Catalytic and Conformational Changes Induced by Limited Subtilisin Cleavage of Bovine Carboxypeptidase A

Beka M. Solomon,‡ Kjeld S. Larsen, and James F. Riordan*

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115

Received February 22, 1990; Revised Manuscript Received April 24, 1990

ABSTRACT: Limited proteolysis of carboxypeptidase A from bovine pancreas with subtilisin Carlsberg generates a stable intermediate, carboxypeptidase S, whose esterase and peptidase activities are increased and decreased, respectively, under standard assay conditions. Carboxypeptidase S was isolated by affinity chromatography. Sequence analysis shows that it is cleaved solely at the Ala^{154}–Gly^{155} bond. Its enzymatic properties were determined under stopped-flow conditions with Dns-Gly-Ala-Phe and its ester analogue Dns-Gly-Ala-OPhe. For both substrates, the K_m values are increased 30–40-fold. The k_{cat} value for peptide hydrolysis is virtually unaffected whereas that for ester hydrolysis is increased 10-fold. The magnitude of the K_m effect is equivalent to a loss of 9 kJ/mol of binding energy and likely reflects a disruption of the network of hydrogen bonds that links Tyr-248 and Arg-145 to the backbone carbonyls of Ala-154 and Gly-155. The difference in k_{cat} effects for the two substrate classes is related to differences in the chemical nature of the rate-determining step. Product release is rate determining for catalytic hydrolysis of ester substrates, and hence, the increase in k_{cat} indicates that dissociation of products is facilitated as a result of the Ala^{154}–Gly^{155} bond scission. The changes in enzymatic activity accompanying limited proteolysis are due to conformational alterations in the vicinity of the active center of the molecule. The affinity of a monoclonal antibody, mAb 100, directed toward the antigenic determinant located between residues 209 and 218 in carboxypeptidase A is diminished considerably for carboxypeptidase S. Presumably, cleavage of the Ala^{154}–Gly^{155} bond induces conformational changes beyond the cleavage region which, in addition to the effect on K_m and k_{cat}, interfere with the accessibility or recognition of mAb 100 to its antigenic epitope.

Ever since the discovery that the conversion of ovalbumin into plakalbumin is the result of limited proteolysis (Linderstrøm-Lang & Ottesen, 1949), proteolytic enzymes have been used widely for structure–function studies of proteins. Subtilisin-catalyzed formation of ribonuclease S from ribonuclease A (Richards, 1958) is one of the best known examples of this approach, but there have been many others [see Neurath (1986)]. Domain structures (Fazel et al., 1983; Edwards et al., 1988), active sites (Monroe et al., 1984), active fragments (Brutlag et al., 1969), local conformation (Harper & Vallee, 1988), and three-dimensional structure (Rupley, 1967) are among the numerous aspects of proteins that have been probed by limited proteolysis.

Carboxypeptidase A (CPD-A)[1] from bovine pancreas is the product of limited proteolysis. It is generated from its parent zymogen by trypsin digestion and is remarkably resistant to further degradation. However, it can be cleaved with subtilisin Carlsberg under specific conditions to yield a derivative, called carboxypeptidase S (CPD-S), whose enzymatic and conformational characteristics are altered (Riordan & Livingston, 1971). The reaction product has now been isolated and characterized by sequence and kinetic analysis. In addition, conformational alterations induced by subtilisin cleavage have been evaluated with a monoclonal antibody (mAb 100) directed to a defined region of CPD-A (Solomon et al., 1989). Site-directed changes in protein structure cause local as well as long-distance perturbations that may not be observed by low-resolution techniques but are easily detected by binding studies with mAbs of this type (Solomon et al., 1989; Collawn et al., 1988). Conformational alterations occurring in CPD-A due to limited proteolysis manifest themselves in corresponding alterations of the binding characteristics of CPD-S to the monoclonal antibody raised against unmodified CPD-A.

Materials and Methods

Carboxypeptidase A from bovine pancreas, prepared according to Cox et al. (1964), was obtained from Sigma Chemical Co. The CPD-A$_\alpha^{Val}$ isozyme[2] was isolated by a minor modification of the procedure developed by Pĕtra and Neurath (1969) followed by affinity chromatography as described by Bazzone et al. (1979). The CPD-A$_\gamma^{Val}$ isozyme was isolated by the same method using the enzyme prepared according to Anson (1937), from Worthington Biochemical Corp. Subtilisin Carlsberg from *Bacillus subtilis* strain Carlsberg (bacterial protease type VIII) was obtained from Sigma.

Enzyme Assays. Routine activity measurements were made spectrophotometrically with the peptide Fa-Phe-Phe (200 μM)

* Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Seeley G. Mudd Building, 250 Longwood Ave., Boston, MA 02115.

‡ Present address: Department of Biotechnology, Tel Aviv University, Tel Aviv 69978, Israel.

[1] Abbreviations: CPD, carboxypeptidase; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CABS-Sepharose, [*N*-(ε-aminocaproyl)-*p*-aminobenzyl]succinyl-Sepharose 4B; Dns or dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; OPhe, L-β-phenyllactate; HPLA, hippuryl-DL-phenyllactic acid; Fa, furanacryloyl; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; APase, alkaline phosphatase.

[2] The isozyme nomenclature was adapted from Pĕtra and Neurath (1969).

