Macromol. Symp. ***145****, 125–136 (1999)*

Conformational changes in proteins at interfaces: from solution to the interface, and back

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SUMMARY: After adsorption, polymer molecules, including proteins, undergo conformational relaxation. It involves a certain degree of spreading of the polymer molecule over the sorbent surface. Adsorption kinetics experiments reveal that the characteristic time of spreading is much longer for globular proteins than for highly flexible polymers. Based on differential scanning calorimetry it is concluded that the degree of spreading of protein molecules decreases with increasing rate of covering the surface with the protein. It is furthermore inferred that the relaxation does not proceed gradually but rather in steps. According to circular dichroism spectra the relaxed protein molecules still contain a large fraction of ordered structure. As a rule, protein molecules that, by homomolecular exchange, are released from the surface re-adopt their original structure; however, an exception to this rule is reported.

Introduction

Protein adsorption at interfaces is omnipresent. Exposure of a protein containing aqueous solution to an interface leads in almost all cases to adsorption of protein(s) onto that interface. The overall protein adsorption process is schematically outlined in Figure 1. It comprises various steps or stages. First, the protein molecule has to be transported from the bulk solution into the sub-surface region, where it interacts with the sorbent surface. That interaction may result in attachment of the protein at the surface, and once it is attached the molecule will try to relax, i.e., to optimize its interaction with the surface. This relaxation process usually leads to some degree of spreading of the molecule over the surface, involving structural rearrangements or, for that matter, conformational changes in the protein.

Depending on the extent of relaxation the protein molecule may detach more or less readily, for instance by exchange with protein molecules in solution (1,2,3,4). The question arises whether the molecules returning from the surface into the solution regain their original structure.

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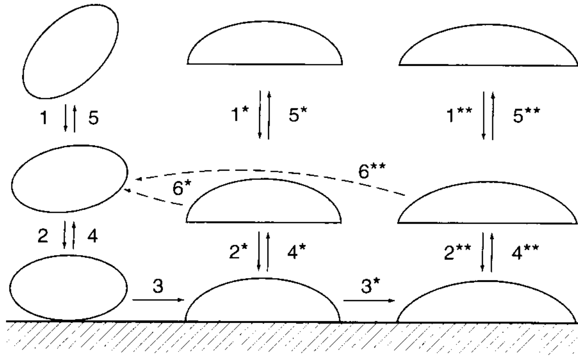

Fig. 1: Schematic presentation of the various stages of the overall protein adsorption process: (1) transport from the bulk solution towards the interface; (2) attachment at the interface; (3) relaxation (spreading) of an adsorbed molecule; (4) detachment from the interface; (5) transport into the bulk solution; (6) restructuring of an exchanged protein molecule.

In this paper we will focus on the spreading after attachment (step 3, in Figure 1) and its consequences for the biological functioning and on the issue of restructuring after release from the surface (step 6, in Figure 1).

Spreading after attachment

It may be clear that the extent of spreading, i.e., the degree of relaxation, depends on the rate of spreading relative to the rate of attachment. In other words, the extent of spreading is determined by the ratio of the characteristic time of spreading τ_s (which is mainly a function of the internal cohesion in the protein molecule) and the characteristic time of filling the sorbent surface τ_f (which is mainly determined by the protein flux, J, towards the surface).
If spreading occurs faster than filling the surface, i.e., if $\tau_s/\tau_f < 1$, the attached protein molecules are allowed to relax completely so that the adsorbed amount at saturation conditions, Γ^{sat}, is independent of J. However, if spreading proceeds slower than, or on a comparable time scale as, filling, i.e., if $\tau_s/\tau_f \gtrsim 1$, the degree of spreading will be affected by the protein flux. As J increases a neighbouring site will be occupied by a newly arriving molecule before the previously adsorbed one is completely relaxed. This results in less spreading, which is reflected in a higher value of Γ^{sat} at higher flux (smaller τ_f).
Relaxation of the proteins at the sorbent surface may be a gradual process, but it might as well occur in distinct steps. If gradual, the adsorbed layer would be rather homogeneous with respect

to the conformational states of the adsorbed protein molecules; if the relaxation process goes in distinct steps the adsorbed layer accommodates a heterogeneous conformation population.
Below, these considerations are illustrated by experimental data for adsorption kinetics obtained in a well-defined stagnation point flow (5,6).

