Supramolecular Self-Assembly of *Escherichia coli* Glutamine Synthetase: Effects of Pressure and Adenylylation State on Dodecamer Stacking†

William M. Atkins‡

Department of Medicinal Chemistry, School of Pharmacy, BG-20, University of Washington, Seattle, Washington 98195

Received July 15, 1994; Revised Manuscript Received September 19, 1994⊗

ABSTRACT: *Escherichia coli* glutamine synthetase is a dodecamer of identical subunits, consisting of two face-to-face hexameric rings. The enzymatic activity of GS is regulated by covalent attachment of an adenylyl group to each subunit, at the edge of the ring structure (Tyr-397). In the presence of Zn^{2+}, Cu^{2+}, Co^{2+}, and other divalent metal ions, the free dodecamers self-organize into protein tabules [Miller et al. (1974) *Arch. Biochem. Biophys. 163,* 155−171]. Here, the temperature dependence and pressure dependence of the kinetics of Zn^{2+}-induced self-assembly of GS tubules have been determined for the adenylylated and unadenylylated GS. The adenylylated enzyme exhibits a bimolecular rate constant for Zn^{2+}-induced stacking that is 3-fold lower than for the unadenylylated GS at temperatures ranging from 0 to 25 °C. The enthalpy of activation, $\Delta H^{\ddagger}$, for both adenylylated and unadenylylated GS increases from approximately 10 kcal/mol of dodecamer interface to 20 kcal/mol of dodecamer interface upon addition of 125 mM KCl to the reaction buffer. The $\Delta H^{\ddagger}$ values for adenylylated and unadenylylated GS are nearly identical, at each concentration of KCl, suggesting that entropic factors are responsible for the differences in rate of stacking for these forms of GS. Hydrostatic pressure markedly inhibits the stacking reaction for both adenylylated and unadenylylated GS. The activation volumes, $\Delta V^{\ddagger}_a$, for stacking are increased from approximately 50 mL/mol of dodecamer interface in the absence of KCl to approximately 65 mL/mol of dodecamer interface in the presence of 125 mM KCl. The kinetics of pressure-induced disassembly of GS tubules were also examined. "Pressure-jump" experiments with preformed GS tubules indicate that rates of dissociation at atmospheric pressure, in the presence of 200 μM Zn^{2+} and 125 mM KCl, are 1.1×10^{-5} s^{-1} and 1.9×10^{-5} s^{-1} for the unadenylylated and adenylylated GS, respectively. The $\Delta V^{\ddagger}_d$ values for disassembly increase from approximately −63 mL/mol of dodecamer interface in the absence of KCl to approximately −55 mL/mol of dodecamer interface in the presence of 125 mM KCl. These results suggest that the encounter complex formed between dodecamers, en route to the stacked complex, is not extensively desolvated. Furthermore, the experimentally determined rate constants for the forward and reverse reactions have been used to calculate apparent equilibrium dissociation constants. These values range from 1.3×10^{-10} M^{-1} to 8.0×10^{-9} M^{-1}, and they are increased by adenylylation and by addition of KCl.

Bacterial glutamine synthetases (GS)[1] are dodecameric enzymes formed from two face-to-face hexameric rings of identical subunits (Valentine et al., 1968). The presumed physiological form of the enzyme has two Mg^{2+} ions bound at each of 12 active sites, although Mn^{2+} ions also supported GS activity *in vitro*. Some bacterial GSs are regulated *in vivo* by adenylylation of a specific tyrosine (Tyr-397 in *Escherichia coli* GS). Adenylylation of GS is accompanied by a change in the metal specificity of the reactions that it catalyzes and by changes in the internal reaction thermodynamics of phosphoryl transfer from ATP to glutamate (Ginsburg et al., 1970; Abel & Villafranca, 1991). In fact, the Mg^{2+} form of the enzyme is essentially inactive when it is adenylylated. The structural basis for functional differences between these forms of the enzyme is not known. X-ray crystal structures have been published for the substrate-free (Almassy et al., 1986; Yamashita et al., 1989) and ligand-complexed forms of the enzyme in the unadenylylated state (Liaw et al., 1993a,b; Liaw & Eisenberg, 1994), but no crystal structure is available for the adenylylated enzyme.

Glutamine synthetases from some bacteria form protein tubules in the presence of divalent metal ions which do not support catalytic activity. The physiological role of this supramolecular assembly is not known. In the accompanying paper (Yanchunas et al., 1994) it is suggested that GS tubules may provide directly useful components of biomaterials and nanostructures. This proposal is based on current widespread interest in self-organizing polymers with periodic structure (Whitesides et al., 1992; Bhatia et al., 1992; Jorgensen, 1993) and on the observation that the supramolecular architecture of naturally occurring proteins can be exploited for non-natural functions. For example, ferritin has been used as a biomineralization template to direct the synthesis of nanocrystalline Fe-S (Meldrum et al., 1991). Similar applications may be envisioned for other proteins with unique supramo-

† W.M.A. is supported by the National Science Foundation (MCB-9305202) and by the Whitaker Foundation (9300272).

‡ Telephone (206) 685-0379; FAX (206) 685-3252.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1994.

[1] Abbreviations: GS, glutamine synthetase; $\Delta V^{\ddagger}_a$, activation volume for dodecamer association; $\Delta V^{\ddagger}_d$, activation volume for dissociation of stacked GS; $\Delta H^{\ddagger}$, activation enthalpy for association of GS dodecamers; ΔV_a, the molar volume change for association of free GS dodecamers into stacked GS tubules; PBA, succinimidyl pyrenylbutyrate; PBA-GS, glutamine synthetase labeled with succinimidyl pyrenylbutyrate; Hepes, 4-(2-hydroxyethyl)piperazineethanesulfonic acid.

0006-2960/94/0433-14965$04.50/0

lecular structure, and GS provides an excellent model for design of self-assembling protein arrays. The metal-induced macromolecular association between *E. coli* glutamine synthetase dodecamers is a dramatic example of molecular recognition which yields supramolecular assemblies of ordered structure. An understanding of this process requires characterization of the changes in protein−protein and protein−solvent interactions that accompany formation of the "encounter complex" leading to a stable aggregate (Pontius, 1993).

Hydrostatic pressure has been used extensively to study stochastic and deterministic equilibira of protein−protein and protein−ligand interactions (Gross & Jaenicke, 1994). Examples include simple dimers (Raun & Weber, 1988), antibody−hapten complexes (Coelho-Sampaio & Voss, 1993), and viral particles (Silva & Weber, 1988). In addition to these equilibria, hydrostatic pressure provides a useful probe for understanding the kinetics of association and dissociation of protein assemblies (Davis, 1985; Swezey & Romero, 1985). As with small molecule association, the rates of protein−protein association reactions depend on rates of formation of an initial encounter complex. However, encounter complexes between proteins usually involve more numerous weak intermolecular interactions than those found in simple bimolecular collisions between small molecules. The potential effects of weak, long-range interactions on the kinetics of protein association reactions have been discussed (Pontius, 1993). In order to understand the processes underlying GS tubule formation, the effects of pressure and adenylylation state on the kinetics of assembly and disassembly of these aggregates have been determined. The studies presented here suggest that there are significant differences in the encounter complexes formed by the adenylylated and the unadenylylated enzyme leading to the stacked complex. Furthermore, the results suggest that the stacked GS complex has an extensively solvated interface, as indicated by the molecular model in the acocmpanying paper (Dabrowski et al., 1994).

