Conformation, structure and activation of bovine cathepsin D

Unfolding and refolding studies

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Cathepsin D is found in the cell in two forms, one a single polypeptide chain (M_r 44000) and the other a non-covalent complex of two peptides of M_r 14000 and 30000. These correspond to the *N*-terminal and *C*-terminal regions of the single chain from which they originate. It has been shown that the two forms of the enzyme are closely similar in secondary-structure content, in aromatic amino acid environment and in denaturation behaviour. The two-chain enzyme has half the specific activity of the single-chain form. The denaturation and renaturation of the single-chain cathepsin D has now been studied by c.d., fluorescence and enzyme activity. Activity is lost irreversibly on unfolding, but the loss of backbone ellipticity and of folded aromatic environment is 75% reversible. The enzyme unfolds in two main stages, and the kinetics of these transitions indicate the existence of at least two intermediate forms between the native and the fully unfolded states. A further form of the enzyme exists in 0.5M-guanidinium chloride. It is characterized by having an activity 40% greater than that of the native state. This increase is not reversed on removing the denaturant. The similarities between cathepsin D and pepsin are discussed.

Cathepsin D (EC 3.4.23.5), purified from bovine spleen by Press *et al.* (1960), is a lysosomal aspartic proteinase that plays a major role in intracellular protein degradation. On extraction from bovine spleen, cathepsin D is obtained as two species each of M_r 44000, as determined by gel filtration. One is the single-polypeptide-chain cathepsin D and the other a non-covalently associated complex consisting of two chains of M_r 28000–30000 and M_r 14000–15000 (Turk *et al.*, 1981). Polypeptides having these three molecular masses are obtained in preparations of cathepsin D isolated from a variety of tissues and species (Ogunro *et al.*, 1980; Whitaker, 1981; Takahashi & Tang, 1981). Rat spleen cathepsin D, however, is reported to be a single chain of M_r 44000 (Yamamoto *et al.*, 1979), whereas cathepsin D isolated from human liver is obtained only in the two-chain form (Barrett, 1979).

Abbreviation used: GdmCl, guanidinium chloride.

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Cathepsin D is formed *in vivo* by proteolytic cleavage of a precursor after glycosylation and phosphorylation (Erickson & Blobel, 1979; Erickson *et al.*, 1981). The two chain species may arise as part of the processing of cathepsin D in the cell (Erickson *et al.*, 1981), possibly by autolysis (Lah & Turk, 1982). Its widespread occurrence suggests that a region of cathepsin D that is particularly sensitive to proteolysis is a feature basic to the enzyme conformation.

We have studied the conformation and denaturation of cathepsin D in order to compare the intact and the cleaved forms, to examine the reversibility of unfolding and to look for possible intermediate conformers.

During the course of the denaturation studies, apparent activation of the enzyme was observed similar to that reported earlier (Wojtowicz & Odense, 1970). A re-investigation of this phenomenon showed, contrary to previous suggestions, that the proteolytic activity of cathepsin D is significantly increased by the action of low concentrations of GdmCl or urea.

Materials and methods

Materials

GdmCl (AristaR grade) was from BDH Chemicals, Poole, Dorset, U.K. Urea (ultra pure and cyanide-free), dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride, sodium dodecyl sulphate, 2-mercaptoethanol, acrylamide (4 × crystallized), *NNN'*-tetramethylethylenediamine and ammonium persulphate were from Serva, Heidelberg, Germany, and Folin-Ciocalteu reagent was from Merck, Darmstadt, Germany. All other chemicals were of analytical grade. Sepharose CL-6B and Sephadex G-100, G-25 and G-10 were from Pharmacia, Uppsala, Sweden. Pepstatin was from the Peptide Institute, Osaka, Japan. Standard marker proteins for gel filtration were from Serva, and those for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis from Pharmacia, Uppsala, Sweden.

Isolation of cathepsin D

Cathepsin D was isolated from bovine spleen (Smith & Turk, 1974; Kregar *et al.*, 1977). This procedure results in three active fractions, D1 consisting of single-chain enzyme, and D2 and D3, each containing a mixture of single-chain and two-chain species. The two forms of cathepsin D studied in the present work are the fractions D1 and D2. They were dialysed against 0.1 M-NaCl/10 mM-Mes (4-morpholine-ethanesulphonic acid)/NaOH buffer, pH 6.6, and concentrated by ultrafiltration. Cathepsin D concentration was determined by using the value of $A_{1\text{cm}}^{0.1\%} = 1.05$ at 280 nm.