0006-2960/90/0429-7303$02.50/0

and the ester HPLA (5 mM) in 0.5 M NaCl/0.05 M Hepes, pH 7.5 and 25 °C. The kinetics of carboxypeptidase-catalyzed hydrolysis of Dns-Gly-Ala-Phe and Dns-Gly-Ala-OPhe were recorded on a Durrum stopped-flow spectrophotometer, Model D13000, equipped with a 75-W mercury–xenon lamp (Illumination Industries, HX-75-7415) and an EMI photomultiplier, Model 9601, type B, interfaced with a low-noise amplifier AR II Analog/Digital converter, PDP 11/34 computer, and VT-55 Decscope (Digital Equipment Corp.) as described (Hanahan & Auld, 1980). The hydrolysis of Dns-Gly-Ala-Phe was monitored via changes in fluorescence properties between substrate and product. The dansyl chromophore was excited with the intense mercury line at 365 nm, and the fluorescence envelope was monitored through a 430-nm cuton optical filter (Larsen & Auld, 1989). Initial rates were either calculated from the initial linear slope of the change in fluorescence intensity vs time or obtained by fitting the entire progress curve to an integrated form of the Michaelis–Menten rate equation (Pollock & Auld, 1982). Steady-state parameters were determined from Lineweaver–Burk plots by least-squares analysis. The plots consisted of at least five different substrate concentrations for which the initial rate was the average of three to five determinations. The time course of carboxypeptidase-catalyzed hydrolysis of the ester substrate, Dns-Gly-Ala-OPhe, was monitored by radiationless energy transfer (excitation 285 nm, emission 340 nm) and steady-state progress curves analysis (Lobb & Auld, 1984). In this recording configuration a 250–400-nm UV band-pass filter (Schott Optical Glass, Inc. Duryea, PA; Model UG-11) was placed in the exit mirror box to eliminate low-intensity visible light from the optical system.

Kinetics of Proteolysis. CPD-A (10 mg/mL) in 2 M NaCl/0.02 M Hepes, pH 7.5, was treated with subtilisin Carlsberg (0.1 mg/mL) at 4 °C, and changes in the enzymatic activity were followed after withdrawing aliquots and treating them with PMSF. The reaction product was subsequently treated with β-mercaptoethanol and analyzed on SDS–polyacrylamide gels (Laemmli, 1970). In control experiments, CPD-A was incubated for up to 5 days with PMSF-inactivated subtilisin Carlsberg but under otherwise identical conditions. Enzymatic activity and the molecular weight of CPD-A were not affected by this treatment.

Preparation of Carboxypeptidase S. Proteolysis was allowed to proceed for 5 days at 4 °C. The reaction was then stopped by addition of 0.01 volume of PMSF (5.2 mg/mL in dioxane), and the products were fractionated by affinity chromatography (Cueni et al., 1980). For this purpose, the reaction mixture was diluted 10-fold with water and adjusted to pH 5.5 with solid Mes. The sample was applied to a column (1.5 × 10 cm) of CABS-Sepharose, equilibrated with 0.2 M NaCl/0.02 M Mes, pH 5.5 at 4 °C. The column was then washed with starting solvent until the absorbance at 278 nm reached the base-line value, and then with 0.02 M Mes, pH 5.5, and 0.02 M Mops, pH 7.0. The column was finally eluted with a 300-mL linear gradient from 0 to 0.5 M NaCl in 0.02 M Mops, pH 7.0. The CPD-S peak was rechromatographed at least twice in the same system, except that a linear gradient from 0 to 0.2 M NaCl was used. Activity was not altered on rechromatography. The rechromatographed products were analyzed on SDS–polyacrylamide gels (Laemmli, 1970) with and without β-mercaptoethanol reduction.

Amino Acid and Sequence Analysis. Amino acid analysis was performed as described (Strydom et al., 1985) using the PicoTag derivatization method (Bidlingmeyer et al., 1984). Automated Edman degradation was done with a Beckman 890 C sequencer with 0.1 M quadrol coupling buffer and Beckman program 121078 as described (Strydom et al., 1985).

Preparation of Monoclonal Antibodies to CPD-A. Mouse monoclonal antibodies to CPD-A were prepared in collaboration with Bio-Yeda, Israel, following the fusion techniques of Köhler and Milstein (1975). The monoclonal antibodies present in ascitic fluids were isolated by precipitation with 50% ammonium sulfate (Goding, 1980) and their binding characteristics with CPD-A and CPD-S were determined by means of a modified ELISA technique (Ball et al., 1982).

ELISA Assays. The antigen-coating solutions (100 μL) containing CPD-A and/or CPD-S (1.0–2.5 μg/mL) in PBS, pH 9.6, were incubated overnight at 4 °C in a polystyrene ELISA plate (Costar, Cambridge, MA). Ascitic fluid (0.1 mL) containing the mAb 100 (Solomon et al., 1989) was diluted 1:1000 to 1:30000 v/v in PBS and added to the plate which was then incubated at 37 °C for 1 h. The apparent amount of mAb bound was determined with β-galactosidase-linked $F(ab)_2$ fragments of sheep anti-mouse IgG (Amersham International, U.K.) (Engvall & Perlmann, 1971; Ball et al., 1982). In another set of experiments, mAb 100 was labeled with APase (Kearney et al., 1979) and its affinity for CPD-A and CPD-S determined in competition assays on polystyrene plates as follows: The plates were coated with CPD-A, 0.1 μg/100 μL, as described above. After washings, 100 μL of a solution containing the same amounts of mAb 100–APase (diluted 1:5000) and increasing amounts of CPD-A or CPD-S (0.01–0.4 μg), preincubated together for 2 h at 37 °C, were added to each of the wells and incubated for another 2 h at 37 °C before determining enzymatic activity (Kearney et al., 1979) of the bound labeled antibody.

Results

Limited Proteolysis of Carboxypeptidase A. Preliminary studies demonstrated that in its native state CPD-A is unaffected by the action of a number of proteolytic enzymes as assessed by their effects on its esterase and peptidase activities (data not shown). With both subtilisin BPN′ and subtilisin Carlsberg, however, time-dependent changes in the two activities were observed to occur concomitant with the formation of low molecular weight peptides. In particular, esterase activity toward the conventional ester substrate HPLA underwent a transient *increase*, suggesting that proteolysis results in the formation of an enzymatically active intermediate species with altered kinetic properties. Conditions for the formation of this species were optimized with subtilisin Carlsberg, the seemingly more specific of the two subtilisins.

