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# Stability and Solvent Accessibility of SecA Protein of *Escherichia coli*[1]

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**It has been shown that many proteins, when converted into partially unfolded structures, interact strongly with a lipid bilayer. SecA protein of *Escherichia coli* is an unusual water-soluble protein which, in the native form, can readily penetrate the membrane and lipid bilayer. In order to see whether the native SecA exhibits partially unfolded characteristics, the stability and solvent accessibility of SecA were studied using various spectroscopic and hydrogen-exchange methods. The results are compared with the reported data for native and molten globule forms of α-lactalbumin (α-LA), as well as those for apocytochrome *c*. The exposure of hydrophobic residues of SecA, as monitored by 8-anilinonaphthalene-1-sulfonic acid (ANS) binding, and the extent of amide hydrogen exchange were comparable to those of native α-LA. On the other hand, equilibrium unfolding experiments showed that SecA is less stable than native α-LA. The results of tryptic digestion and the change of endogenous ATPase activity induced by guanidine hydrochloride were suggestive of the C-terminal half of SecA being more flexible than the rest of the protein. The overall conclusion is that the SecA as a whole has a somewhat open structure due to a relatively unstable C-domain which may facilitate its penetration into a lipid bilayer.**

**Key words: 8-anilinonaphthalene-1-sulfonic acid, ATPase, hydrogen exchange, protein-lipid interaction, SecA.**

According to the fluid-mosaic model proposed by Singer and Nicholson (*1*), the membrane proteins are classified into two categories, integral and peripheral. Since this proposal was made, a number of peripheral proteins (*2-5*) and stable water-soluble proteins (*6-8*) have been found to penetrate membranes under appropriate conditions, blurring the distinction between these two classes of membrane proteins. In fact, it appears that penetration of normally stable proteins into a lipid bilayer under acidic conditions is a common trait of many, if not all, proteins. It was proposed that the membrane-penetrative form of proteins is the molten globule structure (*9*). The molten globule, or compact intermediate state, is an equilibrium and folding form of proteins intermediate between the native and completely unfolded structures (*9*). In this form, the secondary structure of the native form is intact, but the tertiary structure is lost. It has been established that the molten globule state is relatively compact and the motions of aromatic side chains are restricted, indicating the presence of a hydrophobic core. Many proteins, including α-lactalbumin (α-LA) (*2, 3*) (Kim, H., Kim, U.H., Lee, H., and Marsh, D., to be submitted), diphtheria toxin (*10*), and colicin A (*11*), interact strongly with a lipid bilayer only when they are converted into molten globule form at low pH.

Some apoproteins also penetrate the membrane readily even at neutral pH although their holoproteins do not under the same conditions. A representative example is apocytochrome *c*. This apoprotein was found to traverse the lipid bilayer (*12, 13*) and an extensive study by Marsh and his associates using spin-labeled lipid confirmed its deep penetration of the lipid bilayer (*14*). Since the CD spectra of apocytochrome *c* are similar to those of unfolded protein at low ionic strength, it has been assumed that this protein is in the form of a random coil. However, fluorescence resonance energy transfer experiments showed that apocytochrome *c* is rather compact (*15*), being somewhat less structured than the molten globule state. Apolipoprotein A-I also shows molten globule characteristics (*16*).

Except for special cases like these, stable proteins generally do not interact hydrophobically with membranes. One exception is the SecA protein, which functions as the central component of the protein translocation machinery in *Escherichia coli*. This protein, with a molecular mass of approximately 102 kDa, exists as a homodimer and is distributed *in vivo* almost equally between the inner membrane and cytosol, about half of the membrane-bound protein being unextractable with electrolytes (*17*). An appreciable amount of this protein binds even to phos-

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Abbreviations: α-LA, α-lactalbumin; ANS, 8-anilinonaphthalene-1-sulfonic acid; GdnHCl, guanidine hydrochloride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; TAME, *p*-toluenesulfonyl-L-arginine methyl ester.

phatidylcholine vesicles (approximately 20% at 22°C and 50% at 37°C) and the extent of binding to phosphatidylcholine/phosphatidylglycerol vesicles is even greater (more than 80%) (*18*). It was also found that SecA can readily penetrate and traverse the lipid bilayer (*17, 19, 20*), as well as the inner membrane of *E. coli* (*20-22*). This behavior is similar to that of the apocytochrome *c*. Extensive studies on the effect of nucleotides (*19*) and precursor ribose binding protein and its signal peptide on the SecA penetration of a lipid bilayer (*20*) have been made.

The present investigation addresses the question of what characteristics of SecA allow this protein readily to penetrate the lipid bilayer. Emphasis is given to two aspects of this phenomenon: the stability of SecA under various conditions and the solvent accessibility of this protein.

## EXPERIMENTAL PROCEDURES

*Materials*—Cibacron Blue 3G Sepharose and Sephadex G-15 were purchased from Pharmacia LKB Biotechnology. [$^{14}$C]Formaldehyde (10 mCi/mmol) was from Du Pont and tritiated water (5 mCi/ml) was from Amersham. Guanidine hydrochloride (GdnHCl), KI, $Na_2S_2O_3$, ATP, 8-anilinonaphthalene-1-sulfonic acid (ANS), Malachite Green oxalate, tosylphenylalanylchloromethyl ketone-treated trypsin (type XIII), and *p*-toluenesulfonyl-L-arginine methyl ester (TAME) were from Sigma. A stock solution of GdnHCl in buffer A (50 mM potassium phosphate buffer, pH 7.5, containing 1 mM DTT) was prepared daily, and its concentration was determined by using a Bausch & Lomb refractometer after pH adjustment. A 10 mM ANS stock solution was made in buffer A. All solutions were passed through 0.2 $\mu$m Sartorius syringe filters just before the measurements.

*SecA Preparation*—SecA protein was purified from a SecA-overproducing strain (RR1/pMAN400) using a Cibacron Blue 3G Sepharose column (*20*). Fractions containing SecA were dialyzed against buffer A and stored at −75°C. SecA concentration was determined after Bradford (*23*) (Bio-Rad Laboratories).

