

Structural determinants of cold adaptation and stability in a psychrophilic α -amylase

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The heat-labile α -amylase from an Antarctic bacterium is the largest known protein that unfolds reversibly according to a two-state transition, as shown by differential scanning calorimetry. Mutants of this enzyme were produced, carrying intended additional weak interactions of a type found in thermostable α -amylases. It is shown that single amino acid side chain substitutions can significantly modify the melting point T_m , the calorimetric enthalpy ΔH_{cal} , the cooperativity and reversibility of unfolding, the thermal inactivation rate constant, and the kinetic parameters k_{cat} and K_m . Although all mutations were located far from the active site, their overall trend is to decrease both k_{cat} and K_m , probably by making the molecule more rigid, but this protects mutants against thermal inactivation.

Key words: extremophiles, protein engineering, microcalorimetry.

Abbreviations: AHA, *Pseudoalteromonas haloplanktis* α -amylase; PPA, pig pancreatic α -amylase; BAA, *Bacillus amyloliquefaciens* α -amylase; DSC, differential scanning calorimetry.

Introduction

The cold-active and heat labile α -amylase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* is an exceptional case in that it is the largest known protein undergoing a reversible unfolding according to a two-state reaction pathway. It has been proposed that this unusual behavior is dictated by the requirement for an improved flexibility or plasticity of the protein molecule in order to perform catalysis at near-zero temperatures (FELLER et al., 1999). Structural investigations have suggested that this is achieved by decreasing the number and strength of weak interac-

tions stabilizing the native conformation (FELLER et al., 1994; AGHAJARI et al., 1998a,b). Accordingly, one can expect that any mutation designed to improve the stability of this fragile structure will induce significant perturbations of the denaturation pattern, and therefore provide evidence for structural features linked to the unfolding properties. We report here the calorimetric characterization of mutants from the cold-active α -amylase which were designed on the basis of a strong homology with heat-stable mesophilic α -amylases. These mutants reveal the unsuspected contribution of single amino acid substitutions to the thermodynamic parameters characteristic of large,

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heat-stable proteins and provide new insight into the molecular adaptations of enzymes from psychrophilic organisms. A detailed account of these results can be found in D'AMICO et al. (2001).

Stability of α -amylases analyzed by differential scanning calorimetry

Proteins are said to be marginally stable, as the positive Gibbs free energy change for unfolding (or conformational stability) represents a small difference between the large enthalpic and entropic contributions in the temperature range between the cold and hot melting temperatures. Basically, denaturation at high temperature is entropy-driven, as the large positive thermal dissipative force exceeds the corresponding enthalpy change. By contrast, cold unfolding is enthalpy-driven and arises from the negative contribution of the hydration Gibbs energy of protein groups, mainly polar, at low temperatures (MAKHATADZE & PRIVALOV, 1995). Studies of small globular proteins that unfold according to a pure two-state process, i.e. reversibly and without a stable intermediate between the native and the unfolded states, allowed establishment of the thermodynamic stability function accounting for the Gibbs energy change associated with their denaturation.

The thermodynamic studies were mainly performed by differential scanning calorimetry. Four parameters are usually assigned to the unfolding event recorded by differential scanning calorimetry: (i) the melting point of the unfolding transition, (ii) ΔC_p , the difference in heat capacity between the native and unfolded states arising from the transfer of buried groups in the folded protein to water, in the random coil, (iii) ΔH_{cal} , the calorimetric enthalpy corresponding to the total amount of heat absorbed during unfolding and recorded by the calorimeter, and (iv) ΔH_{eff} , the effective or van't Hoff enthalpy calculated from the slope of the transition and describing the cooperativity of the unfolding reaction. In the case of a pure two-state model, ΔH_{cal} and ΔH_{eff} are essentially identical. However, large proteins generally unfold irreversibly, as a result of strong heat-induced aggregation, with large deviations from the two-state model as their multidomain structure induces a stepwise or overlapping unfolding of domains that behave as individual calorimetric units. Figure 1 depicts the microcalorimetric behavior of the recombinant psychrophilic α -amylase from *P. haloplanktis* (AHA) and of heat-stable α -amylases, including porcine pancreatic α -amylase (PPA) used as reference for selection of muta-

