Thermodynamic Analysis of the Potentiometric Titrations of Polypeptides Accompanying the Coil-Beta Conversion

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The stability of uncharged β-structure of polypeptides from potentiometric titrations is examined based on thermodynamic considerations with special references to the aggregation and/or precipitation of polypeptides and the irreversible nature of the titration. It is concluded that most of the reported data on various kinds of polypeptides approximately represent the stability of the β-structure. Diverse stabilities exhibited by reported data can be partly ascribed to different extents of the stacking of the pleated sheets.

The stability of the β-structure, an important secondary structure found in proteins, in aqueous media has been studied.[1–15] For an intermolecularly associated β-structure, the free energy of association is derived from the concentration dependence of the conversion.[1] However, all other studies have evaluated the free energy change based on the potentiometric titrations.[2–15]

The interpretations of the potentiometric titrations accompanied by the coil-β conversion are often ambiguous because of slow attainment of equilibrium and/or irreversible behavior. These complex situations originate mainly, not always, from the aggregation and/or precipitation of the polypeptides. Under this situation, the reported values of the free energy change of the conversion are only nominal and apparent. These values are called here tentatively the apparent free energy change, ΔG_{app}, which is defined as follows in the case of poly (weak acid).[16–18]

$$-\Delta G_{app} = 2.303RT\int_{-\infty}^{pH_1}(\alpha_C - \alpha^*)\,d\,pH$$
$$= 2.303RT\left[\int_0^1 pH^*\,d\alpha^* - \int_0^1 pH_C\,d\alpha_C\right]$$
$$= 2.303RT\int_0^1 (pH^* - pH_C)\,d\alpha \qquad (1)$$

Here, α_c and α^* (pH_c and pH*) represent, respectively, the degrees of ionization (the values of pH) of the reference solution and the actual solution (including the case with phase separation). The upper limit of the integral, pH_1, corresponds to the pH where α_c coincides with α^*. In the reference solution, random coils are titrated without being converted to the β-structure when protonated. In other words, in the reference solution, only random coils are present at any pH. In the actual solution, the β-structure is formed at low values of pH or at low charge densities. The fraction of random coils at zero ionization is assumed to be negligible in the present study.

Potentiometric titrations accompanying the β-coil conversion have been performed on four different groups of polypeptides; poly(L-tyrosine),[2–7] poly(L-lysine) (PLL)[8,9] poly[*S*-(carboxymethyl)-L-cysteine][10–12] (poly[Cys(CH_2COOH)]) and poly[*S*-(2-carboxyethyl)-L-cysteine] (poly[Cys(CH_2CH_2COOH)]),[12,13] and copolypeptides of L-lysine with L-valine[14] and L-isoleucine.[15] The reported values of ($-\Delta G_{app}$) differ markedly; about 1.25 kJ mol^{-1} for poly(L-tyrosine),[3–7] 590 J mol^{-1} for poly(L-lysine),[8] 3.51 kJ mol^{-1} and 4.60 kJ mol^{-1} for poly[Cys(CH_2COOH)] and poly[Cys(CH_2CH_2-COOH)], respectively[12] and 3.76 kJ mol^{-1} and 2.15 kJ mol^{-1} for poly(L-valine)[14] and poly(L-isoleucine), respectively.[15]

It is pertinent, therefore, to examine whether or not these reported diverse values of ($-\Delta G_{app}$) can be accepted as representing different stabilities of the β-structures formed by different kinds of side chains.

In the present study, the relation between the apparent free energy change (ΔG_{app}) and the stability of the β-structure is analyzed at first when the aggregation or the precipitation of polypeptides occurs. Then, a conventional procedure to evaluate ΔG_{app} from titration data as well as the effect of polypeptide concentration on the stability of the β-structure is examined. Finally, as an example of the usefulness of the present approach, a new interpretation is proposed on the stability of the β-structure of poly(L-lysine) from the titration data.

