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# A conserved glutamic acid bridge in serine carboxypeptidases, belonging to the $\alpha/\beta$ hydrolase fold, acts as a pH-dependent protein-stabilizing element

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**Abstract**

Serine endopeptidases of the chymotrypsin family contain a salt bridge situated centrally within the active site, the acidic component of the salt bridge being adjacent to the catalytically essential serine. Serine carboxypeptidases also contain an acidic residue in this position but it interacts through a short hydrogen bond, probably of low-barrier type, with another acidic residue, hence forming a "glutamic acid bridge." In this study, the residues constituting this structural element in carboxypeptidase Y have been replaced by site-specific mutagenesis. It is demonstrated that the glutamic acid bridge contributes significantly to the stability of the enzyme below pH 6.5 and has an adverse effect at pH 9.5. Carboxypeptidase WII from wheat contains 2 such bridges, and it is more stable than carboxypeptidase Y at acidic pH.

**Keywords:** carboxypeptidase; glutamic acid; hydrogen bond; mutagenesis; *Saccharomyces cerevisiae*

An important feature of the active site of chymotrypsin-like serine endopeptidases is the presence of a salt bridge comprising the $\beta$ carboxylate group of an aspartic acid positioned adjacent to the catalytically essential serine and either the N-terminal amino group, e.g., chymotrypsin (Matthews et al., 1967; Birktoft et al., 1976), or a positively charged side chain, e.g., protease A from *Streptomyces griseus* (Brayer et al., 1978). This salt bridge is essential for maintaining the enzyme in the catalytically active form, and it collapses at high pH (Fersht, 1972).

The serine carboxypeptidases also possess an acidic residue, Glu 145, next to the essential serine (Sørensen et al., 1986, 1987, 1989; Breddam et al., 1987; Svendsen et al., 1993). In CPD-Y from *Saccharomyces cerevisiae* and the homologous carboxypeptidase from wheat, CPD-WII, the side chain of this acidic residue interacts with the side chain of Glu 65 and not with a positive charge as in the endopeptidases (Kinemage 1; Liao et al., 1992; Mortensen et al., 1994). The p$K_a$'s of Glu 145 and Glu 65 in the wild-type enzyme are 4.2 and >9, respectively, the high value of the latter being due to ionization of Glu 145, which stabilizes the carboxylic acid form (Mortensen et al., 1994). When both glutamic acid residues are protonated, and when only Glu 145 is deprotonated, a single hydrogen bond exists between the side chains (Mortensen et al., 1994), thus forming a "glutamic acid bridge."

Examination of the tertiary structures of the serine carboxypeptidases has demonstrated that they belong to a newly identified enzyme fold, the $\alpha/\beta$ hydrolase fold (Ollis et al., 1992). Other members of this fold, acetylcholinesterase from *Torpedo californica* (Sussman et al., 1991) and lipase from *Geotrichum candidum* (Schrag et al., 1991), contain a catalytic triad similar to the one found in serine endopeptidases, although the aspartic acid is substituted for a glutamic acid. Interestingly, these enzymes also possess a glutamic acid pair in the center of the active site (Kinemage 2), indicating that this element is of general importance. This study examines the role of the glutamic acid bridge in stabilizing CPD-Y by substituting the involved residues by site-directed mutagenesis.

## Results and discussion

A role of the Glu 65–Glu 145 bridge in serine carboxypeptidases could be to stabilize the active site. In chymotrypsin the salt bridge organizing the active site collapses when the $\alpha$-amino-

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*Abbreviations:* CAPS, 3-(cyclohexylamino)-1-propansulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CPD-Y, carboxypeptidase Y from yeast; CPD-WII, carboxypeptidase II from wheat; FA, furylacryloyl; Gdn-HCl, guanidinium chloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); MES, 2-(*N*-morpholino) ethanesulfonic acid; SGPA, *Streptomyces griseus* protease A.

group is deprotonated at high pH, thus reversibly inactivating the enzyme (Fersht, 1972). In CPD-Y the glutamic acid bridge could assume a similar role. In a previous paper (Mortensen et al., 1994) it was demonstrated that the mutant enzyme E65A + E145A hydrolyzes the ester substrate FA-Ala-OBzl with an efficiency similar to that obtained with the wild-type enzyme. Thus, disruption of the glutamic acid bridge does not lead to the collapse of the active site as it is observed in chymotrypsin.

