Interaction of aspartic acid-104 and proline-287 with the active site of m-calpain

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In an ongoing study of the mechanisms of calpain catalysis and Ca^{2+} -induced activation, the effects of Asp-104 $\rightarrow$ Ser and Pro-287 $\rightarrow$ Ser large subunit mutations on m-calpain activity, the pH-activity profile, Ca^{2+} -sensitivity, and autolysis were measured. The importance of these positions was suggested by sequence comparisons between the calpain and papain families of cysteine proteinases. Asp-104 is adjacent to the active-site Cys-105, and Pro-287 is adjacent to the active-site Asn-286 and probably to the active-site His-262; both Asp-104 and Pro-287 are absolutely conserved in the known calpains, but are replaced by highly conserved serine residues in the papains. The single mutants had approx. 10–15% of wild-type activity, due mainly

to a decrease in k_{cat} , since K_m was only slightly increased. The Pro-287 $\rightarrow$ Ser mutation appeared to cause a local perturbation of the catalytic Cys-105/His-262 catalytic ion pair, reducing its efficiency without major effect on the conformation and stability of the enzyme. The Asp-104 $\rightarrow$ Ser mutation caused a marked narrowing of the pH-activity curve, a 9-fold increase in Ca^{2+} requirement, and an acceleration of autolysis, when compared with the wild-type enzyme. The results indicated that Asp-104 alters the nature of its interaction with the catalytic ion pair during Ca^{2+} -induced conformational change in calpain. This interaction may be direct or indirect, but is important in activation of the enzyme.

INTRODUCTION

The calpains (EC 3.4.22.17) are a family of Ca^{2+} -dependent cytoplasmic cysteine proteinases [1,2]. Two forms (μ - and m-) have been isolated from mammalian tissue, and differ in their *in vitro* requirements for Ca^{2+} , μ -calpain requiring 50–80 μM Ca^{2+} for activity, and m-calpain requiring 300–500 μM Ca^{2+} . In addition to these two widely distributed forms, cDNA sequences for other members of the calpain family have been reported, although the corresponding proteins have not yet been detected *in vivo* [3]. Both μ - and m-calpain consist of an 80 kDa catalytic subunit and a 30 kDa regulatory subunit. On the basis of amino acid sequence, the large subunit was described as containing four domains (I–IV) [4]. The functions of domains I and III are unclear, but domain II contains the active-site residues and shows a limited amino acid sequence similarity to the papain-like proteinases [5,6]; and domain IV is a Ca^{2+} -binding domain, containing several EF-hand motifs. The small subunit contains two domains [7], a glycine-rich N-terminal domain V which is removed during autolysis, and a C-terminal Ca^{2+} -binding domain VI which is similar to domain IV. The regulation of calpain by Ca^{2+} is assumed to involve a conformational change induced by Ca^{2+} binding to domains IV and VI, which in some way not yet understood affects the active-site residues in domain II.

On the basis of sequence comparisons, it seemed likely that the active-site residues in calpain were Cys-105, His-262 and Asn-286 (m-calpain numbering) [4,5,8]. The identity of Cys-105 was first established by labelling with [^{14}C]iodoacetic acid [9], and the importance of all three residues to catalysis has been confirmed by mutagenesis [10]. The three-dimensional structure of calpain is not known, and apart from a few positions around these active-site residues, the sequence identities between calpain domain II and papain are too low for structural modelling on the known three-dimensional structure of papain. This makes it

difficult to predict which other calpain residues may be involved in the active site, but two differences which stand out in the sequence alignments of the calpain and papain families [5] are at residues Asp-104 and Pro-287, which are absolutely conserved in all forms of calpain. In contrast, the equivalent positions in the papain family are occupied by highly conserved serine residues. Asp-104 is located in the primary sequence between Gln-99 (which is assumed to be part of the oxyanion hole) and the active-site Cys-105. As a further reason for believing that Asp-104 might be important to the mechanism of calpain, a kinetic study of the reaction of calpain with dipyrindyl disulphide led to the suggestion that the Cys[−]/ImidH⁺ catalytic ion pair (Cys-105/His-262) was influenced by another dissociating group, either Asp, Glu or His 'in a relatively hydrophobic environment' [11]; Asp-104 is clearly a reasonable candidate for this group. It was of interest therefore to determine the effects of the mutation Asp-104 $\rightarrow$ Ser on calpain, i.e. of changing the conserved calpain Asp-104 to the conserved papain serine residue.