Theoretical

Relation between the Stability of the β-Structure and ΔG_{app}. In the potentiometric titrations with aggregation of polymers, the thermodynamic analysis has shown that ($-\Delta G_{app}$) consists of essentially two contributions.[19]

$$-\Delta G_{app} = \Delta\mu_p(0)/x + (n_w/n_p)\Delta\mu_w(0)/x \qquad (2)$$

and

$$\Delta\mu_p(0) = \mu_p^c(0) - \mu_p^\beta(0) \qquad (3)$$

Here μ_p and μ_w are the chemical potentials of the polymer component (taken as protonated species) and solvent (water), respectively. Numbers of moles of these components are denoted by n_p and n_w. $\Delta\mu_p(0)$ represents the difference between the chemical potentials of the protonated species of random coil (superscript c) and β-structure (superscript β). The second term of rhs of Eq. 2 represents the solvent contribution.[19,20] When the number of dissociable sites per polymer x is not too small, the solvent contribution is shown to be negligible.[19] In the present study, the second term of Eq. 2 is always neglected. This approximation is valid

for synthetic polypeptides considered in the present study. In the case of proteins, however, the solvent contribution of Eq. 2 can be significant in a certain case.[19] In the case of unimolecular conversion, such as the helix-coil conversion, the concentration dependent terms (cratic terms) of the chemical potential are identical for both conformations. Hence, $\Delta\mu_p(0)$ reduces to the corresponding standard part $\Delta\mu_p^*(0)$. When aggregation occurs, on the other hand, $\Delta\mu_p^*(0)$ can be evaluated from $\Delta\mu_p(0)$ after the correction for the cratic part is made. Further, $\mu_p{}^{\beta*}(0)$ itself can vary with concentration if the "crystallinity" of the β-structure depends on the concentration, as is likely in the case of crystalline polymers. In this sense, the effect of aggregation on ΔG_{app} can be said to be dual, even when the solvent contribution is ignored (which represents also the effect of aggregation).

The effect of phase separation on ΔG_{app} has been developed recently for a simple case where neither conformational change nor aggregation occurs in the solution phase and where the potentiometric equation for the polymers in the precipitated phase is given by Eq. 4.[20]

$$d\mu_p = -2.303\,RT\,\alpha_b x\,d\,pH \tag{4}$$

The result is given as follows:

$$-\Delta G_{app} = \Delta\mu_p(0)/x - 2.303(RT/C_p{}^t)\int_{-\infty}^{pH_c} C_p(\alpha-\alpha_b)d\,pH. \tag{5}$$

Here, the integration extends over the entire region of precipitation. In this region, the solution (degree of ionization α) and the precipitates (degree of ionization α_b) are in equilibrium with each other. The solubility C_p increases with pH and eventually coincides with the total concentration $C_p{}^t$ in the single solution region ($pH \geq pH_c$). The second term is suggested to be negligible in ordinary cases.[20] Although the effect of aggregation in the solution phase is not taken into account in the derivation of Eq. 5, the effect can be included in the term $\Delta\mu_p(0)/x$ according to the result of the preceding paragraph. Therefore, for the most of the data encountered in the present study, $-\Delta G_{app}$ can be approximated to $\Delta\mu_p(0)/x$, *i.e.*, Eq. 6 holds.

$$-\Delta G_{app} = \Delta\mu_p(0)/x. \tag{6}$$

However, as discussed later, the effect of phase separation could be significant in the case of poly(L-lysine).

It is important to examine the applicability of Eq. 4 to the β-precipitates. As shown experimentaly in a particular example,[21] β-precipitates contain a considerable amount of water, *i.e.*, the β-precipitates most likely consist of a gel which is uniform like a solution. Consequently, Eq. 4 holds for the β-precipitates to a good approximation. Stacked aggregates are also present in a gel constituting crosslinkages. However, as shown later, titration of β-precipitates occurs approximately for those dissociable sites on the surface of the aggregates and those sites inside the stacked aggregates are not titrated. In the course of titration, pleated sheets are peeled off from stacked aggregates. However, this disintegration process does not affect $\mu_p{}^{\beta}$ but the solubility C_p.

Examination of the Procedure of Evaluating ΔG_{app} from Experimental Data. In the present section, the procedure to evaluate ΔG_{app} from titration curves will be examined.

