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Effects of Ammonium Sulfate on the Unfolding and Refolding of the Variable and Constant Fragments of an Immunoglobulin Light Chain†

Yuji Goto, Naoya Ichimura, and Kozo Hamaguchi*
Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan
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ABSTRACT: The equilibria and kinetics of unfolding and refolding by guanidine hydrochloride of the V_L and C_L fragments of a type κ immunoglobulin light chain were studied in the presence of ammonium sulfate using circular dichroism and tryptophyl fluorescence at pH 7.5 and 25 °C. The unfolding equilibria of the V_L and C_L fragments were described in terms of the two-state transition. The midpoints of unfolding in the absence of ammonium sulfate were at 0.9 and 1.2 M guanidine hydrochloride for the C_L and V_L fragments, respectively. The transition curves were shifted to higher concentrations of guanidine hydrochloride by 1.4 and 1.6 M for the C_L and V_L fragments, respectively, per mole of ammonium sulfate. Unfolding reactions of the V_L and C_L fragments in 3 M guanidine hydrochloride followed first-order kinetics, and the rate constants for the two proteins were both greatly decreased by the presence of ammonium sulfate. The refolding reaction of the C_L fragment in 0.3 M guanidine hydrochloride consisted of two phases, and the rate constants were increased a little by the presence of ammonium sulfate. The refolding reaction of the V_L fragment in 0.3 M guanidine hydrochloride followed first-order kinetics, and the rate was not affected by the presence of ammonium sulfate. These results showed that ammonium sulfate stabilizes the C_L and V_L fragments mainly by decreasing the unfolding rate.

Sulfate ion has long been known to protect the protein structure from unfolding (von Hippel & Wong, 1964), but little is known about the quantitative nature of this effect. Recently, Mitchinson and Pain (1985) studied the effect of $(NH_4)_2SO_4$ on the stability of β-lactamase from *Staphylococcus aureus*. Their results showed that as the destabilizing effect of urea or guanidine hydrochloride (Gdn-HCl)[1] can be described quantitatively, so the stabilizing effect of $(NH_4)_2SO_4$ can also be described quantitatively. In the course of a series of studies on the unfolding and refolding of the C_L and V_L fragments obtained from the immunoglobulin light chain (Goto et al., 1979; Goto & Hamaguchi, 1979, 1981, 1982a,b, 1986a,b, 1987; Ashikari et al., 1984; Kikuchi et al., 1986; Tsunenaga et al., 1987), we found that the C_L and V_L fragments are stabilized by $(NH_4)_2SO_4$.

The immunoglobulin light chain consists of two independently folded structural units, the amino-terminal variable domain (V_L) and the carboxy-terminal constant domain (C_L), each with a molecular weight of about 12000. Each domain consists of two β-sheets and has one intrachain disulfide bond buried in the interior hydrophobic region between the sheets (Beale & Feinstein, 1976; Amzel & Poljak, 1978). Recently, Tsunenaga et al. (1987) succeeded in isolating the V_L and C_L fragments from a type κ light chain by limited proteolysis with clostripain (EC 3.4.22.8) and studied the unfolding and refolding of the light chain and its constituent V_L and C_L fragments. It was found that the unfolding of the type κ light chain can be described by the independent folding of the two domains but that there are some interactions between the V_L and C_L domains in the refolding process. These results are very similar to those obtained for a type λ light chain (Goto et al., 1979; Goto & Hamaguchi, 1982a). It was also found that the unfolding and refolding kinetics of the V_L fragment are different from those of the C_L fragment, in spite of their similar immunoglobulin folding. There might thus be a dif-

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[1] Abbreviations: CD, circular dichroism; Gdn-HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane.

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ference in the effect of $(NH_4)_2SO_4$ between the V_L and C_L fragments, and an understanding of the folding of the V_L and C_L fragments would thus be very useful.

In the present paper, we report the equilibria and kinetics of unfolding and refolding by Gdn-HCl of the V_L and C_L fragments of a type κ light chain in the presence of $(NH_4)_2SO_4$ at various concentrations. $(NH_4)_2SO_4$ stabilizes both the V_L and C_L fragments greatly, and the stabilizing effect can be described quantitatively in terms of the increase in the free energy change of unfolding. The kinetic measurements show that for both proteins, the stabilizing effect is largely due to a great reduction in the unfolding rate in the presence of $(NH_4)_2SO_4$.

Materials and Methods

Materials. Light-chain Oku (type κ) was prepared from the urine of a multiple myeloma patient by precipitation with $(NH_4)_2SO_4$ and ion-exchange chromatography (DE-52), as described previously (Goto et al., 1979). The C_L and V_L fragments were obtained by digestion with clostripain (EC 3.4.22.8), an arginine-specific proteinase, of light-chain Oku in which the C-terminal cystine residue had been reduced and alkylated with iodoacetamide (Tsunenaga et al., 1987). The V_L and C_L fragments used here were fractions II-1 and III-2, respectively, described by Tsunenaga et al. (1987). Gdn-HCl (specially purified grade) was obtained from Nakarai Chemicals, and $(NH_4)_2SO_4$ was from Wako Chemicals.

CD Measurement. The buffer used in the present experiments was 50 mM Tris-HCl buffer at pH 7.5 containing 0.15 M NaCl. All measurements were carried out at 25 °C.

The CD spectra were measured with a Jasco spectropolarimeter, model J-500A, which is equipped with a DP-501 data processor. The details of the CD measurements have been described previously (Goto & Hamaguchi, 1986a). Unfolding transitions of the C_L and V_L fragments by Gdn-HCl were measured in terms of the ellipticity at 218 nm. The protein concentration was 0.05 mg/mL, and a 0.2-cm cell was used.

Fluorescence Measurement. Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, Model MPF-4, equipped with a spectral corrector. Square cells with a 1-cm path length were used. Tryptophyl fluorescence was measured by using 295-nm light for excitation. The protein concentration was 0.05 mg/mL. The temperature was kept at 25 °C by using a thermostatically controlled cell holder.

Unfolding transitions of the C_L and V_L fragments by Gdn-HCl were measured in terms of the fluorescence at 350 nm. The concentrations of the C_L and V_L fragments were 0.05 mg/mL, and the excitation was at 295 nm. The same protein solution was used for both the CD measurements and the fluorescence measurements of unfolding transitions.