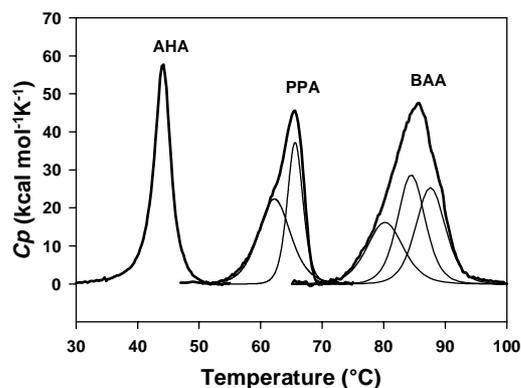


Fig. 1. Thermal unfolding of α -amylases recorded by DSC. The heat-stable PPA and BAA are characterized by higher T_m (top of the transition) and ΔH_{cal} (area under the transition) values, by a flattening of the transition and by the occurrence of calorimetric domains (deconvolution are in thin lines). All thermograms are baseline-subtracted and normalized for protein concentrations.

tions and *Bacillus amyloliquefaciens* α -amylase (BAA).

Selection of potentially stabilizing mutations

AHA belongs to the group of chloride-dependent α -amylases (FELLER et al., 1996). This group includes all known animal α -amylases and enzymes from some Gram-negative bacteria, with a large range of optimum temperatures. The high degree of amino acid sequence identity between chloride-dependent α -amylases allowed us to construct a multiple sequence alignment of the available primary structures (D'AMICO et al., 2000) and to detect about 20% out of 453 residues which are specific to the cold-adapted enzyme. The involvement of these residues in the weak stability of the psychrophilic α -amylase was assessed by comparison of its crystal structure (AGHAJARI et al., 1998a,b) with that of PPA, the closest structural homologue, as well as with the structure of α -amylases from human salivary and pancreatic glands and from the insect *Tenebrio molitor*. Molecular modeling was then used to check the consistency of the mutations aimed at introducing, into the heat-labile AHA, weak interactions found in mesophilic α -amylases. The selected mutations are listed in Table 1, along with the restored interactions. Following production in *Escherichia coli* and purification, the stability and kinetic parameters of 14 mutant enzymes were determined.

Table 1. Selected mutations in this study.

Mutants	Putative restored interactions ^a
K300R	Additional H-bond in the Cl ⁻ binding site (A)
N150D	Salt bridge with K190 (A)
V196F	Double aromatic interaction with Y82 and F198 (A)
N150D/V196F	Double mutant (A)
Q164I/V196F	Double mutant (A)
Q164I	Hydrophobic interaction in a core cluster(A)
T232V	Hydrophobic interaction in a core cluster(A)
N288V	Hydrophobic interaction in a core cluster(A)
M379V	Hydrophobic interaction in a core cluster(C)
R64E	Helix dipole stabilization (A)
L219R	Amino-aromatic interaction with Y298 (A)
D6AA	Deletion of 6 non-constrained residues at the C-terminus possibly involved in unzipping (C)
N12R	Salt bridge with D15 (A)
E279W	Aromatic interaction with F307 (A)

^a (A) and (C) refer to the central (α/β)₈ barrel and to the globular C-terminal domain, respectively.

Enthalpic stabilization by electrostatic interactions

Enthalpic stabilization involves an increase of the denaturation enthalpy and of the melting point. Such stabilization of the heat-labile α -amylase is obtained when a salt-bridge (N150D), polar aromatic interactions (V196F) or an additional H-bond (K300R) are introduced in the protein. Thermal unfolding of some of these mutants is illustrated in Figure 2. These additional electrostatic interactions induce a slightly higher T_m value, associated however with a large increase of the calorimetric enthalpy (ΔH_{cal}), e.g. the T_m value is shifted from 44 °C in the native AHA to 46.4 °C in the double mutant N150D/V196F and ΔH_{cal} is increased from 214 kcal/mol in AHA to 272 kcal/mol in this mutant.