In all the data published up to now, identical values of pK_o were assumed for both random coil and the β-structure. In the case of poly (L-lysine),[8] both β-sheet and random coil limbs are discriminated clearly and they can be reasonably extrapolated to the same point pK_o. In the case of poly[Cys(CH_2COOH)] and its side chain homolog, however, the extrapolation of the β-sheet limb cannot be made unambiguously. In the case of poly(L-tyrosine), the situation is reversed. Extrapolation can be made only from the β-sheet limb and hence $pK_o$is assumed to be identical with $pK_o{}^{\beta}$. When the stacking of the pleated sheets occurs appreciably, the dissociable sites belonging to adjacent pleated sheets in the interior of the stacked aggregates will have a different value of pK_o, $pK_o{}^{\beta}$, from normal one found in the state of random coils. If $pK_o{}^{\beta}$ is greater than pK_o, $-\Delta G_{app}$ will be greater than reported values in each case discussed above.

In the titration of the stacked aggregates, what is likely to occur is such that titration proceeds with the sites located on the surface of the aggregates and that the sites buried in the interior of the aggregates are not titrated. When the surface charge density of the aggregates increases, the aggregates split into small aggregates or pleated sheets are peeled off one by one from the aggregates, or both. Consequently, there are a number of dissociable sites on the pleated sheets which have normal pK_o at any stage of titration. Under this situation, pK_o remains unchanged, on one hand, irrespective of the degree of ionization α. On the other hand, the distribution of charged and uncharged sites becomes highly deviated from a uniform distribution. The degree of ionization of the surface, α', is much higher than the nominal value α. In Fig. 1, titration curves of hypothetical system are schematically drawn, where a well-defined aggregate species is in equilibrium with unstacked pleated sheet. The titration curve of the aggregate (curve d) differs from that of unstacked pleated sheet (curve c), simply because α and α' are not identical. In other words, an important consequence of the difference between α and α' is included in the titration data themselves when integrated. Also, the question about the term $\log[\alpha/(1-\alpha)]$ does not cause any trouble, as seen below. It is obvious that the quantity pK^* ($=pH^*-\log[\alpha/(1-\alpha)]$) along curve (d) and in the transition region no longer represents the same physical meaning as attached to it in ordinary potentiometric titrations. However, the integral $\int_0^1 (pK^*-pK_c)\,d\alpha$ correctly reduces to the integral

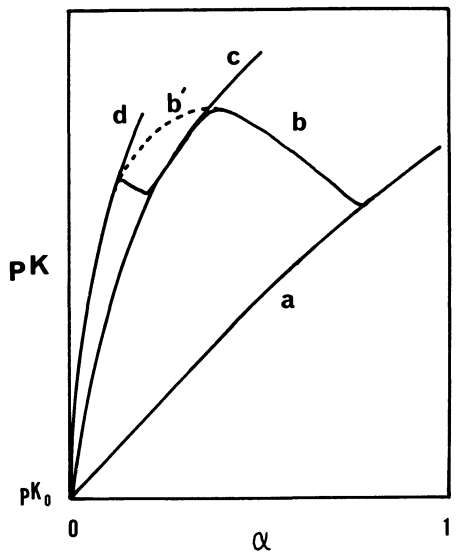

Fig. 1. Schematic titration curves for a hypothetical system where a well-defined stacked aggregate is in equilibrium with unstacked pleated sheet. Titration curves: (a) random coil, (b) actual system (pK^* *vs.* α), (c) single (unstacked) pleated sheet, and (d) stacked aggregate. Dashed curve (b′) represents the titration curve found on poly[Cys(CH_2CH_2COOH)], corresponding to a mixture of various kinds of aggregates.