Flexible polymers

In the case of a polymer that has no, or hardly any, internal coherence, e.g. polystyrene dissolved in decalin, the polymer molecule adopts a swollen, coil-like conformation and it is expected to relax rapidly upon adsorption. Figure 2 shows the adsorbed amount on silica as a function of the scaled time, $J \times t$. Γ^{sat} is essentially independent of J indicating that $\tau_s < \tau_f$. The values for τ_f are derived as Γ^{sat}/J and they vary from 640 s to 64 s. Because Γ^{sat} is invariant over that range it follows that τ_s < 64 s.

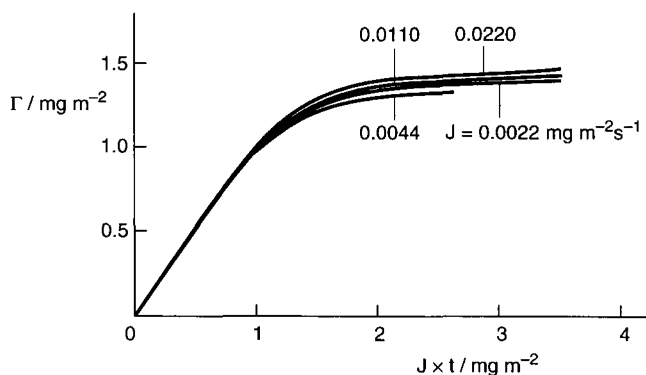

Fig. 2: Kinetics of the adsorption of highly flexible polystyrene molecules from decalin on a silica surface. Influence of the flux, J, of polystyrene to the surface. Data taken from ref. (6).

Globular proteins

Globular protein molecules in an aqueous environment attain compact, densely packed conformations due to a strong internal cohesion. After adsorption, at least at solid surfaces, the structure of such protein molecules remains compact although their 3-dimensional structure may have changed. This applies, for instance, for savinase, a relatively small protein molecule of 28,000 D molar mass (7). For the adsorption of savinase on a silica surface Γ^{sat} varies with τ_f

up to beyond 68 s as can by observed in Figure 3. By interpolation in Γ^{sat} (τ_f) a value of around 100 s is estimated for τ_s.

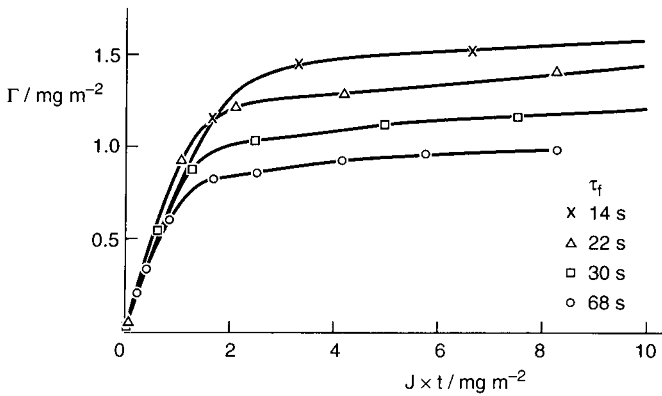

Fig. 3: Kinetics of adsorption of savinase from aqueous solution on a silica surface. Influence of the filling time, τ_f, of the surface by the protein. Data taken from ref. (8).

For IgG, a much larger globular protein molecule (150,000 D), which also adsorbs in a perturbed compact structure (9), Γ^{sat} varies with τ_f over the range 53 s - 925 s, yielding an estimated value of ca. 1000 s for τ_s; see Figure 4.

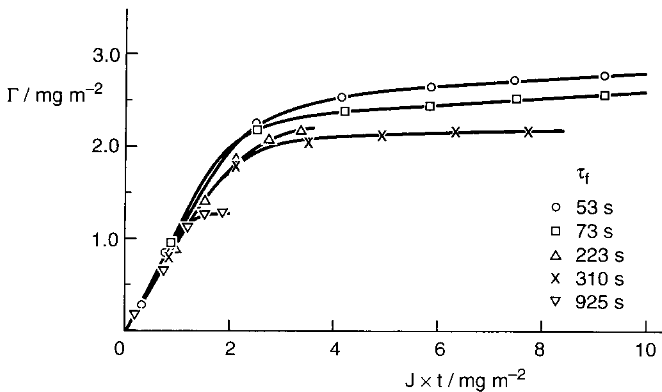

Fig. 4: Kinetics of adsorption of IgG from aqueous solution on a silica surface. Influence of the filling time, τ_f, of the surface by the protein. Data taken from ref. (10).

