophalloidin are 1.0. Dashed curve: bovine serum albumin (2.0×10^{-5} M), with and without phalloidin (2.0×10^{-5} M).

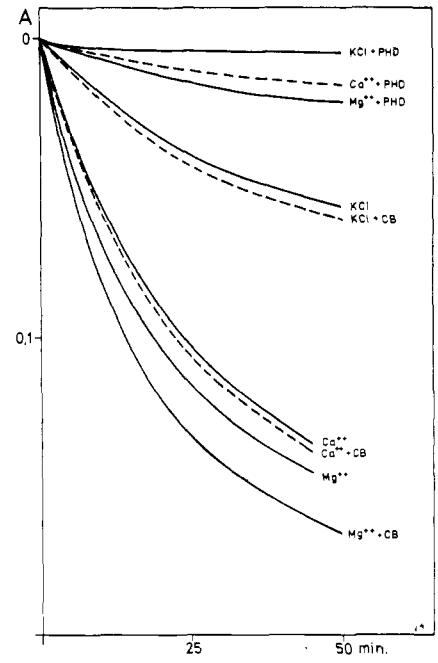

FIGURE 4: Effect of some metal ions and of cytochalasin B (CB) on the degradation of F-actin by subtilisin measured by dual wavelength spectroscopy. F-actin (2.0×10^{-5} M) was obtained by polymerization of G-actin in 0.1 mM ADP containing 1 mM Tris-HCl of pH 7.4 at 4 °C for 15 h in the presence of either one of the following salts: KCl (0.1 M); $MgCl_2$ (0.7 mM); $CaCl_2$ (0.7 mM). Concentration of phalloidin was 2×10^{-5} M, of CB 2×10^{-4} M. CB was added at the beginning of the digestion experiment.

subtilisin is only achieved in presence of K^+ ions (Figure 4). Even after 24 h at 30 °C the viscosity of a solution of F-actin plus phalloidin dropped only by 30% (not shown in Figure 4). The toxin, in a 1:1 ratio, exerts its protective effectivity rather well, but not completely, also in the presence of Ca^{2+} instead of K^+, whereas, with Mg^{2+} as a cation, phalloidin is less effective as a protecting agent.

In order to find out whether the distinct effect of KCl slowing down the rate of proteolysis is due to stabilizing the substrate or inhibiting the enzyme, we investigated the proteolysis of F-actin and of other proteins also by proteases other than subtilisin. We found that KCl equally retards the proteolytic degradation of F-actin by trypsin or by α-chymotrypsin (data not shown). On the other hand, 0.1 M KCl also inhibited the proteolysis of bovine serum albumin by subtilisin as well as trypsin or α-chymotrypsin by a factor of 3 to 4. Lactate dehydrogenase, however, was hydrolyzed by the proteases at the same rate in the absence or presence of 0.1 M KCl. Phalloidin did not influence the rate of proteolysis of proteins other than F-actin by any of the enzymes.

Effect of Adenosine Nucleotides. Whether a retardation of proteolysis is caused by ATP (instead of the usually added ADP) was examined only in the presence of Mg^{2+} ions, because in this milieu where F-actin has a most loosened structure an effect, if any, could be expected. The experiment, however, showed that there is no difference in the hydrolysis rates of F-actin by subtilisin in the presence of adenosine diphosphate or adenosine triphosphate. The slope of the curves was about the same as that of the hydrolysis in the presence of Mg^{2+} alone.

Discussion

The stabilizing effect of phallotoxins on the filamentous structure of F-actin, evident by the resistance against depolymerization by 0.6 M potassium iodide (Löw & Wieland, 1974; Dancker et al., 1975), against denaturation by heat (deVries et al., 1976), against local ruptures either by ultrasonication (Dancker et al., 1975) or by acidic conditions (Löw et al., 1976), or by cytochalasin B (Löw et al., 1975) manifests itself also by a strong inhibition of the proteolytic degradation of F-actin by the toxins. Since also here maximal stability is attained when the molar ratio of phallotoxin to actin protomer is unity the occupation of each G-actin subunit in the filament brings about complete consolidation of the structure. In addition, however, the presence of K^+ ions is indispensable; with Ca^{2+} alone or with Mg^{2+}, the F-actin–phalloidin complex is accessible to the attack of subtilisin although to a small extent. K^+ ions also in the absence of phallotoxins exert a certain protective effect against proteolysis of F-actin. Total resistance of F-actin against rupture by cytochalasin B is also affected by K^+ ions (Löw et al., 1975). The drug induces a reduction of viscosity of a solution of F-actin (Spudich & Lin, 1972) as well as an ATPase activity (as a consequence of reversible ruptures) only in the absence of KCl (presence of Mg^{2+} ions). This reflects the strong polymerizing action of K^+ ions and indicates a stronger adherence of the actin subunits in the presence of K^+ ions rather than Mg^{2+} ions.