Separation of the light chain (M_r 14000) from cathepsin fraction D2

Cathepsin D2 was denatured in 5 mM-Mes buffer, pH 6.6, containing 2.5 M-GdmCl and fractionated on a column (63 cm × 1.5 cm) of Sepharose CL-6B, equilibrated with the same buffer/GdmCl solution.

C.d.

Ellipticity values are reported as mean-residue ellipticities $[\theta]_{\text{m.r.w.}}$ calculated by using a mean residue M_r value of 110. Cells were 1 cm for the near u.v. and 0.05 cm or 0.01 cm for the far u.v. region. The spectral bandwidth was set at 1 nm (near u.v.) and 2 nm (far u.v.). Measurements were made at room temperature on a Jobin-Yvon dichrograph III standardized with epiandrosterone (Jobin-Yvon, Longjumeau, France).

Fluorescence

Fluorescence was measured at 25°C with a Hitachi Perkin-Elmer MPF3 spectrofluorimeter.

The excitation wavelengths were 278 nm and 285 nm and the excitation bandwidths 4 nm and 7 nm for measurements of spectra and unfolding respectively. First-order rate constants and amplitudes of the fast and the slow reactions from bi-phasic processes were calculated as described by Frost & Pearson (1961).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was performed in 12.5% acrylamide in tubes and on slabs as described by Laemmli (1970).

Assay of cathepsin D activity

Proteolytic activity was determined with haemoglobin as substrate in a modification of the method of Anson (1939). Enzyme samples (5–50 μ l) were incubated with 2 ml of 2% (w/v) substrate solution for 5 min at 37°C. Preincubation of substrate with denaturant caused no change in the extent of proteolysis on subsequent incubation with proteinase. Refolding of partially or fully denatured enzyme on dilution into substrate solution was shown not to occur by measuring the time course of proteolysis. A linear increase of trichloroacetic acid-soluble products over the first 5 min was observed with both native and partially denatured enzymes. In a third control enzyme was incubated with substrate for 5 min at 37°C and denaturant or buffer was added immediately before the addition of trichloroacetic acid. Under these conditions denaturant was found to have no effect on the solubility of the products of haemoglobin digestion.

Determination of N-terminal amino acids

This was done by using the dansyl chloride technique of Hartley (1970) (Narita, 1970), with t.l.c. on F-1700 micro polyamide-coated t.l.c.-ready plastic sheets (Schleicher und Schüll, Dassel, Germany).

Denaturation experiments

Samples of filtered stock enzyme solution (3–4 mg of cathepsin D/ml) were diluted into solutions of denaturant. Optical properties and enzyme activity were measured after the mixture had stood at 25°C for from 60 min to 22 h. In kinetic experiments the changes in optical properties were followed within 30 s of the addition of protein to the denaturant solution. The decay of enzyme activity was measured by the withdrawal of samples (5–50 μ l) from the denaturing solution. In renaturation experiments the denaturant was removed by dilution (1:10) into buffer, by dialysis or by gel filtration on Sephadex G-10.

Results

Comparison of cathepsins D1 and D2

The results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on the single-chain species D1 (M_r 44000) and the two-chain species D2 confirm that the complex (M_r 44000) of heavy chains (M_r 29000) and light chains (M_r 14000) is held together by non-covalent interactions only. The absence of any detectable change in the gel pattern of banding in the presence of mercaptoethanol shows that there is little, if any, other proteolytic nicking in either cathepsin D1 or cathepsin D2. *N*-Terminal analysis showed that glycine is at the *N*-terminus of cathepsin D1 and also of the light chain separated from species D2, confirming the position of the light chain at the *N*-terminal end of the cathepsin D molecule (Takahashi & Tang, 1981; Huang *et al.*, 1980). In the other fraction from cathepsin D2, which contains 44000- M_r and 29000- M_r chains, phenylalanine and smaller amounts of valine, lysine and alanine were found in addition to glycine, supporting the suggestion (Lah & Turk, 1982) that the cleavage is autolytic.