Protein adsorption data are often presented in adsorption isotherms, where the adsorbed amount is plotted vs. the protein concentration, c_p, in solution, as shown in Figure 5. At higher c_p the protein flux toward the surface is higher; hence, τ_f decreases with increasing c_p. It is therefore to be expected that ascending along the isotherm the extent of spreading decreases. For various protein/sorbent combinations this expectation has been confirmed using different forms of spectroscopy (11,12,13), calorimetry (11,14,15) and other techniques (16). Here, we present some data obtained with differential scanning calorimetry (DSC).

In a DSC experiment the protein molecule is heated. The heat or, for that matter, the enthalpy, $\Delta_d H$, required for the thermal unfolding of the protein shows up as the peak area in the thermogram. The temperature half-way the peak area is usually defined as the denaturation temperature T_d.

In aqueous solution, proteins, at least all the single domain proteins, show one single transition peak that develops over a rather narrow temperature range. This reflects a two-state transition, which implies that no thermodynamically stable states exist intermediate of the native and the denatured, unfolded state. Thus, at $T = T_d$ half of the protein population is in the native (N) state and the other half in the denatured (D) state. Hence, the thermal denaturation in solution is characterized by well-defined values for T_d and $\Delta_d H$.

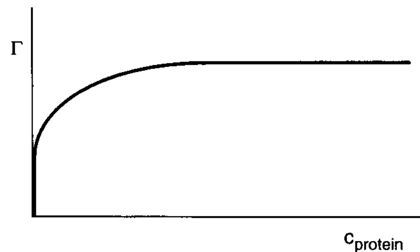

Fig. 5: Typical shape of a protein adsorption isotherm.

How would that be for adsorbed protein? If adsorption induces conformational changes which are the same for all protein molecules, i.e., if a homogeneous population of (partially) relaxed molecules is formed, it is to be expected that further thermal denaturation of the adsorbed molecules occurs at a temperature that deviates from T_d of the dissolved native molecules. However, if incomplete relaxation implies that only a fraction of the molecules is relaxed whereas the remainder is still in a native-like state, one peak at $T = T_d^{\text{native}}$ reflecting an enthalpy effect that is smaller than that for the dissolved protein, and, possibly, one or more other peaks should be observed. Therefore T_d- and $\Delta_d H$-values of the protein in the dissolved and the adsorbed

Table 1. Thermal denaturation of α-chymotrypsin in solution and adsorbed on a polystyrene surface on which oligoethylene oxide moieties are grafted. Data taken from ref. (17).

protein	T_d(°C)	$\Delta_d H$(Jg^{-1})	% N
α- chymotrypsin in solution	49	20.4	100
α- chymotrypsin adsorbed			
Γ = 0.42 mg m^{-2}	47	4.2	20
0.98 mg m^{-2}	49	10.3	50
1.30 mg m^{-2}	50	12.4	61

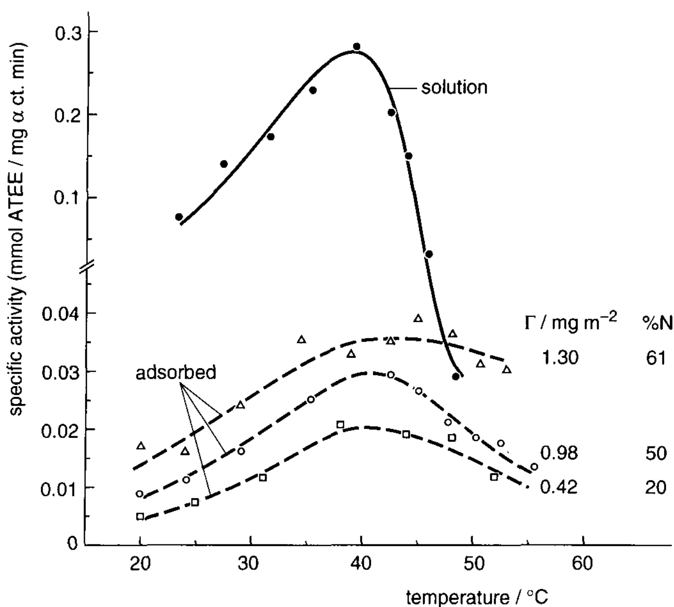

Fig. 6: Specific enzymatic activity of α-chymotrypsin in aqueous solution and adsorbed on polystyrene particles, as a function of temperature. For further explanation is referred to the text. Data taken from ref. (17).