A second marked difference is at residue Pro-287 in m-calpain, which is totally conserved in the sequence Asn-Pro-Trp in all known calpains, while the equivalent sequence in the papain family is almost invariably Asn-Ser-Trp (rarely Asn-Thr-Trp). In m-calpain, Asn-286 is part of the active site, and Trp-288 is thought to contribute either to substrate binding or to stabilization of the active-site His-262 [10,12], so that mutations at position 287 might also be expected to affect catalysis. It was therefore of interest to observe the effects of changing the conserved calpain Pro-287 to the conserved papain serine residue.

We report here a comparison of some properties of recombinant m-calpain with single Asp-104 $\rightarrow$ Ser and Pro-287 $\rightarrow$ Ser mutations, and the double Asp-104 $\rightarrow$ Ser, Pro-287 $\rightarrow$ Ser mutation. The Pro-287 $\rightarrow$ Ser mutation had relatively small effects, but the properties of the Asp-104 $\rightarrow$ Ser mutant suggest that Asp-104, as part of the Ca^{2+} -induced conformational change in

Abbreviations used: DTT, dithiothreitol; β -ME, β -mercaptoethanol; MCA, 7-amino-4-methylcoumarin; Succ, N-succinyl.

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calpain, forms a new interaction with the catalytic ion pair. This interaction may be direct or indirect, but is important in activation of the enzyme.

EXPERIMENTAL

Expression systems and mutagenesis

cDNA clones and expression systems for the rat m-calpain large subunit with a C-terminal His-tag, and for a 21 kDa fragment of the rat calpain small subunit, have been described previously [13,14]. Co-expression of the cDNA constructs pET-24d-80k-CHis₆ (80 kDa large subunit) and pACpET-21k (C-terminal domain of the small subunit, referred to as the 21 kDa subunit) gives rise to an active (80 + 21 kDa) calpain, which is referred to in this paper as (wild-type) wt-80k/21k.

Site-directed mutagenesis was performed on single-stranded DNA from pET-24d-80k-CHis₆, using the antisense primers 5'-P-agccagaagccagcagctgccaagggtccc-3' (Asp-104 → Ser), and 5'-P-tgtccagctgttctgatacg-3' (Pro-287 → Ser). The sequence around the mutated sites was confirmed by DNA sequencing. The Asp-104 → Ser mutant large subunit was co-expressed both with the 21 kDa small subunit (D104S-80k/21k), and with a full-length rabbit calpain small subunit which carried an N-terminal His-tag (D104S-80k/NHis₁₀-30k; J. S. Elce, J. S. C. Arthur and C. Crawford unpublished work). The Pro-287 → Ser single-mutant and the Asp-104 → Ser, Pro-287 → Ser double-mutant large subunits were co-expressed with the 21 kDa small subunit (P287S-80k/21k; D104S, P287S-80k/21k). Purification of wild-type and mutant proteins was achieved by chromatography on columns of DEAE-Sepharose, Ni-NTA-agarose and Spectra/Gel AcA-44 [14]. All procedures with calpain were performed in the presence of 10 mM β-mercaptoethanol (β-ME) and 5 mM EDTA, unless otherwise stated.

Active-site titration

The active-site concentration was titrated using an irreversible inhibitor for calpain, carbobenzoxy-Leu-Leu-Tyr-CHN₂ (kindly provided by Dr. E. Shaw [15]). Calpain was incubated with Ca²⁺ and various concentrations of inhibitor smaller than the concentration of calpain, in 0.1 M NaCl, 10 mM β-ME, 0.1 M Tris/HCl, pH 7.2 (for D104S-80k/21k), or 7.4 (for P287S-80k/21k and wt-80k/21k). After 14 min for P287S-80k/21k and wt-80k/21k, or 45 s for D104S-80k/21k, the reaction was diluted into a casein digestion assay and the residual activity determined over 30 min at 25 °C. Active-site concentrations were obtained by extrapolation from graphs of residual activity against total inhibitor concentration.

