Concentration Dependence of the Conversion between the Intermolecular β-Structure and the Disordered State of Poly(*S*-carboxymethyl-L-cysteine) in Aqueous Solutions

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The effects of polymer concentration on the reversible conversion between the intermolecular β-structure and the disordered state of poly(*S*-carboxymethyl-L-cysteine) were examined by means of circular dichroism at constant pHs in 20 mM $NaClO_4$ solutions. The data were tentatively analyzed based on an assumed all-or-none model. A value of −1.1 kJ (−250 cal) per mole of residues was obtained for the standard free energy of association. It was suggested that the obtained results depended significantly on the molecular weight distribution of the used samples.

An important aspect of the β-structure of polypeptides in solution is the problem of whether it is formed by intermolecular association or by chain folding of a single polymer molecule. It is likely that these two types coexist in proportion to their relative stabilities and that other mixed types are also present. Accordingly, assignment of either of the two types can be done only under favorable conditions. Recently, we have shown that fractionated low molecular weight samples of poly(*S*-carboxymethyl-L-cysteine) form the β-structure only by association.[1,2] Further, the conditions for a reversible β-disorder conversion were explored.[2] The stability of the β-structure relative to the disordered state can be thus estimated based on the free energy of association using low molecular weight samples which form the β-structure entirely by association.

In the present study, the concentration dependence of the β-disorder conversion was examined on the same sample as used in the previous study[2] by circular dichroism (CD) in 20 mM $NaClO_4$ solutions at constant pHs: the condition prerequisite to a quantitative analysis.[2] The stability of the intermolecular β-structure was estimated from the concentration dependence based on an assumed type of association.

Another sample of similar molecular weight but significantly different extent of polydispersity was also used to examine the effects of polydispersity on the β-disorder conversion.

Experimental

Two samples (sample code SMA and SMA-2) were prepared from polydisperse low molecular weight samples (A/I=8, 12, and 16) of poly(*S*-carboxymethyl-L-cysteine) by ion-exchange chromatography. The fractionation procedure will be given elsewhere.[3] A sample SMA, used in a previous study,[2] was obtained as a pooled fraction originating from samples A/I=8 and 12, eluted at an ionic strength range 0.59—0.67 M. Another sample SMA-2 was obtained as a fraction originating from sample A/I=16, eluted at an ionic strength range 0.61—0.71 M.

Circular dichroism (CD) was recorded on a Jasco circular dichrograph J-40 A at 25±1 °C using cells of path lengths, 10, 5, 2, 1, 0.5 and 0.2 mm. Measurements of pH were carried out with a Hitachi-Horiba pH meter Model F-7 SS. Light scattering was measured on a Chromatix low angle light-scattering photometer KMX-6 using a 6—7° annulus which collected the scattered light about 4—5° from the cell. The whole assembly was as follows. A Hamilton syringe (5 ml) containing a 3—4 ml solution was connected to a filter holder (Millipore XX30-012-00) by a teflon tubing (ϕ=2 mm). The filter holder equipped with a membrane filter (0.1 μm pore size) (VC WP 01300) was mounted onto the scattering cell. The weight average molecular weight $\overline{M}_w$ was calculated according to the formula

$$\frac{Kc_p}{\Delta R_o} = \overline{M}_w^{-1} + B_2 c_p, \quad (1)$$

here ΔR_o represents the reduced scattering intensity at zero scattering angle (solvent contribution is subtracted), which was approximated by the value at scattering angle of 4—5°. K denotes the optical constant including the refractive index increment $(\partial n/\partial c_p)$. The refractive index increment at a constant salt concentration of 0.2 M NaCl was determined as 0.191 (ml/g) (unpublished results). The concentration c_p was expressed in g/ml referred to sodium salt of the polymer.

Ion-exchange chromatography was carried out on a DEAE-Sephacel (Pharmacia, particle size 40—150 μm) column (ϕ=1.6 cm, l=90 cm) at 25±0.5 °C. Gel permeation chromatography was carried out on a Sephadex G-75 column (ϕ=1.5 cm, l=85 cm) at 25±2 °C. In both chromatographic runs, the effluent was monitored by ultraviolet absorption at 220—235 nm (depending on the applied amount) on a Shimadzu UV-200 S spectrophotometer using a flow cell of 1 cm light path.

