Reversible denaturation of cyclosporin synthetase by urea

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Abstract The reversible denaturation of the multifunctional polypeptide, cyclosporin synthetase, by urea was analyzed. It is possible to discriminate between at least two stages of enzyme denaturation. While at low urea concentration (up to 0.8 M) cyclosporin A formation is inhibited, synthesis of the diketopiperazine *cyclo*-(D-alanyl-*N*-methylleucyl), a molecule representing a partial sequence of cyclosporin A is still detectable. At higher concentrations of urea the enzyme preparation is totally inactive. This inactivation is a consequence of conformational change(s) of cyclosporin synthetase as shown by fluorescence emission spectra of native and denatured enzyme. These data imply a consecutive folding/defolding mechanism for the different domains forming the multifunctional polypeptide.

Key words: Biosynthesis; Cyclosporin synthetase; *cyclo*-(D-alanyl-*N*-methylleucyl); Fluorescence spectrum; Folding; Multidomain enzyme

1. Introduction

Cyclosporin A (CyA), a powerful drug in human transplantation surgery and the treatment of autoimmune diseases [1–4], is a cyclic undecapeptide produced by the fungus *Beauveria nivea*.

It is synthesized by cyclosporin synthetase, a multienzyme polypeptide. Its molecular mass has been estimated to be about 1,400 kDa [5] by SDS-PAGE and CsCl density gradient centrifugation. Sequencing of the open reading frame of the cyclosporin synthetase gene resulted in a derived molecular mass of 1,689,243 Da [6]. This multifunctional polypeptide catalyzes CyA formation in at least 40 reaction steps in an assembly belt-like mechanism [7]. The enzyme activates all constituent amino acids of CyA as thioesters via aminoacyladenylates [7] and carries out specific *N*-methylation reactions [8].

Studies on chaperone functions have shown that these proteins, assisting in the folding of proteins, form complexes with cavities giving space for globular proteins of about 90 kDa [9,10]. A majority of proteins has molecular masses of about this size and below. Cyclosporin synthetase is so far the largest known polypeptide exerting enzymatic activities. To function adequately it has to be folded in a very specific manner which cannot be assisted by the chaperone/chaperonine machineries in the cell as such. The sequencing of the open reading frame reported in [6] has demonstrated that the enzyme is composed of eleven major domains of about 140 kDa each. One obvious possibility for the folding mechanism of such a multidomain enzyme would be that each domain is folded by its own, assisted by a chaperone/chaperonine machinery. The final arrangement of the folded domains in relation to each other can then be either a spontaneous step or it could be catalyzed by specific enzymes (like the peptidyl-prolyl-*cis*/*trans*-isomerases [11]).

To prove whether there would be a step-by-step defolding mechanism detectable for cyclosporin synthetase we analyzed the reaction products of this enzyme at different low urea concentrations. We could show that there exist conditions under which some reactions are no longer catalyzed, whereas others (early steps of cyclosporin synthesis) are still carried out by the enzyme.

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Abbreviations: Abu, L-2-aminobutyric acid; AdoMet, *S*-adenosyl-L-methionine; [^{14}C]AdoMet, *S*-adenosyl-L-[methyl-^{14}C]methionine; Bmt, (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine; CyA, cyclosporin A; *cyclo*-(D-Ala-MeLeu), *cyclo*-(D-alanyl-*N*-methylleucyl); Me, *N*-methyl-.

2. Materials and methods

2.1. Radioisotopes

Radioactive [^{14}C]D-alanine (specific activity 40 Ci/mol), [^{14}C]L-alanine (155 Ci/mol), [^{14}C]L-leucine (308 Ci/mol), [^{14}C]L-valine (270 Ci/mol), [^{14}C]L-threonine (228 Ci/mol), [^{14}C]glycine (107 Ci/mol) and *S*-adenosyl-L-[methyl-^{14}C]methionine ([^{14}C]AdoMet, 59 Ci/mol) were purchased from Amersham Buchler (Braunschweig, Germany). [^{14}C]D/L-aminobutyric acid ([^{14}C]-Abu, 16 Ci/mol) was from DuPont NEN (Bad Homburg, Germany). ATP was from Boehringer (Mannheim, Germany). Bmt ((4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine) and CyA were donated by Dr. R. Traber, Sandoz Ltd. (Basel, Switzerland).