Casein digestion assay

Casein (2 mg) in 100 mM Tris/HCl, pH 7.2, 10 mM β-ME and either 5 mM EDTA or 5 mM CaCl₂ was incubated with calpain in a final volume of 0.4 ml. After incubation for 30 min at 25 °C the reaction was terminated by addition of 0.3 ml of ice-cold 10 % (w/v) trichloroacetic acid. After standing in ice, the samples were centrifuged at 1200 *g* for 10 min, and the A₂₈₀ of the supernatant was recorded. One unit of activity is defined as a Ca²⁺-dependent increase of 0.1 absorption unit over 30 min at 25 °C.

Ca²⁺ dependence

The Ca²⁺-dependence of calpain activity was measured in 100 mM Mops/NaOH, pH 7.2, and the free Ca²⁺ concentration

was varied between 0 and 12 mM. Data were fitted to the equation for co-operative binding:

$$Y = [\text{Ca}^{2+}]^n / (K + [\text{Ca}^{2+}]^n)$$

where *Y* is the activity expressed as a fraction of the maximum, *n* is the Hill constant, *K* = [Ca²⁺]_{0.5}^{*n*}, and [Ca²⁺]_{0.5} is the Ca²⁺ concentration required for half-maximal activity.

Fluorogenic peptide substrate assay using Succ-Leu-Tyr-MCA (Sigma)

Calpain (10–20 μg of wt-80k/21k or 150–250 μg of D104S-80k/21k and P287S-80k/21k) was incubated with the peptide (0.02 to 1.5 mM) in a final volume of 2 ml of 0.1 M Mops/NaOH, pH 7.2, containing 0.1 % (v/v) Triton X-100, 2 % (v/v) DMSO and 1 mM dithiothreitol (DTT), at 24 °C with constant stirring. For measurement of variation of activity with pH, a mixture of 33 mM Tris, 33 mM Mops and 33 mM Mes was adjusted with HCl or NaOH to the desired pH values. Reaction was started by addition of CaCl₂ to a final concentration of 5 mM, and the change in fluorescence was observed (excitation wavelength = 360 nm; emission wavelength = 460 nm) in a Perkin-Elmer LS50 fluorimeter controlled using Hewlett Packard data acquisition software. Control samples were observed in the presence of 5 mM Ca²⁺ without substrate, and in 5 mM EDTA with substrate, and the machine was calibrated with a standard solution of 7-amino-4-methylcoumarin (MCA).

In the fluorogenic peptide assay, initial rates at various substrate concentrations were measured from the first 10–20 s of the fluorescence traces. To obtain *K_m* and *k_{cat}*, the data were analysed by non-linear regression to the simple Michaelis-Menten equation:

$$v_0 = (k_{\text{cat}} \cdot E_0 \cdot S) / (K_m + S)$$

To estimate p*K* values, a three-protonation-state model was assumed, characterized by only two kinetically important apparent p*K* values, p*K*₁ and p*K*₂, and in which only the species EH can catalyse significant reaction [16]:

In these assays, the substrate concentration (*S*) was held at values ≤ 0.14 × *K_m*, and it was assumed that in these conditions at any given pH:

$$v_0 \approx E_0 \cdot S \cdot (k_{\text{cat}} / K_m)_{\text{obs}}$$

where (*k_{cat}*/*K_m*)_{obs} refers to the values obtained at a given pH value. Even smaller values of [*S*] are desirable to strengthen this assumption, but were not feasible because the observed initial velocities were already at a minimum for accurate measurement. For the three-protonation-state model, apparent p*K* values can be obtained from the equation:

$$(k_{\text{cat}} / K_m)_{\text{obs}} = \frac{(k_{\text{cat}} / K_m)_{\text{lim}}}{\left(\frac{[\text{H}^+]}{K_1} + 1 + \frac{K_2}{[\text{H}^+]} \right)}$$

where (*k_{cat}*/*K_m*)_{lim} is the theoretical specificity constant for the fully active enzyme [16].