Polymer concentration is expressed in residue molarity C_p(N) and 20 mM (1 M=1 mol dm^{-3}) $NaClO_4$ solutions were used as a solvent throughout, unless otherwise stated. Solutions were mostly prepared from the solutions of neutral pH by the addition of HCl. Sometimes, solutions were prepared by dilution. In this case pH changed on dilution and the pH was readjusted to the same value as that before dilution.

Results

Molecular Weight Distributions of Samples. The results of the light scattering measurements on two samples SMA and SMA-2 in their fully charged state in 0.2 M NaCl solutions are given in Fig. 1. Molecular weights and the degrees of polymerization (DP) (in parentheses) were calculated as $(3.1\pm0.2)\times10^3$ (17 ± 1) and $(3.7\pm0.2)\times10^3$ (20 ± 1) for sample SMA and SMA-2, respectively. These values will be increased slightly if the effect of dialysis on the value of the refractive index increment is taken into consideration. Ion-exchange chromatography on DEAE-cellulose and

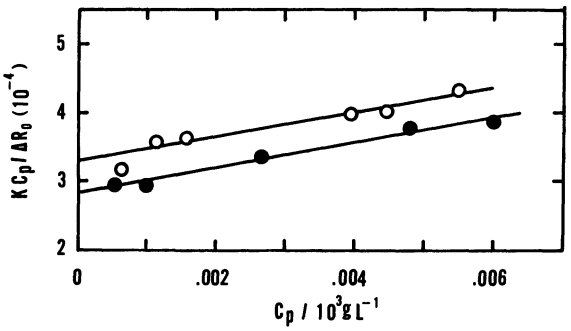

Fig. 1. Light scattering data for sample SMA(○) and SMA-2(●) at pH 7 in 0.2 M NaCl solutions.

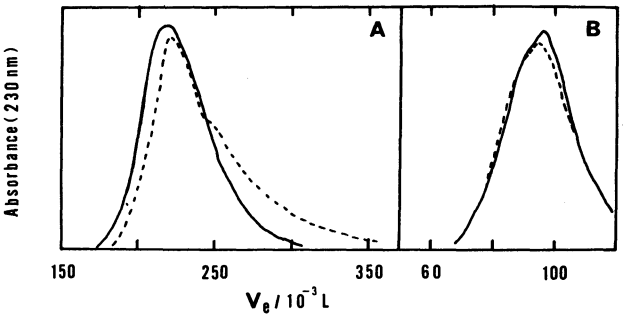

Fig. 2. Elution patterns on a DEAE-cellulose column (A) and sephadex G-75 (B) at pH 7.4. Samples: SMA (——), SMA-2(·····). Ionic strengths: (A) 0.56 M, (B) 0.20 M.

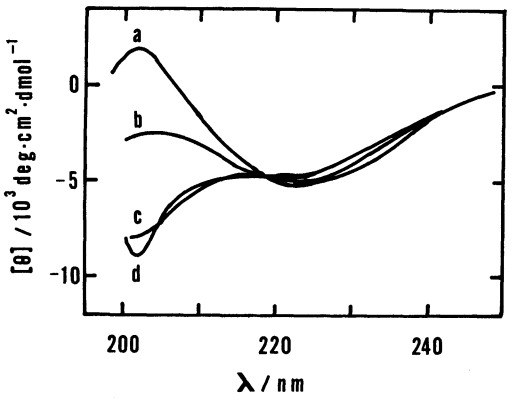

Fig. 3. CD spectra at pH 3.45 in 20 mM $NaClO_4$ solutions. Sample SMA. Concentrations (10^{-4} N): (a) 20, (b) 6.0, (c) 1.5, and (d) 0.50.

gel permeation chromatography on Sephadex G-75 were carried out on these two samples to examine the difference in their molecular weights more closely, although these measurements provide rather qualitative information. In Fig. 2(A), the elution patterns on a DEAE-cellulose column at an ionic strength of 0.56 M (0.55 M NaCl+phosphate buffer pH 7.4) are shown for the two samples. The peak position of sample SMA-2 is slightly shifted toward higher molecular weights as compared with that of sample SMA. A shoulder near V_e=250 ml is significant in the case of sample SMA-2.