MATERIALS AND METHODS

Protein Purification. Purified *E. coli* GS was obtained as described in the accompanying paper (Yanchunas et al., 1994). Fully unadenylylated protein was obtained from the *E. coli* strain YMC21E harboring the plasmid pgln35, as previously described (Chen et al., 1982). This *E. coli* strain lacks a chromosomal gene encoding GS, as well as the ATase gene. The GS preparations obtained from this strain are fully unadenylylated ($n = 0$) on the basis of the γ-glutamyl transferase assay (Woolfolk et al., 1966; Stadtman et al., 1986). Adenylylated GS was obtained from the *E. coli* strain YMC21D which lacks the chromosomal gene encoding the PII protein. This protein regulates ATase activity (Chen et al., 1982). GS preparations obtained from this strain were fully adenylylated ($n = 12$) on the basis of the transferase assay.

Kinetics of GS Stacking. The GS stacking reaction was followed by changes in 90° light scattering intensity with a SLM-Aminco 8000C fluorometer (Urbana, IL). The excitation and emission monochromotors were set at 340 nm. For all reactions in the studies presented here, samples were 0.063 μM subunits GS in 50 mM Tris-HCl, pH 7.0, 1 mM $MnCl_2$, and either 0 mM or 125 mM KCl. Temperature was controlled by a circulating water bath connected to the sample compartment of the fluorometer. Pressure studies were performed on the SLM-Aminco high-pressure system HPSC-3K, which is nearly identical to the system described previously (Paladini, 1986; Paladini & Weber, 1981). The only modification of the apparatus included the use of Teflon O-rings instead of lead O-rings. Teflon rings (Action Grinding, Seattle, WA) were used for 5−10 cycles of compression−decompression before replacement was required. For isothermal experiments, the temperature was 10 °C. Light scattering data for the assembly reaction were analyzed by fitting the data to the integrated bimolecular rate equation

$$I_t = (I_0 + I_\infty[GS]_0kt)/([GS]_0kt + 1)$$

where k is the bimolecular rate constant, I_0 is the light scattering intensity at time = 0, I_∞ is the light scattering intensity at time = ∞, and I_t is the intensity at time = t. Previous results have demonstrated that the kinetics of the GS stacking reaction are described by a simple bimolecular rate law at low extent of reaction under the conditions used here (Yanchunas et al., 1994). For pressures above 0.2 kbar, light scattering data obtained in the first 1500 s were used to fit to the integrated rate expression by nonlinear regression. For pressures = 0.2 kbar and below, data obtained in the first 1000 s were used. Data points which obviously fall in "noisy" regions of the kinetic profiles were not included in the nonlinear regression. Because the rate constants reflect initial rates, at low extent of stacking, the recovered $\Delta V^{\ddagger}_a$ values represent the volume changes for formation of a single dodecamer−dodecamer interface.

For dissociation of stacked GS, light scattering data were fit to a sum of exponential decay rates, according to $I_t = \sum a_i e^{-k_i t}$, where k_i is the dissociation rate constant for the ith component, a_i is the fractional contribution of the ith component and I_t is the intensity above background of scattered light at time t after increasing the pressure. Nonlinear regression of the data was performed with Enzfitter by Biosoft.

The activation volumes for association of GS dodecamers, $\Delta V^{\ddagger}_a$, and the activation volumes for dissociation of prestacked complexes, $\Delta V^{\ddagger}_d$, were determined for the pressure dependence of k_1 or k_{-1} from the relation

$$\mathrm{d} \ln k_1/\mathrm{d}p = -\Delta V^{\ddagger}_a/RT \text{ or } (\mathrm{d} \ln k_{-1}/\mathrm{d}p)/2 = -\Delta V^{\ddagger}_d/RT$$

where k_1 and k_{-1} are the forward and reverse rates of stacking, respectively. Thus, the slopes of plots of ln k_1 vs pressure, or of ln k_{-1} vs pressure, yield $\Delta V^{\ddagger}_a/RT$ or $\Delta V^{\ddagger}_d/RT$, respectively (Morild, 1981). The k_{-1} values are the weighted average rate constants for dissociation. In contrast to the forward (stacking) reaction, the data for the entire reaction were fit to the kinetic model by nonlinear regression. In order to account for the fact that both faces of a stacked GS dodecamer become solvent exposed upon complete disassembly of prestacked tubules, the volume change, $\Delta V^{\ddagger}_d$, obtained from ln k_{-1} vs pressure plots is divided by 2. This affords the volume change per mole of dodecamer interface.

Fluorescence Measurements. Purified GS was labeled with succinimidyl pyrenylbutyrate (PBA; Molecular Probes, Eugene, OR) by incubating 50 μM GS subunits with 200 μM PBA in 50 mM borate buffer, pH 9.2, for 4 h at room

Table 1: Activation Enthalpys for Dodecamer Stacking

adenylylation state[a]	[KCl] (mM)	rate constant, k_1[b] (M^{-1} s^{-1})	$\Delta H^{\ddagger}$[c] (kcal/mol)
$n = 0$	0	$(1.9 \pm 0.4) \times 10^4$	11
$n = 12$	0	$(8.6 \pm 0.5) \times 10^3$	10
$n = 0$	125	$(6.0 \pm 0.6) \times 10^3$	21
$n = 12$	125	$(2.3 \pm 0.3) \times 10^3$	20

[a] Adenylylation state is the number of adenylyl groups per dodecamer. [b] $T = 283$ K, average of duplicate determination. [c] From data in Figure 1 using $\Delta H^{\ddagger} = \text{slope}/R$, where $R = 0.082$ L·atm/mol.

temperature. The mixture was dialyzed extensively against 50 mM Tris, pH 7.0, 100 mM KCl, and 1 mM $MnCl_2$ and then chromatographed on a Sephadex G-25 column equilibrated with the same buffer. The labeling ratio obtained under these conditions is 0.9 ± 0.1 PBA/subunit GS, based on the absorbance at 345 nm (PBA $\epsilon = 40$ mM^{-1} cm^{-1}) and on protein determination by the BCA assay (Pierce, Rockford, IL). Fluorescence polarization measurements were performed with an SLM-Aminco 8000C spectrofluorometer and the HPSC-3K system described above. Measurements were made using the T-format, correcting for sample blank polarization and correcting for pressure-induced changes in the birefringence of the quartz windows of the pressure bomb (Paladini & Weber, 1986; Paladini, 1986). Excitation was at 345 nm. Both emission channels contained KV-370 and NaS_2O_4 filters.

RESULTS

Kinetics of GS Stacking. During the characterization of solution conditions which affect the dodecamer stacking reaction of GS (Yanchunas et al., 1994), it was observed that samples of unadenylylated GS ($n = 0$) exhibited kinetics for this reaction that were markedly different from the preparations of adenylylated GS ($n = 12$). For a range of temperatures, the adenylylated enzyme stacks with a bimolecular rate constant that is 2−4-fold lower than the adenylylated GS. In order to understand this difference, the transition-state parameters $\Delta H^{\ddagger}_a$ and $\Delta V^{\ddagger}_a$ for dodecamer stacking have been determined by measuring the rates of association at several temperatures and pressures. Because ionic strength previously has been shown to affect dramatically the kinetics of GS stacking, the effects of temperature and of pressure on rates of stacking were examined in the absence and presence of KCl (125 mM).