Calorimetric domain acquirement on introduction of additional non-polar groups

Aliphatic side chains were introduced in AHA in order to reconstruct hydrophobic clusters found in the core of mesophilic α -amylases (Q164I, T232V, N288V and M379V). Interestingly, each substitution results in a non-two-state unfolding of the mutant enzymes and in the appearance of two calorimetric units or domains (Fig. 3). As also shown in these thermograms, the separation between both calorimetric units in the mutants can be improved by adjusting the experimental conditions, providing evidence for the coexistence of two protein domains of distinct stability. Deconvolution of the heat capacity function revealed that one domain

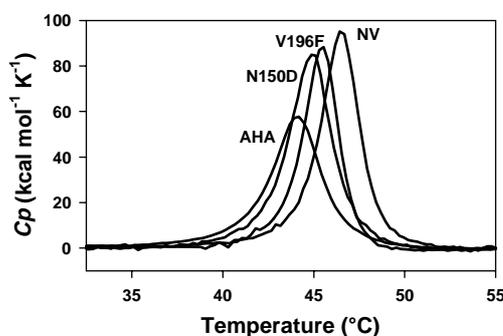


Fig. 2. Enthalpic stabilization. Thermal unfolding of AHA and of single and double mutants (NV: N150D/V196F) characterized by increased T_m and ΔH_{cal} values.

has always an increased T_m compared to that of AHA, underlining that stabilization was induced by the reconstituted hydrophobic cluster. In contrast, the lower T_m of the first domain seems to be the consequence of strain imposed in this domain, which is not compensated in other protein regions. This is supported by the combination of mutations Q164I and V196F resulting in a mutant enzyme displaying two stabilized domains, as far as T_m is concerned. The mutant T232V deserves special comments. Indeed, the shape of its heat capacity function is similar to that of PPA (compare Figs 1 and 3) as well as the relative contribution of each domain to ΔH_{cal} . Therefore, this mutant consistently reproduces the microcalorimetric unfolding pattern of the heat-stable PPA, but at lower temperatures.

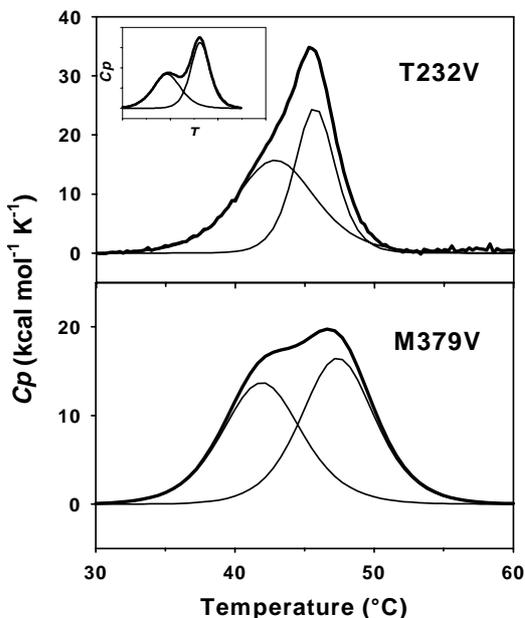


Fig. 3. Thermal unfolding of AHA mutants, providing evidence for calorimetric domains induced by additional non-polar groups in hydrophobic core clusters (inset: T232V at pH 8.0).

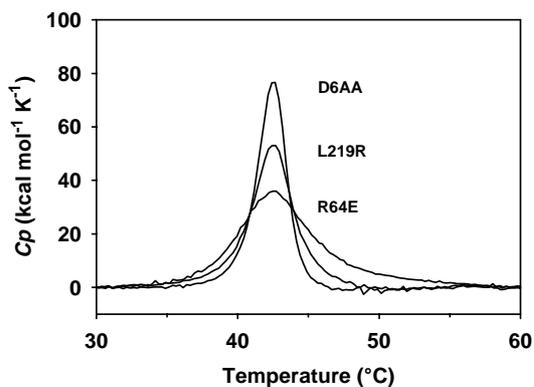


Fig. 4. Thermal unfolding of AHA mutants showing alteration of unfolding cooperativity. Decreasing the steepness of the transition maintains a higher level of native structures at elevated temperatures.