Slow unfolding reactions of the V_L fragment in 3 M Gdn-HCl and slow refolding reactions of the C_L and V_L fragments in 0.3 M Gdn-HCl were measured by using the fluorescence at 350 nm. The unfolding reaction of the V_L fragment was initiated by manually mixing 1.5 mL of protein solution in the absence of Gdn-HCl and $(NH_4)_2SO_4$ at pH 7.5 with 1.5 mL of 6 M Gdn-HCl at pH 7.5 containing $(NH_4)_2SO_4$ at a given concentration. The refolding reactions of the C_L and V_L fragments were initiated by adding 0.2 mL of protein solution in the presence of 4 M Gdn-HCl and in the absence of $(NH_4)_2SO_4$ at pH 7.5 to 2.5 mL of a buffer solution at pH 7.5 containing $(NH_4)_2SO_4$ at a given concentration. Neither the unfolding kinetics nor the refolding kinetics were affected by the presence of $(NH_4)_2SO_4$ in the initial protein solution. The refolding reaction of the C_L fragment was also measured by the pH-jump method. A protein solution in glycine buffer at pH 2.0 containing 0.6 M Gdn-HCl and no $(NH_4)_2SO_4$ was mixed in a 1:1 ratio with Tris-HCl buffer at pH 8.0 containing $(NH_4)_2SO_4$ at a given concentration to give a final pH of 7.5. The C_L and V_L fragments were denatured in 0.6 M Gdn-HCl at pH 2.0 (Tsunenaga et al., 1987).

Stopped-Flow Measurement. Fast unfolding and refolding reactions were measured on a Union Giken stopped-flow spectrophotometer, Model RA-401, using fluorescence detection. The details of the apparatus have been described previously (Goto & Hamaguchi, 1982a). The excitation wavelength was set a 280 nm, and the fluorescence at wavelengths longer than 330 nm were observed. The reservoir and the observation cell were thermostated with circulating water at 25 °C. The final protein concentration was about 0.03 mg/mL. The unfolding was initiated by mixing a solution of the C_L or V_L fragment in the absence of Gdn-HCl and the presence of $(NH_4)_2SO_4$ at a given concentration with 6 M Gdn-HCl solution containing $(NH_4)_2SO_4$ at the same concentration in a 1:1 ratio. The fast refolding reaction of the C_L fragment was measured by using the pH-jump method described above. The refolding was initiated by mixing a solution of the C_L fragment unfolded in glycine buffer at pH 2.0 containing 0.6 M Gdn-HCl with Tris-HCl buffer at pH 8.0 containing $(NH_4)_2SO_4$ at the same concentration in a 1:1 ratio to give a final pH of 7.5.

Protein Concentration. The protein concentration was determined spectrophotometrically. The absorption coefficient at 280 nm for a 1% (w/v) solution in a 1.0-cm cell ($A^{1\%}_{1cm}$) was assumed to be 10.8 for the C_L fragment and 12.7 for the V_L fragment (Tsunenaga et al., 1987).

pH Measurement. pH was measured with a Radiometer PHM 26c at 25 °C.

Results

CD and Fluorescence Spectra. Panels a and b of Figure 1 show the CD spectra of the C_L and V_L fragments, respectively, in the absence and presence of $(NH_4)_2SO_4$ and in the presence of 4 M Gdn-HCl. The spectra of both proteins in the far-ultraviolet region were not affected by the presence of $(NH_4)_2SO_4$ up to 1 M. The negative maximum at 280 nm for the V_L fragment increased with increasing concentration of $(NH_4)_2SO_4$. The change produced by the presence of $(NH_4)_2SO_4$ in the CD spectrum of the C_L fragment in the aromatic absorption region was smaller than that for the V_L fragment, but significant.

Panels a and b of Figure 2 show the fluorescence spectra of the C_L and V_L fragments, respectively, in the absence and presence of $(NH_4)_2SO_4$ relative to the spectrum in the presence of 4 M Gdn-HCl. Each fragment has one tryptophyl residue buried in the interior of the protein molecule and located close to the intrachain disulfide bond. The tryptophanyl fluorescence of each protein in the absence of denaturant was greatly quenched by the disulfide bond, a strong quencher (Cowgill, 1967). The effects of $(NH_4)_2SO_4$ on the fluorescence spectra of the C_L and V_L fragments were negligible.

Equilibrium Studies. Panels a and b of Figure 3 show the equilibrium transition curves of the C_L and V_L fragments, respectively, by Gdn-HCl in the absence and presence of $(NH_4)_2SO_4$ at various concentrations. Unfolding equilibria by Gdn-HCl of the V_L and C_L fragments in the absence of $(NH_4)_2SO_4$ were reversible (Tsunenaga et al., 1987). The transitions were also reversible in the presence of less than 1 M $(NH_4)_2SO_4$. In the presence of more than 1 M $(NH_4)_2SO_4$, the protein solutions became turbid with time. Therefore, the concentrations of $(NH_4)_2SO_4$ used were limited to 1 M or less.

Table I: Parameters for Unfolding Transitions by Gdn-HCl of C_L and V_L Fragments of Light-Chain Oku in the Absence and Presence of $(NH_4)_2SO_4$ at pH 7.5 and 25 °C

		linear extrapolation				
			$-(d[\Delta G_D]/d[\text{Gdn-HCl}])$ (kcal mol^{-1} M^{-1})	Gdn-HCl binding		
$[(NH_4)_2SO_4]$ (M)	C_m[a] (M)	$\Delta G_D^{H_2O}$ (kcal/mol)		$\Delta G_D^{H_2O}$ (kcal/mol)	Δn	method
			C_L Fragment			
0	0.9	3.8	4.1	5.1	31	fluorescence
0	0.9	3.5	3.6	4.7	30	CD
0.3	1.2	4.5	3.6	6.5	33	fluorescence
0.5	1.7	5.5	3.3	7.4	30	fluorescence
0.5	1.7	5.4	3.3	7.6	31	CD
0.7	1.9	6.1	3.2	8.4	30	fluorescence
0.7	2.0	6.4	3.2	8.9	32	CD
			V_L Fragment			
0	1.3	4.7	3.7	6.2	31	fluorescence
0.1	1.5	5.2	3.6	7.0	32	fluorescence
0.25	1.7	5.8	3.4	8.0	32	fluorescence
0.5	2.1	6.5	3.1	8.8	30	fluorescence
0.7	2.4	7.3	3.1	9.8	31	fluorescence

[a] Concentration of Gdn-HCl at the midpoint of the unfolding curve by Gdn-HCl.