As shown in Fig. 2(A), the main components constituting these two samples have nearly identical molecular weights but a considerable fraction of longer chains is

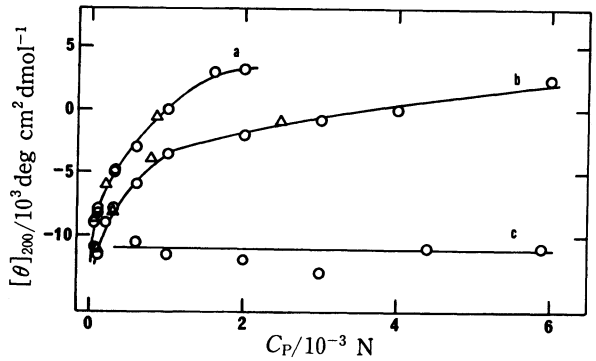

Fig.4. Concentration dependence of the residue ellipticity at 200 nm $[\theta]_{200}$ at constant pH. Sample SMA. pH: (a) 3.30±0.05 (●) and 3.45±0.05 (open symbols) in 20 mM $NaClO_4$, (b) 3.60 in 20 mM $NaClO_4$, and (c) 4.00±0.08 in 10 mM acetate buffer. Triangles refer to the solutions prepared by dilution from the solution of the highest concentration at a given pH.

contained in sample SMA-2. In Fig. 2(B) the elution patterns of the two samples in 0.2 M NaCl solutions on a Sephadex G-75 column are given. The peak positions are V_e=95.4 ml and 94.3 ml for samples SMA and SMA-2, respectively. The distribution shifts only slightly toward higher molecular weights for sample SMA-2 compared to sample SMA. All these three independent measurements, light scattering, ion-exchange chromatography, and gel permeation chromatofraphy, gave consistent results.

Concentration Dependent Conversion at Constant pHs. In Fig. 3, selected CD spectra of sample SMA at a constant pH of 3.45 in 20 mM $NaClO_4$ solutions are shown for different concentrations. An isodichroic point near 218 nm exists at all concentrations examined at pH 3.45 as well as at pH 3.30 and 3.60 (not shown). The presence of an isodichroic point suggests that charges of each polymeric species are kept constant irrespective of the polymer concentration. This result contrasts with that obtained in the previous study where dilution at a constant degree of neutralization caused enhanced ionization at extremely low concentrations.[2] Further, the p.esence of an isodichroic point shows that the stacking of the pleated sheets does not occur appreciably.[4]

In Fig. 4, the residue ellipticities at 200 nm, $[\theta]_{200}$, are shown at three different pHs, 3.30, 3.45, and 3.60, as functions of the polymer concentration. The variation of pH was within 0.05 in every case. In Fig. 4 the data obtained in 10 mM acetate buffer (pH=4.00±0.08) are also shown. Reversible conversion is shown both at pH 3.45 and 3.60, as expected from the results of the previous study.[2] Further, in 20 mM $NaClO_4$ solutions it was shown that electrostatic interaction among polyion species was negligible due to shielding effect.[2] Accordingly, the concentration dependent conversion shown in Fig. 4 deserves quantitative analyses to estimate the free energy of association, which in turn can be related to the stability of the intermolecular β-structure.

As seen in Fig. 4, the β-structure is favored at lower pHs if compared at a constant concentration. At the

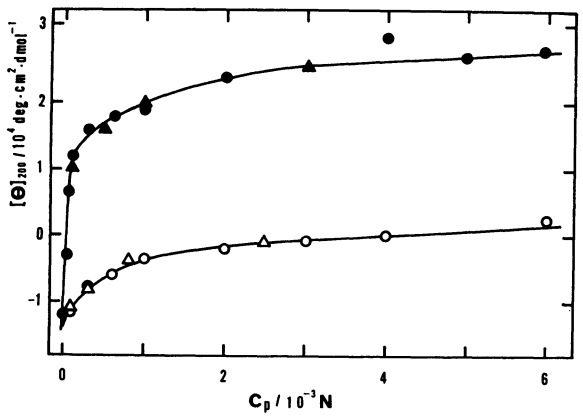

Fig. 5. Concentration dependence of the residue ellipticity at 200 nm at pH 3.60 for two samples. Samples: SMA (open symbols) and SMA-2 (filled symbols). Triangles have the same mening as in Fig. 4.