The specific activities of cathepsin D1 and D2 against haemoglobin at pH 3.5 are 125 ± 5 and

Fig. 2. Unfolding transitions of cathepsin D1 in GdmCl: *c.d.* and activity

Cathepsin D was diluted into GdmCl solutions at the stated concentrations and time-dependent reactions were allowed to go to completion. Values of ellipticity were normalized to the value in zero [GdmCl]. Enzyme activities were calculated relative to the activity of native enzyme stored under the same conditions. No loss of activity was detected for native enzyme over similar periods of time. Δ , Activity; $\bullet$, ellipticity at 291 nm; $\circ$, mean-residue ellipticity at 220 nm. Activity and ellipticities were measured as described in the Materials and methods section.

Fig. 1. Near-u.v. *c.d.* spectra of cathepsins D1 and D2. Spectra were measured with a 1cm-pathlength cell at protein concentration 1mg/ml and 1nm bandwidth. The spectra shown are those of cathepsin D1 in 10mM-Mes buffer, pH 6.6, containing no GdmCl (—), containing 2.9M-GdmCl (.....) and containing 6.2M-GdmCl (----) and of cathepsin D2 in 10mM-Mes buffer, pH 6.6 (— · — ·).

95 ± 4 Anson units/mg respectively. Since cathepsin D2 contains approx. 50% single-chain cathepsin D, and assuming that the latter has the same specific activity as the D1 single-chain enzyme, the specific activity of the two-chain complex is approx. 65 units/mg or half that of the single-chain enzyme, D1.

The *c.d.* and fluorescence spectra of cathepsins

D1 and D2 were compared. The far-u.v. c.d. spectra indicate a very close similarity of secondary structure, with 4% α -helix, 50% β -structure, 18% reverse turn and 28% aperiodic structure (Provencher & Glöckner, 1981). Near-u.v. c.d. spectra show a general decrease in intensity for cathepsin D2 compared with species D1 at the shorter wavelength end, though without loss or shift of the tyrosine and phenylalanine fine structure (Fig. 1). This suggests a small change in tryptophan environment or interaction, associated with the 1L_b transition (Strickland, 1974). There is no significant difference in fluorescence spectra between cathepsins D1 and D2.

Unfolding transitions of cathepsins D1 and D2

Cathepsin D exhibits two consecutive unfolding transitions in GdmCl on the basis of static and kinetic experiments. The first is steep and occurs between 0.5M- and 2M-GdmCl. The loss of enzyme activity is paralleled by loss of ellipticity at 291 nm (Fig. 2) and by a decrease in fluorescence emission at 314 nm (Figs. 3 and 4). There is also a decrease in the ellipticity at 220 nm, corresponding to 60% of the total change in $[\theta]_{220}$ between 0M- and 6M-GdmCl (Fig. 2). The superposition of these transitions measured by changes in enzyme activity, aromatic amino acid asymmetry and backbone conformation indicates co-operative unfolding of a substantial element of the cathepsin D1 structure in 2M-GdmCl.

The molecule is not, however, completely unfolded under these conditions, as shown by the further changes in ellipticity at 220 nm and in

Fig. 3. Fluorescence emission spectra of cathepsin D1. Fluorescence was measured with a 1 cm cell at protein concentrations of 0.07 mg/ml. Bandwidths were 4 nm for the exciting beam at 285 nm and 5 nm for the emitted beam. The fluorescence emission spectra shown are those of cathepsin D1 in 10 mM-Mes buffer, pH 6.6, containing no GdmCl (—), containing 3.0M-GdmCl (---) and containing 5.6M-GdmCl (.....). Fluorescence is given in arbitrary units.