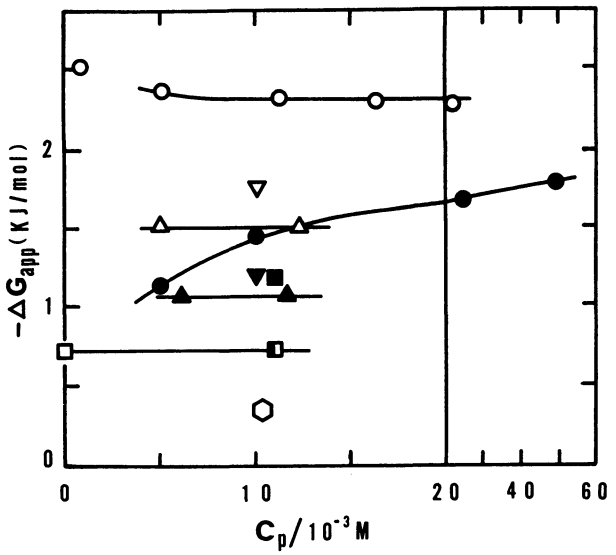

Fig. 2. Dependence of apparent free energy change (ΔG_{app}) on the polypeptide concentration (C_p) at 25 °C in the media of ionic strengths 0.1—0.2 M (with a few exceptions). Poly(L-lysine): (○) (DP=670—837).[8] Poly(L-tyrosine): (□) (DP=445),[6] (◧),[5] and (■) (DP=1370).[3,6] Poly[Cys(CH_2COOH)]: (△) (DP=304) and (▲) (DP=50),[11] (▽) (DP=360) and (▼) (DP=62).[12] Poly[Cys(CH_2CH_2COOH)]: (○) (DP=206),[12] and (●) (DP=97).[13]

$\int_0^1$(pH*−pH$_c$) dα, which is the required quantity in Eq. 1. In this way, the difference between α and α' does not affect the evaluation of ΔG_{app}.

In the titration of poly(L-tyrosine) at high concentrations, titration curves similar to curve (b) in Fig. 1 are reported.[2,4] In the case of poly[Cys(CH_2COOH)], however, a titration curve like curve (b) has never been observed. A distribution about the number of pleated sheets in aggregates is expected and the side-by-side aggregation of pleated sheets is also likely. Under this situation, disintegration of aggregates occurs in diverse ways. As a result of their superposition, the titration curve (b) will be modified as shown by dotted line (b′) in Fig. 1. This kind of titration curve (b′) is observed in poly[Cys(CH_2COOH)] and poly[Cys(CH_2CH_2COOH)]. On the other hand, the titration curves of poly(L-lysine)(PLL) do not show any trace of the stacking as far as this diagnostic tool is concerned. However, fluorescence studies have suggested the stacking of the pleated sheets of PLL.[22-25] Thus, the titration behavior of PLL is different from those of other groups of polypeptide also in this respect.

Effects of Polymer Concentration. Experimentally obtained concentration dependence is summarized in Fig. 2 for the data at 25 °C in 0.1—0.2 M (1 M=1 mol dm^{-3}) ionic strength (with a few exceptions). In the case of poly(L-tyrosine) represented by squares, very close values of ($-\Delta G_{app}$) were obtained at 1.1×10^{-2} M[5] and 2×10^{-4} M.[5] In the case of poly[Cys(CH_2COOH)], negligible concentration dependence was found on two samples (DP=304 and 50).[11] For comparison, results on other samples are also shown (DP=360 and 62).[12] In the case of poly[Cys(CH_2CH_2COOH)], the effect of the concentration was extensively examined. Negligible dependence resulted for a high molecular weight sample (DP=206),[12] while a considerable dependence was found on another sample (DP=62).[13] In summary, concentration dependence of the titration curves is negligible except for one example (poly[Cys[CH_2CH_2COOH), DP=62) even when aggregation and/or precipitation occur with increasing concentration. Although extensive study on poly[Cys(CH_2COOH)] and poly(L-tyrosine) in this respect is desirable, we accept the conclusion for the moment and consider its implication.

The chemical potential of the polymer in a random coil can be generally written as follows:

$$\mu_p^c(0) = \mu_p^{c*}(0) + RT\ln a_c = \mu_p^{c*}(0) + RT\ln C_p^t y_p(0). \quad (7)$$

Here a_c and y_p (0) represent the activity and activity coefficient of uncharged random coils, respectively. Generally, explicit expression for $\mu_p^{\beta}(0)$ is not easily obtained, since detailed knowledge on the β-structure is scarcely available. At first, the simplest case is considered where the β-structure can be characterized as forming a pseudophase like detergent micells, as proved to be in the case of poly[Cys(CH_2COOH)] at a concentration range higher than about 1×10^{-3} M.[26] This situation, although simple, is valid for other polypeptides in the same concentration range. In this case, $\mu_p^{\beta}=\mu_p^{\beta*}$ and $\mu_p^{\beta*}(0)$ depends on the concentration only when "crystal structure" varies with the concentration. The chemical potential difference $\Delta\mu_p(0)/x$ is given by Eq. 8 in this case.