The bimolecular rate constants obtained from the light scattering data in the first 10 min after addition of Zn^{2+} are approximately 3-fold larger for the unadenylylated enzyme, at each temperature in the range 0−25 °C. This was observed with several preparations of enzyme in each adenylylation state. Van't Hoff analyses of the kinetic data obtained in the absence and presence of KCl were performed ($r^2 = 0.986$), and the results are summarized in Table 1. The data demonstrate that rates of GS stacking are dramatically reduced in the presence of salt, and the reduction in rate is essentially identical for both the adenylylated and the unadenylylated proteins. The results suggest that adenylylation of GS inhibits the stacking reaction predominantly through effects on entropic terms, or preexponential factors of the Arrhenius relation, rather than enthalpic contributions (Weber, 1992). In contrast, the decrease in the rate of stacking due to addition of KCl is accompanied by a significant increase in $\Delta H^{\ddagger}_a$. Furthermore, it is apparent that the difference in the rate of stacking between adenylylated and unadenylylated GS remains constant at different concentrations of KCl.

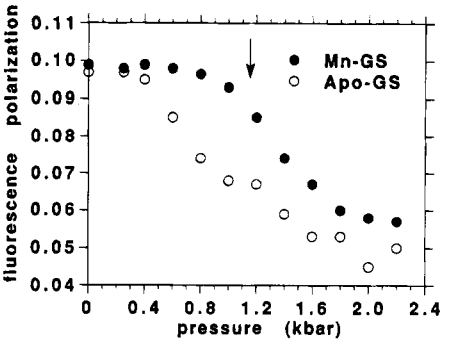

FIGURE 1: Dissociation of GS dodecamers by hydrostatic pressure. The fluorescence polarization of pyrene-labeled GS is monitored with increasing pressure. Symbols: closed circles, Mn-GS; open circles, apo-GS. The arrow indicates the upper limit of pressure used to study the kinetics of GS stacking. The precision of the individual polarization measurements is ±0.002. Conditions were as described in Materials and Methods.

Notably, in the range of temperatures examined in these studies, the extent of stacking after addition of Zn^{2+} did not change with temperature. That is, the equilibrium between free dodecamers and the stacked complex is not altered by changes in temperature under these conditions, and essentially at GS dodecamers become incorporated into GS tubules when 200 μM Zn^{2+} is present. The rates at which this self-assembly occurs, however, are markedly affected by changes in temperature.

Stacking at High Pressure. The kinetics of GS stacking were also found to be extremely sensitive to hydrostatic pressure. One possible explanation for the inhibition of GS stacking at elevated hydrostatic pressure is that the dodecameric GS dissociates, as many other oligomeric proteins do (Morild, 1981; Weber, 1992), and that the resulting subaggregates or subunits do not undergo Zn^{2+}-induced stacking. Therefore, the effect of pressure on dodecameric GS was determined in the absence of Zn^{2+}. This was accomplished using two methods.

GS was labeled with the fluorescent probe succinimidyl pyrenylbutyrate (PBA) at an approximate ratio of 0.9 pyrene/subunit. This probe has a sufficiently long excited-state lifetime (100 ns) to provide rotational relaxation times of the high molecular weight GS dodecamers (624 000). Fluorescence polarization of PBA-labeled GS provides a convenient monitor of pressure-induced dissociation of dodecameric GS. The experiments were done with the Mn^{2+} form of GS and with apo-GS. Two Mn^{2+} ions are located at each of the intersubunit active sites. The apoprotein, therefore, provides a measure of Mn^{2+}-dependent stability of subunit interfaces. After each increment increase in pressure, the sample was allowed to equilibrate for 10 min before the polarization value was recorded. The fluorescence polarization of PBA-labeled Mn^{2+}-GS does not change significantly until approximately 0.9 kbar and then decreases over the range 0.9−2.2 kbar (Figure 1). In contrast, the PBA-labeled apo-GS exhibits a decrease in fluorescence polarization at pressures as low as 0.4 kbar and reaches a lower final polarization value than Mn^{2+}-GS. These results are consistent with pressure-induced dissociation of Mn^{2+}-GS above 0.9 kbar and dissociation of apo-GS at lower

pressures. This is similar to the dissociation of GS in 6 M GdnHCl (Maurizi & Ginsburg, 1982), where ligands bound at the active site stabilize intersubunit interactions. The lower final polarization value obtained with apo-GS compared to Mn^{2+}-GS presumably results from the apo-GS dissociating more completely than the Mn^{2+}-GS. That is, subaggregates may be the products of pressure-induced dissociation when Mn^{2+} stabilizes the subunit interfaces, whereas free monomers or smaller subaggregates are obtained when the Mn^{2+} is absent. In principle, the molecular weight of the particles present at 2 kbar can be calculated from the Perrin equation (Steiner, 1991), which relates the size of a macromolecule to its rotational correlation time. The polarization data are consistent with a model in which the Mn^{2+}-GS dissociates to particles with an average size of trimers or tetramers (MW 189 000), assuming values of 1.194 cP for the viscosity, 100 ns for the excited-state lifetime, 523 500 mL/mol for the hydrated volume of a GS dodecamer, and 0.192 for the limiting polarization, respectively. However, the polarization experiments only provide an average value for the ensemble, and given the complexity of the GS structure it is likely that the solution at 2 kbar is heterogeneous in particle size. Because the extact composition of the particles at elevated pressure is not known, this equilibrium has not been analyzed in detail. The results do indicate, however, that dissociation of Mn^{2+}-GS does not occur at pressures below 0.9 kbar.

The enzymatic activity of GS also was determined at elevated pressures with the γ-glutamyl transferase assay (Woolfolk et al., 1974). The rationale for this experiment is based on the assumption that GS is catalytically inactive if the dodecamer is dissociated. The X-ray crystal structure indicates clearly that the active sites lie between subunits (Almassy et al., 1986; Yamashita et al., 1990). Furthermore, several X-ray crystal structures (Liaw et al., 1993a,b; Liaw & Eisenberg, 1994) and data from numerous site-directed mutations at the subunit interfaces indicate that intraring subunit contacts contribute to the catalytic activity of the wild-type protein (Alibhai & Villafranca, 1994; Liaw et al., 1993a,b). Therefore, any disruption of the oligomeric structure is expected to result in loss of catalytic activity. Samples containing the reaction components of the γ-glutamyl transferase assay and 0.05 μM GS subunits at 10 °C were brought to various hydrostatic pressures, with 1.5 min after initiation of the reaction with ADP. After 30 min at various pressures, the samples were rapidly decompressed, and the reaction was quenched with 0.2 M ferric chloride/acetate solution (pH 1). The product formed during the incubation at each pressure was quantitated colorimetrically (A_{540}). At atmospheric pressure, the rate of product formation was linear vs time during this period (data not shown). Mn^{2+}-GS is completely active at pressures up to 1.0 kbar, indicating that no dissociation of Mn^{2+}-GS has occurred. This result agrees with the fluorescence polarization experiments. At higher pressures, activity does diminish, presumably due to dodecamer dissociation. At 2.0 kbar, the enzymatic activity is 40% of the activity at atmospheric pressure. Together these results provide assurance that, at the pressures which alter the kinetics of the GS stacking reaction, dodecamer dissociation does not occur. The effect of pressure on the kinetics of GS tubule formation does not result from indirect effects on the oligomeric structures of GS. Thus, hydrostatic pressure can be used to determine directly the $\Delta V^{\ddagger}_a$ for formation of the encounter complex leading to a stacked dodecameric aggregate.