RESULTS

Expression

The various mutant and wild-type forms of calpain were all expressed as stable heterodimeric complexes in *Escherichia coli*,

Table 1 Kinetic constants observed at pH 7.2 and 24 °C with the substrate Succ-Leu-Tyr-MCA

Calpain form	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
wt-80k/21k	0.015 ± 0.0005	0.174 ± 0.016	86.2
D104S-80k/21k	0.0023 ± 0.0001	0.286 ± 0.042	8.04
P287S-80k/21k	0.0049 ± 0.0002	0.238 ± 0.034	20.5

Table 2 Apparent pK values and $(k_{\text{cat}}/K_{\text{m}})_{\text{lim}}$ values of calpains measured at 21 °C with the substrate Succ-Leu-Tyr-MCA

The Table shows these values, and the value of $(\text{pK}_2 - \text{pK}_1)$, obtained by the best fit of the rate data (Figure 1) to the equation for the pH-activity profiles based on the three-protonation-state model [16].

Calpain form	pK_1	pK_2	$\text{pK}_2 - \text{pK}_1$	$(k_{\text{cat}}/K_{\text{m}})_{\text{lim}}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
wt-80k/21k	5.69 ± 0.07	8.16 ± 0.07	2.47	65.0 ± 2.4
D104S-80k/21k	6.45 ± 0.17	7.20 ± 0.15	0.75	10.9 ± 2.1
P287S-80k/21k	6.10 ± 0.09	7.21 ± 0.09	1.11	33.0 ± 3.1

Figure 1 pH-activity profiles of (A) D104S-80k/21k and wt-80k/21k calpain, and (B) P287S-80k/21k calpain

Succ-Leu-Tyr-MCA was used at 0.04 mM, and initial rates were measured and analysed as described in the Experimental section; all data points are shown and the curves are the best fit to the three-protonation-state model [16]. In (B) the fitted curve (but not the data points) for the wild-type enzyme is reproduced from (A) for comparison. Key to symbols: ∇ , wt-80k/21k; $\bullet$, D104S-80k/21k; $\square$, P287S-80k/21k. The pK values and $(k_{\text{cat}}/K_{\text{m}})_{\text{lim}}$ values derived from curve fitting are shown in Table 2. Note that several different y-axis ranges are used.

and behaved identically during purification by ion-exchange, nickel-affinity and gel-filtration chromatography. The yields were also similar in terms of protein, giving 3–4 mg of purified calpain per litre of culture, and the products had 1:1 subunit stoichiometry, as judged by laser densitometry of Coomassie Blue-stained SDS gels. In the casein assay at pH 7.2, the specific activities of D104S-80k/21k, P287S-80k/21k and D104S-80k/NHis₁₀-30k were all in the range of 200–250 units/mg, approx. 11–14% of the specific activity of wt-80k/21k (results not shown). The double mutant D104S,P287S-80k/21k showed 0.5–1% of wt-80k/21k activity in the casein assay, and was not further examined.

Active-site titrations were performed with carbobenzoxy-Leu-Leu-Tyr-CHN₂, since its reaction with calpain is much faster than that of E-64 [15]. This was particularly important for the D104S mutant as short reaction times had to be used due to the very rapid autolysis of the enzyme (see below). Values of 0.7–1.3 mol of active site per mol of calpain were observed for all the enzymes.

Kinetic constants

The values of k_{cat} and K_{m} at pH 7.2 obtained from plots of v_0 versus concentration of Succ-Leu-Tyr-MCA are given in Table 1, and show that the K_{m} values of the single mutants for the peptide were only slightly larger (1.4-fold and 1.6-fold) than that of the wild-type enzyme, but the k_{cat} values were significantly smaller (P287S-80k/21, 3-fold; D104S-80k/21k, 6.5-fold) than the wild-type value.

pH dependence

The single mutants had apparent pH optima close to pH 7.2 with casein as substrate, whereas the wild-type enzyme had a broad pH optimum from pH 7.2 to 8 (results not shown). The full pH range could not, however, be studied with casein because of its poor solubility at lower pH values. In the pH/activity measurements with Succ-Leu-Tyr-MCA (which remains soluble throughout the desired pH range and has no pK value within this range), the mutant calpains displayed pH optima at 6.7, slightly lower than that of the wild-type enzyme at 6.8–7.0; but the P287S-80k/21k curve was narrower than the wild type, and the D104S-80k/21k curve was even more narrow (Figure 1). The apparent pK values obtained by fitting of the pH-activity data for the fluorescent substrate to the equation for a three-protonation-state model [16] are given in Table 2. The differences between pK_1 and pK_2 were 0.75 for D104S-80k/21k, 1.11 for P287S-80k/21k and 2.47 for wt-80k/21k (Table 2).