*Heat-Induced Conformational Change*—Conformational change of SecA induced by heat was monitored by measuring the far-UV CD spectra, Trp fluorescence and ANS fluorescence. SecA concentrations for far-UV CD and fluorescence measurements were 0.5 and 0.2 $\mu$M in buffer A, respectively. Spectra of unfolded SecA were obtained after incubating this protein for 2 h at 30°C in buffer A containing 3 M GdnHCl. Far-UV CD spectra were recorded on a JASCO J-720A spectropolarimeter over 250-200 nm using a 1-mm pathlength cell. Scan speed was 50 nm/min and 10 scans of each sample were averaged. The cell holder was thermostated using a Neslab RTE-111 water-bath. For the temperature scanning of CD, the ellipticity at 222 nm was measured with a thermostated 1-mm pathlength cell at 0.1°C intervals when the temperature was raised at a constant rate of 0.33°C/min. Fluorescence measurements were carried out on a Shimadzu RF-5301PC spectrofluorometer equipped with a constant-temperature cell holder. For Trp fluorescence, the excitation wavelength was 295 nm (1.5 nm slit) and the emission was recorded from 320 to 450 nm (5 nm slit). For the temperature scanning of fluorescence of 0.5 $\mu$M SecA, the Trp fluorescence intensities, excited at 295 nm (1.5 nm slit) and measured at 340 nm (5 nm slit), and the ANS fluorescence intensities, excited at 374 nm (3 nm slit) and measured at 480 nm (5 nm slit), were collected at every 2°C increment. The ANS concentration was 200 $\mu$M.

*GdnHCl-Induced Equilibrium Denaturation and Renaturation*—Sample solutions containing SecA were incubated at 30°C with various concentrations of GdnHCl in buffer A for 12 h. The fluorescence intensities of Trp and ANS and the ellipticity were measured as described above. To determine the effect of Mg-ATP on denaturation, SecA was pre-incubated with this nucleotide for 1 h at 30°C before the denaturation. Equilibrium renaturation was performed with SecA that had been incubated with 4 M GdnHCl for 2 h at 30°C. The denatured SecA was diluted 40-fold with solutions of appropriate GdnHCl concentration, and the mixtures were incubated at 30°C for 12 h.

*Hydrogen-Tritium Exchange*—In order to facilitate the concentration determination, SecA was labeled with [$^{14}$C]-formaldehyde as described (*24*). SecA stock solution (1 ml, 2 mg/ml) was added to a solution containing 3 mCi of tritiated water and 40 $\mu$g of [$^{14}$C]SecA. This solution was mixed with stock buffer solution and distilled water to make 2 ml of final solution in which the concentrations of buffer components are the same as in buffer A.

Complete tritiation of all labile hydrogens was achieved by incubating the protein solution for 5 h at 37°C. A preliminary experiment established that the isotope exchange is completed within 4 h under the conditions used. The solution was re-incubated for 1 h at 30°C, then the exchange rate was determined by the two-column separation technique using Sephadex G-15 columns (*25, 26*). The activities (cpm) of $^{14}$C and $^{3}$H were counted on a Beckman LS 6000LL liquid scintillation counter to determine the SecA concentration and the tritium content, respectively. Protein concentrations were determined from the $^{14}$C counting of protein standards, of which the concentration had been established previously by the Bradford assay. The columns and buffers were incubated at 4°C, to minimize tritium exchange during gel filtrations. The hydrogen exchange rate of unfolded SecA was determined in 3 M GdnHCl using the same method as above. The number of hydrogens remaining unexchanged per protein molecule at time $t$, $H(t)$, was calculated using equation (*25*)

$$H(t) = \frac{\text{cpm}(t)\,W}{\text{cpm}(0)\,IP}$$

where cpm(0) and cpm($t$) are the specific $^{3}$H activity per unit volume of the solutions at time 0 and time $t$, respectively, $W$ is the number of moles of solvent hydrogen per unit volume (111 mol/liter), $I$ is the isotopic enrichment factor (1.21) to correct for the preferential binding of tritium by peptide groups (*26*) and $P$ is the number of moles of protein in the sample.

*ANS Binding to SecA*—The number of ANS molecules bound to a SecA molecule was determined by following the procedures described in the literature (*27, 28*). The ANS fluorescence intensity was measured at 30°C as described above. The inner filter effect of ANS was corrected using the method described in the literature (*29*). The number of molecules of ANS bound to a SecA monomer and the dissociation constant ($K_d$) for SecA-ANS complex were estimated from a Scatchard plot of the fluorescence intensities.

*Iodide Quenching of Trp-Fluorescence*—Quenching of SecA Trp-fluorescence by iodide was measured according to the method given in the literature (*30*). A 2 M KI stock solution and a 2 M KCl stock solution were prepared in buffer A. A small amount of $Na_2S_2O_3$ (0.1 mM) was added to the KI stock solution to prevent $I_3^-$ formation. Sample solutions of native SecA and unfolded SecA in 3 M GdnHCl, containing increasing amounts of KI (0–0.2 M), were prepared by mixing SecA solution with the stock solution of KI. The ionic strength was kept constant at 0.2 by adding appropriate amounts of KCl solution. The solutions were incubated at 30°C for 30 min and Trp fluorescence intensities were measured as described above.

*Limited Proteolysis of SecA by Trypsin*—Proteolysis of 0.2 mg/ml SecA was performed in the presence of 0.0, 0.5, 1.0, 1.5, or 2.0 M urea in buffer A. All solutions were incubated for 6 h at 30°C. The decrease in the trypsin activity with increasing urea concentration was compensated by increasing trypsin concentration to the level of trypsin activity without urea. For this, the decrease in the trypsin activity with increasing urea concentration was determined spectrophotometrically as the initial rate of hydrolysis of the chromogenic substrate, TAME. Digestion of native SecA in the absence of urea was performed at 30°C at trypsin/SecA ratio of 1/50 (by weight). After 5, 10, and 20 min, proteolysis was stopped by mixing aliquots with a solution comprising 4 mM PMSF and 4×electrophoresis sample buffer (0.25 M Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate, 20% 2-mercaptoethanol, 20% glycerol, and 0.008% bromophenol blue). All samples were boiled for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis. The N-terminal sequences of the main fragments were analyzed by an Applied Biosystems 476A Protein Sequencer after the bands had been transferred to PVDF membrane.