Fig. 4. Unfolding and refolding transitions of cathepsin D1 followed by fluorescence

Fluorescence, calculated as the emission at 314 nm relative to the emission for native enzyme, is plotted against increasing concentrations of GdmCl (unfolding transition) (●) and against decreasing concentrations of GdmCl, obtained by diluting enzyme from 3.0M-GdmCl (○). Also, intensity of fluorescence emission at 348 nm is plotted as a fraction of change with increasing concentration of GdmCl (□). All solutions contained 0.07 mg of protein/ml in 10 mM-Mes buffer, pH 6.6, and were measured at 25°C. $\lambda_{exc.}$ is 278 nm; bandwidths are 7 nm and 6 nm for excitation and emission respectively.

fluorescence emission at 348 nm that take place between 2M- and 6M-GdmCl. This second transition is broad and much less co-operative than is the first transition. The kinetics of these changes (see below) show them to be associated with the unfolding of molecular structure.

The unfolding of cathepsin D2 is closely similar to that of cathepsin D1 (Table 1). The specific cleavage in cathepsin D2, besides decreasing the asymmetry of one or more tryptophan residues (Fig. 1), also renders the remaining tryptophan asymmetry more sensitive to low concentrations of GdmCl. The small 'pre-transition' detected by measurements of ellipticity at 291 nm accounts for the increased breadth and lower average midpoint of the first transition (Table 1). Similar changes in

Table 1. Comparison of the first unfolding transition in GdmCl for cathepsins D1 and D2

The range of GdmCl concentration covering each transition, the concentration of GdmCl at which 50% of the transition has taken place (C_m) and the fraction of the total change between native and fully unfolded enzyme are listed. Transitions were measured at pH6.6.

	Range of GdmCl concn. (M)	C_m (M)	Percentage change	Parameter
Cathepsin D1	0.8–2.0	1.1	100	Ellipticity at 291 nm
Cathepsin D1	0.8–2.2	1.3	60	Ellipticity at 220 nm
Cathepsin D1	0.5–2.3	1.1	100	Fluorescence at 314 nm
Cathepsin D1	0.8–2.0	1.1	100	Enzyme activity
Cathepsin D2	0.2–2.1	0.6	100	Ellipticity at 291 nm
Cathepsin D2	1.0–2.0	1.4	66	Ellipticity at 220 nm
Cathepsin D2	0.6–2.1	0.95	100	Enzyme activity

backbone ellipticity occur on denaturation of cathepsins D1 and D2 in GdmCl. Since the heavy chains and light chains separate in 2.5M-GdmCl (see the Materials and methods section), the remaining structure in 2M-GdmCl must exist in one or both of the isolated chains.

Activation at low GdmCl concentrations

When cathepsin D1 is incubated in concentrations of GdmCl below the first main transition and samples are diluted into haemoglobin substrate solution and assayed, the specific activity is found to be significantly increased (Fig. 2). Enzyme incubated under the same conditions, but without GdmCl, maintains the native specific activity. At 0.5M-GdmCl the activity was 140% of that of native enzyme, reaching this maximal value only after 10min incubation. Controls, described in the Materials and methods section, show that this increase in specific activity is not due to increased susceptibility of haemoglobin or decreased precipitation of substrate by trichloroacetic acid, as was suggested for the apparent activation observed at much higher concentrations of denaturant (Schlamowitz *et al.*, 1961; Wojtowicz & Odense, 1970). This activation of cathepsin D is not reversible within the period of the assay (see the Materials and methods section). Both near-u.v. c.d. (Fig. 2) and fluorescence (Fig. 4) are decreased significantly in intensity at concentrations of GdmCl less than 1M. These observations suggest that the activation of cathepsin D is associated with a separate conformational transition, since the main transition involves loss of activity (Fig. 2).

Reversibility of denaturation

Activity. Solutions of cathepsin D denatured in GdmCl concentrations up to 6M were adjusted back to low or zero concentrations of GdmCl by dilution, by gel filtration and by dialysis. In no case was any recovery of activity observed. The rate of

Fig. 5. Comparison of the decay of fluorescence and of activity for cathepsin D1 in 2.32M-GdmCl

Fluorescence emission at 314nm (■) and enzyme activity values (○) were normalized and plotted against time after placing the enzyme in 2.32M-GdmCl. Activity was measured by diluting samples 10-fold into haemoglobin substrate solution. Cathepsin D was 0.05mg/ml for fluorescence and 0.25mg/ml for activity measurements, both at 25°C.

loss of activity on denaturation was measured by diluting samples of enzyme unfolding in 2.32M-GdmCl into excess substrate solution. The resulting decay curve coincides with the decrease in fluorescence measured in 2.32M-GdmCl over the same time period (Fig. 5), demonstrating the lack of reversibility to native enzyme in the presence of substrate. A similar experiment, but with the mixture left to stand overnight before measurement of the activity, led to the same curve, confirming the complete inability of the denatured enzyme to recover activity.