Cooperativity of unfolding

The comparison of ΔH_{cal} and ΔH_{eff} values allowed us to analyze the unfolding cooperativity by microcalorimetry. Several mutations listed in Table 1 affect the unfolding cooperativity without

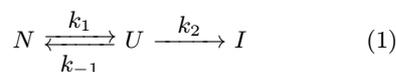
noticeable change of ΔH_{cal} . Thermal unfolding of mutants D6AA, L219R and R64E (Fig. 4) clearly illustrates the effect of mutations on the steepness of the transition. Furthermore, the thermogram slope of mutants L219R and R64E is decreased considerably and thus approached the profile of large and stable proteins such as BAA (Fig. 1).

Folding and unfolding reversibility

In these experiments, the mutant enzymes were 100% unfolded and then allowed to refold during a cooling period of 15 min at 15°C. The reversibility was calculated by the recovery of ΔH_{cal} during a second scan. Substitution of a single amino acid side chain is sufficient to drastically alter this critical parameter of protein folding, as demonstrated by the mutant N12R which only displays 25% renaturation. Mutants Q164I, V196F and Q164I/V196F which are stabilized by hydrophobic groups also decrease the unfolding reversibility significantly to about 50–60%. Under these same conditions, unfolding of the heat-stable PPA and BBA is completely irreversible.

Thermal inactivation of activity

Determination of inactivation rate constants usually requires the recording of the residual enzyme activity after incubation at high temperature. In the case of AHA and its mutants, the model of LUMRY & EYRING (1954) is valid:



where k_2 is the first order rate constant of thermal inactivation. However, the various degrees of reversibility noted for the mutants (kinetically characterized by k_{-1}) strongly impair the validity of the results obtained by conventional methods. We have therefore devised a new method using isothermal titration calorimetry and recording activity at 45°C as the heat released by the hydrolysis of starch glycosidic bonds. This method provides a direct and continuous monitoring of activity at the denaturing temperature (D'AMICO et al., 2001). It is significant that ten out of 14 mutants are protected against thermal inactivation and that mutants carrying newly introduced electrostatic interactions display the lowest inactivation rate constants. A plot of unfolding reversibility versus thermal inactivation (Fig. 5A) shows that these parameters for the mutant enzymes are

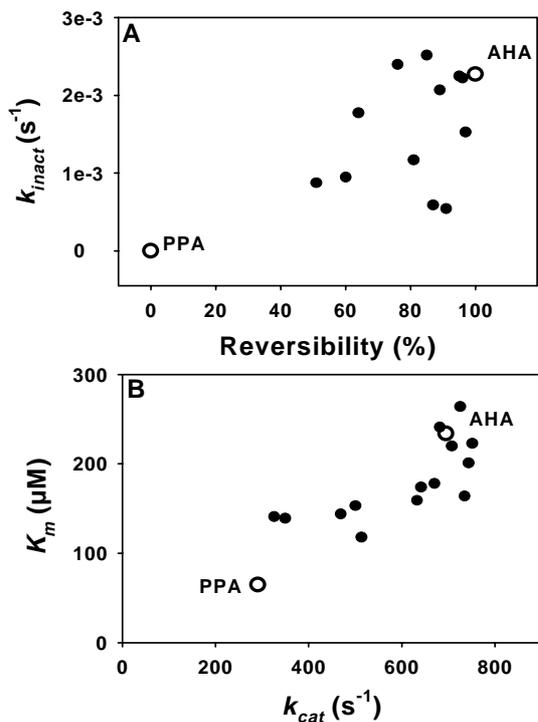


Fig. 5. Correlation of stability (A) and kinetic (B) parameters for wild-type (\circ) mutants (\bullet) α -amylases.

clustered between the respective values for AHA and PPA.

Kinetic parameters

The kinetic parameters k_{cat} and K_m were determined using a chromogenic maltoheptaose oligosaccharide (Fig. 5B). From these results, three main aspects should be pointed out. i) As previously reported, AHA possesses a higher activity (k_{cat}) than PPA in order to compensate for the slow chemical reaction rates at low temperature. ii) The general trend of the mutations is to decrease both k_{cat} and K_m . Figure 5B shows that both of these kinetic parameters for mutants fall within a narrow region of the plot, mostly between the values of AHA and PPA. iii) Most mutations protecting the enzymes against thermal inactivation also concomitantly decrease the k_{cat} value.