*Change of ATPase Activity*—After 0.2 μM SecA was incubated at 30°C for 12 h with various concentrations of GdnHCl in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT, 5 mM $MgCl_2$, an equal volume of 2 mM ATP in each concentration of GdnHCl was added to each sample. After further incubation at 30°C for 1 h, the ATPase activity of SecA was measured by a modified Malachite Green method as described (*31*). The absorbance at 650 nm was measured immediately after incubation in a Perkin Elmer Lambda 16 UV/VIS spectrophotometer. The average activity of 6 repeats at each GdnHCl concentration was normalized to take account of the difference between the absorbances of native SecA and a blank.

## RESULTS

*Thermal Unfolding*—Figure 1 shows the far-UV CD spectra and Trp emission spectra of SecA obtained at different temperatures. The minima around 208 and 222 nm of the CD spectra (Fig. 1A) are indicative of significant α-helix content. As was observed by Ulbrandt *et al.* (*18*), there was a gradual decrease in the α-helix content when the temperature was raised from 10 to 37°C followed by an abrupt change between 37 and 45°C. The unfolding is apparently an irreversible process, since lowering the temperature from 45 to 30°C did not cause the spectrum to revert to that of 30°C (data not shown). The estimated secondary structure obtained by curve-fitting the spectra at 37°C, for example, is 32% α-helix, 12% β-sheet, 13% β-turn, and 44% random coil.

Figure 1B compares the thermal unfolding behavior of native SecA and SecA in 3 M GdnHCl as monitored by Trp fluorescence. At 60°C, the fluorescence intensities of these two solutions became about the same, indicating that the extent of denaturation by heat beyond this temperature is about the same as that of GdnHCl-induced denaturation.

Figure 2 compares the thermal unfolding curves obtained from the far-UV CD and Trp fluorescence data mentioned above, as well as the ANS binding. The extent of unfolding given in this figure was determined from the Trp fluorescence at 340 nm, normalized by setting the intensities of the native and unfolded SecA above 50°C to be 0 and 1, respectively, after proper correction for the temperature dependence of the fluorescence intensity. This fluorescence-temperature curve is essentially identical to the one

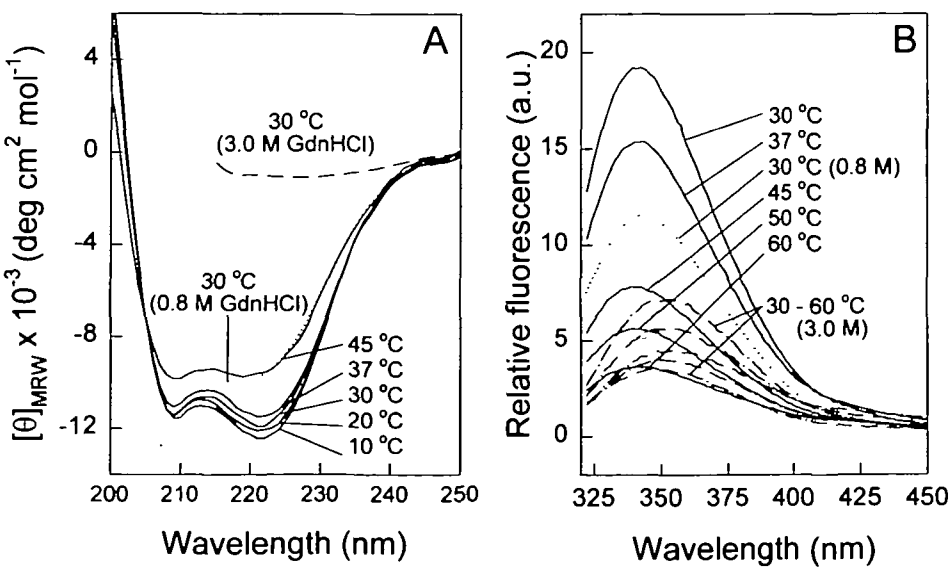


Fig. 1. **Temperature-dependent far-UV CD spectra (A) and Trp fluorescence (B) of SecA.** (A) CD spectra of 0.5 μM SecA in 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM DTT were obtained at several temperatures. SecA was incubated for 5 min at each temperature prior to the measurements. Ten scans each were averaged and smoothed. Dotted and dot-and-dashed lines show the spectra of unfolded SecA in 0.8 M and 3.0 M GdnHCl at 30°C, respectively. (B) Trp fluorescence intensities (arbitrary unit) of 0.2 μM SecA in the same buffer measured at several different temperatures. Solid lines represent the spectra obtained in the absence of GdnHCl and dot-and-dashed lines indicate those obtained in the presence of 3.0 M GdnHCl. The dotted line is the spectrum of unfolded SecA in 0.8 M GdnHCl at 30°C.

obtained by Ulbrandt *et al.* (*18*). The unfolding appears to be a one-step reaction between two states. The irreversible nature was apparent here, too, because cooling after the heating at 40°C did not bring the fluorescence intensity back to the original value (data not shown). In fact, the SecA solution became opaque when heated beyond 40°C. The $T_m$ value from the fluorescence curve is around 38°C, which indicates that the tertiary structure of SecA is partially broken at the physiological temperature. Also, the $T_m$ value of SecA is appreciably lower than those of most proteins. As an example, the $T_m$ value of holo-$\alpha$-LA is 82°C at neutral pH (*32*). The $T_m$ value of this protein, however, becomes 37°C at pH 3.4, at which pH value this protein is largely in the molten globule form (*33*). The $T_m$ value for apocytochrome *c* was found to be 35.5°C at pH 7.5 (Song, M. and Kim, H., unpublished data), while that of its holoprotein is 83°C (*32*).