The hydrogen bond length between the glutamic acid residues is only 2.71 Å (Mortensen et al., 1994), and this is indicative of a strong, low-barrier hydrogen bond in which the proton is shared between the 2 groups (Cleland, 1992). Such a strong bond could have a profound influence on the stability of the protein. The glutamic acid bridge is predicted to exist when both glutamic acids are protonated and when only one of them is charged. No favorable interactions can be expected when both of them are charged, rather this may destabilize the enzyme due to charge repulsion between the carboxylate groups of the 2 Glu residues.

To test this hypothesis, the stability of a mutant enzyme without the possibility to form a bridge (E65A + E145A) and that of one possessing this capacity but without the ability to ionize (E65Q + E145Q) was investigated. The catalytic parameters for the hydrolysis of the ester substrate FA-Ala-OBzl using E65Q + E145Q were: $k_{cat} = 530 \pm 10$ min$^{-1}$, $K_M = 0.019 \pm 0.001$ mM, and $k_{cat}/K_M = 28{,}000 \pm 2{,}200$ min$^{-1}$ mM$^{-1}$, compared with $k_{cat} = 9{,}100$ min$^{-1}$, $K_M = 0.054$ mM, and $k_{cat}/K_M = 170{,}000$ min$^{-1}$ mM$^{-1}$ using the wild-type enzyme (Breddam, 1984). Thus, the E65Q + E145Q double mutation results in an enzyme with a 6-fold reduced catalytic activity. The low activity of E65Q + E145Q could be due to the altered hydrogen bonding capacity inducing a slight structural change, perturbing the orientation of the adjacent catalytically essential serine (Ser 146).

The rates of inactivation of the wild-type enzyme, of E65A + E145A, and of E65Q + E145Q were determined at pH 3.5, 6.5, and 9.5. At all pH values, the loss of activity with time could be described by first-order kinetics with the 3 enzymes investigated (Fig. 1), indicating the absence of substantial quantities of active intermediates during inactivation. In contrast to chymotrypsin, inactivation of CPD-Y at pH 3.5 and 9.5 was irreversible because the lost activity could not be recovered when the enzyme was transferred to pH 6.5, where CPD-Y was completely stable.

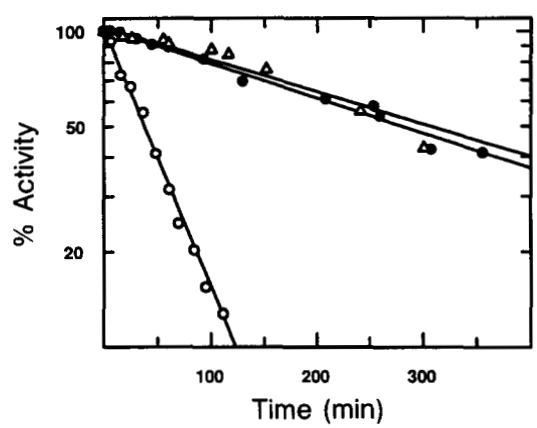


**Fig. 1.** A semilogarithmic plot of the decrease of activity at 25 °C of the wild-type enzyme (●), E65Q + E145Q (△), and E65A + E145A (○) versus incubation time at pH 4.0. Activity was normalized to 100% at time = 0 for each of the enzymes.