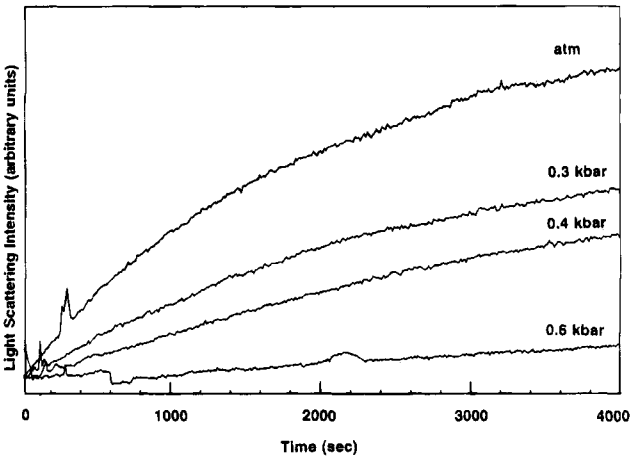

FIGURE 2: Inhibition of GS stacking by hydrostatic pressure. After addition of Zn^{2+}, light scattering intensity was monitored at atmospheric pressure, 0.3 kbar, 0.4 kbar, and 0.6 kbar. Conditions were as described in Materials and Methods.

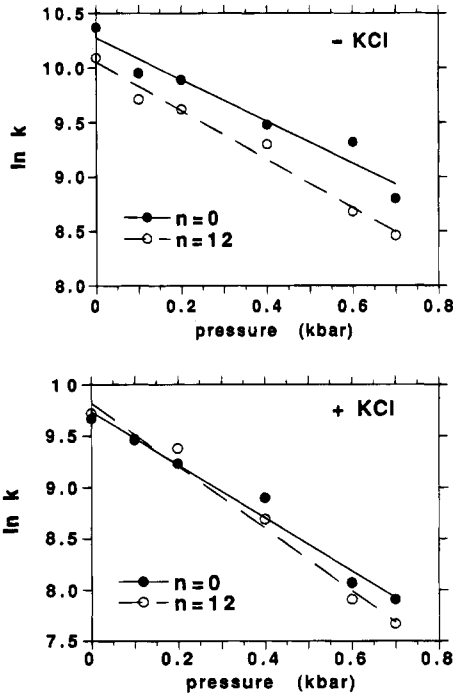

FIGURE 3: Activation volume for GS stacking. The ln k values are plotted vs pressure. The slope yields $\Delta V^{\ddagger}_a/RT$ as reported in Table 2. GS samples were either fully adenylylated, $n = 12$, or fully unadenylylated, $n = 0$. Conditions were as described in Materials and Methods.

The kinetics of GS stacking are remarkably sensitive to pressure. A pressure of only 0.2 kbar is sufficient to decrease the bimolecular rate constant by 3-fold. Progress curves for GS stacking ($n = 0$) at several pressures, in the presence of 125 mM KCl, are shown in Figure 2. Similar progress curves are obtained in the absence of KCl, although the stacking reaction is faster in the absence of KCl at each pressure examined. Plots of the ln k vs pressure are shown in Figure 3, and the values for $\Delta V^{\ddagger}_a$ are summarized in Table 2. Both adenylylated and unadenylylated GS exhibit an increase in $\Delta V^{\ddagger}_a$ upon addition of KCl. As with the temperature dependence, at the pressures examined here, the extent of reaction is not altered. Even at 0.6 kbar, after

Table 2: Activation Volumes for Dodecamer Stacking

adenylylation state[a]	[KCl] (mM)	$\Delta V^{\ddagger}_a$ (mL/mol)
$n = 0$	0	46 ± 6
$n = 12$	0	52 ± 7
$n = 0$	125	62 ± 5
$n = 12$	125	72 ± 6

[a] Adenylylation state is the number of adenylyl groups per dodecamer. $\Delta V^{\ddagger}$ values are the average of duplicate determination, expressed as millilters per mole of dodecamer interface.

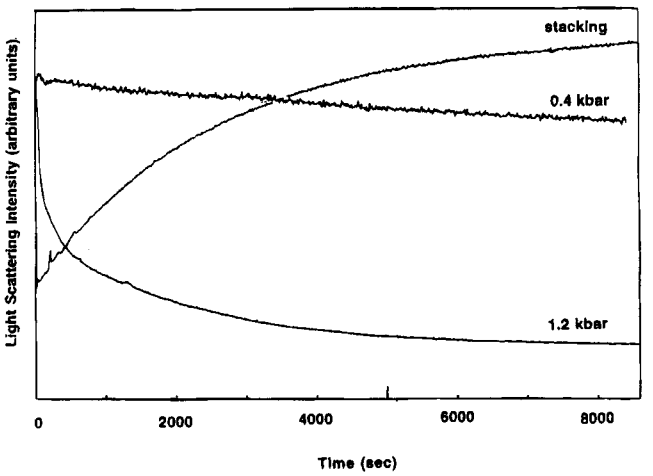

FIGURE 4: "Pressure-jump" dissociation of GS tubules. Samples containing GS were allowed to stack in the presence of Zn^{2+}, at atmospheric pressure. A typical light scattering curve is shown (stacking). The pressure was then increased to the indicated levels, and light scattering intensity was monitored. Conditions were as described in Materials and Methods. The rate constants recovered for several pressures are reported in Table 3.

several hours, the extent of stacking is the same as in samples at atmospheric pressure, within experimental error. At higher pressures, sufficient to decrease the extent of reaction, stacking is sufficiently slow that dissociation of the dodecamer complicates interpretation of the light scattering data.

Kinetics of Dissociation of GS Tubules. In addition to the stacking reaction, the kinetics of the reverse reaction were studied by application of hydrostatic pressure to preformed GS tubules. GS samples were allowed to stack in the presence of 200 μM Zn^{2+} at atmospheric pressure, and the pressure was subsequently increased. After increasing the pressure, the decrease in light scattering intensity was monitored. In effect, these experiments represent pressure-jump conditions because the time required to raise the pressure of the samples is approximately 30 s, compared to the half-times for dissociation of several minutes to several hours. Examples of typical changes in light scattering following addition of Zn^{2+} and followed by subsequent increase in pressure are shown in Figure 4. When preformed stacks of GS dodecamers are kept in the pressure bomb at atmospheric pressure for several hours, no decrease in light scattering is observed.