Stability of large proteins

The magnitude of the contribution, as recorded by microcalorimetry, of a single amino acid side chain

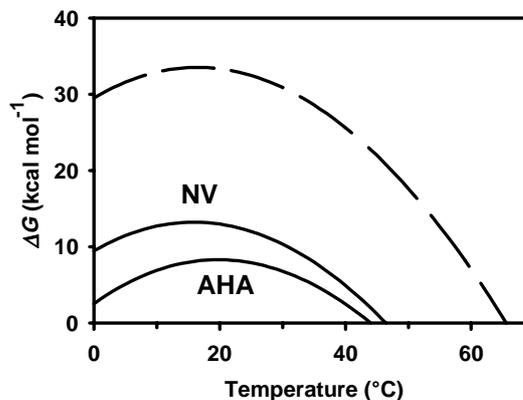


Fig. 6. Stability curves of AHA and of the double mutant N150D/V196F (NV). Dashed line: theoretical curve for a heat-stable α -amylase, using data from FUKADA et al. (1987).

to the stability parameters of a large protein is one of the most significant features revealed by engineering the heat-labile α -amylase. Such individual contributions are generally masked in stable proteins by the complex network of interactions involved in the native state conformation and by the fact that most frequently, heat-induced unfolding of large proteins is kinetically driven as a result of pronounced irreversibility. Our results show that the selected electrostatic interactions provide the largest contribution to stability *per se*, by increasing both T_m and ΔH_{cal} and by protecting mutants against thermal inactivation. Thermal unfolding of the double mutant N150D/V196F follows the two-state transition model and is essentially reversible. Accordingly, its thermodynamic stability can be analyzed using the modified Gibbs-Helmholtz equation:

$$\Delta G(T) = \Delta H_m(1 - T/T_m) + \Delta C_p(T - T_m) - T\Delta C_p \ln(T/T_m) \quad (2)$$

where ΔH_m is the melting enthalpy recorded as ΔH_{cal} in microcalorimetry and ΔC_p is the difference in heat capacity between the native and unfolded states. This function corresponds to the energy required to disrupt the native state (PRIVALOV, 1979) and is illustrated in Figure 6. One can note that despite a modest increase of the melting point, the conformational energy of the double mutant is twice that of the wild-type AHA around $10^{\circ}C$. Similarly, the contribution at $20^{\circ}C$ of the ion pair in N150D can be estimated as 4.4 kcal/mol, of the H-bond in K300R as 0.7 kcal/mol, and of a polar aromatic interaction in

V196F (assuming 2 interplanar interactions) as 0.8 kcal/mol. These values are in good agreement with data obtained by ab initio calculations (BURLEY & PETSKO, 1988) or by experimental studies (ANDERSON et al., 1990; JAENICKE, 1999). These free energy gains, however, originate from very large enthalpic-entropic compensation effects. The denaturation enthalpy and entropy can be calculated according to the relations (PRIVALOV, 1979):

$$\Delta H(T) = \Delta H_m + \Delta C_p(T - T_m) \quad (3)$$

$$\Delta S(T) = \Delta H_m/T_m + \Delta C_p \ln(T/T_m) \quad (4)$$

For example, the denaturation enthalpy at 20°C for AHA is 10.5 kcal/mol and the entropic term ($T\Delta S$) amounts to 2.2 kcal/mol. The corresponding values for mutant N150D are 60.9 kcal/mol and 48.2 kcal/mol, respectively. Therefore, the gain of 4.4 kcal/mol in conformational energy provided by the ion pair arises from a 6-fold increase of the denaturation enthalpy balanced by a 20-fold increase of the entropic cost. It should be added that mutant N150D most probably restores a surface salt bridge. Although the net contribution of solvent-exposed ion pairs has been frequently questioned (STROP & MAYO, 2000), our results demonstrate that such interactions can provide a substantial increment of conformational stability. This is especially relevant for thermophilic and hyperthermophilic proteins which are characterized by an abundance of surface ion pairs (CAMBILLAU & CLAVERIE, 2000), in some instances organized in interconnected networks (PAPPENBERGER et al., 1997; VETRIANI et al., 1998). On the other hand, about 8 ion pairs, 15 arginine residues, and 10 aromatic interactions are lacking in the crystal structure of AHA when compared with the heat-stable PPA (AGHAJARI et al., 1998a). The disappearance of these electrostatic-based features is obviously a main determinant for both the low T_m and melting enthalpy of the psychrophilic α -amylase.