Interestingly, F-actin is not the sole protein whose resistance against proteolytic enzymes is enhanced by K^+ ions. The degradation of bovine serum albumin was also found to be retarded by a factor of 3 to 4 in the presence of 0.1 M KCl. That this is not due to an inhibition of the proteolytic enzymes (subtilisin, trypsin, α-chymotrypsin) by the metal ion is evident from the observation that the rate of proteolysis of lactate dehydrogenase (pig muscle) is not influenced by KCl.

Phallacidin, a mushroom component equally toxic as phalloidin, likewise protects F-actin from degradation by subtilisin. The nontoxic *S* sulfoxide which is not able to prevent depolymerization of F-actin by 0.6 M KI (Löw & Wieland, 1974) shows some effectiveness in retarding the proteolysis. This is in accordance with our observation of a limited protective effect against denaturation of F-actin by heating (deVries et al., 1976). There it could be demonstrated by ultraviolet difference spectroscopy that the *S* sulfoxide of phalloidin binds to F-actin with a dissociation constant of the complex about ten times greater than that of the F actin–phalloidin complex. Secophalloidin, a nontoxic derivative which has no affinity to F-actin, has practically no protecting effect against degradation of the protein by subtilisin. G-actin

does not combine with phalloidin and consequently is not protected by phallotoxins from proteolytic degradation.

Phalloidin exerts its protective activity of F-actin against subtilisin at least for 24 h (not measured after longer incubation times). This confirms also former observations in our laboratory that the bicyclic peptide itself is not hydrolyzed by any of numerous protein or peptide cleaving enzymes investigated. The specific protection of F-actin from proteolytic degradation enables an easy removal of non-actin proteins by treatment with proteolytic enzymes and so recovering resistant proteins from various protein mixtures.

Acknowledgment

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Complete Amino Acid Sequence of the Major Component Myoglobin of Finback Whale (*Balaenoptera physalus*)†

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ABSTRACT: The complete amino acid sequence of the major component myoglobin from finback whale, *Balaenoptera physalus,* was determined by the automated Edman degradation of several large peptides obtained by specific cleavages of the protein. Three easily separable peptides were obtained by cleaving with cyanogen bromide at the two methionine residues and one large peptide was isolated after cleavage with (2-*p*-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine. More than 60% of the covalent structure was established by the sequential degradation of three of these peptides and the apomyoglobin. An additional 30% of the primary sequence was established with peptides obtained from tryptic digestion of both the apomyoglobin and the acetimidoapomyoglobin, and the final 10% of the sequence was completed after digestion of the two larger cyanogen bromide peptides with *S. aureus* strain V8 protease. This myoglobin differs from that of the sperm whale, *Physeter catodon,* at 15 positions, from that of the arctic minke whale, *Balaenoptera acutorostrata,* at 3 positions, and from that of the California gray whale, *Eschrichtius gibbosus,* at 4 positions. All of the substitutions observed in this sequence fit easily into the three-dimensional structure of the sperm whale myoglobin.

This report presents the determination of the primary structure of the myoglobin from the finback whale, *Balaenoptera physalus.* In preceding papers, the complete amino acid sequences of several cetacean myoglobins were reported (Dwulet et al., 1975, 1977; Bogardt et al., 1976; Jones et al., 1976; Lehman et al., 1977; Wang et al., 1977). The analytical procedures developed in these papers were utilized in this sequence determination. The finback whale myoglobin was found to differ from that of the arctic minke whale, *Balaenoptera acutorostrata,* at 3 out of the 153 sequence positions.

Experimental Section

Materials

The major component of finback whale myoglobin was

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47401. *Received December 12, 1977.* This is the 93rd paper in a series dealing with coordination complexes and catalytic properties of proteins and related substances. For the preceding paper, see Dwulet & Gurd (1978). This work was supported by U.S. Public Health Service Research Grant HL-05556.

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