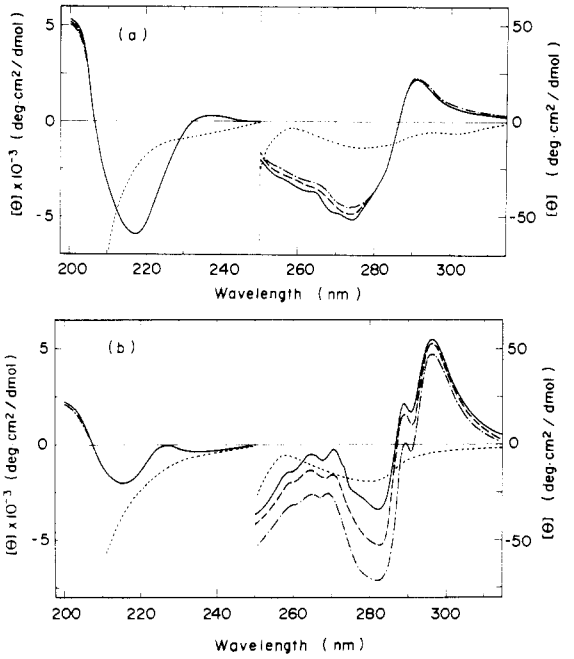


FIGURE 1: CD spectra of the C_L fragment (a) and V_L fragment (b) of light-chain Oku in the absence (solid line) and presence of 0.5 M (dashed line) and 1.0 M $(NH_4)_2SO_4$ (dashed–dotted line) at pH 7.5 and 28 °C. Dotted lines indicate the spectra in the presence of 4 M Gdn-HCl.

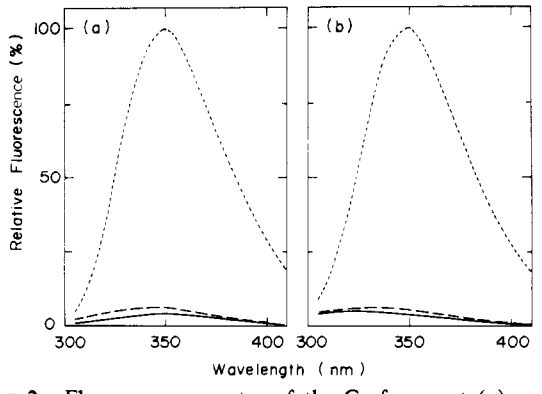


FIGURE 2: Fluorescence spectra of the C_L fragment (a) and V_L fragment (b) of light-chain Oku in the absence (solid line) and presence (dashed line) of 1.0 M $(NH_4)_2SO_4$ relative to the fluorescence in the presence of 4 M Gdn-HCl (dotted line) at pH 7.5 and 25 °C. Excitation wavelength was 295 nm.

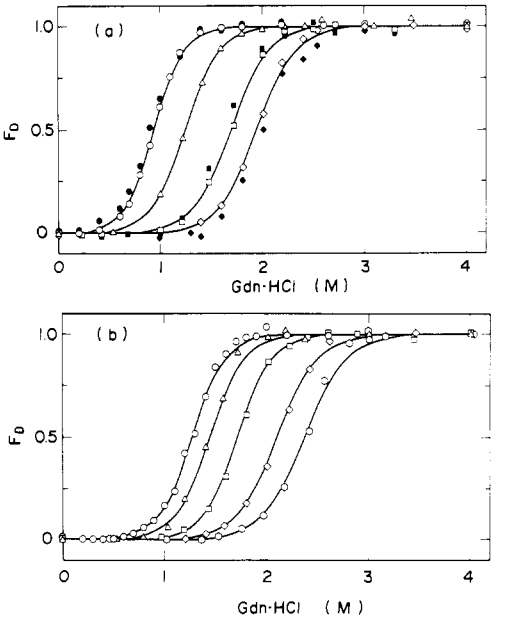


FIGURE 3: Unfolding transitions by Gdn-HCl of the C_L fragment (a) and V_L fragment (b) of light-chain Oku in the absence and presence of $(NH_4)_2SO_4$ at pH 7.5 and 25 °C: (a) 0 (○, ●), 0.3 (△), 0.5 (□, ■), and 0.7 M $(NH_4)_2SO_4$ (◇, ◆); (b) 0 (○), 0.1 (△), 0.25 (□), 0.5 (◇), and 0.7 M $(NH_4)_2SO_4$ (○). The ordinate indicates the fraction of the unfolded protein. Open symbols indicate the data obtained by fluorescence measurements at 350 nm and closed symbols the data obtained by CD measurements at 218 nm. The solid lines indicate the theoretical curves constructed by assuming the linear dependence of ΔG_D on Gdn-HCl concentration and using the values given in Table I. Protein concentrations for the C_L fragments were both 0.05 mg/mL.

The transition curves were normalized, assuming that the signals (fluorescence or CD) for the native and unfolded proteins which were observed before and after the cooperative transition zone, respectively, could be extrapolated linearly into the transition zone. The transition curves for the C_L fragment obtained by measuring the CD at 218 nm and the fluorescence at 350 nm were the same. In the case of the V_L fragment, the change in the ellipticity at 218 nm on unfolding was small (Figure 1b), and only the fluorescence at 350 nm was used to determine the unfolding transitions. The Gdn-HCl concentration at the midpoint of the unfolding curve in the absence of $(NH_4)_2SO_4$ was 0.9 M for the C_L fragment and 1.3 M for the V_L fragment (Table I). While the C_L fragment molecules exist as the monomer, the V_L fragment molecules are in a