The first question was whether the single-charged or uncharged glutamic acid bridge would contribute to the stability of the enzyme or not. At pH 3.5, the fraction of CPD-Y containing a glutamic acid bridge in the double-protonated form dominates (Mortensen et al., 1994). Values of $t_{1/2}$ were determined at pH 3.5 for the wild-type enzyme and compared to the corresponding values obtained with the 2 mutant enzymes. The wild-type enzyme and E65Q + E145Q were found to be equally stable at low pH, with $t_{1/2}$ values of 270 min and 290 min, respectively. E65A + E145A was much less stable, with a $t_{1/2}$ value of only 37 min. Thus, it is evident that the hydrogen bond between the glutamic acids in the wild-type enzyme contributes significantly to the stability of the enzyme and that a similar bond exists between the glutamines in E65Q + E145Q.

At intermediate pH (6.5), the single-charged form of the glutamic acid bridge dominates in the wild-type enzyme. Unfortunately, at this pH the contribution of the glutamic acid bridge to the stability could not be evaluated because a 4-h incubation of the wild-type enzyme, E65A + E145A, or E65Q + E145Q resulted in less than 10% reduction in the activity.

The second part of the hypothesis predicts an adverse effect on stability of the 2 glutamic acids when they both are ionized due to charge repulsion, consistent with previous observations that serine carboxypeptidases generally are rather unstable at basic pH (Breddam et al., 1983, 1987; Breddam, 1988). Unfortunately, the p$K_a$ of the second deprotonation cannot be determined by kinetic investigations, and this prevents studying the stability at a pH where a defined proportion of the enzyme is in the double-deprotonated form. At pH 9.5, the following decreasing order of half lives was determined: E65A + E145A > E65Q + E145Q > wild-type (Table 1). Thus, substitution of the glutamic acid pair for residues without the capacity to ionize results in enzymes that are significantly more stable at high pH compared to the wild-type enzyme. We interpret this as indicative of substantial amounts of the double-ionized form of the wild-type enzyme at pH 9.5. Interestingly, the enzyme without hydrogen bonding capacity at these positions (E65A + E145A) was much more stable at high pH compared to the enzyme possessing the potential to form a stable bridge even at high pH (E65Q + E145Q). The results obtained at acidic pH suggested the existence of a hydrogen bond between the 2 glutamines in E65Q + E145Q. If it also exists at pH 9.5, the results suggest different routes of denaturation at acidic and basic pH values. Alternatively, other structural changes prevent the formation of the hydrogen bond and, under such circumstances, 2 adjacent glutamines may have an adverse effect on stability.

The observation that the glutamic acid bridge, which exists at low and intermediate pH, contributes to the stability of the

**Table 1.** *Values of $t_{1/2}$ (in minutes) for CPD-Y as well as for the derivatives E65A + E145A and E65Q + E145Q at pH 3.5, 6.5, and 9.5*

| | pH 3.5 | pH 6.5 | pH 9.5 |
|---|---|---|---|
| Wild-type CPD-Y | 270 | Stable | 142 |
| E65A + E145A | 37 | Stable | 760 |
| E65Q + E145Q | 290 | Stable | 379 |

enzyme and, when disrupted at high pH, acts as a destabilizing element, warranted further investigations. The pH range in which the glutamic acid pair stabilizes CPD-Y was investigated by subjecting the wild-type enzyme and E65A + E145A to thermal denaturation at different pH values. However, with CPD-Y a low signal (excess heat)-to-noise ratio prevented accurate determinations of heat-induced transitions. Instead, the influence of pH on Gdn-HCl-induced denaturation of the wild-type enzyme and E65A + E145A was investigated by determination of the fraction of unfolded protein ($F_u$) in the pH range 3.5–10. The curves for the pH range 4–8 show a sharp transition of the enzymes from folded to fully denatured state as a function of increasing Gdn-HCl (Fig. 2), indicating that denaturation follows a simple 2-state transition model. However, at high pH with both enzymes and at pH 3.5 with the wild-type enzyme, the denaturation curves appeared complicated, indicating the existence of intermediates on the denaturation pathway.