When monitored by far-UV CD, the unfolding was more gradual with a $T_m$ value near 45°C, but below and beyond the transition region, there was a gradual increase in the mean residue ellipticity with temperature. It should be noted that the $T_m$ value obtained by CD coincides with the end of the transition monitored in terms of the Trp fluorescence. The $T_m$ obtained by CD is some 8 degrees higher than the value from the Trp fluorescence. The changes in the ellipticity of far-UV CD and in the intensity of Trp fluorescence are not parallel. This pattern is akin to that of a typical molten globule structure when the $[\theta]$ values in the near-UV and far-UV are compared. Here, a CD experiment in the near-UV range was not possible because the necessary high concentration of SecA resulted in aggregation. Another interesting feature of Fig. 2 is that the temperature increase from 20 to 37°C brought about a reduction of 40% in the Trp fluorescence while the $[\theta]$ value remained practically the same. This suggests that SecA has a partially unfolded conformation at the physiological temperature.

The ANS fluorescence intensity as a function of temperature was also normalized by setting the intensity of the base line below 30°C to be zero and the maximum value at 45°C to be one. The general bell shape of the curve is that of a typical equilibrium unfolding with a molten globule intermediate (*28*). It is of interest that $T_m$ from the far-UV CD measurement is the same as the temperature which gives the maximum intensity of ANS fluorescence. This is also a typical feature of equilibrium unfolding with a molten globule intermediate (*28*).

Although the thermal unfolding exhibits some features typical of the molten globular intermediate, the complication arising from the irreversible unfolding at high temperature makes it difficult to reach a definite conclusion. It is clear, however, that pure SecA is already somewhat unfolded even at the physiological temperature.

*Unfolding by Denaturants*—The equilibrium unfolding of SecA by GdnHCl was also monitored in terms of Trp fluorescence, ANS fluorescence, and far-UV CD (Fig. 3). At 3.0 M GdnHCl, SecA was fully unfolded (show Fig. 1, A and B). Unlike the curves of thermal denaturation shown in Fig. 2, these curves obtained by measurements of Trp fluorescence and far-UV CD appear to suggest a two-step unfolding process. The presence of an apparent intermediate, however, coincides with aggregate formation as shown by

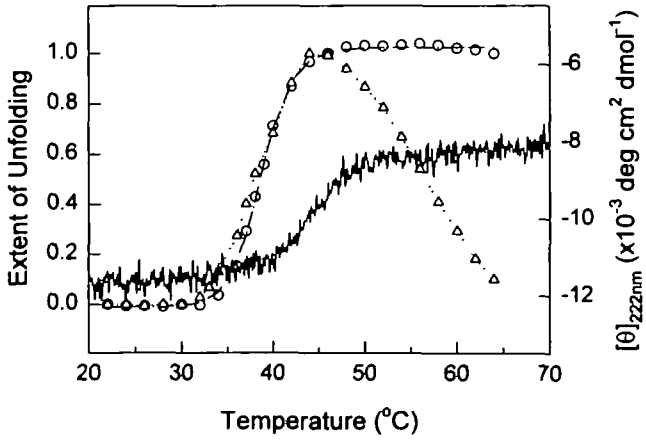


Fig. 2. **Thermal unfolding of SecA monitored by Trp fluorescence, ANS fluorescence, and CD.** *Changes in the fluorescence and* ellipticity (at 222 nm, solid line) of 0.5 $\mu$M SecA were recorded as described under "EXPERIMENTAL PROCEDURES." Intensities of Trp (○) and ANS (△) fluorescence of native and unfolded SecA were arbitrarily set as 0 and 1, respectively. Dot-and-dashed lines represent *two-state curve-fitting of the temperature-corrected Trp fluorescence.* ANS concentration was 200 $\mu$M.

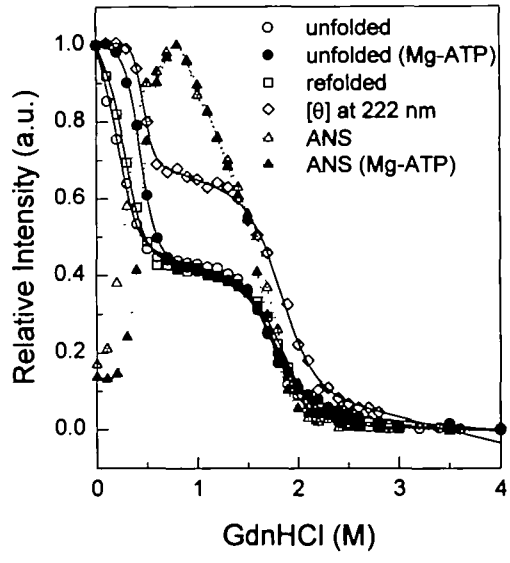


Fig. 3. **Comparison of the equilibrium denaturation profiles monitored in terms of the change of the ellipticity at 222 nm and the intensities of Trp and ANS fluorescence at 30°C.** *Equilibrium* denaturation was performed by incubation of SecA in buffer A for 12 h with increasing GdnHCl concentrations at 30°C. Denaturation of 0.5 $\mu$M SecA with (open symbols) or without (closed symbols) 1 mM Mg-ATP was monitored in terms of the change of the intensities of *Trp (circles) fluorescence measured at 340 nm (5 nm slit) and excited* at 295 nm (1.5 nm slit). The intensities of ANS (dotted lines and triangles) fluorescence were measured at 480 nm (5 nm slit) and excited at 374 nm (3 nm slit). Denaturation of 0.5 $\mu$M SecA without ATP was also monitored in terms of the change of the ellipticity (◇) *at 222 nm. Renaturation (□) without ATP as monitored by following* the change of the Trp fluorescence intensity was also examined for comparison. The intensities were corrected to relative intensities (arbitrary unit) by setting the intensities of the minimum and maximum at each condition to be 0 and 1, respectively. The far-UV *CD and Trp fluorescence data were fitted to the equation for a two-step* transition model [Eq. 5 in Morjana *et al.* (*38*)] (—).