Table II: Dependence on $(NH_4)_2SO_4$ Concentration of Parameters for Unfolding and Refolding of C_L and V_L Fragments of Light-Chain Oku at pH 7.5 and 25 °C

protein	$d[C_m]/d[(NH_4)_2SO_4]$	$d[\Delta G_D^{H_2O}]/d[(NH_4)_2SO_4]$ (kcal mol^{-1} M^{-1}) linear extrapolation	Gdn-HCl binding	$d[\Delta G_D^{1.5M}]/d[(NH_4)_2SO_4]$ (kcal mol^{-1} M^{-1})	$d[\Delta G_D^{1.8M}]/d[(NH_4)_2SO_4]$ (kcal mol^{-1} M^{-1})	$d[\log k_U]/d[(NH_4)_2SO_4]$ at 3 M Gdn-HCl [log (s)/M]	$d[\log k_r]/d[(NH_4)_2SO_4]$ at 0.3 M Gdn-HCl [log (s)/M] fast phase	slow phase
C_L	1.4	3.6	5.1	5.4		−1.5	0.7	0.5
V_L	1.6	3.6	4.9		5.4	−2.2		0

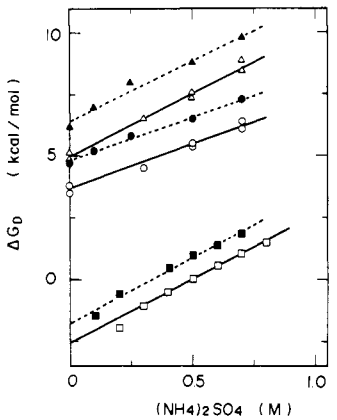


FIGURE 4: Dependence on $(NH_4)_2SO_4$ concentration of the free energy change of unfolding of the C_L fragment (open symbols) and V_L fragment (closed symbols) at pH 7.5 and 25 °C. (○, ●) ΔG_D values in the absence of Gdn-HCl ($\Delta G_D^{H_2O}$) obtained by linear extrapolation; (△, ▲) $\Delta G_D^{H_2O}$ values obtained by assuming the Gdn-HCl binding model; (□) ΔG_D values in the presence of 1.5 M Gdn-HCl for the C_L fragment; (■) ΔG_D values in the presence of 1.8 M Gdn-HCl for the V_L fragment.

monomer–dimer equilibrium, and the dimer conformation seems to contribute to the stability of the V_L fragment (Tsunenaga et al., 1987). The presence of $(NH_4)_2SO_4$ shifted the transition curve to a higher concentration of Gdn-HCl per mole of $(NH_4)_2SO_4$ by 1.4 M for the C_L fragment and by 1.6 M for the V_L fragment (Table II).

Assuming the two-state transition, we obtained the free energy change of unfolding at a given concentration of Gdn-HCl (ΔG_D) from the equation:

$$\Delta G_D = -RT \ln [f_D/(1 - f_D)] \quad (1)$$

where f_D is the fraction of the unfolded molecule. We then estimated the free energy change of unfolding in the absence of Gdn-HCl, $\Delta G_D^{H_2O}$, by using two methods of extrapolation [see Pace (1986)]. In one method (linear extrapolation), the values of ΔG_D were plotted against the concentration of Gdn-HCl, and the value of $\Delta G_D^{H_2O}$ was obtained by linear extrapolation to zero concentration of Gdn-HCl. In the other method (Gdn-HCl binding model), we estimated $\Delta G_D^{H_2O}$ on the basis of the binding of Gdn-HCl to the native and unfolded molecules using the equation proposed by Tanford (1970):

$$\Delta G_D = \Delta G_D^{H_2O} - \Delta nRT \ln (1 + ka_{\pm}) \quad (2)$$

where Δn is the difference in the number of binding sites between unfolded and folded states, k is the average binding constant of the sites, and $a_{\pm}$ is the mean ion activity of Gdn-HCl. We used 0.6 M^{-1} as the value of k (Pace, 1986). The respective values of $\Delta G_D^{H_2O}$ determined by the two methods are plotted against the concentration of $(NH_4)_2SO_4$ in Figure 4. For both the C_L and V_L fragments, the values of $\Delta G_D^{H_2O}$ increased linearly with an increase in the concentration of $(NH_4)_2SO_4$, but the slope differed depending on the method of extrapolation (Table II). For both proteins, the slope, $d[\Delta G_D^{H_2O}]/d[(NH_4)_2SO_4]$, obtained on the basis of the Gdn-HCl binding model [5.1 kcal mol^{-1} (M $(NH_4)_2SO_4)^{-1}$ for the C_L fragment of 4.9 kcal mol^{-1} (M $(NH_4)_2SO_4)^{-1}$ for the V_L fragment] was larger than that obtained by linear extrapolation [3.6 kcal mol^{-1} (M $(NH_4)_2SO_4)^{-1}$ for both proteins].

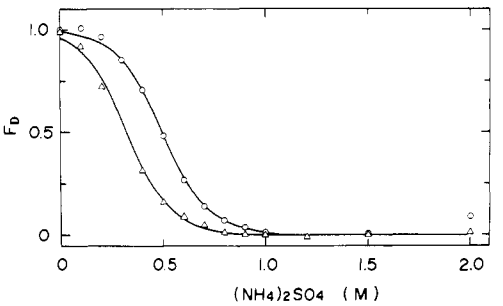


FIGURE 5: Refolding transitions by $(NH_4)_2SO_4$ of the C_L fragment in 1.5 M Gdn-HCl (circles) and of the V_L fragment (triangles) in 1.8 M Gdn-HCl at pH 7.5 and 25 °C. The ordinate indicates the fraction of the unfolded molecule. Protein concentrations were both 0.05 mg/mL.

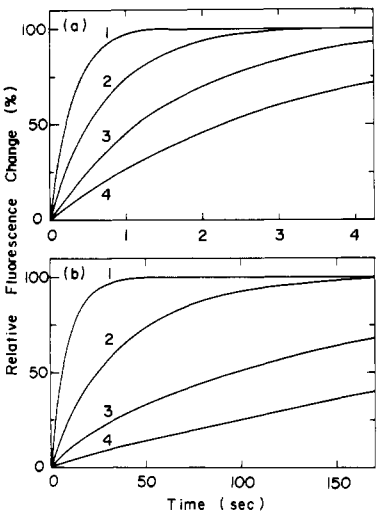


FIGURE 6: Unfolding kinetics of the C_L fragment (a) and V_L fragment (b) in 3 M Gdn-HCl in the absence and presence of $(NH_4)_2SO_4$ at pH 7.5 and 25 °C. $(NH_4)_2SO_4$ concentration: 1, 0 M; 2, 0.25 M; 3, 0.5 M; 4, 0.7 M. Protein concentrations were 0.03 mg/mL.