$$-\Delta G_{app} = \Delta\mu_p(0)/x = \Delta\mu_p^*(0)/x + (RT/x)\ln C_p^t y_p(0), \quad (8)$$

In this way, $(-\Delta G_{app})$ or $\Delta\mu_p(0)/x$ has to vary with C_p^t through the second term of rhs of Eq. 8, aside from the possible concentration dependence of $\Delta\mu_p^*(0)/x$. However, the second term changes only by $(RT/x)\times 2.303\times 2 \simeq 5RT/x$, which amounts to 125 J mol^{-1} for $x=100$ when C_p^t is changed by 100 times. It is now clear that for the high molecular weight samples shown in Fig. 2, the variation of the second term is practically negligible either because x is sufficiently large or because the examined concentration range is not wide enough. Therefore, the apparent inconsistency is solved, which is found between the formal dependence on C_p^t predicted by Eq. 8 and the actually observed independence on C_p^t. Moreover, it is inferred that the "crystal structure" remains constant irrespective of the concentration in these examples. The constant values of thus $(-\Delta G_{app})$ correspond to $\Delta\mu_p^*(0)/x$ under this situation.

$$-\Delta G_{app} = \Delta\mu_p^*(0)/x. \quad (9)$$

However, the values of $(-\Delta G_{app})$ differ considerably for polypeptides of different side chains as well as for different chain lengths, as exhibited in Fig. 2. Different values of the standard chemical potential difference can be partly ascribed to different "crystal structures." For example, the stacking of the pleated sheets is suggested to occur in the case of poly[Cys(CH_2COOH)] under the conditions shown in Fig. 2.[26,27] The extent of the stacking becomes negligible at the concentration range around 1×10^{-4} M[26] or lower.[27] Since the tendency of the stacking of the pleated sheets is stronger in the case of poly[Cys(CH_2CH_2COOH)],[28,29] the observed values of $(-\Delta G_{app})$, which is equivalent to $\Delta\mu_p^*(0)/x$, for these two polypeptides represent the stabilities of the stacked pleated sheets.

In the case of poly(L-tyrosine), the β-structures at the two concentrations differ considerably. At 1.1×10^{-2} M, the approximation of pseudophase is probably good, based on the results on the molecular weight measurements.[3] Hence, Eq. 9 is expected to hold at this concentration. On the other hand, unimolecular β- structure without aggregation is suggested at 2×10^{-4} M.[6,30] The observed values of $(-\Delta G_{app})$ at this concentration can be also regarded as $\Delta\mu_p^*(0)/x$ because the concentration terms in the chemical potentials cancel out each other just as in the case of the helix-coil transition, when activity coefficients of uncharged polypeptide in the two conformations are approximated to be identical. Similar values of $\Delta\mu_p^*(0)/x$ for both aggregated and unaggregated pleated sheets indicate either that the aggregation at 1.1×10^{-2} M does not involve the stacking of pleated sheets or that the single pleated sheet at 2×10^{-4} M is folded over on itself (self-stacking) as proposed by Auer *et al.*[6,7,30,31] The idea of self-stacking is interesting and it is hoped that the idea will be developed both experimentally and theoretically.

In Eq 9, $(-\Delta G_{app})$ is experimentally obtained and hence independent of the concentration scale employed, while μ_p^{c*} and hence $\Delta\mu_p^*(0)$ depend on it. If the concentration scale is changed from C_p^t to $C_p^{t\prime}=C_p^t\,\xi$, ξ being the conversion factor, $\Delta\mu_p^*(0)/x$ also changes to $[\Delta\mu_p^*(0)/x]'=\Delta\mu_p^*(0)/x+(RT/x)\ln\xi$. The difference $[(RT/x)\ln\xi]$ can be again very small owing to the factor x, unless an extraordinary conversion factor is used such as $\xi=10^x$. Consequently, it can be that $\Delta\mu_p^*(0)/x$ depends, in principle, on the concentration scale but the difference arising from the change of the concentration scale is practically negligible.

Contrary to many examples discussed above, on the other hand, the titration curves are dependent on the polymer concentration for a low molecular weight sample of poly[Cys(CH_2CH_2COOH)],[13] as shown in Fig. 2. Judging from the molecular weight of the used sample, the β-structure is formed by intermolecular association of extended chains to a considerable extent.[26] However, judging from the CD spectra, the fraction of the β-structure is large, very close to the completion, and hence the concentration dependence cannot be ascribed to the effect on the association equilibrium. Therefore, the observed concentration dependence can be understood as representing the situation that the extent of the stacking of the pleated sheets increases with C_p^t.