2.2. Growth of organism, enzyme preparation, enzyme assays and thin layer chromatography

These were carried out as described elsewhere [8,12].

2.3. In vitro cyclosporin A formation in the presence of urea

100 μl of enzyme preparation (pooled active HW 55 fractions) were preincubated in the presence of different concentrations of urea (calculated for the final volume of 170 μl) dissolved in buffer B (0.1 M Tris-HCl, pH 7.8; 4 mM EDTA; 4 mM DTE; 15% (w/v) glycerol) in a total volume 145 μl for 10 min. Cyclosporin A synthesis reaction was initiated by the addition of 25 μl reaction mixture containing (final concentration in 170 μl) 6 mM $MgCl_2$, 2.5 mM ATP, 0.25 μCi of [^{14}C]AdoMet, a mixture of 0.5 mM of each of Leu, D-Ala, Val, Abu, Gly and Ala, 0.12 mM Bmt [12]. The reactions were carried out for 10 min at 25°C. Further treatment was done as described elsewhere [12].

2.4. Determination of thioester-bound amino acids

90 μl of cyclosporin synthetase were incubated together with 25 mM $MgCl_2$, 22 mM ATP, each of the constituent amino acids of CyA in ^{14}C-radiolabeled form (0.6 μCi (15 nM); Bmt: 0.3 μCi [^{14}C]AdoMet (15 nM) and 15 nM Bmt; [^{14}C]-D/L-Abu: 0.24 μCi (15 nM)) and urea dissolved in buffer B in different concentrations in a total volume of 204 μl. After incubation for 10 min at 25°C reaction was stopped by adding

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1 ml of 7% trichloroacetic acid. Further sample treatment was done according to [13].

3. Results

3.1. Reversible denaturation of cyclosporin synthetase

The overall reaction of CyA synthesis can be divided in at least 40 partial reaction steps: 11 aminoacyladenylation reactions, 11 transthiolation reactions, 7 *N*-methylation reactions, 10 elongation reactions and a final cyclization reaction. All steps are catalyzed by cyclosporin synthetase, a single polypeptide chain [7,8] consisting of eleven amino acid activating domains [6].

To investigate whether specific reaction steps of CyA formation could be abolished by low urea concentrations, we preincubated the enzyme with various concentrations of urea. Once an equilibrium state of unfolding has been reached (after approximately 10 min, see below) the cyclosporin A synthesis reaction was started by addition of the reaction's substrates (ATP, Mg^{2+}, [^{14}C]AdoMet, and a mixture of all amino acids constitutive of CyA). After stopping the reaction the overall incorporation of radiolabelled methyl-groups (derived from [^{14}C]AdoMet in our incubation mixture) into the synthesized peptides extractable with EtOAc was determined. Under these conditions, at concentrations of urea up to 2 M we found that the enzyme activity was exponentially decreased depending on urea concentration (Fig. 1). In the presence of 0.4 M urea the catalytic activity of cyclosporin synthetase was already approximatively 50% (52.3 ± 1.8%) that of the original activity. The time course (Fig. 1; insert) shows that urea at a concentration of 0.4 M needs some time (at least 5 min) to develop its full inhibitory