Figure 5 shows the refolding transitions by $(NH_4)_2SO_4$ for the C_L fragment in the presence of 1.5 M Gdn-HCl and for the V_L fragment in the presence of 1.8 M Gdn-HCl. Both proteins were completely unfolded under these conditions (see Figure 3). From these curves, the free energy change of unfolding (ΔG_D) was estimated at a given concentration of $(NH_4)_2SO_4$, and the values were plotted against the concentration of $(NH_4)_2SO_4$, as shown in Figure 4. The slope was 5.4 kcal mol^{-1} (M $(NH_4)_2SO_4)^{-1}$ for the C_L and V_L fragments (Table II). For both proteins, the value was close to the value in the absence of denaturant, $d[\Delta G_D^{H_2O}]/d[(NH_4)_2SO_4]$, obtained by assuming the Gdn-HCl binding model.

Unfolding Kinetics. Panels a and b of Figure 6 show the unfolding kinetics measured by tryptophyl fluorescence of the C_L and V_L fragments, respectively, in 3 M Gdn-HCl in the absence and presence of $(NH_4)_2SO_4$ at various concentrations.

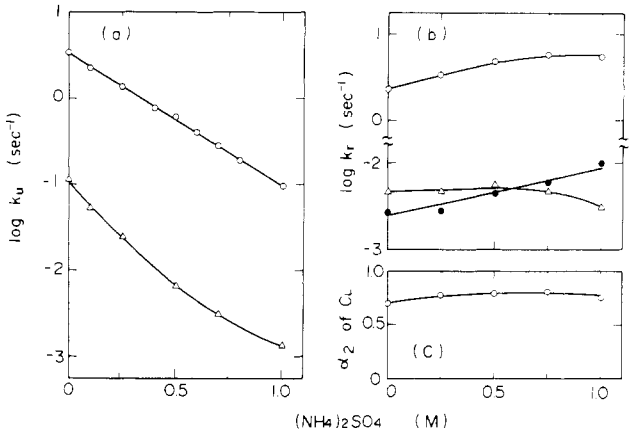


FIGURE 7: Dependence on $(NH_4)_2SO_4$ concentration of the unfolding (a) and refolding (b) rate constants of the C_L fragment (open circles, fast phase; closed circles, slow phase) and V_L fragment (triangles) and the relative amplitude of the fast phase for the refolding kinetics of the C_L fragment (c) at pH 7.5 and 25 °C. The conditions were as described in the legends to Figures 6 and 8.

All the unfolding reactions followed first-order kinetics. In the absence of $(NH_4)_2SO_4$, the rate constant for the unfolding of the V_L fragment (0.11 s^{-1}) was 300 times smaller than that of the C_L fragment (3.3 s^{-1}). The presence of $(NH_4)_2SO_4$ greatly decreased the rate of unfolding of both proteins. As shown in Figure 7a, the plot of the logarithms of the first-order rate constant (k_U) for the C_L fragment against the concentration of $(NH_4)_2SO_4$ was linear, and the slope, d[log k_u]/d-[$(NH_4)_2SO_4$], was found to be −1.53 per mole of $(NH_4)_2SO_4$. The plot for the V_L fragment was not linear, and the initial slope below 0.25 M $(NH_4)_2SO_4$ was −2.20 per mole of $(NH_4)_2SO_4$ (Table II).

Refolding Kinetics. Panels a and b of Figure 8 show the refolding kinetics of the C_L and V_L fragments, respectively, in 0.3 M Gdn-HCl containing $(NH_4)_2SO_4$ at various concentrations. The refolding kinetics of the C_L fragment were obtained by the pH-jump method and those of the V_L fragment by the dilution method (see Materials and Methods).

As reported previously (Tsunenaga et al., 1987), the refolding kinetics of the C_L fragment consisted of three phases: a fast phase with an apparent rate constant of $k_{r,2}$ and a relative amplitude of α_2, a slow phase with an apparent rate constant of $k_{r,1}$ and a relative amplitude of α_1, and an additional intermediate phase with an apparent rate constant of $k_{r,3}$ and a relative amplitude of α_3, where $\alpha_1 + \alpha_2 + \alpha_3 = 1$. The values of α_1, α_2, and α_3 in the presence of 0.3 M Gdn-HCl were 0.2, 0.7, and 0.1, respectively (Tsunenaga et al., 1987). We did not analyze the additional phase further, because of its small amplitude (about 0.1). The slow refolding process of the C_L fragment obtained by dilution from 4 M Gdn-HCl to a lower concentration was the same as that obtained by the pH-jump method. As shown in Figure 8a, the refolding of the C_L fragment was accelerated a little in the presence of $(NH_4)_2SO_4$. The logarithms of the rate constants for the fast phase ($k_{r,2}$) and slow phase ($k_{r,1}$), and the amplitude of the fast phase (α_2), are plotted against the concentration of $(NH_4)_2SO_4$ in Figure 7b and Figure 7c, respectively. The presence of $(NH_4)_2SO_4$ increased the rates of both the fast and slow phases, but the effect was much less compared with the marked effect of $(NH_4)_2SO_4$ on the unfolding rate (Figure 7a and Table II). The amplitude of the fast phase was increased slightly by the increase in the concentration of $(NH_4)_2SO_4$.

The refolding reaction of the V_L fragment followed first-order kinetics [see also Tsunenaga et al. (1987)]. No significant change in the refolding kinetics of the V_L fragment in 0.3 M Gdn-HCl was observed by the presence of $(NH_4)_2SO_4$ (Figures 7b and 8b).

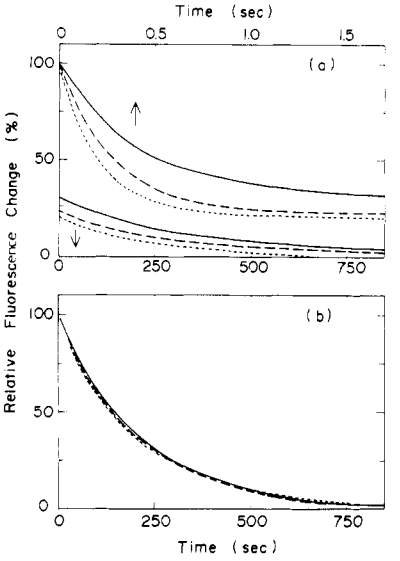


FIGURE 8: Refolding kinetics of the C_L fragment (a) and V_L fragment (b) in the absence and presence of $(NH_4)_2SO_4$ in 0.3 M Gdn-HCl at pH 7.5 and 25 °C. $(NH_4)_2SO_4$ concentration: solid line, 0 M; dotted line, 0.25 M; dashed line, 0.5 M. Protein concentrations were both 0.03 mg/mL.