Figure 2 Ca^{2+} requirement for activity of mutant and wild-type calpains

Casein assays were carried out in triplicate at 25 °C in 100 mM Mops/NaOH, pH 7.2, 10 mM β -ME, using free Ca^{2+} concentrations from 0 to 12 mM. Key to symbols: ∇ , wt-80k/21k; $\bullet$, D104S-80k/21k; $\square$, P287S-80k/21k.

Ca^{2+} -dependence

The Ca^{2+} -dependence of activity was measured at pH 7.2 with casein. The results (Figure 2) showed that D104S-80k/21k required 0.82 ± 0.017 mM Ca^{2+} for 50% of maximum activity, 9-fold greater than the value of 0.088 ± 0.002 mM Ca^{2+} required for the wild-type enzyme. The Ca^{2+} requirement of P287S-80k/21k, 0.067 ± 0.002 mM Ca^{2+} , was not greatly different from that of the wild-type enzyme, although the co-operativity of the curve was slightly altered. Hill constants of 1.75 ± 0.07 for P287S-80k/21k, 2.0 ± 0.08 for D104S-80k/21k and 2.4 ± 0.14 for wt-80k/21k were obtained. (These measurements were made in conditions of low ionic strength, and the Ca^{2+} requirements are lower than normally reported for m-calpain; we have found recently that the Ca^{2+} requirements of all these calpains are greater at higher NaCl concentrations, but that the differences shown in Figure 2 are maintained; J. S. Elce, C. Hegadorn and J. S. C. Arthur, unpublished work).

Autolysis

On incubation without substrate at 20 °C in the presence of 4 mM Ca^{2+} , both D104S-80k/21k and wild-type enzymes autolysed and lost activity. In both cases, the amount of enzyme activity remaining at a given time correlated with the remaining amount of apparently intact 80 kDa subunit measured by densitometry of a gel (Figure 3). However, the rate of loss of activity and of apparently intact 80 kDa subunit from D104S-80k/21k was much greater than from wt-80k/21k, even though the catalytic efficiency of D104S-80k/21k is much less than that of wt-80k/21k. The pattern and amounts of autolysis products in the range of 25–75 kDa derived from the 80 kDa subunits also differed between the two enzymes (Figure 4). While the D104S-80 kDa subunit was lost more rapidly, the 21 kDa subunit, which is identical in both enzymes and is usually thought to be relatively proteinase-resistant, was cleaved more extensively during wt-80k/21k autolysis than during D104S-80k/21k autolysis (Figure 4). This is presumably because the more rapid degradation of the

Figure 3 Rate of inactivation by autolysis, and of degradation of the large subunit

Calpain (0.5 mg/ml) was incubated with stirring in 4 mM Ca^{2+} , 100 mM NaCl, 10 mM β -ME, 100 mM Tris/HCl, pH 7.3, at room temperature. At 0, 1, 2, 3, 6, 12 and 24 min, samples were removed and autolysis stopped by addition of excess EDTA. Residual activity (open symbols) was determined using the casein assay, and the extent of large subunit degradation was determined by laser densitometry of the 80 kDa band on a Coomassie Blue-stained SDS gel (closed symbols). Error bars represent the S.D. of three observations for each assay. (∇ , $\bullet$), wt-80k/21k; ($\circ$, $\bullet$), D104S-80k/21k.

Figure 4 Autolysis products of D104S-80k/21k and wt-80k/21k calpains

SDS gel of wt-80k/21k and D104S-80k/21k before and after incubation in 4 mM Ca^{2+} , 100 mM NaCl, 10 mM β -ME, 100 mM Tris/HCl, pH 7.3, at 20 °C for 24 min. The tracks contain: lanes 1 and 6, molecular mass standards (from the top, 97, 66, 45, 31, 21 and 14 kDa); lane 2, wt-80k/21k, not autolysed; lane 3, wt-80k/21k, autolysed; lane 4, D104S-80k/21k, autolysed; lane 5, D104S-80k/21k, not autolysed.