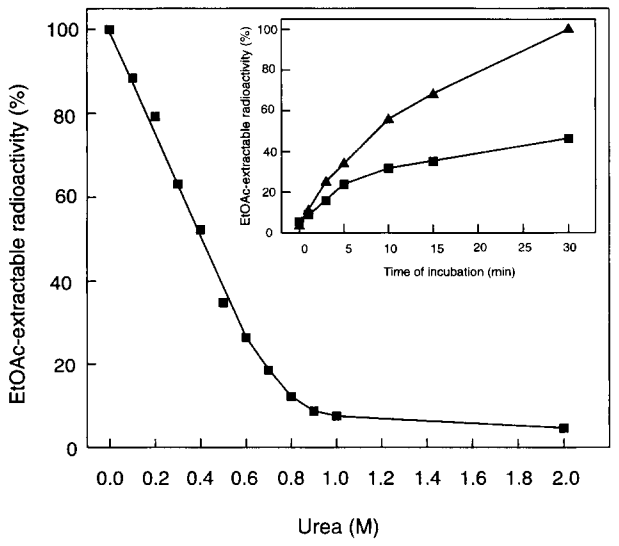

Fig. 1. Denaturation of cyclosporin synthetase by urea. 100 μl of enzyme preparation were preincubated together with the indicated urea concentrations for 10 min. The cyclosporin A synthesis reaction was initiated by the addition of the necessary co-factors and substrate amino acids and was carried out for 10 min at 25°C. Samples were worked up as described in section 2. 100% was set as 3,044 counts/minute. Insert: Time course of enzyme activity in the presence of 0.4 M urea. Cyclosporin synthetase was incubated together without (▲) and with 0.4 M urea (■) and the necessary cofactors and substrate amino acids at 25°C. At indicated times aliquots were removed and the EtOAc-extractable radioactivity was measured. 100% was set as 7,428 counts/minute.

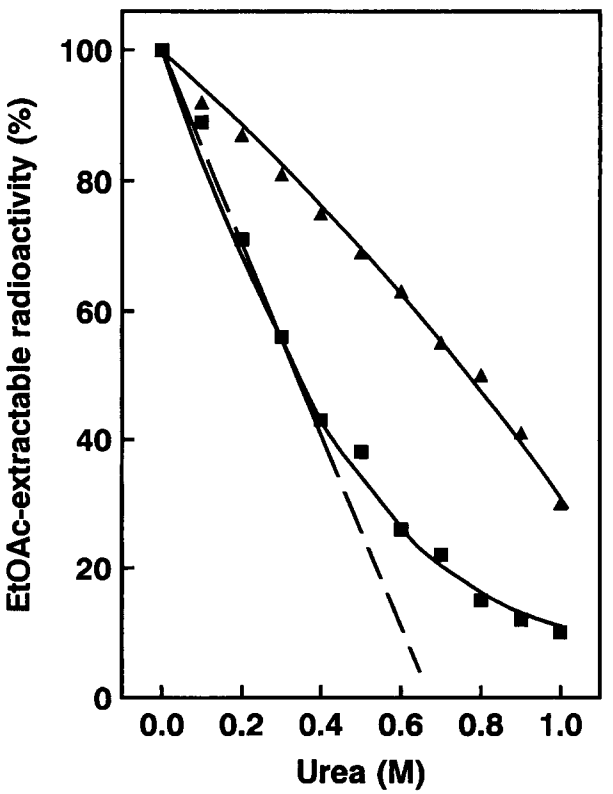

Fig. 2. Comparison of overall catalytic activity and *cyclo*-(D-Ala-MeLeu) formation. Enzyme preparations were incubated for 10 min at 25°C in the presence of urea with different substrate mixtures. As radiolabel [^{14}C]AdoMet was used. For determination of the overall catalytic activity (■) a mixture of all constituent amino acids of CyA, ATP and $MgCl_2$ was used. For diketopiperazine synthesis (▲) only D-Ala and Leu (0.6 mM of each amino acid) and the necessary cofactors were used. 100% in overall catalytic activity represents 3,896 counts/minute. In the case of diketopiperazine formation 100% was set as 984 counts/minute.

effect on enzyme activity reflecting the kinetic rate of unfolding of cyclosporin synthetase.

When we analyzed the reaction products of these incubations by thin layer chromatography we could not detect any CyA formation at urea concentration higher than 0.8 M whereas the enzyme was still able to synthesize the diketopiperazine *cyclo*(D-alanyl-*N*-methylleucyl) (*cyclo*-(D-Ala-MeLeu)) (not shown). This molecule represents the first two amino acids of the growing peptide chain of CyA and therefore needs only the first two domains of the enzyme for its formation [7,13].