Discussion

Unfolding Equilibria. We have described the effects of $(NH_4)_2SO_4$ on the conformations, stabilities, and unfolding and refolding kinetics of the C_L and V_L fragments of light-chain Oku (type κ). The effects of $(NH_4)_2SO_4$ on the conformations of the C_L and V_L fragments are small judging from the CD and fluorescence spectra (Figures 1 and 2), but $(NH_4)_2SO_4$ greatly increases the stabilities to Gdn-HCl of both proteins (Figure 3).

To characterize the effect of $(NH_4)_2SO_4$, we estimated the free energy change of unfolding in the absence of denaturant and in the presence of $(NH_4)_2SO_4$ at various concentrations using two methods. As shown in Figure 4 and Table II, for both proteins in the absence of $(NH_4)_2SO_4$, the value of $\Delta G_D^{H_2O}$ was obtained by assuming that the denaturant binding model was larger by 1.5 kcal/mol than the value obtained by linear extrapolation, and the slope of the dependence of $\Delta G_D^{H_2O}$ on $(NH_4)_2SO_4$ concentration for the former was larger than that for the latter. It is unknown at present, however, which method is more appropriate for estimating the value of $\Delta G_D^{H_2O}$ (Pace, 1986). The effects of $(NH_4)_2SO_4$ on the C_L and V_L fragments measured in terms of the value of $\Delta G_D^{H_2O}$ were very similar.

Previously, Goto et al. (1987) studied the effect of $(NH_4)_2SO_4$ on the unfolding by Gdn-HCl of the reduced C_L fragment of Oku protein. The values of $\Delta G_O^{H_2O}$ for the reduced C_L fragment were estimated from the Gdn-HCl binding model to be 0.4 kcal/mol in the absence of $(NH_4)_2SO_4$ and 3.0 kcal/mol in the presence of 0.5 M $(NH_4)_2SO_4$. This is comparable with the stabilization effect of $(NH_4)_2SO_4$ for the intact C_L fragment (see Table I).

We also measured the refolding transitions by $(NH_4)_2SO_4$ of the C_L fragment in 1.5 M Gdn-HCl and of the V_L fragment in 1.8 M Gdn-HCl (Figure 5). In the absence of $(NH_4)_2SO_4$, the C_L and V_L fragments were denatured completely in 1.5 and 1.8 M Gdn-HCl, respectively (Figure 3a,b). $(NH_4)_2SO_4$ has the ability to refold the unfolded C_L and V_L fragments. A similar observation was reported by Mitchinson and Pain (1985) for the effect of $(NH_4)_2SO_4$ on the unfolding by urea of β-lactamase. As they suggested, our findings may also be

well explained in terms of additive effects of $(NH_4)_2SO_4$ and Gdn-HCl. The dependence of the value of ΔG_D on the concentration of $(NH_4)_2SO_4$ determined for the C_L fragment in the presence of 1.5 M Gdn-HCl and that for the V_L fragment in the presence of 1.8 M Gdn-HCl (Figure 4) were both about 5 kcal/mol per mole of $(NH_4)_2SO_4$ (Table II). This value is close to that in the absence of Gdn-HCl obtained by assuming the denaturant binding model rather than linear extrapolation. As shown in Table I, the destabilizing effect of Gdn-HCl was 3–4 kcal/mol per mole of Gdn-HCl for both proteins. Therefore, compared at unit molar concentration, the stabilizing effect of $(NH_4)_2SO_4$ is greater than the destabilizing effect of Gdn-HCl.

The stabilizing effect of $(NH_4)_2SO_4$ on proteins is due to sulfate ion, and its effect is similar to that of Na_2SO_4 (von Hippel & Wong, 1964). Nandi and Robinson (1972a,b) measured the free energy changes in the transfer of nonpolar amino acid side chains and the peptide group from water to aqueous solutions of various salts. They estimated the difference in the free energy change of unfolding of ribonuclease A between water and a salt solution using the equation proposed by Tanford (1970), these free energy changes, the number of hydrophobic side chains and the peptide group, and the average change in the fractional exposure [0.35 (Tanford, 1970)]. By use of the same method, the stabilizing effects of 1 M Na_2SO_4 on the V_L and C_L fragments were estimated to be 5.8 and 4.2 kcal/mol, respectively. These values are consistent with the values obtained here (Table II). In this estimate, the contributions of the ionizable and hydrophilic side chains are neglected, because these side chains are largely exposed to solvent in both the native and unfolded states. The agreement of the estimated values with the observed values indicates that the stabilizing effect of $(NH_4)_2SO_4$ is due largely to an increase in hydrophobic interactions. Timasheff and his co-workers (Arakawa & Timasheff, 1982a,b) have shown that preferential hydration is involved in the stabilizing effect of Na_2SO_4 on the protein molecule.

Kinetics of Unfolding and Refolding. The apparent stability of the protein molecule is determined by the balance of unfolding and refolding rate constants, and thus the kinetics may yield more useful information than thermodynamics with regard to the mechanism of the stabilization effect of $(NH_4)_2SO_4$. We studied the effects of $(NH_4)_2SO_4$ on the unfolding and refolding kinetics of the C_L and V_L fragments.

Tsunenaga et al. (1987) studied in detail the unfolding and refolding kinetics by Gdn-HCl of the C_L and V_L fragments used here. The unfolding and refolding kinetics of the C_L fragment inside and above the transition zone were explained on the basis of the three-species mechanism:

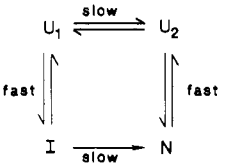
mechanism 1

$$U_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} U_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} N$$

where N is the native protein, U_1 and U_2 are the slow folding and fast folding species, respectively, of the unfolded protein, and k_{12}, k_{21}, k_{23}, and k_{32} are the rate constants for the respective processes. The equilibrium constant ($K_{21} = k_{21}/k_{12}$) between U_1 and U_2 is assumed to be 6 at all the concentrations of Gdn-HCl studied. The unfolding and refolding kinetics of the C_L fragment inside the transition zone are expressed by two exponential terms, and those above the transition zone are expressed by a single-exponential term. The apparent rate constant of unfolding above the transition zone corresponds to the microscopic rate constant, k_{32}. Therefore, the unfolding rate constant, k_u, measured in the presence of 3 M Gdn-HCl (Figure 6) corresponds to k_{32}.