light-scattering (data not shown). The aggregation formation may be due to exposure of hydrophobic residues at an intermediate state (*34*). The equilibrium renaturation was also studied in terms of the Trp fluorescence. The denaturation and renaturation curves almost overlap, suggesting the reversible nature of the process. Transition in the secondary structure appears to occur at higher GdnHCl concentration than in the tertiary structure. The CD spectra in the far-UV region of SecA at 30°C in the presence of two different concentrations of GdnHCl have already been shown in Fig. 1A. The denaturation was also monitored in terms of the ANS fluorescence. ANS binding to the intermediate state was higher than to the native and unfolded states, which may be caused by ANS binding to the intermediate state with more exposed hydrophobic region. Although these results are suggestive of the existence of a molten globule intermediate (*28*), it is also possible that SecA consists of at least two domains with different stability, as the subsequent experiments of tryptic digestion and the effect of the GdnHCl on the ATPase activity indicate. It is of interest that even the native SecA binds ANS appreciably.

It was shown that ATP converts SecA into a more compact form and retards the SecA insertion into the membrane (*19*). However, the effect of ATP on the GdnHCl-induced unfolding is apparent only at low GdnHCl concentration. Table I gives the values of standard free energy change ($\Delta G°$) obtained from GdnHCl-induced unfolding and refolding profiles. For calculating the $\Delta G°$ values, the data were curve-fitted to an equation for the two-step transition model (*38*), where $\Delta G_1°$ and $\Delta G_2°$ represent the Gibbs free energy change for the first and second transitions, respectively. Figure 3 (solid-lines) shows that the data points can be adequately represented by the two-step model. Table I also lists the $\Delta G°$ values of some other proteins for comparison. $\Delta G_1°$ for SecA is smaller than those of other proteins, underlining the unstable nature of this protein.

Denaturation of SecA at 30°C by urea, as monitored by Trp and ANS fluorescence, is similar to that by GdnHCl (data not shown). The stable intermediate was also observed in the range of 1.0 to 3.0 M urea and the maximum binding of ANS was observed at about 2.0 M urea.

*Hydrogen-Tritium Exchange*—Figure 4 shows the time courses of hydrogen-tritium exchange of native and denatured SecA at pH 7.5 and 30°C. The hydrogen-deuterium exchange curves for $\alpha$-LA (*39*) and lysozyme (*40*) are also given for comparison. There is an initial stage of exchange which is too fast to measure. This is followed by a moderately fast stage with a measurable rate and finally the exchange becomes very slow. The measurable exchanges were curve-fitted to the equation

$$\%H(t)_{rem} = A_1\exp(-k_1 t) + A_2\exp(-k_2 t)$$

where $\%H(t)_{rem}$ is the % number of hydrogens per molecule that remain unexchanged at time $t$, and $A_1$ and $A_2$ are the numbers of hydrogens per molecule characterized by the exchange constants $k_1$ and $k_2$, respectively. The exchange rates and the % number of exchanged hydrogens thus obtained are given in Table II. The hydrogens involved in the initial very fast exchange and the hydrogens exchanged at a moderately fast rate ($A_1$) of the native SecA amount to

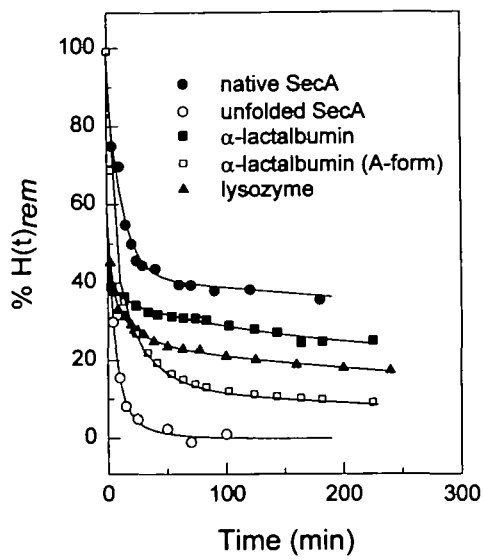


Fig. 4. **Hydrogen exchange kinetics.** Hydrogen-tritium exchanges of native (●) and unfolded (○) SecA at pH 7.5 and 30°C were measured as described in the "EXPERIMENTAL PROCEDURES." For comparison, the hydrogen exchange profiles of the native state (■) at pH 5.5 and the A-state (□) at pH 2.0 of $\alpha$-LA at 20°C [Fig. 3 of Chyan *et al.* (*39*)], and native lysozyme (▲) at pH 7.5 and 35°C [Fig. 1 of Gregory *et al.* (*40*)] are also presented. The fitted curves are represented by solid lines. $\%H(t)_{rem}$ means the % number of remaining hydrogens at time $t$.

TABLE I. **Standard free energy change, $\Delta G°$, of equilibrium unfolding of SecA by GdnHCl.**

| Protein | $\Delta G_1°$ (kcal/mol) | $\Delta G_2°$ (kcal/mol) | Solution conditions | Reference |
|---|---|---|---|---|
| SecA | 1.4 | 7.6 | pH 7.5, 30°C[a] | |
| | 1.7 | 7.8 | pH 7.5, 30°C, renaturation[a] | |
| | 5.6 | 7.0 | pH 7.5, 30°C[b] | |
| | 3.4 | 7.6 | pH 7.5, 30°C, with 1 mM Mg-ATP[a] | |
| RNase A | 7.5 | – | pH 7.0, 25°C[c] | *35* |
| Lysozyme | 8.9 | – | pH 7.0, 25°C[c] | *35* |
| $\alpha$-Lactalbumin | | | | |
| N↔D | 3.8 | – | pH 7.0, 25°C[b,d], with $Ca^{2+}$ | *36* |
| N↔D | 7.2 | – | pH 7.0, 25°C[b,d], with 1 mM $Ca^{2+}$ | *36* |
| A↔D | 1.4 | – | pH 7.0, 25°C[b,d] | *36* |
| Myoglobin | 7.6 | – | pH 6.6, 25°C[c] | *35* |
| Apomyoglobin | 2.8 | – | pH 8.0, 20°C[a] | *37* |
| Protein disulfide isomerase | 5.4 | 7.6 | pH 7.5, 23°C[a] | *38* |

[a]Fluorescence. [b]Far-UV CD. [c]UV absorbance. [d]Near-UV CD.

about 60% of total exchangeable hydrogens. The remaining 40% of hydrogen was exchanged more slowly with a rate approximately 100-fold smaller than that of the rapid exchange. It is possible that approximately 60% of the backbone amides of native SecA is located within flexible structures with ready access to the solvent. This value is smaller than those for native $\alpha$-LA (~75%), A form of $\alpha$-LA (~90%), and lysozyme (~80%).