As shown in Figure 1, changes in enzymatic activities accompany the time course of subtilisin cleavage of $CPD\text{-}A_{Anson}$. At 4 °C with a subtilisin to CPD-A weight ratio of 0.01, esterase activity increases progressively, reaching a maximum of about 300%, relative to the native enzyme, over a period of 4 days and declining thereafter. Peptidase activity, on the other hand, decreases to about 50% over the same time period. Changes in pH (from 6.8 to 8.2) or ionic strength (0.5–2.5) had little effect on the results.

The activity vs time profile for subtilisin cleavage of CPD-A is markedly dependent on temperature. While it takes 5 days for esterase activity to reach a maximum when digestion is carried out at 4 °C, it only requires 1 day at 10 °C and about an hour at 25 °C. However, the maximum is not as high nor does it persist for very long at the latter two temperatures. Indeed, at 37 °C, esterase activity does not increase at all but instead decreases, although not as rapidly as peptidase activity. In order to minimize this nonspecific degradation, which predominates with increasing temperature, subsequent ex-

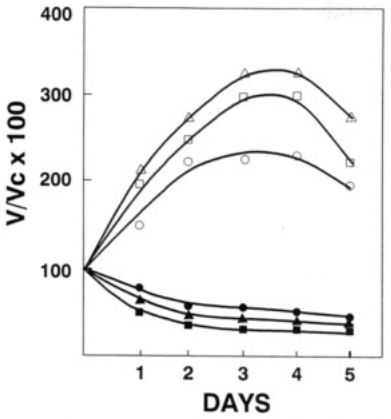

FIGURE 1: Changes in esterase (open symbols) and peptidase (closed symbols) activities during digestion of carboxypeptidase A (10 mg/mL) with subtilisin Carlsberg (0.1 mg/mL) in 2 M NaCl/0.02 M Hepes, pH 7.5 at 4 °C. CPD-A_{Anson} (△, ▲); CPD-A_{γ}^{Val} (□, ■); CPD-A_{α}^{Val} (○, ●).

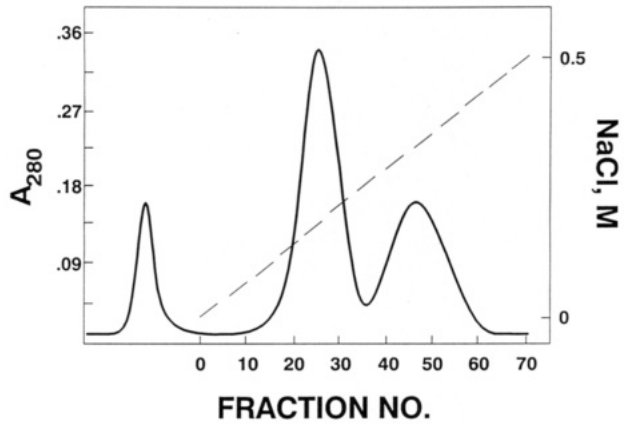

FIGURE 2: CABS-Sepharose chromatography of subtilisin-treated CPD-A_{α}^{Val}. After sample application, the column was washed with starting buffer (0.2 M NaCl/0.02 M Mes, pH 5.5) until the A_{280} returned to base line. After further washing (see Materials and Methods), the bound enzyme was eluted with a linear gradient (0–0.5 M) of NaCl at pH 7.0. The material eluting with a peak at fraction 24 is CPD-S; that at fraction 44 is CPD-A_{α}^{Val}.

periments were typically carried out at 4 °C.

Subtilisin Cleavage of Carboxypeptidase Isozymes. Commercial preparations of CPD-A are not homogeneous but consist of two allotypic variants (Bargetzi et al., 1964) each of which occurs as a mixture of α, β, and γ forms having different N-terminal residues (Neurath et al., 1970). Since N-terminal heterogeneity could confuse the result of amino acid sequence analysis, we decided to separate the isozymes by ion-exchange chromatography. CPD-A_{α}^{Val} and CPD-A_{γ}^{Val} were obtained in the greatest yield, and hence they were tested for their response to subtilisin. Both undergo essentially the same changes in esterase and peptidase activities with the same time course as does unfractionated CPD-A_{Anson} (Figure 1).

Treatment of CPD-A_{α}^{Val} with subtilisin generates a chromatographically distinct species which elutes from the CABS-Sepharose affinity column ahead of the native enzyme (Figure 2). The breakthrough fraction is devoid of enzymatic activity and consists of low molecular weight, dialyzable peptides. The first peak (fraction 24) that elutes after the start of the NaCl gradient is clearly not CPD-A as judged by its enzymatic characteristics whereas that centered at fraction 44 corresponds to native CPD-A_{α}^{Val}. This new, early eluting form of carboxypeptidase is referred to as CPD-S.

Polyacrylamide gel electrophoresis of fractions 24 and 44 in the presence of SDS indicates that both contain a predominant species corresponding to a molecular weight of 34 000 (Figure 3). In the presence of SDS and β-mercaptoethanol, the migration of fraction 44 is unchanged while fraction 24 still has a single band but it now corresponds to a molecular weight of 17 000. Similar results were obtained with CPD-A_{γ}^{Val} (not shown).

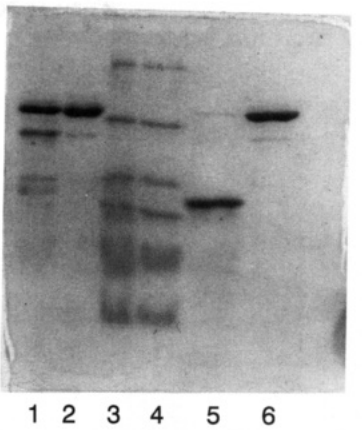

FIGURE 3: SDS–polyacrylamide gel of affinity-purified CPD-A and CPD-S in the absence and presence of β-mercaptoethanol (ME). Lane 1, CPD-A + ME; lane 2, CPD-A − ME; lanes 3 and 4, molecular weight markers; lane 5, CPD-S + ME; lane 6, CPD-S − ME.