state, respectively, are compared. This has been done for various combinations of proteins and surfaces (11,17).

By way of example, the results for α- chymotrypsin adsorbed on a modified polystyrene surface are presented. The adsorption isotherm resembles the one depicted in Figure 5, showing a well-

defined plateau-value of ca. 2.4 mg m^{-2} (obtained after 16 hours incubation at 22°C in a phosphate buffered medium of 0.01 M and pH 7). DSC experiments were performed with α- chymotrypsin in solution and at the polystyrene surface for loadings of 0.42 mg m^{-2}, 0.98 mg m^{-2} and 1.30 mg m^{-2}. Results of the DSC experiments are given in Table 1. In solution as well as in the adsorbed state the thermograms display only one transition peak. Moreover, the denaturation temperature of the adsorbed protein is not significantly different from that of the protein in solution. However, the values for $\Delta_d H$ are lower after adsorption, the more so the lower the coverage of the sorbent surface by the protein. These results strongly suggest a heterogeneous population in the adsorbed layer with a fraction of the molecules in a N-like state and the remainder in at least one perturbed state, that is thermally stable up to 90°C. It is furthermore inferred that the fraction in the N-like state increases with increasing amount adsorbed, i.e., with decreasing τ_f.

How does this affect the biological activity of adsorbed α-chymotrypsin? Figure 6 shows that adsorption causes a reduction of the specific enzymatic activity (activity per unit mass of protein), the more so the smaller the fraction of adsorbed molecules in the N-like state is. It is furthermore remarkable that the specific activity-temperature profile of the adsorbed enzyme is less pronounced, so that at elevated temperatures, say beyond 50°C, the specific activity in the adsorbed state may be higher than in solution. This observation could be of importance when immobilized enzymes are applied in systems that operate at relatively high temperatures, for instance in laundry-detergents.

Release from the adsorbed state

In many applications the number of protein molecules is in excess of the available sorbent surface area. It is documented that under such conditions the situation at the surface is dynamic; protein molecules are continuously exchanged between the solution and the sorbent surface (1,2,3,4). The exchange rate typically is of the order of minutes to hours so that, for a not too large excess of protein molecules, after, say, overnight incubation essentially all protein molecules have been adsorbed, exchanged, and so on.

Whether or not the protein molecules that have a perturbed structure in the adsorbed state regain their original, native conformation after returning into the solution is highly relevant for both (a) practical and (b) academic reasons:

a. The biological performance of the exchanged proteins may be affected. Examples are purification of proteins by chromatographic methods, hemodialysis, drug targeting and controlled drug release.

b. A perturbed structure of the protein molecule in solution after being released from the surface is stabilized in a "local" Gibbs energy minimum. This minimum may be lower than the Gibbs energy minimum stabilizing the native conformation. The native conformation is not necessarily the most stable one. Hence, the sorbent surface may act as a catalyst to transfer the protein molecule from the one (semi-)stable structure into the other.

Experimental results are presented for two different proteins, bovine serum.albumin (BSA) which has a great conformational adaptability ("soft" protein) and hen's egg lysozyme (LSZ) that has a much stronger internal coherence ("hard" protein). These proteins are incubated with hydrophobic, negatively charged teflon particles and with hydrophilic, negatively charged silica particles. Relevant physico-chemical characteristics of the components, at conditions of incubation, are summarized in Table 2.

The protein solution and the sorbent dispersion were incubated for 16-18 hours and, after reaching steady state conditions, the ratio between adsorbed and dissolved portein is ca. 1:1.