The formation of *cyclo*-(D-Ala-MeLeu) could also be achieved when the incubation mixture containing all constituent amino acids of CyA was replaced by a mixture containing only D-Ala and Leu as substrate amino acids. We have compared the overall catalytic activity of cyclosporin synthetase (using an incubation mixture containing all constituent amino acids of CyA) with the synthesis of *cyclo*-(D-Ala-MeLeu) (using as substrates D-Ala and Leu) catalyzed by the enzyme in the presence of urea. Whereas the dependence of *cyclo*-(D-Ala-MeLeu) formation from the urea concentration is almost linear, the EtOAc-extractable radioactivity shows a biphasic dependence on urea concentration when the incubation mixture is designed for CyA biosynthesis (Fig. 2). As our TLC analyses show there is no CyA, but only *cyclo*-(D-Ala-MeLeu) synthesis

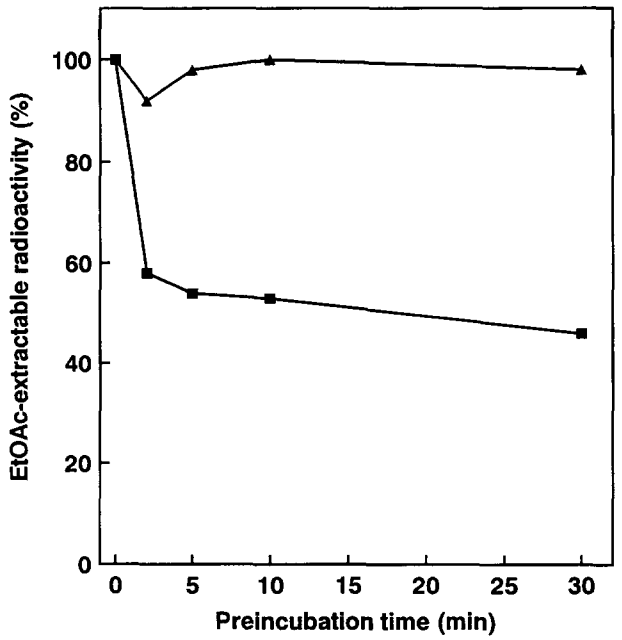

Fig. 3. Reversible denaturation of cyclosporin synthetase. The catalytic activity of the preparation in the absence of urea was set as 100% corresponding to 3,947 cpm. Enzyme preparation was preincubated with 0.4 M urea at 0°C for various times. At the indicated times aliquots were removed, diluted four times with buffer containing 0.4 M urea and tested for CyA activity (■) or diluted four times with an urea free buffer (▲), resulting in a final urea concentration of 0.1 M, and kept at 0°C for further 30 min. Thereafter the CyA formation activity was measured as described in section 2.3.

at urea concentrations of about 0.8 M and above. So it is obvious that under these conditions the first phase reflects CyA synthesis, whereas the second phase reflects *cyclo*-(D-Ala-MeLeu) synthesis. These results suggest that the first two domains of the enzyme which are responsible for the synthesis of *cyclo*-(D-Ala-MeLeu) are unfolded at higher urea concentration than some of the other nine domains. In the presence of low urea concentrations cyclosporin synthetase changes its catalytic activity leading to an enzyme which has a similar behaviour as the cyclosporin synthetase isolated from blocked mutant of *Beauveria nivea*, namely strain YP 582. This mutant enzyme does not catalyze the synthesis of CyA but the formation of the cyclic dipeptide [13].

To analyze the reversibility of the defolding of cyclosporin synthetase we preincubated the enzyme in the presence of 0.4 M urea at 0°C. After dilution of urea concentration to 0.1 M we allowed the enzyme to refold for 30 min at 0°C and determined thereafter the catalytic activity of the enzyme. Under these conditions we observed a fully reversible process of denaturation (Fig. 3).

To prove that conformational changes of the polypeptide chain of cyclosporin synthetase are responsible for the loss of catalytic activity in the presence of urea, we recorded fluorescence emission spectra of the enzyme in the presence of different urea concentrations. The native enzyme, purified by glycerol gradient ultracentrifugation, when excited at 280 nm showed an emission maximum at 337.2 nm (Fig. 4). It shifted gradually to 341.0 nm at an urea concentration of 4 M (Fig. 4). A similar shift of the emission maximum was obtained when we analyzed the enzyme in the presence of 6 M urea (data not shown). In no case catalytic activity of cyclosporin synthetase could be detected (Fig. 1).