Conformation. In contrast with enzyme activity, the loss of fluorescence emission at 314nm on denaturation was rapidly though only partially reversed when denaturant was removed by dilution. Fig. 4 shows the values of fluorescence inten-

Fig. 6. Kinetics of unfolding of cathepsin D1 with GdmCl at 314 nm

Fluorescence emission at 314 nm (F) was used to follow the unfolding of cathepsin D in 1.1 M-GdmCl (●), 1.89 M-GdmCl (■), 2.42 M-GdmCl (○) and 3.02 M-GdmCl (△), each in 10 mM-Mes buffer, pH 6.6. Protein concentration was 0.088 mg/ml. Results are plotted according to first-order kinetics.

sity measured between 30 and 60 s after dilution from 3 M-GdmCl into the indicated concentration of denaturant.

Ordered secondary structure as indicated by ellipticity at 220 nm is also recovered on renaturation, again with a half-time of less than 1 min. Under certain conditions, the recovery of fluorescence and far-u.v. ellipticity is followed by slow changes with high-order kinetics, indicating aggregation of the partially refolded polypeptide. Extrapolation of these kinetics to zero time shows that approx. 75% of native fluorescence and ellipticity are recovered by rapid refolding.

Examination of the denatured enzyme by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that, although a small amount of autolytic cleavage can occur during the unfolding process even at pH 6.6, more than 90% of the polypeptide chain retains its covalent integrity. The amount of autolysis decreases with increased speed of unfolding at higher GdmCl concentrations. The inability of the denatured enzyme to refold is therefore not due to cleavage of covalent bonds.

Fig. 7. Unfolding of cathepsin D1: dependence of the rate constants and amplitudes on GdmCl concentration

Biphasic rate plots (Fig. 6) were decomposed (Frost & Pearson, 1961) to give amplitudes (a) and first-order rate constants (b) for the fast phase and the slow phase. Amplitudes of the fast phase are plotted as a percentage of the total amplitude of fluorescence change at 314 nm. Rate constants (s^{-1}) for unfolding were calculated from fluorescence at 314 nm (○ and ●), ellipticity at 291 nm (△ and ▲) and ellipticity at 220 nm (□ and ■). Open and filled symbols refer to the fast phase and the slow phase respectively. The lines for the rate constants are least-squares-regression slopes for the values obtained from fluorescence experiments. Kinetics were measured at 25°C for fluorescence and approx. 22°C for c.d.

Kinetics of denaturation of cathepsin D

The progress of denaturation of cathepsin D1 was followed by fluorescence emission at 314 nm, by c.d. and by enzyme activity. The first-order rate plots are biphasic within the region of the first unfolding transition, becoming monophasic at higher concentrations of GdmCl (Fig. 6). The rate constants and relative amplitudes for the two phases are plotted as a function of GdmCl concentration (Fig. 7). The relative amplitude of the faster phase is strongly dependent on GdmCl concentration, as are the rate constants for each phase. The values of

Fig. 8. Kinetics of unfolding of cathepsin D1 with GdmCl monitored by fluorescence at 348nm

Fluorescence emission at 348 nm was monitored as a function of time for cathepsin D (0.08 mg/ml) in 10 mM-Mes buffer, pH 6.6, containing 3.17 M-GdmCl (●) or containing 4.19 M-GdmCl (□). The experiment in 3.17 M-GdmCl was also monitored at 314 nm for comparison (○). Results are plotted according to first-order kinetics.

$d(\log k)/d(\log [\text{GdmCl}])$ obtained by least-squares-regression analysis of the fluorescence results are 4.4 and 5.3 for the slow phase and fast phase respectively.