*ANS Binding to SecA*—Figure 5A shows the binding isotherm of ANS to SecA at 30°C, corrected for the inner filter effect by using the following equation (*29*)

$$F_{cor} = 2.3F_{obs}10^{A_{480}/2}A_{374}/(1-10^{-A_{374}})$$

where $F_{obs}$ is the observed fluorescence intensity, $F_{cor}$ is the fluorescence intensity corrected for the inner filter effect, and $A_{374}$ and $A_{480}$ are the measured absorbances at 374 and 480 nm, respectively. The Scatchard plot given in Fig. 5B shows that there are apparently two binding sites. The $K_d$ value of the high-affinity sites is comparable to that of $\alpha$-LA (*27*). Native $\alpha$-LA, as well as apo $\alpha$-LA, has only one ANS binding site (*27*).

*Quenching of the Intrinsic Fluorescence of Trp*—For both native and denatured SecA, the Trp-fluorescence intensity at 340 nm was measured with increasing iodide concentration. Addition of iodide to SecA resulted in quenching of Trp fluorescence without shifting the emission. Figure 6 shows a plot of $F_o/(F_o-F)$ against the reciprocal of iodide concentration. These straight lines were fitted to the Lehrer equation (*30*),

$$F_o/(F_o-F) = 1/\alpha + 1/(\alpha K_{sv}[Q])$$

where $F_o$ and $F$ are the fluorescence intensities in the absence and in the presence of the quencher, respectively, $\alpha (0 \leq \alpha \leq 1)$ is a quenchable fraction with Stern-Volmer constant $K_{sv}$, and [Q] is quencher concentration. The $\alpha$ value for the native SecA was 0.75, while that of denatured SecA was 1.00. This result shows that 75% of the 7 Trp residues in SecA are accessible to the solvent. It is interesting that this exposed Trp fraction is much larger than that for native $\alpha$-LA and somewhat larger than that for the A

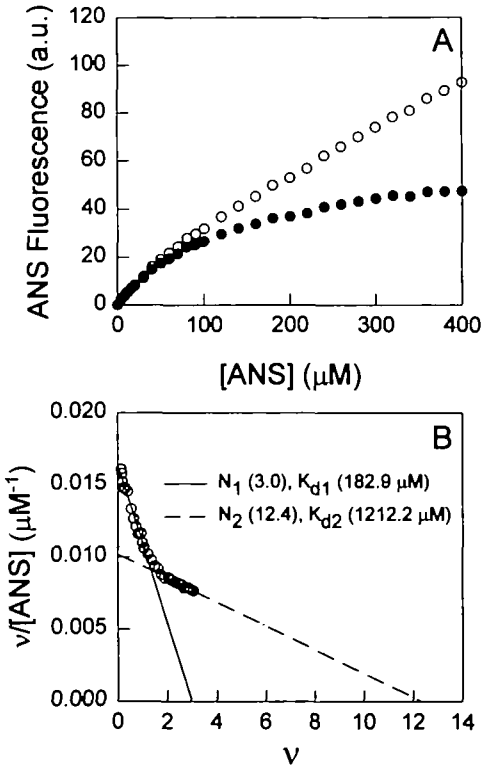


Fig. 5. **ANS binding to SecA.** (A) Binding isotherm of ANS to SecA at 30°C. (○), observed ANS fluorescence intensity; (●), after correction for inner filter effect. (B) Scatchard plot of ANS binding to SecA. The number of high-affinity ANS binding sites ($N_1$) and low-affinity binding sites ($N_2$) on SecA and the dissociation constant ($K_d$) were estimated from the binding data obtained at pH 7.5 and 30°C as described in "EXPERIMENTAL PROCEDURES." $\upsilon$ is the number of ANS bound per SecA and [ANS] is the free ANS concentration.

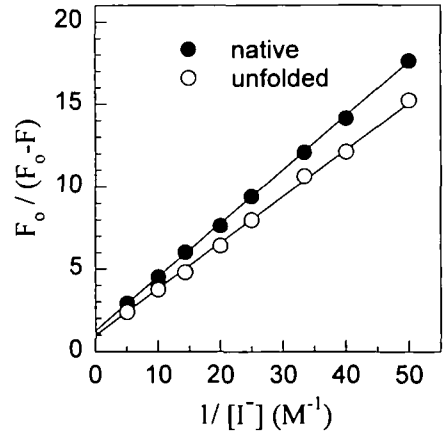


Fig. 6. **Modified Stern-Volmer plot of quenching of Trp fluorescence.** Quenching of Trp fluorescence intensities of native (●) and unfolded (○) SecA in 3 M GdnHCl by iodide were compared. Trp fluorescence was quenched with 0.0–0.2 M KI at pH 7.5 and 30°C. KCl was added to keep the ionic strength constant. The reciprocal of the $y$-intercept indicates the quenchable fraction ($\alpha$) of the fluorescence intensity.