D104S-80 kDa subunit had abolished activity of the mutant before it had time to degrade its 21 kDa subunit, while the wild-type enzyme, of which part remained active for much longer, had more time in which to degrade the 21 kDa subunit. These differences were observed equally, whether the mutant and wild-type enzymes were incubated in conditions of equal protein concentration or of equal activity concentration. P287S-80k/21k autolysis appeared to be very similar to that of the wild-type enzyme and was not further studied.

Figure 5 Thermal stability of D104S-80k/21k and wt-80k/21k calpains

Calpain (0.75 mg/ml) was incubated in 100 mM Mops/NaOH, pH 7.4, 100 mM NaCl, 5 mM EDTA, 10 mM β -ME at 55 °C. Samples were removed at 0, 1, 2, 4 and 6 min intervals into ice-cold casein assay mixture with Ca^{2+} or EDTA. The remaining activity was measured by incubation for 30 min at 25 °C. Error bars represent the S.D. of three observations. Key to symbols: ∇ , wt-80k/21k; $\bullet$, D104S-80k/21k.

Thermal stability

Since the D104S-80k/21k mutant was highly unstable to autolysis in the presence of Ca^{2+} , it was of interest to compare the thermal stabilities of the D104S-80k/21k and wild-type enzymes in the presence of EDTA, i.e. in the unactivated state. Thermal stabilities were measured by incubating the enzymes (0.75 mg/ml) at 55 °C in 0.1 M Mops/NaOH, pH 7.4, 0.1 M NaCl, 2 mM EDTA, 10 mM β -ME, and measuring the remaining activity at 25 °C with casein at various times. Mops buffer, adjusted to pH 7.4 at 21 °C, was used since its pK value is relatively insensitive to temperature change ($\Delta pK_a/^\circ\text{C} = -0.006$ [17]). D104S-80k/21k was found to be very slightly more stable to heat treatment in the presence of EDTA than the wild-type enzyme (Figure 5).

DISCUSSION

The ability to express active recombinant calpain has created the opportunity to probe the active site of this heterodimeric system by site-directed mutagenesis. In the absence of three-dimensional structural information on calpain, the work is necessarily based on comparisons among highly conserved residues within families of cysteine proteinases. In this study we have focused on Asp-104 and Pro-287, since these residues are adjacent to the active-site Cys-105 and Asn-286, respectively, and are absolutely conserved in calpain, while the corresponding residues in most other cysteine proteinases are highly conserved as serines [5].

k_{cat} and K_m values

The mutants were well expressed in *E. coli*, and could be purified by the standard protocols developed for wt-80k/21k, giving active heterodimeric products with the expected 1:1 stoichiometry. The specific activity of both single mutants was approx. 13 % of that of wt-80k/21k in the casein assay at pH 7.2. Consistent with this result, with the substrate Succ-Leu-Tyr-MCA the values of (k_{cat}/K_m) for the mutants were approx. 10 %

(D104S-80k/21k) and 25 % (P287S-80k/21k) of that of wt-80k/21k. Since the K_m values were only slightly increased, while the k_{cat} values were decreased 6.5-fold and 3-fold, it appears that binding of substrates to the enzymes is not very much affected by the mutation, but that subsequent hydrolysis of the scissile bond is impaired.

pH-activity measurements

Although extensive studies of the variation of activity with pH in papain-like enzymes have been described, derivation of pK values from pH-activity curves remains difficult [18–22]. In similar work in the calpain system, there are severe experimental limitations. Calpain assays are complicated by aggregation and autolysis of calpain in the presence of Ca^{2+} , increasing with time, and by the intrinsically low catalytic efficiency of calpain: typical values of $(k_{\text{cat}}/K_m)_{\text{lim}}$ for the most active calpains with fluorogenic peptides are 60–100 $\text{M}^{-1}\cdot\text{s}^{-1}$, and for mutant calpains 1–10 $\text{M}^{-1}\cdot\text{s}^{-1}$, whereas those for papain are in the range 10^3 – 10^5 $\text{M}^{-1}\cdot\text{s}^{-1}$. As a result, the calpain data are not sufficient to define additional parameters in kinetic models with multiple macroscopic pK_a values.