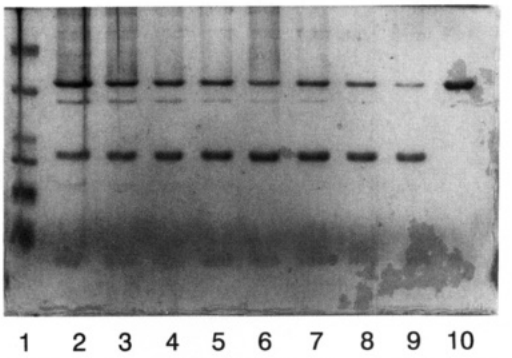

FIGURE 4: Limited proteolysis of CPD-A by subtilisin monitored by SDS–polyacrylamide gel electrophoresis. Molecular weight markers (from Sigma Chemical Co.) were run in lane 1, and untreated CPD-A was run in lane 10. Lanes 2–9 are aliquots of the digestion mixture taken at days 1–8.

The time course of subtilisin digestion of CPD-A_{α}^{Val} was followed by SDS–PAGE under reducing conditions (Figure 4). There is a progressive decrease in the 34-kDa band and a corresponding increase in the 17-kDa band which seems to reach a maximum after 5 or 6 days at 4 °C. After 8 days, the 34-kDa band has almost disappeared, and the 17-kDa band has lost intensity.

Kinetic Properties of Carboxypeptidase S. The ester substrate HPLA, typically used for monitoring proteolysis of CPD-A by subtilisin, exhibits numerous kinetic anomalies that preclude unambiguous interpretation. We therefore selected a matched ester–peptide pair devoid of these problems to evaluate the kinetic consequences of subtilisin cleavage. The steady-state parameters for the hydrolysis of the peptide substrate, Dns-Gly-Ala-Phe, and the corresponding ester substrate, Dns-Gly-Ala-OPhe, catalyzed by the various forms of CPD-A and CPD-S, are listed in Table I. There is little difference between CPD-A_{α}^{Val} and CPD-A_{γ}^{Val} in terms of their response to limited proteolysis. For both, the major change in peptidase activity is a 30–40-fold increase in K_m accompanied by a much smaller (30%) decrease in k_{cat}. The resulting catalytic efficiency for peptide hydrolysis of CPD-S relative to CPD-A is about 2%. For esterase activity, K_m also increases

Table I: Steady-State Parameters[a] for Hydrolysis of the Peptide Substrate, Dns-Gly-Ala-Phe, and the Ester Substrate, Dns-Gly-Ala-OPhe, in 0.5 M NaCl/0.02 M Hepes, pH 7.5, at 25 °C

activity	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^6$ M^{-1} s^{-1})
peptidase[b]			
CPD-A_α^{Val}	190 ± 5.4	20 ± 1.4	9.5
CPD-S_α^{Val}	140 ± 9.4	600 ± 56	0.23
CPD-A_γ^{Val}	160 ● 3.5	21 ± 0.96	7.6
CPD-S_γ^{Val}	134 ± 9.0	710 ± 66.0	0.18
esterase[c]			
CPD-A_α^{Val}	14 ± 0.32	1.0 ± 0.073	14
CPD-S_α^{Val}	180 ± 9.4	36 ± 5.2	5
CPD-S_γ^{Val}	16 ± 0.25	1.3 ± 0.084	12
CPD-S_γ^{Val}	170 ± 9.0	44 ± 6.3	3.9

[a] The estimates of error around the mean were calculated from the standard deviation using the t distribution at the 90% confidence level. [b] From direct excitation recordings. [c] From radiationless energy transfer recordings.

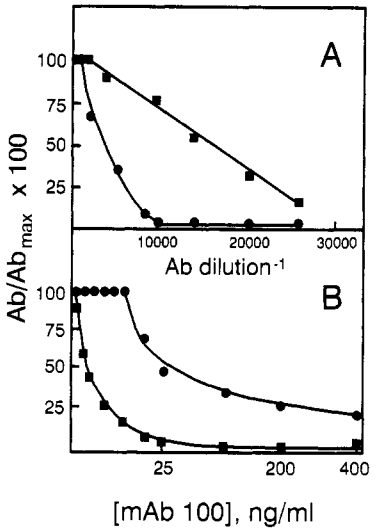

FIGURE 5: Immunochemical characterization of CPD-A and CPD-S using mAb 100. (A) Direct ELISA measurements. (B) Inhibition binding of CPD-A absorbed on the ELISA plates to mAb 100 by either native CPD-A (■) or CPD-S (●) as measured by competitive ELISA. (For details, see Materials and Methods.)

30–40-fold, but in this case, there is a 10-fold increase in k_{cat} as well. As a consequence, the resulting catalytic efficiency for ester hydrolysis of CPD-S relative to CPD-A is 20–40%.

Immunochemical Characterization of Carboxypeptidase S. The changes in catalytic activity associated with subtilisin cleavage of CPD-A are also reflected in antibody binding. The purified monoclonal antibody, mAb 100, raised against the native enzyme, has been shown to bind to an antigenic determinant encompassing residues 209–218 of CPD-A (Solomon et al., 1989). This antibody does not affect the activity of the native enzyme and binds with a relatively high binding constant ($K_{app} = 10^9$ M^{-1}). The interaction of mAb 100 with CPD-A and CPD-S is illustrated in Figure 5. Subtilisin cleavage clearly diminishes binding as measured both by direct and by indirect ELISA. The apparent binding constants in the direct ELISA were derived from the reciprocal of the free monoclonal antibody concentrations at which 50% of the maximal binding of antigen occurs (Pinkard & Weir, 1978). Half-maximal binding for CPD-A was obtained with an antibody dilution of 1:14 800, corresponding to a $K_{app} = 2.2 \times 10^9$ M^{-1}. For CPD-S, this required a dilution of 1:3800, corresponding to a $K_{app} = 5.7 \times 10^8$ M^{-1}. Competitive ELISA experiments carried out with mAb 100 labeled with alkaline phosphatase confirm that binding to CPD-S is weaker than to CPD-A. Inhibition with CPD-S requires about 5 times higher concentration that with CPD-A.