3.2. Formation of enzyme substrate complexes in the presence of urea

During CyA biosynthesis substrate amino acids are activated in a two step mechanism. In the first step all substrate amino acids are activated as aminoacyladenylates. In the second step, these activated amino acids bind to specific active centers on the enzyme as thioesters under AMP liberation [7,8]. Cloning of the corresponding coding region of the cyclosporin synthetase gene has shown that it contains eleven amino acid activating domains [6]. Our results obtained by fluorescence emission analysis have demonstrated that the polypeptide chain of the enzyme underwent conformational changes in the presence of urea. These experiments do not give informations about which and how many of the eleven amino acid activating domains of cyclosporin synthetase are denaturated by a specific urea concentration.

To differentiate between native (active) and denatured (inactive) domains we analyzed the formation of enzyme substrate complexes in the presence of urea. We incubated cyclosporin synthetase with one of the substrate amino acids of CyA each in radiolabelled form, the necessary cofactors and urea in concentrations up to 1.0 M. (Covalent binding of Bmt to the enzyme was measured indirectly by formation of [^{14}C]MeBmt using [^{14}C]AdoMet and unlabelled Bmt.) In all cases examined,

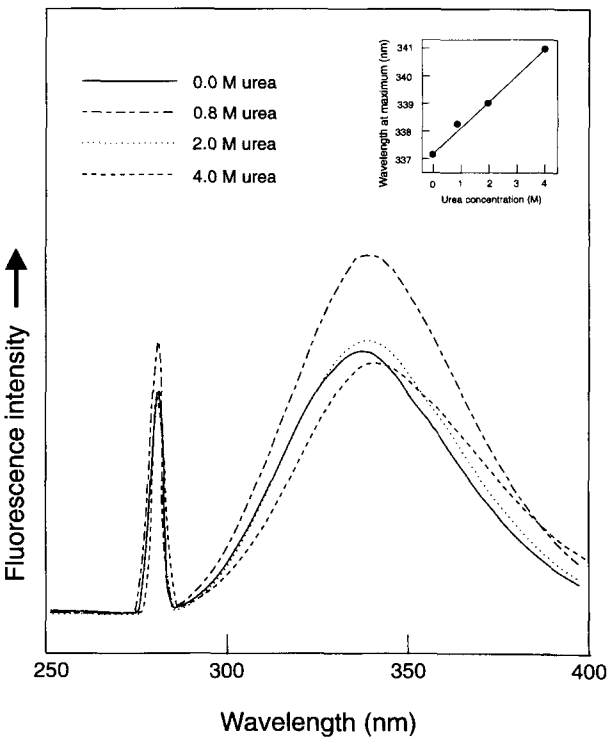

Fig. 4. Fluorescence emission spectra of native and denaturated cyclosporin synthetase. Spectra of native enzyme (solid line), purified by glycerol gradient ultracentrifugation [8] and enzyme denaturated by 0.8 M urea (dashed and dotted line), 2.0 M urea (dotted line) and 4.0 M urea (dashed line) (dissolved in 100 mM Tris-HCl pH 7.8, 4 mM ETDA, 37.5% (w/v) glycerol and 50 mM KCl) were measured with a fluorescence spectrophotometer Hitachi F4000. Excitation wavelength was 280 nm. The insert shows the wavelength of the maxima plotted against the urea concentrations.