TABLE II. **Peptide hydrogen exchange parameters.** The exchange data of native and unfolded SecA were fitted to the equation, $\%H(t)_{rem} = \sum_1^n A_i \exp(-k_i t)$, where $\%H(t)_{rem}$ is the % number of hydrogens per molecule that remain unexchanged at time $t$, and $A_i$ is the number of hydrogens per molecule characterized by exchange constant $k_i$.

| Protein | Rate constant ($min^{-1}$) | | | % of exchanged hydrogen | | |
|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | $k_3$ | $A_1$ | $A_2$ | $A_3$ |
| Native SecA | 0.08 | $7.0\times10^{-4}$ | – | 50 | 42 | – |
| Unfolded SecA | 0.16 | 0.04 | – | 42 | 9 | – |
| $\alpha$-Lactalbumin[a] | 0.092 | $5.9\times10^{-3}$ | $8.5\times10^{-5}$ | 5 | 15 | 20 |
| $\alpha$-Lactalbumin (A-form)[a] | 0.24 | 0.039 | $1.8\times10^{-3}$ | 52 | 34 | 14 |
| Lysozyme[b] | 0.32 | 0.04 | $1.4\times10^{-3}$ | 20 | 12 | 24 |

[a]From Fig. 3 of Chyan *et al.* (*39*). [b]From Fig. 1 of Gregory *et al.* (*40*).

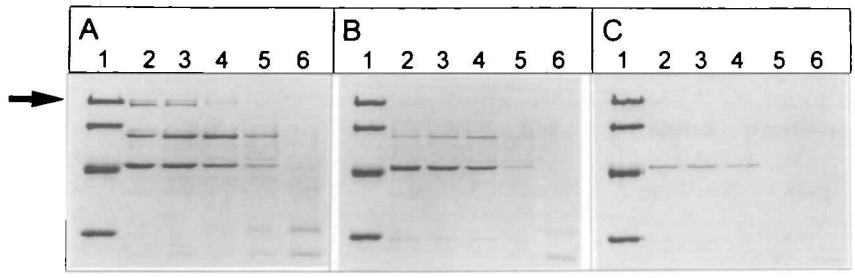


Fig. 7. **Limited proteolysis of SecA by trypsin.** Digestion was performed at pH 7.5 and 30°C in the presence of different concentrations of urea. SecA solution (0.2 mg/ml) was incubated with trypsin (weight ratio, 50 : 1). After 5 (A), 10 (B), and 20 min (C) of incubation, aliquots were taken from the reaction mixtures and put into the sample buffer for electrophoresis (containing 4 mM PMSF). Molecular weight size markers in lane 1 were 94, 67, 43, and 30 kDa. The concentrations of urea were 0.0 (lane 2), 0.5 (lane 3), 1.0 (lane 4), 1.5 (lane 5), and 2.0 M (lane 6), respectively. The arrow indicates the SecA protein.

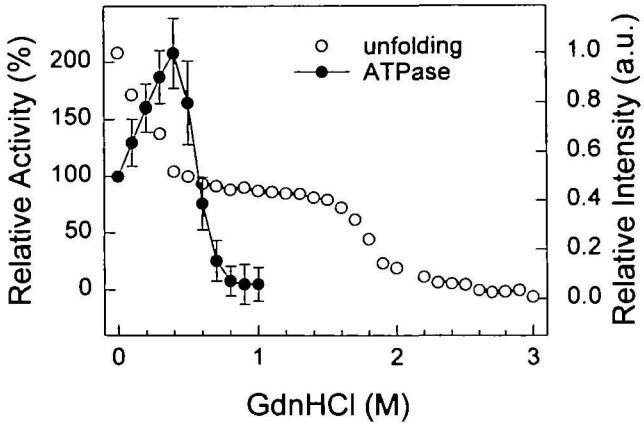


Fig. 8. **Dependence of the ATPase activity of SecA on the GdnHCl concentration.** The ATPase activity (●) was measured by a modified Malachite Green method after incubation of SecA at 30°C for 12 h in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT, 5 mM $MgCl_2$, and various concentrations of GdnHCl. After this, the solution, containing 0.1 $\mu$M SecA and 1 mM ATP, was incubated at 30°C for 1 h. Malachite Green reagent was then added to each solution after the addition of perchloric acid. Absorbance at 650 nm was measured. The unfolding profile (○) was redrawn from Fig. 3. The average ATPase activity of 6 repeats at each GdnHCl concentration was normalized with respect to the difference between the absorbances of native SecA and a blank.

form of $\alpha$-LA (*41*). It is closer to that of lysozyme at pH 2 (*30*).

*Tryptic Digestion*—The tryptic digestion patterns of SecA with increasing urea concentration are shown in Fig. 7. There was about 10% decrease in trypsin activity when the urea concentration was increased to 2 M (data not shown) and this was compensated by increasing the trypsin concentration as the urea concentration was increased. The N-terminal residues of the three main fragments (about 95, 65, 45 kDa) obtained were analyzed. Interestingly, their N-terminal sequences starting from the same ninth aminoacyl residue of the SecA were identical (VFGSRNDRTL). It is clear that the digestion of the SecA by trypsin started from the C-terminal region, which suggests that the C-terminal portion of SecA is more susceptible to tryptic digestion than the N-terminal half containing the high-affinity nucleotide binding site and the precursor binding site. Using differential scanning calorimetry, den Blaauwen *et al.* (*42*) also demonstrated that the C-terminal segment is more flexible than the N-terminal segment. This resistance of the N-terminal region, however, disappears abruptly beyond the urea concentration of 1 M. In this regard, it is of interest that the molten globule form of $\alpha$-LA is partially digested but the native form is resistant to digestion (*43*). A similar band pattern was obtained upon digestion with endoproteinase Arg-C (data not shown) (*44*). Earlier, Price *et al.* (*45*) obtained an N-terminal 65 kDa fragment and a C-terminal 30 kDa fragment by limited proteolysis of SecA at pH 8 and 0°C. In the present case, the C-terminal 30 kDa unit was not observed and the initially observed 65 kDa unit was further degraded into the 45 kDa fragment. This difference may arise from the higher temperature and the presence of urea here.