Table II: N-Terminal Sequence Analysis of CPD-A_α^{Val}, CPD-S_α^{Val}, and CPD-S_γ^{Val}

	identification[a]		
cycle	CPD-A_α^{Val}	CPD-S_α^{Val}	CPD-S_γ^{Val}
1	Ala (846)	Asn (165) + Gly (107)	Asn (371) + Gly (1134)
2	Arg (595)	Thr (36) + Ala (160)	Tyr (727) + Ala (1250)
3	Ser (60)	Phe (236) + Ser (18)	Ala (576) + Ser (69)
4	Thr (85)	Asn (160) + Ser (44)	Thr (78) + Ser (27)
5	Asn (488)	Tyr (263) + Ser (23)	Tyr (423) + Ser (24)
6	Thr (73)	Ala (188) + Pro (42)	

[a] PTH-amino acids were identified as described elsewhere (Strydom et al., 1985). The yield of PTH-amino acid in picomoles is given in parentheses.

Chemical Characterization of Carboxypeptidase S. The structure and composition of CPD-S, purified by affinity chromatography, were examined in detail. Its amino acid composition is indistinguishable from that of the uncleaved enzyme isolated from the same chromatographic run. Direct amino-terminal sequencing of CPD-S prepared from CPD-A_α^{Val} through six cycles reveals two sequences (Asn-Thr-Phe-Asn-Tyr-Ala and Gly-Ala-Ser-Ser-Ser-Pro) (Table II) corresponding to residues 5–10 and 155–160. Sequencing of CPD-A_α^{Val} through six cycles gives a single sequence (Ala-Arg-Ser-Thr-Asn-Thr), as expected from the known sequence of the protein (Bradshaw et al., 1969). Thus, subtilisin cleavage of CPD-A_α^{Val} occurs in two places: between residues 4 and 5 and between residues 154 and 155. Since CPD-A_γ^{Val} lacks the first seven residues of CPD-A_α^{Val}, the changes in enzymatic activity, which are seen with both the α and γ forms of the enzyme, must be due to cleavage of the Ala^{154}–Gly^{155} peptide bond. This was confirmed by direct sequencing of CPD-S prepared from CPD-A_γ^{Val}. Two sequences were obtained (Asn-Tyr-Ala-Thr-Tyr and Gly-Ala-Ser-Ser-Ser) corresponding to residues 8–12 and 155–159. Since the N-terminal residue of CPD-A_γ^{Val} is Asn-8, these results are consistent with a single internal cleavage between Ala-154 and Gly-155.

We have no direct evidence other than amino acid analysis to show that CPD-S_γ^{Val} is composed exclusively of CPD(8–154) and CPD(155–307). It seems unlikely that subtilisin has generated any additional amino termini, but some amino acids could have been lost from the carboxy terminus of either fragment. Attempts to establish this by carboxy-terminal analysis were unsuccessful. No free amino acids were released on incubating affinity-purified CPD-S at pH 7.5 for up to 5 days.

Discussion

A notable feature of carboxypeptidase A is its tenacious resistance to proteolytic degradation. Indeed, one of the early methods for purification of the enzyme calls for slices of fresh frozen bovine pancreas to thaw and autolyze on a rack in a cold room for 5 days (Neurath, 1955). After the exuded drippings are neutralized, the enzyme is precipitated and crystallized. It is remarkable that, despite the presence of large amounts of proteases, carboxypeptidase can be obtained intact from the exudate while essentially all other proteins are hydrolyzed.

The present study was initially undertaken to determine if a broad specificity protease, subtilisin, could generate an altered form of CPD-A by limited proteolysis. Preliminary results (Vallee & Riordan, 1968) had shown that both sub-

tilisin BPN′ and subtilisin Carlsberg could induce time-dependent changes in the esterase and peptidase activities of CPD-A. Similar changes were also observed with a derivative of CPD-A in which Tyr-248 was coupled with diazotized arsanilic acid (Riordan & Livingston, 1971).

Initial attempts to characterize the product of subtilisin cleavage of CPD-A were hampered by the lack of affinity-purified enzyme and the scarcity of purified isozymes. In addition, both the substrates and the assay methods then in use were inadequate for definitive kinetic interpretation. Microsequencing and structural analysis with monoclonal antibodies, which have been critical to this investigation, became available only much later. Once all of these tools were in place, however, it was evident that they could be applied conjointly to this problem to great advantage, thereby creating a unique opportunity for studying the structure–function relationships of CPD-A.

Optimal conditions for limited subtilisin cleavage of CPD-A are 4 °C for 5 days with 1% by weight of subtilisin Carlsberg. Higher temperatures are particularly detrimental and result in extensive, nonspecific degradation of carboxypeptidase, perhaps because it is thermally unstable and many of its peptide bonds become susceptible to proteolysis. Alternatively, the product of limited proteolysis, CPD-S, may be thermally unstable and be rapidly degraded when the temperature exceeds 10 °C. In this regard, it should be noted that if zinc is removed from commercial preparations of CPD-A and the apoenzyme is kept in solution, it slowly loses its capacity to be reconstituted. This is due to the action of contaminating serine proteases on the metal-free protein which is much less stable than the metal-containing protein (Bicknell et al., 1985). Under otherwise identical conditions, the zinc enzyme is completely stable, and if the serine proteases are inhibited by PMSF or removed by affinity chromatography, the apocarboxypeptidase remains fully capable of being reconstituted.

Commercial preparations of CPD-A are generally mixtures of isozymes—α, β, and γ—of the two allotypic variants Leu and Val (Bargetzi et al., 1964), which can be separated by ion-exchange chromatography (Pétra & Neurath, 1969). All of these appear to be susceptible to subtilisin cleavage, as has been demonstrated specifically for both CPD-A_α^{Val} and CPD-A_γ^{Val} (Figure 1). Since the time course and magnitude of the changes in activity are about the same for the two forms, they would be expected to be equally prone to limited proteolysis.