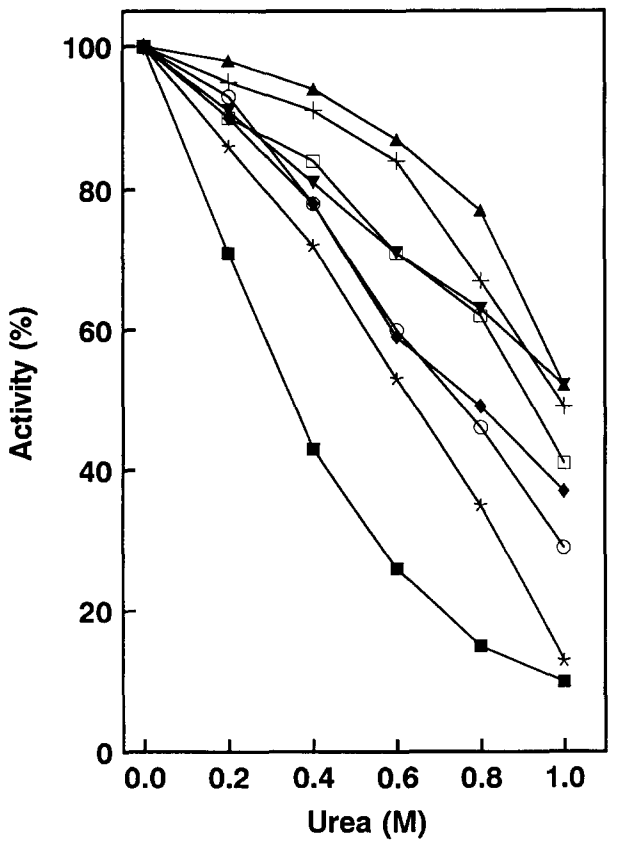

Fig. 5. Formation of enzyme substrate complexes in the presence of urea. Enzyme preparations were incubated with one of the substrate amino acids of CyA in radiolabeled form (in the case of Bmt: [^{14}C]AdoMet and unlabeled Bmt were used) and urea at 25°C for 10 min. The reaction was stopped by adding 7% trichloroacetic acid and the filter bound radioactivity was measured in a scintillation counter. For D-Ala (□) 100% represents 80.3 pmol, for Leu (+) 131.2 pmol, for Val (▲) 108.7 pmol, for MeBmt (♦) 74.6 pmol, for D/L-Abu (○) 325.9 pmol, for Gly (★) 41.0 pmol and for Ala (▼) 40.1 pmol. For detection of the overall catalytic activity of cyclosporin synthetase (■) it was incubated together with all constituent amino acids of CyA and the necessary cofactors at 25°C for 10 min. After stopping the reaction the EtOAc extractable radioactivity was measured. 100% were set as 3,804 counts/minute.

the amount of enzyme bound substrate amino acid decreased depending on the concentration of urea, whereby each substrate amino acid showed an individual inhibition pattern (Fig. 5), suggesting that each of the eleven amino acid activating domains of the enzyme can be individually denaturated by specific urea concentrations. So, for example while at an urea concentration of 1 M the formation of the enzyme glycine complex is reduced below 20% of the original activity the formation of the enzyme D-Ala complex still is above 40% of the original activity.

When we analyzed the biosynthetic activity of cyclosporin synthetase we observed that inhibition of CyA formation was inhibited by lower urea concentration than the thioester-binding of substrate amino acids to the enzyme. As can be seen further from Fig. 5, D-Ala and Leu-thioester formation is inhibited by 1 M urea to only 50% or less, such, *cyclo*-(D-Ala-MeLeu) formation is still possible under conditions where CyA formation is totally inhibited.

4. Discussion

Biosynthesis of the cyclic undecapeptide CyA is catalyzed by cyclosporin synthetase a single multienzyme polypeptide [8] existing of eleven amino acid activating domains, seven of which habour *N*-methyltransferase activities [6]. In the presence of increasing amounts of urea we found a reduction of the catalytic activity of this enzyme. Responsible for this is (are) conformational change(s) of the polypeptide chain of cyclosporin synthetase which could be demonstrated by fluorescence emission analysis. At low concentrations of urea (less than about 0.8 M) the CyA formation activity decreases with increasing urea concentrations, at medium (about 0.8 M) urea concentrations only a minor product is formed which was identified to be *cyclo*-(D-Ala-MeLeu) while the enzyme does no longer synthesize CyA. The cyclic dipeptide represents the first two amino acids (D-Ala in position 8 and MeLeu in position 9) of the growing peptide chain of CyA [7,13]. Formation of *cyclo*-(D-Ala-MeLeu) shows that medium urea concentrations are able to unfold some of the other nine domains of the enzyme whereas the two amino acid activating domains 8 and 9 are still functional. As shown by our studies on the formation of specific enzyme substrate complexes each amino acid activating domain of cyclosporin synthetase is unfolded by urea with specific kinetics suggesting a defolding of rather the single domains than the whole enzyme.

Such cyclosporin synthetase could possibly be developed to a model system for studying the folding/defolding mechanism of multi domain enzymes and of the function of chaperone/chaperonine machineries and/or specific accessory enzymes like peptidyl-prolyl-*cis*/*trans*-isomerases in this process.

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