The dissociation data were fit to several kinetic models, including a simple first-order decay and the sum of exponential decays. Attempts to fit the disassembly data to a first-order decay yielded poor correspondence between the data and the model ($\chi^2 > 25$). Thus, it is clear that the pressure-induced disassembly of GS tubules is complex and cannot be described by a model with homogeneous decay kinetics. A double-exponential decay model more closely

Table 3: Kinetics of Pressure-Induced Disassembly of GS Tubules

system[a]	pressure (kbar)	rate constant[b] (s^{-1})	fractional intensity	χ^2
$n = 0$, 125 mM KCl	1.2	5.9×10^{-3}	0.29	2.11
		4.1×10^{-4}	0.56	
		1.2×10^{-6}	0.16	
		1.9×10^{-3} (av)		
	1.0	7.8×10^{-4}	0.77	2.09
		6.0×10^{-5}	0.22	
		6.1×10^{-8}	0.01	
		6.1×10^{-4} (av)		
	0.8	6.1×10^{-4}	0.63	1.89
		9.0×10^{-6}	0.30	
		5.1×10^{-6}	0.06	
		3.8×10^{-4} (av)		
	0.6	3.8×10^{-5}	0.56	1.99
		1.8×10^{-4}	0.30	
		9.0×10^{-8}	0.14	
		7.5×10^{-5} (av)		
	0.4	1.6×10^{-4}	0.31	2.03
		1.7×10^{-6}	0.19	
		2.2×10^{-6}	0.50	
		5.0×10^{-5} (av)		
	0.2	7.1×10^{-8}	0.66	1.68
		7.0×10^{-7}	0.33	
		6.8×10^{-6}	0.01	
		3.5×10^{-6} (av)		
$n = 12$, 125 mM KCl	1.2	2.1×10^{-4}	0.56	1.91
		2.1×10^{-3}	0.33	
		5.0×10^{-6}	0.01	
		8.1×10^{-4} (av)		
	1.0	7.8×10^{-4}	0.82	1.50
		4.3×10^{-5}	0.09	
		2.1×10^{-7}	0.09	
		6.3×10^{-4} (av)		
	0.8	4.9×10^{-4}	0.68	2.02
		3.2×10^{-6}	0.20	
		7.1×10^{-7}	0.12	
		3.7×10^{-4} (av)		
	0.6	4.0×10^{-5}	0.71	1.98
		6.8×10^{-5}	0.20	
		2.1×10^{-8}	0.09	
		9.7×10^{-5} (av)		
	0.4	9.9×10^{-6}	0.44	2.41
		6.8×10^{-6}	0.40	
		5.1×10^{-4}	0.12	
		4.3×10^{-5} (av)		
	0.2	6.1×10^{-7}	0.49	2.01
		5.8×10^{-6}	0.32	
		6.1×10^{-8}	0.19	
		2.2×10^{-6} (av)		

[a] In Table 3, only data obtained in the presence of 125 mM KCl are included. [b] Weighted average is based on fractional intensities of the individual rate constants.

approximated the data, but three decay rates were required to obtain reasonable fits at each of the pressures used ($\chi^2 < 2$). The recovered rate constants for decay of adenylylated and unadenylylated GS tubules in the presence of KCl and their associated fractional intensities are summarized in Table 3. A similar analysis was performed for the adenylylated and the unadenylylated GS in the absence of KCl (not shown). At each pressure, and for each of the GS preparations at both KCl concentrations, there is one rate constant that dominates the observed rate of disassembly. The heterogeneity of decay kinetics is not unexpected, given the complexity of the stacked GS tubules (Erijman & Weber, 1990; Weber, 1992). However, the source of this hetero-

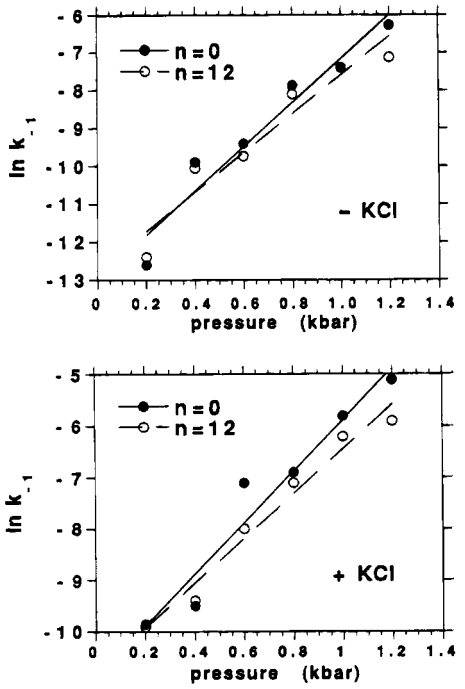

FIGURE 5: Activation volumes for dissociation of stacked GS. The weighted average ln k_{-1} values are plotted vs pressure. The slope yields $2\Delta V^{\ddagger}_a/RT$ as reported in Table 4. The adenylylation state is either fully adenylylated, $n = 12$, or fully unadenylylated, $n = 0$.

Table 4: Activation Volumes for Pressure Dissociation of Stacked GS

adenylylation state	[KCl] (mM)	$\Delta V^{\ddagger}_d$[a] (mL/mol)	k_{-1}, atm[b] (s^{-1})
$n = 0$	0	−67	2.3×10^{-6}
$n = 12$	0	−60	2.9×10^{-6}
$n = 0$	125	−58	1.8×10^{-5}
$n = 12$	125	−51	1.9×10^{-5}

[a] Calculated from d ln k_{-1}/dp at 283 °C; see Figure 6. The $\Delta V^{\ddagger}_d/RT$ obtained from the slope of ln k_{-1} vs pressure has been divided by 2, so that the units are milliliters per mole of dodecamer interface. [b] The dissociation rate constant, k_{-1}, at atmospheric pressure is from the *Y*-intercept of linear regression of data in Figure 5.

geneity is not gleaned from the experiments described here.

Although the individual rate constants obtained from the multiexponential decay analysis (Table 3) cannot be assigned to specific molecular processes, it is reasonable to assume that each rate reflects dissociation of a population of stacked GS tubules with slightly different characteristics. It is useful to consider an average relaxation rate for the dissociation in order to estimate the volume change associated with dissociation of an "average" dodecamer−dodecamer complex. The weighted average rate constants were determined from the individual decay components, and these average values were plotted vs pressure for each GS preparation (Figure 5). The slopes of these plots yield $\Delta V^{\ddagger}_d$ for dissociation of stacked GS dodecamers, and the *Y*-intercepts provide values of k_{-1} at atmospheric pressure for each adenylylation state and salt concentration (Morild, 1981). These values are summarized in Table 4. In principle, the value of k_{-1} at atmospheric pressure (obtained from the pressure dependence of the dissociation) and the directly measured second-order rate constant for assembly measured at the same temperature (10 °C) can be used to calculate the equilibrium dissociation constant (Scheme 1), assuming a two-state model. This estimate assumes that the rate of dissociation of a dodecamer−dodecamer interface is independent of the length of the starting structure.