Subtilisin generates a new species of carboxypeptidase, CPD-S, which can be detected by either affinity chromatography (Figure 2) or SDS gel electrophoresis (Figure 3). In the absence of a reducing agent, CPD-S has the same electrophoretic mobility as CPD-A, indicating that there is no substantial change in molecular weight. This is consistent with the fact that CPD-A and CPD-S also have the same amino acid composition within the limits of the method. However, in the presence of β-mercaptoethanol, CPD-S migrates as a single band with a molecular weight of about 17000, half that of the native enzyme (Figure 3). This indicates that proteolytic cleavage occurs within the single disulfide loop, i.e., between Cys-138 and Cys-161. Given that CPD-A has 300–307 amino acids (depending on the isozyme), cleavage in this region would generate two fragments, each having about 150 amino acids, of virtually the same size.

N-Terminal sequence analysis demonstrates that subtilisin cleaves the bond between Ala-154 and Gly-155 when it acts on CPD-A_γ^{Val}. This bond is also cleaved in CPD-A_α^{Val} along with the Thr^4–Asn^5 bond. Since the change in activity is the same for both forms of the enzyme, and this change correlates with the appearance of a 17-kDa species, it is compelling to conclude that cleavage of the Ala–Gly bond is the critical event. This bond is located at the surface of the protein, as would be expected, and is also close to the active center (Figure 6). There does not seem to be anything intrinsic to these two amino acids that would favor selective cleavage. More likely, it is the combination of accessibility, regional motility, and sequence of amino acids on either side of this bond that would condition its unusual susceptibility to hydrolysis by subtilisin. Kinetic studies have shown that subtilisin has broad specificity (Ottesen & Svendsen, 1970), and when it has been employed for limited proteolysis in other cases, the bonds cleaved have been quite varied. In both ovalbumin and ribonuclease, an Ala–Ser bond is hydrolyzed (Ottesen & Villee, 1951; Richards & Vithayathil, 1959), but in thermolysin, it is Thr–Gln as well as Gln–Asp (Vita et al., 1985), and in fructose 1,6-bisphosphatase, it is Ala–Gly and Thr–Asn (Botelho et al., 1975). Interestingly, a second Ala–Gly bond in carboxypeptidase between residues 149 and 150 is *not* cleaved by subtilisin, even though it is nearby in the same region of the protein molecule and seemingly exposed (Figure 6). Indeed, this region is devoid of standard secondary structure and is part of a "random" region that extends from residue 122 to residue 174. Although it contains the disulfide bridge between residues 138 and 161, it is a relatively flexible region wherein most of the conformational changes accompanying substrate binding occur (Ludwig & Lipscomb, 1973).

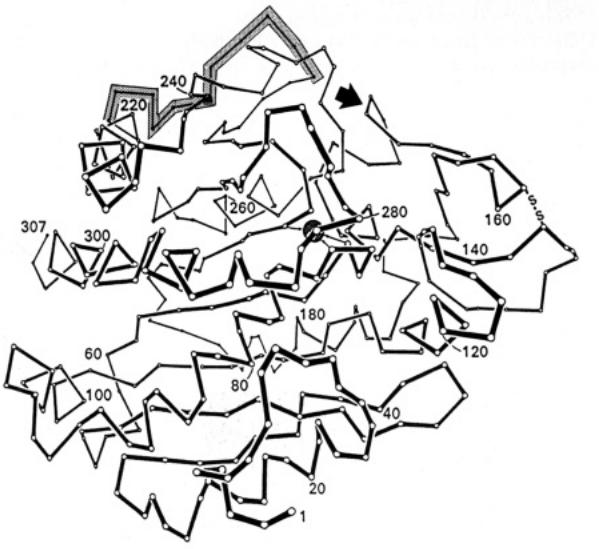

FIGURE 6: Structure of CPD-A [from Lipscomb et al. (1968)] indicating the site of cleavage (arrow) by subtilisin and the recognition region (stippling) of mAb 100.

The subtilisin-induced changes in esterase and peptidase activities are reminiscent of those that occur on chemical modification of tyrosine or arginine in carboxypeptidase (Vallee et al., 1983). Tyr-248 and Arg-145 are both involved in substrate binding and are located close to the Ala^{154}–Gly^{155} peptide bond, as is readily apparent from Figure 6. Moreover, in the free enzyme, a system of hydrogen bonds actually links this peptide bond to Tyr-248 and Arg-145 (Lipscomb et al., 1968). Thus, the guanidinium group of Arg-145 is H-bonded to the peptide carbonyl group of Gly-155. The adjacent carbonyl of Ala-154 is H-bonded to Gln-249, which in turn interacts via a water molecule with the hydroxyl group of Tyr-248. A further interaction of Arg-145 extends to the zinc ligand His-69 via Asp-142. While it is not known what conformational changes result from cleaving the Ala^{154}–Gly^{155}

bond, it seems reasonable that some change should occur and that it would perturb the active site. Indeed, this has been observed via the azophenol moiety of arsanilazotyrosyl-248 carboxypeptidase which is an optically active, environmentally sensitive, active-site probe. Cleavage of azotyrosyl-CPD-A with subtilisin generates esterase and peptidase activity changes that correlate with those in the circular dichroic spectrum of the probe, indicating an alteration in the environment and/or conformation of Tyr-248 (Riordan & Livingston, 1971). Similar alterations no doubt occur with unmodified CPD-A.

The monoclonal antibody mAb 100 recognizes an epitope within residues 208–218 of CPD-A (Solomon et al., 1989) and also binds to CPD-S (Figure 5) but more weakly than it does to the native enzyme. This indicates that subtilisin cleavage perturbs the conformation of this region of carboxypeptidase although residues 208–218 are not directly involved in the proteolytic event. However, they are contained in a loop that is immediately adjacent to the disulfide loop (Figure 6) and is probably close enough to sense a conformational change in that structure.