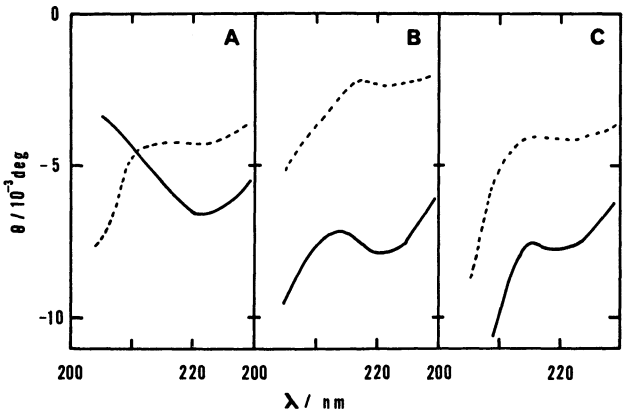

Fig. 6. CD spectra of the solutions at low pHs in 20 mM $NaClO_4$ before and after filtration by membrane filter. Sample: SMA. Solid curves: solutions before filtration Dotted curves: solutions after filtration. Ordinate gives the ellipticity. (A) Initial concentration 5.0×10^{-4} N, pH 3.45, cell length 5 mm. (B) Initial concentration 1.5×10^{-4} N, pH 3.45, cell length 1 cm. (C) Initial concentration 1.5×10^{-4} N, pH 3.60. cell length 1 cm.

same time, solubility decreases from about 6×10^{-3} N at pH 3.60 to about 2×10^{-3} N at pH 3.45 and 3—5×10^{-4} N at pH 3.30. It is to be noted that in Fig. 4 the data obtained at pH 3.30 and at 3.45 are identical within experimental error. This coincidence indicates that the effects of electrostatic interactions on the conversion can be approximately neglected at these pHs, although solubility still depends on the value of pH. On the other hand, it is necessary to separate the electrostatic contribution from the data at pH 3.60 in order to obtain the nonelectric interactions responsible for the β-formation.

The concentration dependent conversion at pH 3.60 was examined for two samples of slightly different molecular weights, SMA and SMA-2. A large difference was found between these two samples as is shown in Fig. 5. As shown in Fig. 2(A), sample SMA-2 contains longer chains much more than sample SMA. These components can form the intermolecular β-structure not only with themselves but also with shorter chains. In other words, the association occurs among chains of different lengths and long chain components act as nuclei for short chain components. This result suggests that the conversion is sensitive to the molecular weight distribution of used samples. From these results, we have to conclude that the extent of the polydispersity of sample SMA-2 is too broad to allow an evaluation of the type of assocation as well as an exact free energy change. It is also uncertain whether the molecular weight distribution of sample SMA is narrow enough for these evaluations.

The light scattering measurements were also carried out on the solutions of sample SMA at low pHs, 3.60 and 3.45, in order to evaluate the aggregation numbers However, a considerable decrease of the polymer concentration was found after solutions were passed through the measuring assembly.

In Fig. 6(A) results obtained for the solution (pH= 3.45, $C_p=5.0\times10^{-4}$ N) are shown. After filtration, the CD spectra (a dashed curve) show a disordered state. while the CD spectra of the solution before filtration show the presence of the β-structure. This kind of change was thought due to the presence of large aggreates which were filtered off. Such an interpretation has been widely accepted since the report of Li and Spector on poly(L-lysine).[5] However, in the present samples, we can prepare non-charged polymer in disordered state and hence we can examine the effects of charge density independently of the aggregation. Such examination cannot be carried out for long chain polymers. At the polymer concentration of 1.5×10^{-4} N most of the polymers are in disordered state at pH 3.45, as easily confirmed from Fig. 4. The decrease of concentration was even pronounced for this solution (pH= 3.45, $C_p=1.5\times10^{-4}$ N) as shown in Fig. 6(B). It is now clear that the decrease of polymer concentration in the present case is a direct consequence of low charge densities rather than due to the presence of large aggregates. From the data of Figs. 6(A) and (B), we calculated the final concentrations as 3.6×10^{-4} N and 4.4×10^{-5} N, respectively. Accordingly, the extent of the concentration decrease became relatively small as the initial concentration increased. In Fig. 6(c), the results on the solution (pH=3.60, $C_p=1.5\times10^{-4}$ N) are shown. The final concentration was 8.1×10^{-5} N. When the results in Figs. 6(B) and (C) are compared, the concentration decrease becomes considerably small as pH increases from 3.45 to 3.60. At neutral pH, the light scattering measurements can be performed without trouble as revealed in Fig. 1.