Table 5: Kinetically Determined Equilibrium Parameters for GS Dissociation

system[a]	$\Delta V^{\ddagger}_a$[b] (mL/mol)	K_d[c] (M)
$n = 0$, 0 KCl	113	1.2×10^{-10}
$n = 12$, 0 KCl	112	3.3×10^{-10}
$n = 0$, 125 mM KCl	120	3.0×10^{-9}
$n = 12$, 125 mM KCl	123	8.0×10^{-9}

[a] n is the adenylylation state or number of adenylyl groups per dodecamer. [b] From $\Delta V^{\ddagger}_a - \Delta V^{\ddagger}_d$ values in Tables 1 and 4. [c] Calculated as k_{-1}/k_1 at 283 °C.

Scheme 1

$$n[GS] \rightleftharpoons [GS]_n \qquad K_d = [GS]^n/[GS]_n = k_{-1}/k_1$$

The kinetically determined equilibrium constants are included in Table 5. In addition, the molar volume change for association of the free dodecamers to afford the stacked complex can be calculated from $\Delta V^{\ddagger}_a - \Delta V^{\ddagger}_d = \Delta V_a$, assuming microscopic reversibility. These values are also summarized in Table 5. It is striking that the ΔV_a values are unaffected by adenylylation and KCl concentration, in marked contrast to the kinetic parameters.

DISCUSSION

The temperature dependence and pressure dependence of the kinetics of the GS stacking reaction have been determined from the adenylylated and the unadenylylated proteins. The kinetics of the pressure-induced dissociation of stacked GS tubules also have been analyzed for both forms of GS. These analyses assume that the heat capacity and compressibility of GS do not change significantly over the range of temperature and pressure used in these studies. Given the narrow range of temperature and pressure used here, this is a reasonable assumption (Morild, 1981; Gross & Jaenicke, 1994). Together, the results provide an initial model for the factors contributing to observed rates of these processes under different conditions and for GS preparations of varying adenylylation state. In addition, the kinetic analyses of both reactions provide an estimate of the equilibrium constant, K_d, for dissociation of the stacked complex. Equilibrium parameters for this self-assembly process have not been obtainable from other methods, presumably because the stacked complex is extremely stable. The stacked GS complex is not in dynamic equilibrium with free dodecamers in the presence of Zn^{2+} concentrations sufficient to initiate stacking. The kinetic analysis, however, provides a measure of the affinity of dodecamers within the stacked complex. Moreover, the kinetic parameters $\Delta V^{\ddagger}_a$ and $\Delta H^{\ddagger}_a$ provide information about the extent of desolvation and noncovalent interaction between dodecamers in the initially formed encounter complex, with either proceeds to form a stable stacked complex or dissociates to free dodecamers.

The first observation of interest is that the adenylylated GS forms stacked complexes with an apparent second-order rate constant that is 2−4-fold lower than that of the unadenylylated enzyme under all the conditions studied. On the basis of the location of Tyr-397, which is the residue that is specifically adenylylated, this result is unexpected. Bacterial GSs are dodecameric assemblies consisting of

identical subunits arranged as two face-to-face hexameric rings. Tyr-397 of each subunit lies on the outer perimeter of the double-layered hexagonal ring. Although an X-ray crystal structure is not available for the adenylylated GS, molecular dynamics calculations and fluorescence studies (Atkins et al., 1991) and NMR experiments (Villafranca et al., 1978) suggest that the adenylyl group attached to one subunit makes direct contact with the Trp-57 loop in the adjacent subunit within the same hexameric ring. The adenylyl group is not, apparently, buried in a close-packed protein interior (Atkins et al., 1993; Villafranca et al., 1978). Thus, the available data are consistent with the adenylyl group residing near the perimeter of the ring structure, albeit unlikely that it extends directly away from the protein surface, into bulk solution. It is not apparent immediately that adenylyl groups would affect the rate of formation of the stacked complex which requires face-to-face interactions between dodecamers; no specific interaction between edges of the dodecameric rings would be expected to contribute to the stability of the stacked GS complexes. Therefore, the data suggest that subtle conformational changes accompanying adenylylation are transmitted to the "stacking face" of GS. Alternatively, the negatively charged phosphoryl groups on the adenylyl moieties at the edge of the ring structure may provide an electrostatic field which affects rates of GS stacking. Notably, the symmetry of the ring structure would lend itself to cooperative interactions, where either a conformational change or an electrostatic interaction originating in each individual subunit may be amplified and result in a large change in the character of the stacking face. For reasons discussed below, electrostatic repulsion between adenylylated GS dodecamers is not likely.

The temperature dependence of the rate of stacking is nearly identical for the adenylylated and unadenylylated forms of GS. The Arrhenius activation energy calculated from the Van't Hoff analysis in the presence of 125 mM KCl, 21 kcal/mol, is nearly identical to the value obtained by Maurizi et al. (1986), and the $\Delta H^{\ddagger}_a$ value is reasonable for formation of an encounter complex between two large aggregates. There is an apparent decrease in $\Delta H^{\ddagger}_a$ when KCl is excluded from the reaction, and on the basis of the Van't Hoff analysis, this is accompanied by an increase in the $\Delta S^{\ddagger}_a$. It is strikingly that the difference in rates of stacking between the unadenylylated and the adenylylated GSs is attributed to entropic factors. This may arise from differences in solvation or local dynamics of surface residues for the two forms of the protein. However, without a precise determination of $\Delta S^{\ddagger}_a$ based on calorimetric measurements, it is difficult to interpret the temperature dependence of the reaction under varying conditions.

One striking feature of these studies is the relative sensitivity of the stacking reaction to pressure. A pressure of 0.4 kbar, which typically does not cause dissociation of large oligomeric proteins (Weber, 1992), is sufficient to slow the rate of GS stacking by 3-fold. The kinetics of assembly of other large protein aggregates, including actin and myosin (Swezey & Romero, 1985; Davis, 1985), are dramatically slowed by pressures comparable to those which exhibit GS stacking. Collectively, the results presented here and the data obtained with these other complex aggregates support the recent proposal of Erijman and Weber (1991) that oligomers become increasingly sensitive to pressure as the number of subunits increase, due to increased rates of dissociation of the subunits. The observation that stacked tubes of GS readily dissociate at pressures well below those required to dissociate individual dodecamers supports their proposal, although the thermodynamic effects for dissociation of GS dodecamers into subaggregates have not been completely quantitated.

The complexity of the self-assembly reaction leading to formation of GS tubules, and of the disassembly reaction, limits the interpretation of the kinetic data, and the numerical values for activation volumes must be considered to be estimates only. Thus, these experiments are not likely to yield physically interpretable values of $\Delta V^{\ddagger}_a$ and of $\Delta V^{\ddagger}_d$ which can, in turn, lead to predictions concerning the number of salt bridges or hydrogen bonds involved in formation of an encounter complex. However, the differences in these values which result from changes in salt concentration and adenylylation state provide useful insights into this reaction.