The principal kinetic consequence of limited proteolysis is a 30–40-fold increase in K_m for both ester and peptide substrates (Table I). Since Gly-155 is one of the residues constituting the substrate binding pocket of the enzyme and Tyr-248 and Arg-145—the two residues with which the Ala^{154}–Gly^{155} peptide bond interacts—are also involved in substrate binding, such a change in K_m is reasonable. It is equivalent to a loss of 9 kJ/mol of binding energy, a value consistent with the disruption of a hydrogen bond. The k_{cat} for peptide hydrolysis is not greatly affected by subtilisin cleavage. The rate-determining step for peptidase activity is bond breaking (Galdes et al., 1986) and apparently does not involve any residues in the segment around Ala-154 and Gly-155. In contrast, the rate-determining step for esterase activity has been shown to be the release of reaction products (Galdes et al., 1986). If product release is also rate limiting for CPD-S, then cleavage of the Ala–Gly bond must facilitate this process. Thus, both substrate binding and product binding are weakened in CPD-S.

Carboxypeptidase has been the object of intense study for over half a century. Steady-state and rapid kinetics, X-ray crystallography, chemical modification, site-directed mutagenesis, and spectroscopic methods, among others, have all been brought to bear to discern its mechanism of action, and the resultant literature is extensive [for recent reviews, see Vallee et al. (1983) and Christianson and Lipscomb (1989)]. No doubt, this multidisciplinary approach has had a synergistic effect on the current level of understanding, since spectral and kinetic data must be examined and interpreted in terms of the known three-dimensional structure and vice versa. It has been suggested that caution should be exercised when extrapolating from the dynamic events occurring in solution to specific interactions in crystals. Nevertheless, the kinetic properties of CPD-S demonstrate that the region around Ala-154 and Gly-155 contributes to the chemical potential of the active site of carboxypeptidase. Perhaps a structural analysis of CPD-S will provide insight and hasten the asymptotic approach to mechanistic truth.

References

Anson, M. L. (1937) *J. Gen. Physiol. 20*, 663–669.

Ball, J. W., Schwartz, A., & Lessard, J. L. (1982) *Biochim. Biophys. Acta 719*, 413–423.

Bargetzi, J.-P., Thompson, E. O. P., Sampath Kumar, K. S. V., Walsh, K. A., & Neurath, H. (1964) *J. Biol. Chem. 239*, 3767–3774.

Bazzone, T. J., Sokolovsky, M., Cueni, L. B., & Vallee, B. L. (1979) *Biochemistry 18*, 4362–4366.

Bicknell, R., Schaeffer, A., Auld, D. S., Riordan, J. F., Monnanni, R., & Bertini, I. (1985) *Biochem. Biophys. Res. Commun. 133*, 787–793.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Botelho, L. H., El-Dorry, H. A., Crivellaro, O., Chu, D. K., Pontremoli, S., & Horecker, B. L. (1977) *Arch. Biochem. Biophys. 184*, 535–545.

Bradshaw, R. A., Ericsson, L. H., Walsh, K. A., & Neurath, H. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1389–1394.

Brutlag, D., Atkinson, M. R., Setlow, P., & Kornberg, A. (1969) *Biochem. Biophys. Res. Commun. 37*, 982–989.

Christianson, D. W., & Lipscomb, W. N. (1989) *Acc. Chem. Res. 22*, 62–69.

Collawn, J. F., Wallace, C. J. A., Proudfoot, A. E. I., & Paterson, Y. (1988) *J. Biol. Chem. 263*, 8625–8634.

Cox, D. J., Bovard, F. C., Bargetzi, J.-P., Walsh, K. A., & Neurath, H. (1964) *Biochemistry 3*, 44–47.

Cueni, L. B., Bazzone, T. J., Riordan, J. F., & Vallee, B. L. (1980) *Anal. Biochem. 107*, 341–349.

Edwards, L. A., Tian, M. R., Huber, R. E., & Fowler, A. U. (1988) *J. Biol. Chem. 263*, 1848–1854.

Engvall, E., & Perlmann, E. (1971) *Immunochemistry 8*, 871–874.

Fazel, A., Müller, K., LeBras, G., Garel, J. R., Vêron, M., & Cohen, G. N. (1983) *Biochemistry 22*, 158–165.

Galdes, A., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 646–651.

Goding, J. W. (1980) *Anal. Biochem. 135*, 221–229.

Hanahan, D., & Auld, D. S. (1980) *Anal. Biochem. 108*, 86–95.

Harper, J. W., & Vallee, B. L. (1988) *J. Protein Chem. 7*, 355–363.

Kearney, J. F., Radbruch, A., Liesegang, B., & Rajewsky, K. (1979) *J. Immunol. 123*, 1548–1550.

Köhler, G., & Milstein, C. (1975) *Nature (London) 256*, 495–497.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Larsen, K. S., & Auld, D. S. (1989) *Biochemistry 28*, 9620–9625.

Linderstrøm-Lang, K. U., & Ottesen, M. (1949) *C. R. Trav. Lab. Carlsberg, Ser. Chim. 26*, 403–454.

Lobb, R. R., & Auld, D. S. (1984) *Experientia 10*, 1197–1206.

Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quiocho, F. A., Bethge, P. H., Ludwig, M. L. Steitz, T. A., Muirhead, H., & Coppola, J. C. (1968) *Brookhaven Symp. Biol. 21*, 24–90.

Ludwig, M. L., & Lipscomb, W. N. (1973) *Inorg. Biochem. 1*, 438–487.

Monroe, D. M., Noyes, C. M., Lundblad, R. L., Kingdon, H. S., & Griffith, M. J. (1984) *Biochemistry 23*, 4565–4572.

Neurath, H. (1955) *Methods Enzymol. 2*, 77–83.

Neurath, H. (1986) *Chem. Scr. 27B*, 221–229.

Neurath, H., Bradshaw, R. A., Pêtra, P. H., & Walsh, K. A. (1970) *Philos. Trans. R. Soc. London 257*, 159–176.

Ottesen, M., & Villee, C. (1951) *C. R. Trav. Lab. Carlsberg, Ser. Chim. 27*, 421–435.

Ottesen, M., & Svendson, I. (1970) *Methods Enzymol. 19*, 199–215.

Pêtra, P. H., & Neurath, H. (1969) *Biochemistry 8*, 5029–5036.