A Simple Analysis of the Concentration Dependence of the CD Data. A simple analysis of the CD data given in Fig. 4 is considered in the present section. These data give the first example for the conversion between the intermolecular β-structure and the disordered state in aqueous solutions.

We consider mainly the data of sample SMA at pH 3.30 and 3.45, which can be approximated to the conversion of noncharged polymers. For the growth of the pleated sheets consisted of noncharged polypeptides, it is a reasonable assumption that the association takes place with a single association constant K

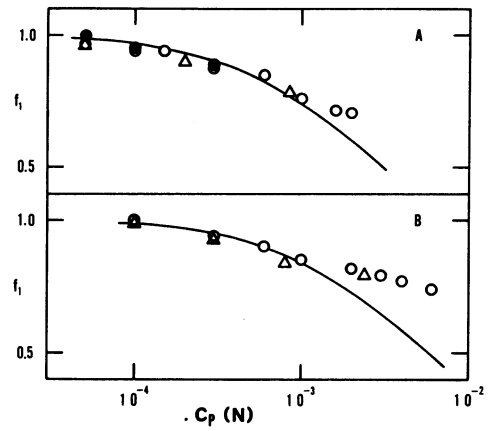

Fig. 7. Fraction of unassociated polymer f_1 as functions of polypeptide concentration C_p at constant pHs in $NaClO_4$ solutions. Sampe: SMA. pH: (A) 3.30(●) and 3.45 (open symbols) and (B) 3.60. Triangles have the same meaning as in Fig. 4. Solid curves are drawn according to Eq. 3 for $K=1.82\times10^2$ N^{-1} (A) and 1.00×10^2 N^{-1} (B).

for any successive addition except dimerization.

$$A_{i-1} + A \overset{K}{=} A_i \qquad (i \geqq 3)$$
$$2A \overset{K^2}{=} A_2 \qquad (i = 2) \qquad (2)$$

The dimerization constant is here approximated as K^2, neglecting the additional contribution for dimerization Then, the fraction f_1 of unassociated polymer is given as follows.[6]

$$KC_p = KC_pf_1 + (KC_p)^2f_1^2(2-KC_pf_1)(1-KC_pf_1)^{-2} \qquad (3)$$

Accordingly, if f_1 is observed as a function of C_p, then we can find a value of K from the concentration C_p^* which satisfies a relation $KC_p^*=1$. At $C_p=C_p^*$, $f_1=(3-5^{1/2})/2$.

With the assumption that all aggregates are in register, we can put $f_1=f_c$, where f_c denotes the fraction of residues in the disordered state. This all-or-none model represents one of the simplest pictures of the pleated sheet proposed by Pauling and Corey.[7] The fraction f_c can be calculated as follows from the CD data.

$$f_1 = f_c = \frac{3.8\times10^4 - [\theta]_{200}}{4.9\times10^4} \qquad (4)$$

Here 3.8×10^4 stands for the residue ellipticity of the β-structure at 200 nm. This value was chosen when available data on the β-structure of the pesent polypeptide were taken into consideration.[1,2,4,8] The residue ellipticity for the disordered state at 200 nm for this pH range (3.30—3.45) was chosen as -1.1×10^4, as shown in Figs. 4 and 5. Accordingly, the figure 4.9×10^4 represents the difference in the residue ellipticity at 200 nm between the β-structure and the disordered state.

In Fig. 7(A), f_1 is plotted against log C_p for the data at pH 3.45, (open symbols) and pH 3.30 (closed symbols). The solid line is drawn assuming that K is 1.82×10^2 N^{-1}. The fit is rather satisfactory. If we take the degree of polymerizationas 20, then K is 3.6×10^3 M^{-1}. The corresponding standard free energy change per polymer ΔG° and that per residue ΔG°(res) are:

$$\Delta G^\circ = -RT\ln K = -20.5 \text{ kJ/mol}$$
$$(-4.9\times10^3 \text{ cal/mol})$$
$$\Delta G^\circ(\text{res}) = \Delta G^\circ/20 = -1.05 \text{ kJ/mol}$$
$$(-2.5\times10^2 \text{ cal/mol}).$$

It is to be noted that the value of ΔG° depends on the concentration unit employed.