It is generally agreed that the hydrophobic effect is the main factor in stabilizing folded conformations (MATTHEWS, 1993). As shown in Figure 3, the insertion of a non-polar group into a hydrophobic core cluster creates a stabilization center in the protein and in the peculiar case of AHA promotes the appearance of calorimetric domains. Unfortunately, large deviations from the two-state model preclude any reliable calculation for these mutants. It should be noted, however, that the increases of T_m for the stabilized transition are of the same magnitude or even higher (M379V) than

that provided by electrostatic interactions. Analysis of the hydrophobic clusters in AHA shows that 20 substitutions decrease the hydrophobicity index of the involved residues relative to PPA. As indicated by the calorimetric behavior of the mutations selected for this work (Fig. 3), the overall low core hydrophobicity of the heat-labile AHA accounts to a large extent for the lack of discrete unfolding intermediates or domains. When protein adaptation to temperature is considered, a practical implication of the flattening of the unfolding transition in thermostable α -amylases such as BAA (Fig. 1) can be deduced to be the maintenance of a higher level of native conformation at elevated temperatures. Figure 4 shows that the same holds true between 45 and 55°C for mutants of the heat labile α -amylase with altered cooperativity. The relation between unfolding reversibility and thermal inactivation (Fig. 5A) is another unsuspected result gained from the α -amylase mutant analysis. Referring to Eq [1], mutants protected against thermal inactivation are also those that refold less efficiently, mimicking the behavior of thermostable α -amylases. This could be explained by the large number of all types of weak interactions required for proper folding and stability in mesophilic and thermophilic enzymes. As a consequence, there will be more possibilities of side reactions during refolding, either by intramolecular mismatch, or by aggregation due to replacement of the regular intramolecular pairing by intermolecular interactions (JAENICKE, 1999).

Cold adaptation

It was shown that all 24 side chains in the active site that participate in H-bonding to a large pseudosaccharide inhibitor in a conformation mimicking that of the transition state are strictly conserved in both AHA and PPA crystal structures (AGHAJARI et al., 1998a; QIAN et al., 1994). As a consequence, changes in the primary structure that occur far from the active site should be responsible for the high specific activity of AHA. As a matter of fact, all mutations in this study designed to restore a mesophilic character are located far from the catalytic center and tend to decrease both k_{cat} and K_m (Fig. 5B). It has been convincingly argued that increases in flexibility of cold-adapted enzymes lead to a broader distribution of conformational states, translated into higher K_m values for enzymes devoid of adaptive changes within the active site (HOLLAND et al., 1997; FIELDS & SOMERO, 1998). In this context, making the molecule or part of the molecule more

rigid because of the newly engineered weak interactions in the psychrophilic α -amylase should contribute to decrease K_m values by reducing the conformational entropy of the binding-competent species. The kinetic parameters of the mutants (Fig. 5B) give experimental support to the earlier hypothesis that temperature-adaptive increases in k_{cat} occur concomitantly with increases in K_m (FIELDS & SOMERO, 1998).

Our structure-based approach also demonstrates that natural mutations occurring in the cold-active α -amylase associate high activity and low stability. This result underlines that the lack of selective pressure for stable proteins at low temperatures does not entirely account for the instability of psychrophilic proteins. On the contrary, heat-lability or conformational plasticity seem to be intimately connected to sustained activity in cold environments.

Concluding remarks

This mutational analysis of a large protein demonstrates that the psychrophilic α -amylase has lost numerous weak interactions during evolution to reach the proper conformational flexibility at low temperatures. These adaptive adjustments contribute to improve k_{cat} without alteration of the catalytic mechanism, as the active site architecture is not modified, but at the expense of a weaker substrate binding affinity. On the other hand, thermophilic enzymes strengthen the same type of interaction to gain in structural stability at high temperatures but at the expense of a poor activity at room temperature (VARLEY & PAIN, 1991; ZAVODSZKY et al., 1998; KOHEN et al., 1999). These aspects underline the continuum in the strategy of temperature adaptation in proteins and reinforce the concepts of "compromise" (SOMERO, 1995) between activity and stability leading to "corresponding states" (JAENICKE & BOHM, 1998) of enzymes adapted to different thermal environments.

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