At higher concentrations of GdmCl the fluorescence emission at 348 nm changes (Fig. 3). The kinetics of unfolding, followed at this wavelength from 3 M-GdmCl upwards, show biphasic first-order kinetics, the main, slower, phase of which has a positive dependence on GdmCl concentration $\{d(\log k)/d(\log [\text{GdmCl}]) = 1.5\}$. The fast phase has rates similar to the monophasic rates measured at 314 nm at the same concentrations of GdmCl (Fig. 8).

Discussion

The two-chain form of cathepsin D from bovine spleen has been shown to be very similar to the

single-chain enzyme in its conformation and in its denaturation behaviour. It possesses a lower but significant proteolytic activity. The structural perturbation resulting from limited cleavage at a relatively highly accessible point in the intact polypeptide chain is thus slight, and this form of the enzyme is capable therefore of having a physiological role, as suggested by Erickson *et al.* (1981).

Cathepsin D loses activity concomitantly with the unfolding of a major part of the molecule (Figs. 2 and 5). All attempts to reverse the unfolding and to recover activity, paying special attention to factors that may affect the ability of a polypeptide chain to refold, including renaturation time, presence or absence of substrate and protein concentration, have failed. In no case has activity been recovered.

Although the possibility that refolding is inhibited by post-translational modification (Erickson *et al.*, 1981; Hasilik & von Figura, 1981; Hasilik *et al.*, 1982) cannot be excluded, the results are in keeping with the fact that cathepsin D originates from a precursor by proteolytic cleavage with the loss of an *N*-terminal peptide (Hasilik & Neufeld, 1980; Erickson *et al.*, 1981; Puizdar & Turk, 1981; Rosenfeld *et al.*, 1982).

The denaturation studies show a highly co-operative transition between 0 M- and 2 M-GdmCl in which enzyme activity, aromatic amino acid environment and backbone structure are affected in a concerted manner. The kinetics of this transition are biphasic, with the fast phase taking over at higher GdmCl concentrations. Extrapolation of these kinetics to zero time shows that the observed phases account for the complete transition. This behaviour is not due to proline isomerization (Brandts *et al.*, 1975), since the slow phase is firstly two orders of magnitude slower than the rate of isomerization of proline residues and secondly is strongly dependent on GdmCl concentration. The biphasic kinetics therefore reflect the presence of an intermediate species either on or off the unfolding pathway between the native state and the partially unfolded state that exists in 2 M-GdmCl. The fact that both phases are positively dependent on GdmCl concentration indicates that the intermediate species has a conformation intermediate between those of the native state and the partially unfolded state (Ikai & Tanford, 1973).

At concentrations of GdmCl higher than 2 M, the further transition shown by ellipticity at 220 nm and fluorescence emission at 348 nm results in loss of the remaining backbone structure and interactions of tryptophan residues. The kinetics are again biphasic. Since the fast phase at 348 nm is similar in rate to the monophasic rates measured at 314 nm, this phase can be assigned to the change of fluorescence occurring between 0 M- and 3 M-

GdmCl (Fig. 3), i.e. to the first transition. The slow phase relates then to the subsequent change between 3M- and 6M-GdmCl, which characterizes the second transition.

A simple model that can reconcile the partial reversibility to a non-native state with the kinetic behaviour is:

where D_1 is transient, D_2 exists at 2M-GdmCl and U is the fully unfolded protein. The denaturation behaviour is indicative of a molecular structure that unfolds in stages and with only partial reversibility. In these general properties cathepsin D is similar to pepsin (Ahmad & McPhie, 1978; Lah *et al.*, 1980; Privalov *et al.*, 1981), which possesses a marked domain structure (Andreeva & Gustchina, 1979).

When the unfolding of cathepsin D was followed by measurements of proteolytic activity against haemoglobin, a further transition was found at low concentrations of denaturant (Fig. 2). Earlier reports have suggested that the autolytic activity of pepsin may be raised in the presence of low concentrations of urea or GdmCl (Perlmann, 1956; Blumenfeld *et al.*, 1960), and its activation against haemoglobin substrate by low concentrations of urea has been characterized (Lah *et al.*, 1980). The activation of cathepsin D by GdmCl reported in the present paper closely parallels that found for pepsin. These activated states of pepsin and cathepsin D may correspond to one of the two states of pepsin demonstrated by Kozlov *et al.* (1979) at temperatures below the main thermally induced transition.

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