In papain, deprotonation of the catalytic thiol group occurs with a pK value of 3.3, and of the catalytic histidine side chain with a pK value close to 8.2 [20,21,23]. One or more deprotonations with pK values in the 4–6 range are required for development of catalytic activity, both in papain [21] and in calpain [11]. The pK_2 value of 8.2 in wt-80k/21k almost certainly reflects deprotonation of His-262, but the pK_1 value in the range 5.7–6.5 cannot yet be ascribed to a specific group. It may represent a perturbed pK value for an Asp, Glu or His residue, or possibly the macroscopic effect of more than one ionization.

Pro-287 → Ser

The Pro-287 → Ser mutation led to a narrowing of the pH profile by about 1.4 pH units (Table 2), indicating alterations in catalytically important pK values. In contrast to the Asp-104 → Ser mutation (see below), the Pro-287 → Ser mutation did not significantly alter autolysis or Ca^{2+} requirement, and caused only a 3-fold reduction in $(k_{\text{cat}}/K_m)_{\text{lim}}$, which strongly suggests that the overall conformation is not greatly changed. The small change in the Ca^{2+} curve (Figure 2) is very similar to that recorded for the mutation in the adjacent residue, Trp-288 → Tyr [10,12]. It may be recalled that Asn-Pro²⁸⁷-Trp in calpain corresponds to Asn-Ser¹⁷⁶-Trp in papain [5], where both Asn-286 and Trp-288 are thought to interact with the catalytic His-262 [10,12,22]. The details of the alterations in pK values differ in the two cases, but the effects of the Pro-287 → Ser mutation in calpain and the Ser-176 → Ala mutation in papain both appear to involve local conformational changes which alter the environment and the pK value of the catalytic histidine [18]. In papain, Ser-176 → Ala mutation causes a loss of a hydrogen bond to Gln-19, resulting in an altered conformation of the adjacent Asn-175 and Trp-177 [12,18]. In calpain, the side chain of Pro-287 cannot form a hydrogen bond, but the bond angle constraints of a proline residue are relaxed in the Pro-287 → Ser mutant, again permitting altered conformations of Asn-286 and Trp-288, which are likely to influence His-262 [10]. This is supported by the observation that the principal effect of Pro-287 → Ser mutation in calpain was a reduction of pK_2 , which is assumed to be that of His-262.

Asp-104 → Ser

In the casein assay, the specific activity of D104S-80k was the

same whether co-expressed with the 21 kDa subunit or the NHis₁₀-30k subunit. A similar observation has been made with the two calpain forms wt-80k/NHis₁₀-30k and wt-80k/21k (J. S. Elce, C. Hegadorn and J. S. C. Arthur, unpublished work), suggesting that the binding between the large and small subunits is not affected by the D104S mutation.

The pH optima measured with casein were distorted by the reduced solubility of casein at the lower pH values required to complete the curves. With Succ-Leu-Tyr-MCA, D104S-80k/21k showed a marked narrowing of the pH profile, by about 1.7 pH units, when compared with wt-80k/21k (Table 2). Previous work [11] had suggested that the catalytic ion pair in calpain was influenced by a group supplied by an Asp, Glu or His residue. If this group, possibly provided by Asp-104, interacts with the ion pair (directly or indirectly), then removal of this group should destabilize the ion pair relative to the wild-type enzyme. It has been argued on theoretical grounds that mutations which adversely affect active-site ionizations should lead to a narrowing of the pH-activity profile [18,24]. The differences in the pH-activity profiles of D104S-80k/21k and wt-80k/21k are therefore consistent with the idea that Asp-104 supplies a stabilizing interaction within the active site following activation of the enzyme by Ca²⁺, and that Ser-104 is unable to provide this interaction. The groups with which Asp-104 interacts are not known, but several possibilities may be proposed, all of which would be expected to alter pK values. In view of its sequence proximity to Cys-105, Asp-104 could have a close charge interaction with the catalytic ion pair. Alternatively, Asp-104 could interact with Gln-99. However, in view of the autolysis and Ca²⁺-titration data, the observed results of the Asp-104 → Ser mutation are perhaps most consistent with an alteration of conformation in the presence of Ca²⁺, with indirect effects on the active site.