The refolding kinetics of the C_L fragment were complex, and an intermediate, I, had to be added to mechanism 1 (Tsunenaga et al., 1987):

mechanism 2



The stability of I, which is in rapid equilibrium with U_1, increases with decreasing concentration of Gdn-HCl. In 0.3 M Gdn-HCl, the refolding kinetics are described almost entirely by two exponential terms, although an additional phase with a small amplitude appears. In the initial unfolding conditions, the fraction of U_1 is 0.86 on the basis of $K_{21} = 6$. We assumed that the fast phase of refolding consists of two folding processes, $U_1 \rightarrow I$ and $U_2 \rightarrow N$, and that the slow phase of refolding consists two isomerization processes, $U_1 \rightarrow U_2$ and $I \rightarrow N$ (Goto & Hamaguchi, 1982a; Tsunenaga et al., 1987). We suggested that the isomerization reactions of Pro-143 of type λ C_L and the corresponding Pro of the type κ C_L or other domains are responsible for the appearance of the slow phase for the immunoglobulin domains ($U_1 \rightarrow U_2$ and $I \rightarrow N$).

As shown in Figure 7, the unfolding rate constant (k_u) of the C_L fragment in 3 M Gdn-HCl was decreased greatly by the presence of $(NH_4)_2SO_4$, while the two refolding rate constants were increased only slightly and the relative amplitude was affected slightly by the presence of the salt. This indicates that $(NH_4)_2SO_4$ decreases the microscopic unfolding rate constant (k_{32}) greatly but affects both the microscopic refolding rate constants and the rate constants of the interconversion between the two unfolded species to only an insignificant extent.

The unfolding and refolding kinetics of the V_L fragment of light-chain Oku were described by a single-exponential term at all the concentrations of Gdn-HCl studied (Tsunenaga et al., 1987). The results of the double-jump experiment, however, suggested that there is an isomerization reaction in the unfolded molecule and that a mechanism more complex than the two-state transition is necessary to explain the folding kinetics of the V_L fragment, although the details are unknown (Tsunenaga et al., 1987). The V_L fragment molecules are known to be in an equilibrium between the dimer and monomer (Cathou & Dorrington, 1975; Azuma et al., 1978; Maeda et al., 1976, 1978). The dimer may contribute to increasing the stability. As reported previously (Tsunenaga et al., 1987), however, the unfolding equilibria (0.04–0.2 mg of proteon/mL), unfolding kinetics (0.04–0.2 mg/mL), and refolding kinetics (0.014–0.177 mg/mL) by Gdn-HCl of the V_L fragment do not depend on the protein concentration in the concentration range used. As shown in Figure 7, the presence of $(NH_4)_2SO_4$ greatly decreased the unfolding rate constant of the V_L fragment in 3 M Gdn-HCl but not the refolding rate constant in 0.3 M Gdn-HCl. These results are similar to those for the C_L fragment. Insensitivity of the refolding rate constant to the presence of $(NH_4)_2SO_4$ for the V_L fragment suggests that the refolding rate does not reflect the direct folding process but reflects the isomerization reaction in the unfolded molecule. Further studies of the effects of $(NH_4)_2SO_4$ on the stability and folding kinetics of the V_L fragment at different protein concentrations should be important to understand the effect of the monomer–dimer equilibrium on the stability and folding mechanism.

The contributions of the change in the unfolding and refolding rate constants to the free energy change of unfolding and refolding are expressed by $-RT[d(\ln k_u)/d[(NH_4)_2SO_4]$ and $RT[d(\ln k_r)/d[(NH_4)_2SO_4]$. The values for the C_L fragment are calculated to be 2.1 kcal mol $(M^{-1}\ (NH_4)_2SO_4)^{-1}$ using the unfolding rate constant and 0.9 and 0.7 kcal mol^{-1} $(M\ (NH_4)_2SO_4)^{-1}$ using the refolding rate constants of the fast and slow phases, respectively. The values for the V_L fragment are calculated to be 3.0 and 0 kcal mol^{-1} $(M\ (NH_4)_2SO_4)^{-1}$ using the unfolding and refolding rate constants, respectively. The sum of these contributions to ΔG_D is 3.7 kcal mol^{-1} $(M\ (NH_4)_2SO_4)^{-1}$ for the C_L fragment and 3.0 kcal mol^{-1} $(M\ (NH_4)_2SO_4)^{-1}$ for the V_L fragment. These values are smaller than the observed value (5.4 kcal/mol) for the C_L fragment in 1.5 M Gdn-HCl and the value (5.4 kcal/mol) for the V_L fragment in 1.8 M Gdn-HCl. Because the conditions used for the measurements of unfolding kinetics, refolding kinetics, and the unfolding equilibrium are all different, it is difficult to clarify the reason for this discrepancy. The difference between the observed and calculated values for the V_L fragment is particularly large. It is possible that a phase (or phases) that we failed to observe due to its small amplitude is affected by the presence of $(NH_4)_2SO_4$. Although there is a difference between the observed value and the value calculated by using kinetic data, we may well conclude that the increase in the stability of the C_L and V_L fragments by the presence of $(NH_4)_2SO_4$ is due largely to the decrease in the microscopic unfolding rate in the presence of $(NH_4)_2SO_4$.