At low pH, at the concentrations of Gdn-HCl where complete denaturation had not been achieved, $F_u$ values obtained with E65A + E145A always exceeded those obtained with the wild-type enzyme. Accordingly, E65A + E145A is much more sensitive to Gdn-HCl than the wild-type enzyme at low pH. At high pH, the picture is reversed, the $F_u$ values being lower with E65A + E145A compared to those obtained with the wild-type enzyme.

Thus, dependent on pH, the 2 glutamic acids at positions 65 and 145 can act as either a stabilizing or a destabilizing element. In this context, the E65A + E145A mutant is considered independent of pH because the 2 ionizable groups have been removed. Thus, when this mutant is the more stable, it signifies that the presence of 2 glutamic acids in the wild-type enzyme due to charge repulsion adversely affects its stability. On the other hand, when the wild-type enzyme is the more stable, the 2 glutamic acids contribute to the stability. When the 2 enzymes are equally stable, the adverse effect of charge repulsion balances the beneficial effect due to bridge formation. The denaturation curves shown in Figure 2 suggest that this is the situation at approximately pH 6.5. However, this value might be slightly overestimated if the degree of destabilization caused by the truncation of the side chains by changing the glutamic acids to alanines cannot be disregarded.

If it is correct that a glutamic acid bridge is able to stabilize an enzyme at low pH, additional such elements in an enzyme might enhance this effect. The homologous carboxypeptidase CPD-WII contains an additional glutamic acid bridge, i.e., Glu 64–Glu 398, which is situated in the vicinity of the Glu 65–Glu 145 bridge (Kinemage 1; Liao et al., 1992). This was investigated by determination of the sensitivity of CPD-WII to Gdn-HCl. In the pH range 3.5–6 the curves for CPD-WII were essentially identical. Consistent with the model, CPD-WII was significantly more stable than CPD-Y at pH values below 6.0, as illustrated for pH 4.0 in Figure 3. In contrast, at pH 6.0, CPD-Y and CPD-WII were equally stable in Gdn-HCl. However, it should be noted that in comparisons between 2 enzymes, other structural differences may contribute to the observed difference in stability.

## Conclusions

The present study shows that the stability of a protein, rather unexpectedly, can be significantly increased by a pair of acidic