Pinkard, R. N., & Weir, D. M. (1978) in *Handbook of Experimental Immunology* (Weir, D. M., Ed.) Chapter 16, pp 16.1–16.20, Blackwell Scientific, Oxford.
Pollack, S. E., & Auld, D. S. (1982) *Anal. Biochem. 127*, 81–88.
Richards, F. M. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 162–166.
Richards, F. M., & Vithayathil, P. J. (1959) *J. Biol. Chem. 234*, 1459–1465.
Riordan, J. F., & Livingston, D. M. (1971) *Biochem. Biophys. Res. Commun. 44*, 695–701.
Rupley, J. A. (1967) *Methods Enzymol. 11*, 905–917.
Solomon, B., Koppel, R., Kenett, D., & Fleminger, G. (1989) *Biochemistry 28*, 1235–1241.
Strydom, D. J., Fett, J. W., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., & Vallee, B. L. (1985) *Biochemistry 24*, 5846–5494.
Vallee, B. L., & Riordan, J. F. (1968) *Brookhaven Symp. Biol. 21*, 91–119.
Vallee, B. L., Galdes, A., Auld, D. S., & Riordan, J. F. (1983) in *Metal Ions in Biology* (Spiro, T. G., Ed.) Vol. 5, pp 25–75, Wiley, New York.
Vita, C., Dalzoppo, D., & Fontana, A. (1985) *Biochemistry 24*, 1798–1806.

Mechanism for Activation of the 4-Nitrobenzo-2-oxa-1,3-diazole-Labeled Sarcoplasmic Reticulum ATPase by Ca^{2+} and Its Modulation by Nucleotides†

Shigeo Wakabayashi and Munekazu Shigekawa*
Department of Molecular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan
Received January 3, 1990; Revised Manuscript Received April 25, 1990

ABSTRACT: The mechanism for activation of sarcoplasmic reticulum ATPase by Ca^{2+} was investigated in 2 mM $MgCl_2$ and 0.1 M KCl at pH 6.5 and 11 °C by using enzyme preparations in which a specific amino acid residue (Cys-344) was labeled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) [Wakabayashi, S., Imagawa, T., & Shigekawa, M. (1990) *J. Biochem. (Tokyo) 107*, 563–571]. We compared the kinetics of binding and dissociation of Ca^{2+} from the enzyme with those of the accompanying NBD fluorescence changes. The fluorescence rise following addition of Ca^{2+} proceeded monoexponentially. At 2–100 μM Ca^{2+} and in the absence of nucleotides, the Ca^{2+}-induced fluorescence rise and Ca^{2+} binding to the enzyme proceeded at similar rates, which were almost independent of the Ca^{2+} concentration. In contrast, the fluorescence decrease induced by Ca^{2+} removal was slower than the Ca^{2+} dissociation, and both of these processes were inhibited markedly by increasing medium Ca^{2+}. ATP by binding at 1 mol/mol of the phosphorylation site markedly accelerated both the Ca^{2+}-induced fluorescence rise and Ca^{2+} binding, ADP and AMPPNP but not GTP also being effective. In contrast, ADP minimally affected the NBD fluorescence decrease and the Ca^{2+} dissociation. These data are consistent with a reaction model in which binding of Ca^{2+} occurs after the conformational transition of the free enzyme from a state (E_2) having low affinity for Ca^{2+} to one (E_1) having high affinity for Ca^{2+} and in which ATP bound at the catalytic site of E_2, whose affinity for ATP is about 30-fold less than that of E_1, accelerates this conformational transition.

It is well established that the sarcoplasmic reticulum (SR)[1] Ca^{2+}-ATPase is activated by binding of two moles of Ca^{2+} to its high-affinity sites (de Meis & Vianna, 1979; Martonosi & Beeler, 1983). This Ca^{2+} binding is accompanied by a change in the enzyme conformation, which has been detected by changes in the spectral parameters (Inesi et al., 1980; Dupont & Leigh, 1978; Guillain et al., 1980, 1981; Pick & Karlish, 1980), in tryptic digestion patterns (Imamura et al., 1984; Andersen et al., 1985), and in reactivity of amino acid residues of the enzyme (Ikemoto et al., 1978; Murphy, 1978). This Ca^{2+}-induced conformational transition in the Ca^{2+}-ATPase has been shown to be influenced by changes in the experimental conditions such as pH and presence or absence of ATP and/or Mg^{2+} (Inesi et al., 1980; Guillain et al., 1980, 1982; Scofano et al., 1979; Pick & Karlish, 1982; Champeil et al., 1983; Froud & Lee, 1986).

The transient kinetics of ATPase activation by Ca^{2+} have been studied by following enzyme phosphorylation by ATP or P_i (Guillain et al., 1981; Scofano et al., 1979; Sumida et al., 1978; Petithory & Jencks, 1988a,b), a change in the intrinsic fluorescence of the ATPase (Dupont & Leigh, 1978; Guillain et al., 1980; Champeil et al., 1983; Dupont, 1982; Fernandez et al., 1984) and direct binding of radioactive calcium to the enzyme (Dupont, 1982, 1984). The data obtained in these studies showed that Ca^{2+} binding is a complex, multistep process involving enzyme isomerization. According to these and other data (Ikemoto et al., 1981; Inesi, 1987), the two calcium sites on the enzyme are not kinetically identical, and binding and release of Ca^{2+} follow an ordered process.

According to the "E_1–E_2" (or E–E*) model for the Ca^{2+}-ATPase (de Meis & Vianna, 1979), which has been widely used to explain the experimental results, the enzyme activation involves Ca^{2+}-induced transition of the enzyme conformation

†This work was supported by Grant-in-Aid for Scientific Research on Priority Areas (62617524) from the Ministry of Education, Science and Culture of Japan.

[1] Abbreviations: SR, sarcoplasmic reticulum; FITC, fluorescein 5′-isothiocyanate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDANS, *N*-acetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine.

0006-2960/90/0429-7309$02.50/0