A New Interpretation of the Titration Data of Poly(L-lysine). As shown in Fig. 2, the stability of the β-structure of PLL is considerably smaller than that of other polypeptide consisting of tyrosine or cysteine derivatives, in spite of a contribution from the hydrophobic interaction which has been suggested for the β-structure of PLL.[8,9,14,15,32] Instead of discussing possible sources for this low stability of β-PLL, a completely different point of view is presented in this section, according to which the stability at 25 °C can be greater than what is given by $(-\Delta G_{app})$.

At 25 °C, an irreversible β-to-coil conversion of PLL takes place when titrated from uncharged to charged states, while a reversible coil-to-helix conversion occurs when titrated from charged to uncharged states. Marked time dependence of pH was reported during the titration from β to coil,[8] showing a slow dissociation of β-aggregates in comparison to helix-coil interconversion. The titration curve does not represent a simple β-to-coil conversion, but it represents a complex conversion from the β-structure to the mixture of the α-helix and random coils: The relative populations of these two states varying with pH. This should be the case, otherwise the titration could be reversible. For the potentiometric titration with separation of phases, Eq. 5 can be rewritten in the case of poly (weak bases) as follows.

$$-\Delta G_{app} = \Delta\mu_p(0)/x - 2.303(RT/C_p^t)\int_{pH_c}^{\infty}(\alpha-\alpha_b)C_p\,d\,pH \quad (10)$$

Here α and α_b represent the degree of ionization or protonation of the solution and the precipitates, respectively. The reason why the second term is negligible in ordinary cases resides in the respect that the solubility varies sharply with pH and hence the integrand remains finite only for a very narrow pH range. The effect of the equilibrium between the helix and random coil is dual. First, it will increase the solubility more than what is expected for the case that only random coils are present. Secondly, the increased solubility will vary gradually with pH, simply because the addition of HCl not only dissociates the β-aggregates but also it converts the α-helices to random coils. The latter process keeps the solubility unchanged. As a result of these two effects, the integrand is expected to remain finite over a wide range of pH. These considerations lead to the interpretation that the second term of Eq. 10 can contribute significantly in the irreversible titration of PLL and hence the term $(-\Delta G_{app})$ underestimates $\Delta\mu_p(0)/x$ considerably. Therefore, it is not unreasonable to expect a value of $\Delta\mu_p(0)/x$ larger than 590 J mol^{-1} $(-\Delta G_{app})$ at 25°C from the titration data of PLL.[8] This consideration predicts that $(-\Delta G_{app})$ increases with temperature (positive ΔH_{app}) even in the case that $\Delta H=0$, simply because both irreversible conversion and phase separation become significant as temperature decreases. A value of 3.64 kJ mol^{-1} was obtained for ΔH_{app} from the temperature dependence of ΔG_{app} including the data corresponding to irreversible titrations.[8] On the other hand, a small temperature dependence (ΔH_{app}=540 J mol^{-1}) was obtained from the titrations accompanying the coil-to-β conversion and limited within soluble range.[9] The present analysis can explain reasonably a large difference between these two values of ΔH_{app}. Conversely, the present point of view based on Eq. 10 is experimentally supported.

Summary

1. Most of the reported values of ΔG_{app} evaluated from the potentiometric titrations accompanying the β-coil conversion, can be approximately accepted as representing $\Delta\mu_p^*(0)/x$, the standard chemical potential difference (per residue) of uncharged polypeptides between the β-structure and random coil state.

2. The values of $\Delta\mu_p^*(0)/x$ vary markedly depending on the kind of side chains.

3. Different values of $\Delta\mu_p^*(0)/x$ are best understood as arising from different extents of "crystallinity" rather than from different extents of the conversion from disordered to the β-conformation. In the case of the β-structure, "crystallinity" corresponds to the extent of the stacking of the pleated sheets.

4. In the case of poly(L-lysine), present analysis suggests that the apparent free energy change $(-\Delta G_{app})$ from irreversible titration with precipitation underestimates the stability of the β-structure.

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