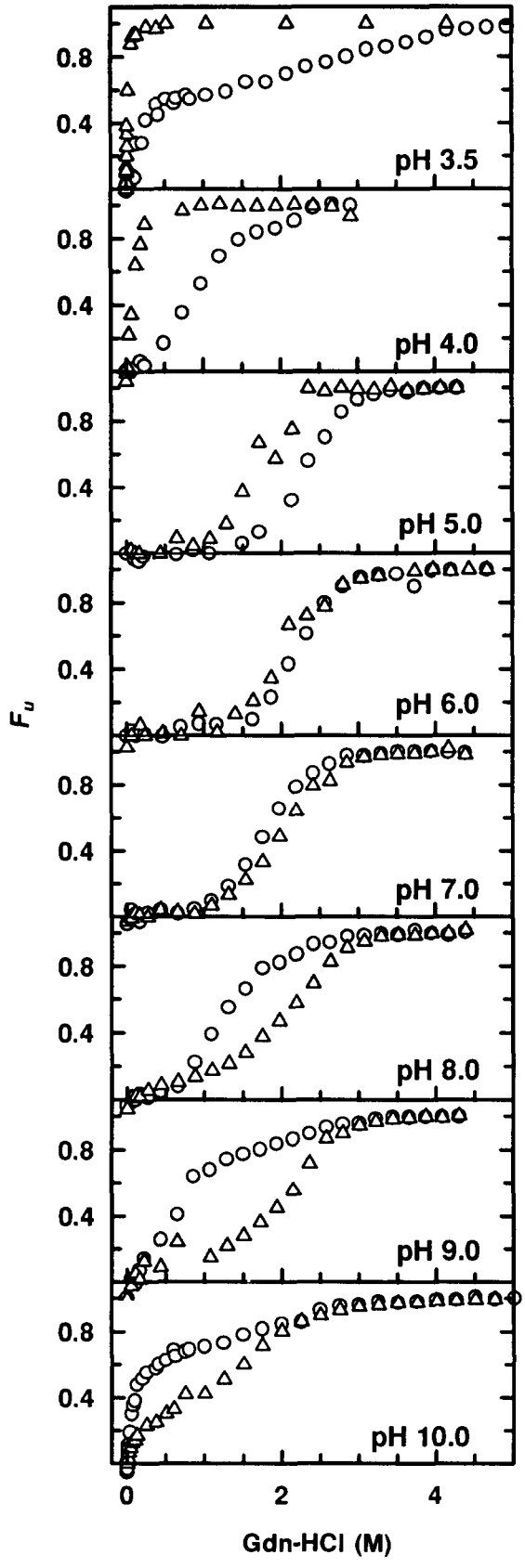


**Fig. 2.** Denaturation of wild-type CPD-Y (○) and E65A + E145A (△) in the pH range 3.5–10 at 25 °C, as determined by measurements of $F_u$ at different concentrations of Gdn-HCl.

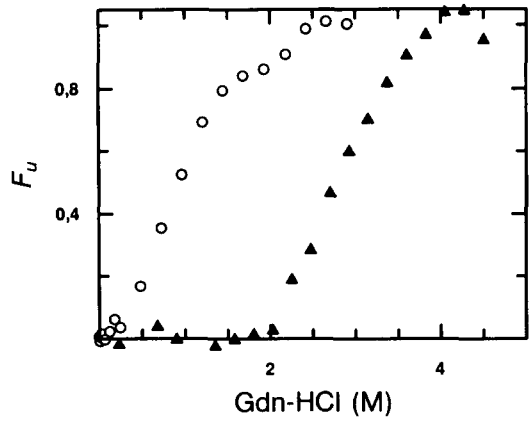


**Fig. 3.** Denaturation of CPD-Y (○) and CPD-WII (▲) at pH 4.0 and 25 °C, as determined by measurements of $F_u$ at different concentrations of Gdn-HCl.

amino acid residues provided that they form a hydrogen bond via their acidic side chains. Thus, even at pH values close to pH 6.5, which is far above the normal p$K_a$ of an acidic side chain, a beneficial effect on the stability of CPD-Y is observed. However, at high pH, the glutamic acid bridge tends to act as a destabilizing element due to charge repulsion. The presence of an additional hydrogen bond in CPD-WII has a further stabilizing effect. In this context, it should be pointed out that numerous serine carboxypeptidases are found in the acidic lysosomes (Breddam, 1986) and, under these conditions, the Glu–Glu bridge may be essential for their stability. Thus, in serine carboxypeptidases the Glu–Glu bridge serves 2 roles because Glu 145 is involved in the recognition of the C-terminal carboxylate group of peptide substrates (Mortensen et al., 1994).

The active site of homologous esterases also contains a glutamic acid pair in the center of the active site, and it has been suggested that they also are involved in stabilization of the protein structure (Schrag & Cygler, 1993). Furthermore, a low-barrier hydrogen bond has been proposed to stabilize transferrin in a pH-dependent manner (Dewan et al., 1993). However, this bond is between 2 unprotonated lysines that stabilize at basic pH and destabilize at acidic pH when they are protonated, i.e., the situation is the reverse of that in CPD-Y.

## Materials and methods

### *Materials*

MES, HEPES, CAPS, and CHES were from Sigma (St. Louis, Missouri). FA-Phe-OMe was from Bachem (Bubendorf, Switzerland). Gdn-HCl was from Fluka (Buchs, Switzerland), and FA-Ala-OBzl and FA-Phe-Ala-OH were synthesized as previously described (Breddam, 1984). Wild-type CPD-Y was from Carlbiotech (Copenhagen), and CPD-WII was prepared as previously described (Breddam et al., 1987).