Table 2. Physico-chemical properties of the proteins and the sorbent materials. Medium: 0.01 M phosphate buffer pH 7.0; 22°C.

protein	*BSA*	*LSZ*
molar mass (D)	67,000	14,600
isoelectric point (pH-units)	4.7	11.1
denaturation temperature (°C)	57	73
denaturation enthalpy (J g^{-1})	11±1	30±3
sorbent surface (supplied as colloidal dispersion)	*silica*	*teflon*
hydrophobicity (contact angle with water)	0°-5°	96°
electrokinetic potential determined by electrophoresis (mV)	−48	−47

As an example, Figure 7 compares the DSC thermograms of BSA before adsorption, adsorbed on teflon particles and after being released from such particles. The denaturation of BSA in solution (ca. 2 mg/ml) occurs at 57°C with a value of 11 J g^{-1} for $\Delta_d H$. The thermogram of BSA adsorbed on the teflon particles does not show a transition peak which could imply either complete unfolding upon adsorption (so that no further heat-induced unfolding can occur) or a more or less ordered structure that is thermostable up to 90°C. For the exchanged BSA the thermogram shows a transition again, but it is broader and it is shifted to a higher temperature. It implies that after returning from the surface into the solution the molecule does not regain its original conformational characteristics: the conformation of the exchanged protein is more thermostable and it

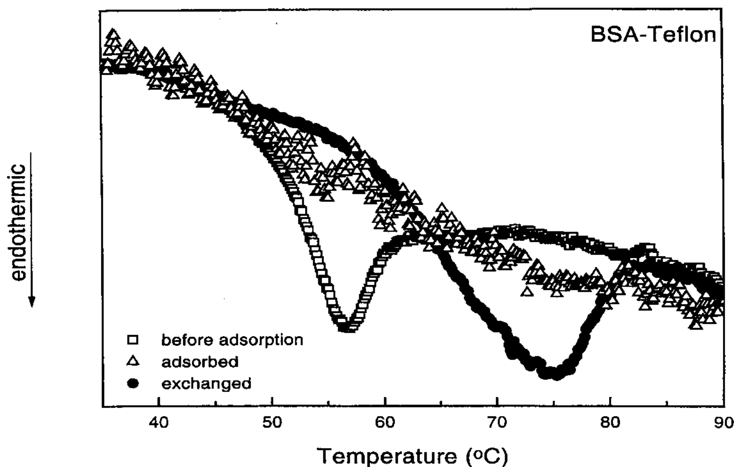

Fig. 7: Thermal denaturation thermograms for BSA (□) in solution before adsorption, (Δ) adsorbed on teflon particles and (●) in solution after being released from the teflon surface. Medium: phosphate buffer 0.01 M, pH 7.0. The arrow indicates the endothermic heat flow expressed in J s^{-1}.

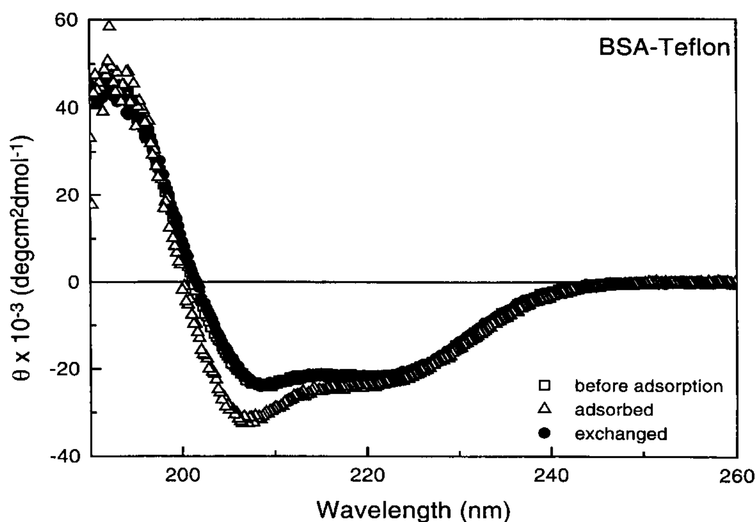

Fig. 8: Circular dichroism spectra of BSA in solution and adsorbed on teflon particles. Symbols and medium as in Figure 7.