Of these alternatives, one possibility was an interaction with Gln-99 in the oxyanion hole. Using the known rat cathepsin B structure [25] (or the papain structure which in this respect is essentially identical) as a speculative model for the protease domain of calpain, it appeared that Asp-104 in calpain would be able to form a hydrogen bond only to Gln-99, which is almost certainly one of the oxyanion-binding residues. In this model, the OH group of a hypothetical Ser-104 was too far from Gln-99 to be able to form a hydrogen bond. In other cysteine proteinases, the oxyanion-binding glutamine residue is hydrogen-bonded to a different conserved serine [18], at a position corresponding to Pro-287 in calpain. The proline side chain clearly cannot form hydrogen bonds, and we postulated that the loss of an Asp-104-Gln-99 hydrogen bond in Asp-104 → Ser calpain might be compensated for by a new Gln-99-Ser-287 hydrogen bond. This suggestion was, however, found to be incorrect, since calpain containing the double mutation Asp-104 → Ser, Pro-287 → Ser was almost completely inactive. In the papain system, uncharged mutations of this glutamine residue did not affect the pH-activity profile of the mutants [26], so the pH results in D104S-80k/21k are also more consistent with effects on active-site pK values than on oxyanion binding.

A further possibility was that the effects of the Asp-104 → Ser mutation were due to alteration of the overall conformation of the enzyme. The thermal stabilities of the wild-type and Asp-104 → Ser mutant enzymes in the presence of EDTA were not greatly different, suggesting that the conformation of this mutant in the resting state was not altered from that of the wild-type enzyme. However, cleavage of the large subunit in D104S-80k/21k in the presence of Ca²⁺, in spite of the lower specific activity of this enzyme, was much more rapid than cleavage of the large subunit in wt-80k/21k, and gave some different products (Figures 3 and 4). We interpret these results to mean that the Ca²⁺-activated

form of D104S-80/21k, while retaining some catalytic activity, is not in a fully native conformation in the presence of Ca²⁺, with the result that more sites in the large subunit are accessible to autolytic cleavage than in the wild-type enzyme.

The conformational effect in D104S-80k/21k in the presence of Ca²⁺ is clearly linked to the difference in Ca²⁺ sensitivity of the mutant and wild-type enzymes. The Asp-104 → Ser mutant had a 9-fold greater Ca²⁺ requirement for activity than the wild-type enzyme, indicating that the Ca²⁺-induced conformational change is energetically less favourable in the mutant enzyme. The dependence of any form of calpain on Ca²⁺ shows that Ca²⁺ binding to domains IV and VI leads to a change in conformation around the active site in domain II, although how this is achieved is not yet clear. Conversely, the Asp-104 → Ser mutation clearly alters the nature of the permitted conformational change at the active site, since Ser-104 is unable to provide an important interaction in the presence of Ca²⁺. This in turn alters the overall conformation of the Ca²⁺-bound form of the enzyme, and therefore changes the measured Ca²⁺ requirement of the enzyme.

In summary, the mutations Asp-104 → Ser and Pro-287 → Ser were undertaken on the basis of conserved residues in sequence alignments with the papain-like cysteine proteinases, and because of their proximity in the primary sequence to known active-site residues. The results indicate, however, that the structure of domain II of calpain will not fit very closely to the well-conserved structure of the papain-like proteinases. The mutation Pro-287 → Ser affected the ion pair, probably by way of local perturbation of Asn-286, Trp-288, and His-262, but did not appear to alter the overall conformation and stability of the enzyme. In contrast, the mutation Asp-104 → Ser had more pronounced consequences: following Ca²⁺ binding to the enzyme, it is clear that Asp-104 forms a new and catalytically important interaction which directly or indirectly stabilizes the catalytic ion pair. A final interpretation of these results requires crystal structure information which is not yet available, but the results provide a first step in understanding the Ca²⁺-induced conformational changes which are involved in calpain activation.

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