### *Preparation of mutant enzymes*

E65A + E145A was constructed as published elsewhere (Mortensen et al., 1994), and the same procedure was employed to introduce E65Q + E145Q into the structural gene of CPD-Y by substituting oligo E65A and oligo E145A with CTGTTCTTTC AATTAGGACCC (oligo E65Q) and GGCGTAGGATTGCCC AGCGAT (oligo E145Q) in the PCR reactions. Nucleotides underlined are different from wild type. Mutant enzymes were produced as previously described (Mortensen et al., 1994) and purified by affinity chromatography (Johansen et al., 1976). Both mutant enzyme preparations were homogeneous as determined by SDS-PAGE.

### *Determination of kinetic parameters and $t_{1/2}$ values*

The $k_{cat}$ and $K_M$ values for the hydrolysis of FA-Ala-OBzl with E65Q + E145Q were determined at 25 °C in 50 mM HEPES, 2.5% (v/v) $CH_3OH$, 1 mM EDTA, pH 7.5. Hydrolysis rates were measured spectrophotometrically at 329 nm using a Perkin Elmer λ9 spectrophotometer thermostated to 25 °C. The inactivation of enzymes with time was followed by transferring samples of 500 μL enzyme-stock solution incubated in 50 mM buffer, 1 mM EDTA, into 500 μL assay solution consisting of the same buffer containing 0.4 mM substrate. Incubation of enzymes as well as activity measurements were performed at 25 °C. Assays at pH 9.5 were performed with CAPS as buffer and FA-Phe-OMe as substrate and at pH 3.5 and pH 6.5 with formic acid and MES as buffers, respectively, using FA-Phe-Ala-OH as substrate. The use of an ester substrate at pH 9.5 was dictated by the low peptidase activity at high pH (Breddam, 1984). Values of $t_{1/2}$ were estimated by fitting the results to the following equation:

$$A_t = A_0 \exp[(-\ln 2/t_{1/2})t],$$

where $A_t$ is activity at a given time and $A_0$ is the activity at 0 time, using the nonlinear regression data analysis program Grafit 3.01 (Erithacus Software, London).

The reversibility of the denaturation process was investigated by incubation of an enzyme stock solution in 50 mM formic acid, 1 mM EDTA, pH 3.5, and 50 mM CAPS, 1 mM EDTA, pH 9.5, until approximately 90% of the activity was lost. The resulting enzyme preparation was then diluted 50-fold into 50 mM MES, 1 mM EDTA, pH 6.5. The activity was determined immediately after dilution and followed over 24 h of incubation at 25 °C.

### *Fluorescence measurements*

Fifty microliters of a 0.4–1.2-mg/mL stock of CPD-Y or mutant enzyme was added to 1 mL of 50 mM buffer, 1 mM EDTA containing various concentrations of Gdn-HCl, and incubated for 1 h. The following buffers were used: formic acid (pH 3.5 and 4.0), acetic acid (pH 5.0), MES (pH 6.0), HEPES (pH 7.0 and 8.0), CAPS (pH 9.0), and CHES (pH 10.0). The intrinsic fluorescence of each enzyme sample was monitored using a Perkin Elmer luminescence spectrometer LS50 by measuring emission at 320 nm following excitation at 275 nm at 25 °C, as described by Winther and Sørensen (1991). Gdn-HCl-induced denaturation of CPD-WII was determined in the same way as with CPD-Y except that the emission was measured at 324 nm. The fraction of unfolded protein $F_u$ was calculated using the equation

$$F_u = (Y_f - Y_{obs})/(Y_f - Y_u),$$

where $Y_{obs}$ is the observed intrinsic fluorescence at a given GdnHCl concentration and $Y_f$ and $Y_u$ are the values of fluorescence of the folded and unfolded conformations, respectively. Values of $Y_f$ and $Y_u$ were obtained by extrapolation of the linear portions of the pre- and posttransition regions of the unfolding curve, respectively.

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