assumes a wider distribution. However, the denaturation enthalpy is not much different from that of the protein before adsorption. The thermodynamic approach is complemented by circular dichroism (CD) spectroscopy, which provides information about the secondary structure of the protein. Results for the BSA-teflon system are presented in Figure 8. The CD spectrum of BSA before adsorption shows the characteristic negative ellipticity at 208 nm and 222 nm for proteins having a high α-helix content. At the teflon surface the spectrum shifts to even higher intensities at these two wavelengths, indicating an induction of helical structure. Helix formation by adsorption on hydrophobic surfaces has been previously reported (7,11,17) and it could result from increased intramolecular hydrogen bonding in the adsorbed protein, as hydrogen bonding with the hydrophobic sorbent surface is not possible. In view of the absence of a transition peak in the DSC thermogram it is concluded that the conformation of the adsorbed BSA is thermostable up to 90°C. The CD spectrum of the exchanged BSA is indistinguishable from the one before adsorption, indicating that after release from the teflon surface the BSA molecule adopts the same secondary structure as in the native, dissolved state before adsorption. This is in agreement with the minor difference in $\Delta_d H$-values for the protein in solution before adsorption and after exchange, respectively. The increased thermal stability of the exchanged BSA may then be due to a change in the tertiary structure (folding of the secondary structured polypeptide chain). Indeed, further evidence for different folding of the exchanged BSA molecules is obtained from dynamic light scattering experiments from which a more spherical shape (decreased aspect ratio) of the exchanged molecules is inferred.

In Table 3 the results obtained for the four systems, BSA-teflon, BSA-silica, LSZ-teflon and LSZ-silica, are summarized. The helical contents are qualitatively indicated, relative to those of the proteins in solution before adsorption. BSA adsorbed on hydrophilic silica particles does not show a thermal transition up to 90°C. At this surface the α-helix content is lowered. It has been

Table 3. DSC and CD results for various protein-sorbent systems. Medium: 0.01 M phosphate buffer pH 7.0.

	BSA						LSZ					
	teflon			silica			teflon			silica		
	sol	ads	exc	sol	ads	exc	sol	ads	exc	sol	ads	exc
T_d (°C)	57	>90	73	57	>90	57	73	>90	73	73	63	73
$\Delta_d H$ (J/g)	11	NT	13	11	NT	11	30	NT	30	30	13	30
α-helix		↑	=		↓	=		↑	=		ND	=

sol: solution before adsorption; ads: adsorbed; exc: exchanged; NT: no thermal transition; ND: no data; ↑ higher than in sol; ↓ lower than in sol; = equal to that in sol

reasoned that a decrease in ordered structure is the main driving force for adsorption of a protein on a like-charged, hydrophilic surface (18). After release from the hydrophilic silica surface the BSA molecules re-adopt the same conformational characteristics as before adsorption, as probed by both DSC and CD.

Thermal denaturation of LSZ in solution before adsorption occurs at 73°C with an enthalpy effect of 30 J g^{-1}. For LSZ as well adsorption on the hydrophobic teflon promotes helix formation and stabilization of the surface-induced conformation. However, unlike for BSA, LSZ returns to its original conformation after returning from the teflon particles into the solution. Adsorbed on the hydrophilic silica LSZ is more heat-sensitive; T_d and $\Delta_d H$ are lower than before adsorption. Here again it cannot be ruled out that a fraction of the adsorbed LSZ population has become less thermostable whereas the remainder is stable up to 90°C. Unfortunately, additional CD data are lacking for the LSZ-silica system because the colloidal stability is too low to obtain reliable CD spectra. After exchange from the surface the LSZ molecules recover their original conformation.

Conclusions

Adsorption-induced conformational relaxation of globular protein molecules occur at a much slower rate (~ hundreds of seconds) than in highly flexible polymers. The conformational rearrangement does not proceed gradually but rather in distinct steps. Relaxation at the surface causes a decrease in the biological activity; however in the adsorbed state that activity may be less sensitive to temperature variation.

The protein conformation at the solid/liquid interface is nòt an unfolded one. It still contains a large amount of ordered secondary structure. In most cases the conformation in the adsorbed state is more thermostable than in solution.

After release (by homo-molecular exchange) from the sorbent surface into the solution globular proteins re-adopt their native conformation as it was before adsorption, with exception of the proteins having a weak internal coherence (great conformational adaptability) and that return from a hydrophobic teflon surface.

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