Moreover, the magnitude of $\Delta V^{\ddagger}_a$ is informative. The average values of $\Delta V^{\ddagger}_a$ for GS in the absence and the presence of KCl are 49 mL/mol of dodecamer face and 67 mL/mol of dodecamer face, respectively. The experimentally measured values of $\Delta V^{\ddagger}_a$ are significantly lower than values of ΔV_a for association of large, macromolecular complexes (Bonafe et al., 1994; Pedrosa & Ferreira, 1994). Also, the experimentally measured values of $\Delta V^{\ddagger}_a$ for GS stacking are much lower than the calculated ΔV_a for the same reaction, 113−123 mL/mol of dodecamer interface. The major contribution to the increase in the system volume when oligomeric proteins associate is the release of ordered solvent from the exposed protein surfaces when the aggregate forms. Therefore, the value of $\Delta V^{\ddagger}_a$ for GS stacking suggests that the encounter complex formed between two free GS dodecamers is not extensively dehydrated. That is, the encounter complex may represent an "early transition state" for assembly of the complex, where close-packed protein interfaces have not formed and solvent has not been completely displaced displaced from the stacking surface.

The second noteworthy aspect of the pressure studies is that the $\Delta V^{\ddagger}_a$ values increase with ionic strength. This indicates that either the molar volume of the free dodecamer is smaller or the molar volume of the encounter complex is larger in the presence of KCl than in its absence. Unfortunately, these possibilities cannot be distinguished from these experiments. It is interesting to speculate, however, that in the presence of KCl the encounter complex is more completely desolvated, resulting in a "later" transition state than in the absence of KCl. That is, more solvent needs to be released from the free dodecamer surface before the encounter complex is committed to formation of the stacked GS aggregate. If solvent includes K^+ and Cl^- ions, then it is possible that these ions shield favorable electrostatic/polar interactions between dodecamers, and an encounter complex is only reached after these ions are released with solvent. In contrast, a low dielectric solvent trapped in the encounter complex would allow complementary charges to form stable salt bridges and facilitate stacking.

The dissociation of stacked GS tubules also may be analyzed by the pressure dependence of the reaction. Notably, increasing the temperature to 50 °C or raising the salt concentration to 0.5 M at atmospheric pressure does not lead to disassembly of stacked GS tubules, so pressure provides a valuable probe of the dissociation process. The

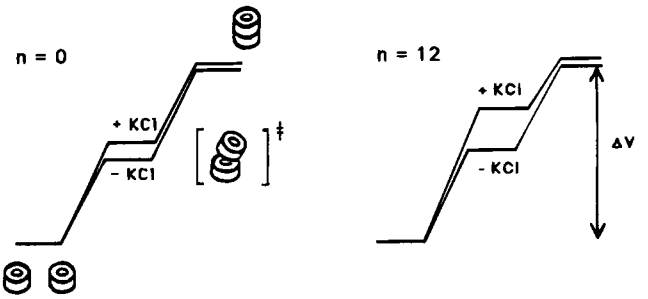

FIGURE 6: Volume changes during formation of a stacked GS complex. Two GS dodecamers are represented as rings which form a face-to-face complex. The encounter complex is represented by the bracketed structure. The extent of reaction is not affected by addition of 125 mM KCl. However, the encounter complex is formed further along the reaction coordinate in the presence of KCl, for both the unadenylylated and adenylylated enzyme.

kinetics of pressure-induced dissociation of GS tubules are complex. Three decay rates are required to fit the data to an exponential decay process. It is possible that disassembly of prestacked GS tubules occurs with sequential dissociation of dodecamers from free tubule ends. It also is possible that pressure causes internal breaks within the stacked complexes, thus generating an increase in the number of free ends with increasing time at elevated pressures. Although the exact mechanism of dissociation is not known, the disassembly data do indicate that there is an increase in $\Delta V^{\ddagger}_{d}$ for dissociation (less negative) when KCl is present and that salt destabilizes the GS tubules. In contrast to the effect of adenylylation on the rate constants for Zn^{2+}-induced association of GS, adenylylation increases slightly the calculated values of k_{-1}.

Assuming microscopic reversibility, the directly measured values of k_1 and the calculated values of k_{-1}, obtained from extrapolation of the pressure dissociation curves, can be used to estimate the equilibrium constant for dissociation of the stacked complex, $k_{-1}/k_1 = K_d$. Whereas KCl clearly destabilizes the stacked complex, adenylylation has a negligible effect. The adenylylated enzyme forms a stacked complex that is 2−3-fold less stable, based on the K_d values, and the kinetic data indicate that this is attributed to a decrease in the association rate of free dodecamers. The calculated values for K_d of 10^{-9}−10^{-10} M indicate a very tight binding between GS proteins in the presence of Zn^{2+}. However, without introduction of a denaturant, pressure experiments provide the activation volumes for both assembly and disassembly of stacked GS dodecamers, as well as the equilibrium dissociation constant for the reaction. The $\Delta V^{\ddagger}_{d}$ and $\Delta V^{\ddagger}_{a}$ values represent the volume change required for formation of the encounter complex between two free dodecamers and from preformed GS tubules, respectively. This is summarized in Figure 6. This scheme indicates that the encounter complex between free dodecamers is attained further along the reaction coordinate when KCl is present, and this effect is more pronounced with the adenylylated enzyme. This result indicates that electrostatic repulsion between adenylyl groups, as suggested above, is not the cause for a decrease in k_1 for the adenylylated enzyme. If electrostatic repulsion occurred, then addition of KCl would be expected to reverse the effect of adenylylation. Instead, the effects of KCl and adenylylation are additive.

It is important to note that changes in the solvation of the Zn^{2+} ions which drive dodecamer stacking may contribute to $\Delta V^{\ddagger}_{a}$, $\Delta V^{\ddagger}_{d}$, and ΔV_a. It is reasonable to expect that the free GS dodecamers plus fully hydrated Zn^{2+} ion occupy a smaller volume than the protein-ligated Zn^{2+} plus six unligated water molecules. Therefore, elevated pressure may favor dissociation of the Zn^{2+} ions from GS and therefore lead to dodecamer dissociation. If the altered solvation of Zn^{2+} ions contributes to the pressure-dependent dissociation of GS dodecamers, this would be additive with the volume change for dissociation of the protein aggregate, and the resulting volume change would be larger than for a simple protein dissociation. The small values of the volume changes obtained experimentally suggest that the changes in metal ion hydration are not the major contribution to the pressure dependence of GS stacking.