In Fig. 7(B) the data at pH 3.60 are analyzed similarly. Deviation from the assumed model is pronounced. This is reasonable, since electrostatic energy of the aggregate cannot be expected to increase linearly with its aggregation number. In other words, the "isodesmic" model is a poor approximation for the aggregation of charged particles.

In the previous study,[2] the converion was examined at a constant degree of neutralization α' rather than at a constant pH. When these data were analysed in the same way, they gave a good fit as shown in Fig. 8. At present, it is not certain whether the good fit is only incidental or not. For the assumed degree of polymerization of 20, the obtained values of $K(10^3$ $M^{-1})$ were 3.8, 3.2, and 2.2 for $\alpha'=0$, 0.15 and 0.25, respectively.

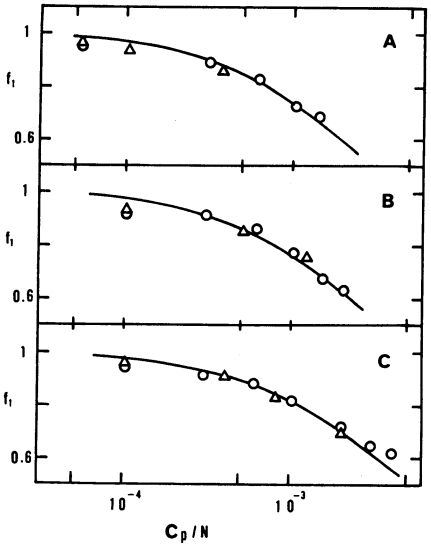

Fig. 8. Fraction of unassociated polymer f_1 as functions of polypeptide concentration C_p at constant degrees of neutralization in 20 mM $NaClO_4$ solutions. Sample SMA. Degrees of neutralization: (A) 0, (B) 0.15, and (C) 0.25. Triangles have the same meaning as in Fig. 4. Solid curves are drawn according to Eq. 3 for $K=1.92\times10^2$ N^{-1} (A), 1.67×10^2 N^{-1} (B), and 1.11×10^2 N^{-1} (C).

Discussion

The non-electrostatic free energy difference between the β-structure and random coils has been estimated from potentiometric titrations of ionizable polypeptides.[9-11] Reported values (per mole of residue) are: −1.25 kJ (−300 cal) for poly(L-tyrosine),[9] −590 J (−140 cal) for poly(L-lysine),[10] and −3.09 kJ (−740 cal) and −4.10 kJ (−980 cal) for poly(*S*-carboxymeth-

yl-L-cysteine) and poly(*S*-carboxyethyl-L-cysteine).[11] In most cases, however, the free energy change evaluated from the potentiometric titrations includes the contributions from various processes; chain folding and aggregation occur often simultaneously and stacking of the pleated sheets occurs as the amount of the β-structure increases. Accordingly, the free energy change cannot be identified with the standard free energy change of any single process, unless the conversion is confirmed to occur as the chain folding process in the absence of aggregation or as the intermolecular association without chain folding process. Plausible but indirect arguments for the absence of aggregation have been provided only for the case of poly(L-tyrosine)[9,12] and poly(*S*-carboxymethyl-L-cysteine).[1]

The stability of the β-structure can be best evaluated if the equilibrium constant for a unimolecular transconformation is obtained in the absence of aggregation. However, this has not been done until now.

In the present study, a value of about −1.1 kJ (−350 cal) was obtained as the standard free energy of association. With this value alone, it is difficult to separate the contribution of a propagation step (or equivalently the formation of a number of hydrogen bonds between peptide groups) from that of an association step. However, when the standard free energies of association are obtained with other samples of different chain lengths, the contributions from the two steps can be separated.

In the case of the intermolecular β-structure, the type of association has to be determined in order to define proper equilibrium constants. It is necessary, therefore to measure average aggregation number in addition to the weight fraction of the residues in the β-structure. Nevertheless, the study of the intermolecular β-structure seems promising for the evaluation of the stability of the β-structure, in contrast to the difficulties to avoid aggregation in the study of the intramolecular β-structure.

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