Other Proteins. Mitchinson and Pain (195) studied in detail the effect of $(NH_4)_2SO_4$ on the stability of β-lactamase from *Staphylococcus aureus*. The unfolding of β-lactamase by urea proceeds through a thermodynamically stable intermediate H. $(NH_4)_2SO_4$ shifts both the N ↔ H and H ↔ U transitions to higher urea concentration by 5.3 M mol of $(NH_4)_2SO_4$. This effect is much larger than that obtained in our present study, although in our study we used Gdn-HCl as the denaturant. The stabilizing effects of $(NH_4)_2SO_4$ on the N ↔ H and H ↔ U transitions of β-lactamase by urea were determined to be 9.8 and 4.8 kcal/mol per mole of $(NH_4)_2SO_4$, respectively, and the effect of the total N ↔ U transition amounted to 14.6 kcal/mol per mole of $(NH_4)_2SO_4$. This value is much larger than the value obtained here [3.6–5.4 kcal/mol per mole of $(NH_4)_2SO_4$]. This suggests that the effect of stabilization by $(NH_4)_2SO_4$ differs depending on the protein species. Mitchinson and Pain also measured the refolding kinetics for the H ↔ N transitions at various concentrations of $(NH_4)_2SO_4$ and estimated the unfolding rate constants using the equilibrium constant for the N ↔ H transition. They found that the unfolding rate constant is decreased by a factor of 10^{11} on addition of 1.4 M $(NH_4)_2SO_4$ and concluded that the stabilizing effect of $(NH_4)_2SO_4$ is reflected almost exclusively in the decrease in the unfolding rate. Although the effect of $(NH_4)_2SO_4$ on the unfolding rate constant of β-lactamase seems to be too large, their conclusion that the stabilizing effect of $(NH_4)_2SO_4$ on the protein is due largely to a decrease in the unfolding rate, and not to an increase in the refolding rate, is the same as that which we have reached.

The effect of $(NH_4)_2SO_4$ on the transition temperature of the thermal unfolding of ribonuclease A has been studied by von Hippel and Wong (1964). The effect of $(NH_4)_2SO_4$ on the refolding kinetics of ribonuclease A has been studied by Cook et al. (1979), Lin and Brandts (1983, 1987), Schmid (1981, 1986), Schmid et al. (1986), and Krebs et al. (1985). The refolding kinetics of ribonuclease A are described by three exponential phases. In 0.3 M urea at pH 5 and 10 °C, the refolding kinetics detected by absorbance change consist of a fast phase (20%, τ = 28 ms), a slow phase (50%, τ = 22 s), and an additional slow phase (30%, τ = 85 s) (Lin & Brandts, 1983). The fast phase is attributed to the direct folding process, but the interpretation of the two slow phases differs depending on the investigators, and the role of the cis–trans isomerization of proline residues has been discussed (Lin & Brandts, 1983, 1987; Schmid, 1983, 1986; Schmid et al., 1984). According to Lin and Brandts (1983), $(NH_4)_2SO_4$ increases the apparent rate constants for the slow phase but does not affect the rate constants for the fast phase and additional slow phase. The same observation was made irrespective of the method used. According to Schmid (1981, 1983) and Krebs et al. (1985), acceleration by $(NH_4)_2SO_4$ of the rate constants for the two slow phases was observed by absorbance measurement but was not significant by measurement of fluorescence.

The stabilizing effects of $(NH_4)_2SO_4$ on protein structures thus seem to differ depending on protein species. Although we have been able to study the stabilizing effect of $(NH_4)_2SO_4$ on proteins quantitatively, further data on other proteins and on interactions between sulfate ions and protein molecules must be gathered in order to understand how the salt stabilizes protein conformations.

Registry No. Guanidine hydrochloride, 50-01-1; ammonium sulfate, 7783-20-2.

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Amino Acid Structures of Multiple Forms of Amyloid-Related Serum Protein SAA from a Single Individual†

Francis E. Dwulet,‡ David K. Wallace, and Merrill D. Benson*

Departments of Medicine and Medical Genetics, Indiana University School of Medicine, and Rheumatology Section A772, Richard Roudebush Veterans Administration Medical Center, 1481 West 10th Street, Indianapolis, Indiana 46202

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ABSTRACT: Multiple forms of the acute-phase serum protein SAA were isolated from the lipoprotein fraction of plasma from a single individual. These protein forms were purified by size-exclusion, ion-exchange, and reverse-phase high-pressure liquid chromatography, and then the tryptic peptides were subjected to amino acid sequence analysis. A total of three distinct 104-residue proteins were identified. Two of these proteins differed only by having either an arginine or a histidine at position 71 while the third protein had seven amino acid differences. Each of these proteins has a 103-residue companion protein where the amino-terminal arginine has been removed. Two of these protein sequences match the two human SAA cDNA structures reported in the literature. The presence of three unique amino acid sequences in one individual is proof that there must be a minimum of two genes for SAA in humans.

In many chronic inflammatory and infectious diseases a severe complication is the development of systemic amyloidosis. In this condition, aggregates of fibril structures are deposited or formed in the extracellular spaces of the individual's tissue with kidney, spleen, and liver often being severely involved (Cohen, 1967; Benditt & Eriksen, 1971). The major protein constituent of these fibril structures is amyloid protein A (AA).[1] Comparison of protein AA from human and animal fibril preparations reveals polypeptides with similar molecular masses (7000–10 000 daltons) and amino acid sequences that are highly conserved, especially in the middle of the polypeptide (Hermondson et al., 1972; Levin et al., 1972; Waalen et al., 1980; DiBartola et al., 1985; Benson et al., 1985). Antibodies to amyloid protein AA have been found to cross-react with a serum α-globulin with a molecular mass of 100–200 kilodaltons which was later identified as high-density lipoprotein (HDL). Treatment of these HDL complexes with denaturing agents yielded a 12 000-dalton fraction that contained all the AA-cross-reacting material and that was named serum amyloid A (SAA) (Eriksen & Benditt, 1980). Since its identification this protein has been found to be an acute-phase protein. In an unstimulated individual, SAA serum levels are in the range of 1–5 μg/mL. However, during an infection or inflammatory episode serum levels of the protein may increase up to several hundred times the normal value and then quickly return to normal levels upon recovery. Recently, it has been shown that when human SAA is administered to a mouse undergoing casein-induced amyloid formation, the resulting fibrils contain both mouse and human AA proteins, thus

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* Address correspondence to this author at the Richard Roudebush Veterans Administration Medical Center.

‡ Present address: Boehringer Mannheim Biochemicals, Indianapolis, IN.

[1] Abbreviations: AA, amyloid A protein; SAA, serum amyloid A; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; ELISA, enzyme-linked immunosorbent assay; Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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