*Effect of GdnHCl on the ATPase Activity*—The SecA protein has an endogenous ATPase activity in the absence of any translocation components (*46*). Figure 8 shows the change in the activity with increasing GdnHCl concentration. The GdnHCl-induced equilibrium unfolding as monitored by Trp fluorescence (Fig. 3) is also shown for comparison. The ATPase activity increases twofold as compared to the endogenous activity and then is lost completely when the GdnHCl concentration reaches 0.8 M. The GdnHCl concentration which gives the maximum activity is 0.4 M, where the first GdnHCl-induced unfolding transition occurs.

## DISCUSSION

It has been demonstrated that stable water-soluble proteins can penetrate the lipid bilayer under appropriate conditions. The prerequisite for the penetration appears to be that the proteins have to be partially unfolded. The protein penetration may be just superficial or may reach at least the end of the acyl chains of phospholipids in the bilayer. Even for the same protein, the depth of penetration may vary depending on the phospholipid composition of the bilayer.

Perhaps the most extensively studied protein from the viewpoint of bilayer penetration is $\alpha$-LA. It was observed that the hydrophobic binding of $\alpha$-LA to lipid vesicles occurs only below pH 5 (*2*), which correlates well with the pH-dependent reduction of the tertiary structure of $\alpha$-LA into molten globule structure (*47*). Both hydrophobic labeling (*3*) and ESR with spin-labeled phospholipid showed that $\alpha$-LA penetrates deep into PS and PE/PS vesicles (Kim, H., Kim, U.H., Lee, H., and Marsh, D., to be submitted). A prominent feature of a molten globule as epitomized by $\alpha$-LA is an extensive secondary structure without specific tertiary structure, while maintaining an overall compact structure. Hydrogen exchange experiments, among others, established that the A form of $\alpha$-LA has a hydrophobic core (*39*). NMR and disulfide-rearrange-

ment experiments, however, indicated that residues within this core are rather flexible (*48, 49*). Also, fluorescence and hydrophobic labeling studies showed there is increased exposure of hydrophobic residues to the surface when going from the native to molten globule form (*50*). Thus, it is likely that the flexibility of surface residues as well as the residues within the hydrophobic core of the A form of $\alpha$-LA increases the chance of hydrophobic residues contacting transiently exposed acyl chains of the phospholipids of a bilayer, initiating protein penetration of the lipid bilayer.

Another protein for which the interaction with phospholipid vesicles has been extensively studied is apocytochrome *c*. Dumont and Richards (*12*) and Rietveld *et al.* (*13*) demonstrated that apocytochrome *c* traverses the lipid bilayer from experiments with vesicles with entrapped trypsin. Later, this was corroborated by extensive ESR spin-label experiments in Marsh's laboratory (*51*). The apocytochrome *c* in solution as studied by CD appeared to be structureless (*52*). Recent studies with low angle X-ray scattering and resonance energy transfer, however, showed that this apoprotein is more compact than in 4 M GdnHCl (*15*). It is possible, therefore, that SecA also has a hydrophobic core without any secondary structure.

The common denominator for the ready penetration of lipid bilayer by a protein, thus, seems to be the availability of a flexible outer part with exposed hydrophobic residues. It is, therefore, paradoxical that SecA, which is in many respects as compact as the native $\alpha$-LA, judging from ANS binding and hydrogen exchange, can penetrate the bilayer on a par with the molten globule form of $\alpha$-LA and apocytochrome *c*. One should, however, note that native SecA at the physiological temperature exhibits partially unfolded characteristics. When the GdnHCl-induced unfolding was monitored in terms of ANS fluorescence (Fig. 3), a higher fluorescence intensity was seen at zero GdnHCl concentration than in the completely unfolded state. This higher intensity implies more accessible hydrophobic regions of native SecA and a loose tertiary structure. The quenching of Trp fluorescence also implies a somewhat open structure. It was observed that the change in tertiary structure preceded that of secondary structure during thermal as well as GdnHCl-induced equilibrium unfolding. This is a typical feature of the molten-globule intermediate state during unfolding. The low $\Delta G^{\circ}$ value of the first transition of GdnHCl-induced unfolding shows the fragile nature of SecA.

The largely irreversible nature of the thermal unfolding and the formation of aggregates during the GdnHCl-induced unfolding prevented reliable interpretation of the structural feature of SecA at physiological temperature. However, the observations made here suggest that the protein is highly unstable and does have some characteristics of open structure.

Since SecA is a large protein, it is likely that it consists of two or more domains with different stability. This is borne out by the tryptic digestion and ATP hydrolysis experiments here, as well as the results of other investigators (*42, 45*). The initial increase in the ATPase activity of SecA as the GdnHCl concentration is increased (Fig. 8) is of considerable interest in connection with the results obtained by Price *et al.* (*45*), who observed a tremendous increase in the ATPase activity when SecA was fragmented into a 65 kDa N-domain segment and a 30 kDa C-domain segment. They also demonstrated that the C-domain segment penetrates the membrane of inverted vesicles of *E. coli* (*21, 45*). It is possible that the unfolding of the C-domain by a low concentration of GdnHCl may free the N-domain with the ATPase activity center from the inhibitory effect of the C-domain. Also, den Blaauwen *et al.* (*42*) observed that ADP binding to the N-terminal segment stabilized the C-terminal segment strongly, suggesting a mutual influence between the two domains. Further increase in the GdnHCl concentration should unfold the remaining N-terminal fraction as well, thus abolishing the ATPase activity. The picture emerging from our present investigation and the results of others are that SecA has two domains with different stability, the N-terminal domain being more stable than the C-terminal domain. Although the extent of exchangeable hydrogen in SecA is much less than in $\alpha$-LA and lysozyme, it is possible that the percentage of exchangeable hydrogen in the C-domain of SecA is comparable to that of the A form of $\alpha$-LA. The high degree of Trp fluorescence quenching by iodide may be due to the fact that 6 out of 7 tryptophans of SecA are located within the C-terminal one-third segment. It is possible, therefore, that only the C-domain, which is unstable, rather than the whole SecA, can readily penetrate the lipid bilayer.

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