Finally, the ΔV_a value obtained from these experiments may be compared to an expected value based on the molecular model for the stacked GS complex in the accompanying paper (Dabrowski et al., 1994). As pointed out above, the experimentally measured value is quite low compared to other protein aggregates of comparable size. Given the surface area of a single dodecamer face that must be buried upon formation of a close-packed dodecamer−dodecamer complex [>6000 Å^2; see Dabrowski et al. (1994)], and based on ΔV_a values obtained with other protein aggregates, the ΔV_a for formation of the final GS complex would be expected to be at least 250−300 mL/mol of dodecamer interface, even if only a few salt bridges are formed when GS stacks (Weber, 1992; Newman et al., 1973). The much lower experimental value suggests one obvious possibility: formation of the stacked dodecamer does not lead to complete desolvation of the stacking face of GS, and solvated pockets are retained at the dodecamer interface. The molecular model of stacked GS dodecamers clearly indicates that this is a likely possibility. The stacking surface of the GS dodecamer is remarkably nonpolar, with Asp, Glu, His, Ser, Cys, Arg, Lys, Gln, or Asn comprising 65% of the solvent-accessible surface. The only hydrophobic residues at the stacking surface lie on one side of the N-terminal amphipathic helix (residues 1−15). Even if the dodecamers within a stacked complex were closely packed, the interface is very polar. Thus, solvent pockets would not be expected to be extremely thermodynamically unfavorable. Furthermore, it is essential to note that the driving force for formation of the stacked complex likely is provided by an intermolecular metal binding site. The optimal geometry of metal ligands provided by the amino acid side chains at the dodecamer interface occurs when the two dodecamers do not make extensive van der Waals contact. When maximum van der Waals contact is established, by "forcing" the dodecamers together, the metal binding site is destroyed. That is, the metal binding site is formed at the expense of an incompletely desolvated, loosely packed protein interface. The molecular model of GS indicates that formation of a stacked complex occurs with a change in solvent-accessible surface area of only ~3000 Å^2 for each dodecamer face, which corresponds to only 15%−20% of the total surface area at the dodecamer interface (Dabrowski et al., 1994). On the basis of the ΔV_a values obtained for other protein aggregates, this change in solvent-accessible surface area is expected to yield a ΔV_a value of ~140 mL/mol of dodecamer interface (Weber, 1992), in close agreement with the experimentally obtained value. Moreover, reexamination of the low-resolution electron density map obtained from

electron diffraction (Frey et al., 1975) clearly demonstrates large interstitial spaces between dodecamers in a stacked complex. Taken together, these observations and the results presented here provide experimental support for the molecular model which is proposed in the accompanying paper: there are solvent pockets between dodecamers in a stacked GS complex.

ACKNOWLEDGMENT

The authors acknowledge Dr. Claudia Jochheim for helpful discussions and for help in preparation of the figures.

REFERENCES

Abel, L. M., & Villafranca, J. J. (1991) *Biochemistry 30*, 1413−1418.
Alibhai, M., & Villafranca, J. J. (1994) *Biochemistry 33*, 682−686.
Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N. H., & Eisenberg, D. (1986) *Nature 323*, 304−309.
Atkins, W. M., Cader, B. M., Hemmingsen, J., & Villafranca, J. J. (1993) *Protein Sci. 2*, 800−813.
Bhatia, S. K., Hickman, J. J., & Ligler, F. S. (1992) *J. Am. Chem. Soc. 114*, 4432−4433.
Bonafe, C. F. S., Araijo, J. R., & Silva, J. (1994) *Biochemistry 33*, 2651−2660.
Chen, Y. M., Backman, K., & Magasanik, B. (1982) *J. Bacteriol. 150*, 214−220.
Coelho-Sampaio, T., & Voss, E. W., Jr. (1993) *Biochemistry 32*, 10929−10935.
Coelho-Sampaio, T., Ferreira, S. T., Benaim, G., & Vieyra, A. (1991) *J. Biol. Chem. 266*, 22266−22272.
Colombo, G., & Villafranca, J. J. (1986) *J. Biol. Chem. 261*, 10587−10591.
Dabrowski, M. J., Yanchunas, J., Jr., Villafranca, B. C., Dietze, E. C., Schurke, P., & Atkins, W. M. (1994) *Biochemistry 33*, 14957−14964.
Davis, J. S. (1985) *Biochemistry 24*, 5263−5269.
Erijman, L., & Weber, G. (1991) *Biochemistry 30*, 1595−1599.
Frey, T. G., Eisenberg, D., & Eiserling, F. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3402−3406.
Frink, R. J., Eisenberg, D., & Glitz, D. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5778−5782.
Ginsburg, A., Yeh, J., Hennig, S. B., & Denton, M. D. (1970) *Biochemistry 9*, 9633−9646.
Gleiter, H. (1992) *Adv. Mater. 4*, 474−480.
Gross, M., & Jaenicke, R. (1994) *Eur. J. Biochem. 221*, 617−630.
Jorgensen, W. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1635−1636.
Karlin, K. D. (1993) *Science 261*, 701−708.
Liaw, S.-H., & Eisenberg, D. (1994) *Biochemistry 33*, 675−681.
Liaw, S.-H., Pan, C., & Eisenberg, D. (1993a) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4996−5000.
Liaw, S.-H., Villafranca, J. J., & Eisenberg, D. (1993b) *Biochemistry 32*, 7999−8003.
Maurizi, M. R., & Ginsburg, A. (1982) *J. Biol. Chem. 257*, 4271−4278.
Maurizi, M. R., Kasprzyk, P. G., & Ginsburg, A. (1986) *Biochemistry 25*, 141−151.
McGrath, K. P., Fournier, M. J., Mason, T. L., & Tirrel (1992) *J. Am. Chem. Soc. 114*, 727−733.
Meldrum, F. C., Wade, V. J., Nimmo, D. L., Heywood, B. R., & Mann, S. (1991) *Nature 349*, 684−685.
Merkler, D. J., Srikumar, K., Marchese-Ragone, S. P., & Wedler, F. C. (1988) *Biochim. Biophys. Acta 952*, 101−114.
Miller, R. E., Shelton, E., & Stadtman, E. R. (1974) *Arch. Biochem. Biophys. 163*, 155−171.
Morild, E. (1981) *Adv. Protein Chem. 34*, 92−166.
Newman, R. C., Kauzman, W., & Zipp, J. (1973) *J. Phys. Chem. 77*, 2687−2691.
Paladini, A. A., Jr. (1986) *Methods Enzymol. 130*, 493−518.
Paladini, A. A., Jr., & Weber, G. (1981) *Biochemistry 20*, 2587−2593.
Pontius, B. W. (1993) *Trends Biochem. Sci. 18*, 181−186.
Raun, K., & Weber, G. (1988) *Biochemistry 27*, 3295−3301.
Rowe, W. B., Ronzio, R. A., & Meister, A. (1969) *Biochemistry 8*, 2674−2679.
Silva, J., & Weber, G. (1988) *J. Mol. Biol. 199*, 149−161.
Stadtman, E. R., Smyrniotis, P. Z., Davis, J. N., & Wittenberg, M. E. (1986) *Anal. Biochem. 82*, 275−281.
Steiner, R. F. (1991) in *Topics in Fluorescence Spectroscopy* (Lakowicz, J., Ed.) Vol. 2, Plenum Press, New York.
Swezey, R. R., & Romero, G. N. (1985) *Biochemistry 24*, 852−860.
Valentine, R. C., Shapiro, B. M., & Stadtman, E. R. (1968) *Biochemistry 7*, 2143−2152.
Villafranca, J. J., Rhee, S. G., & Chock, P. B. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1255−1259.
Weber, G. (1992) in *Protein Interactions*, Chapman & Hall, New York, NY.
Whitesides, G. M., Mathias, J. P., & Seto, C. T. (1992) *Science 254*, 1312−1319.
Woolfolk, C. A., Shapiro, B. M., & Stadtman, E. R. (1966) *Arch. Biochem. Biophys. 116*, 171−192.
Yamashita, M. M., Almassy, R. J., Janson, L. A., Lasek, D., & Eisenberg, D. (1989) *J. Biol. Chem. 264*, 17681−17689.
Yanchunas, J., Jr., Dabrowski, M. J., Schurke, P., & Atkins, W. M. (1994) *Biochemistry 33*, 14949−14956.