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Cavity Defects in the Procapsid of Bacteriophage P22 and the Mechanism of Capsid Maturation

Pedro Caetano de Sousa Jr[1], Roman Tuma[2], Peter E. Prevelige Jr[2] Jerson L. Silva[1] and Debora Foguel[1]*

[1]*Programa de Biologia Estrutural, Departamento de Bioquímica Médica - ICB Centro Nacional de Ressonância Magnética Nuclear de Macromoléculas, Universidade Federal do Rio de Janeiro 21941-590, Rio de Janeiro, RJ Brazil*

[2]*Department of Microbiology University of Alabama at Birmingham, Birmingham, AL 35294, USA*

**Corresponding author*

Bacteriophage P22 belongs to a family of double-stranded DNA viruses that share common morphogenetic features like DNA packaging into a procapsid precursor and maturation. Maturation involves cooperative expansion of the procapsid shell with concomitant lattice stabilization. The expansion is thought to be mediated by movement of two coat protein domains around a hinge. The metastable conformation of subunit within the procapsid lattice is considered to constitute a late folding intermediate. In order to understand the mechanism of expansion it is necessary to characterize the interactions stabilizing procapsid and mature capsid lattices, respectively. We employ pressure dissociation to compare subunit packing within the procapsid and expanded lattice. Procapsid shells contain larger cavities than the expanded shells, presumably due to polypeptide packing defects. These defects contribute to the metastable nature of the procapsid lattice and are cured during expansion. Improved packing contributes to the increased stability of the expanded shell. Comparison of two temperature-sensitive folding (tsf) mutants of coat protein (T294I and W48Q) with wild-type coat revealed that both mutations markedly destabilized the procapsid shell and yet had little effect on relative stability of the monomeric subunit. Thus, the regions affected by these packing defects constitute subunit interfaces of the procapsid shell. The larger activation volume of pressure dissociation observed for both T294I and W48Q indicates that the decreased stability of these particles is due to increase of cavity defects. These defects in the procapsid lattice are cured upon expansion suggesting that the intersubunit contacts affected by tsf mutations are absent or rearranged in the mature shell. The energetics of the *in vitro* expansion reaction also suggests that entropic stabilization contributes to the large free energy barrier for expansion.

Keywords: bacteriophage P22; volume of activation; virus maturation; protein folding; cavity

Introduction

The capsids of many viruses undergo profound morphological transformations during their life-cycle. These structural changes are important for virus infectivity, cell entry and disassembly. For example, proteolysis of retrovirus Gag protein results in transformation of the provirion with spherical core into the infectious capsid with a conical core (reviewed by Weldon & Hunter, 1997). In the case of rotaviruses, disassembly of the outer core during cellular entry activates the inner core of the polymerase complex (reviewed by Prasad & Estes, 1997). For polio virus, receptor binding triggers a conformational change resulting in a 135 S particle that displays membrane-binding properties and can release the RNA (Fricks & Hogle, 1990). The morphogenetic pathway of many of the double-stranded (ds) DNA viruses, including herpesviruses, adenoviruses and dsDNA bacteriophages, includes the formation of a procapsid,

Abbreviations used: wt, wild-type; ts, temperature-sensitive, tsf, temperature-sensitive folding; ds, double-stranded; bis-ANS, bis-(8-anilino naphthalene-1-sulfonate).

E-mail address of the corresponding author: foguel@bioqmed.ufrj.br

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which is the precursor for DNA packaging. Concomitant with DNA packaging the procapsid undergoes an expansion that greatly stabilizes the resulting mature virion lattice. Structural information describing the procapsid and mature capsid states is available for herpes simplex virus (HSV-1) and bacteriophages P22, lambda, and HK97. However, relatively little is known about the mechanism of the transformation itself. In all cases, capsid expansion reflects changes in the packing of the subunits within the lattice and therefore may involve volume changes (Prasad *et al*., 1993; Galisteo & King, 1993; Dokland & Murialdo, 1993; Conway *et al*., 1995; Tuma *et al*., 1998).

Bacteriophage P22 is a dsDNA virus of *Salmonella typhimurium* whose morphogenetic pathway encompasses a procapsid to capsid maturation and whose assembly serves as a prototype for assembly of other dsDNA viruses. The procapsid is formed by copolymerization of 415-420 molecules of coat protein (gp5, 47 kDa) with approximately 300 molecules of scaffolding protein (gp8, 33 kDa) and 12 copies of the portal protein (gp1, 80 kDa) that form a portal protein complex located at one vertex (King & Casjens, 1974; Prevelige *et al*., 1988). The phage DNA is packaged into the procapsid through the portal complex (Bazinet *et al*., 1988). During DNA packaging, the scaffolding protein exits and the procapsid shell matures into the larger but thinner shell of the virion (Prasad *et al*., 1993; Figure 1). Empty procapsid shells of coat protein can be prepared from procapsids by extraction of the scaffolding protein with 0.5 M GuHCl (Fuller & King, 1981). These shells are composed primarily of coat protein with minor contribution of the portal dodecamer and are structurally identical with the procapsids containing scaffolding protein (Thuman-Commike *et al*., 1998). Additionally, an assembly active monomeric coat protein can be prepared (Prevelige *et al*., 1988; Prevelige & King, 1993), thus permitting comparison of the structural and thermodynamic properties of both assembled and monomeric subunits (Galisteo & King, 1993). Monomeric coat protein undergoes denaturation at relatively low pressures (<1.5 kbar; 1 bar $= 10^5$ Pa) with a large volume change (Prevelige *et al*., 1994). On the other hand, subunits within the procapsid shell are pressure stable at room temperature and can be dissociated only at low temperature (Prevelige *et al*., 1994). Thus, the subunit within the procapsid lattice is more compact than the coat protein monomer, which resembles an expanded folding intermediate (molten globule; Vidugiris *et al*., 1995). The temperature-dependence of stability suggests that the procapsid lattice is entropically stabilized.

Previous studies have shown that the procapsid to capsid transformation results in increased stability of the coat protein subunit (Galisteo & King, 1993). Additionally, expansion leads to increased protection of the polypeptide chain against hydrogen-isotope exchange similar to the protection due to the formation of a folding core (Tuma *et al*., 1998). It was proposed that the rearrangement of

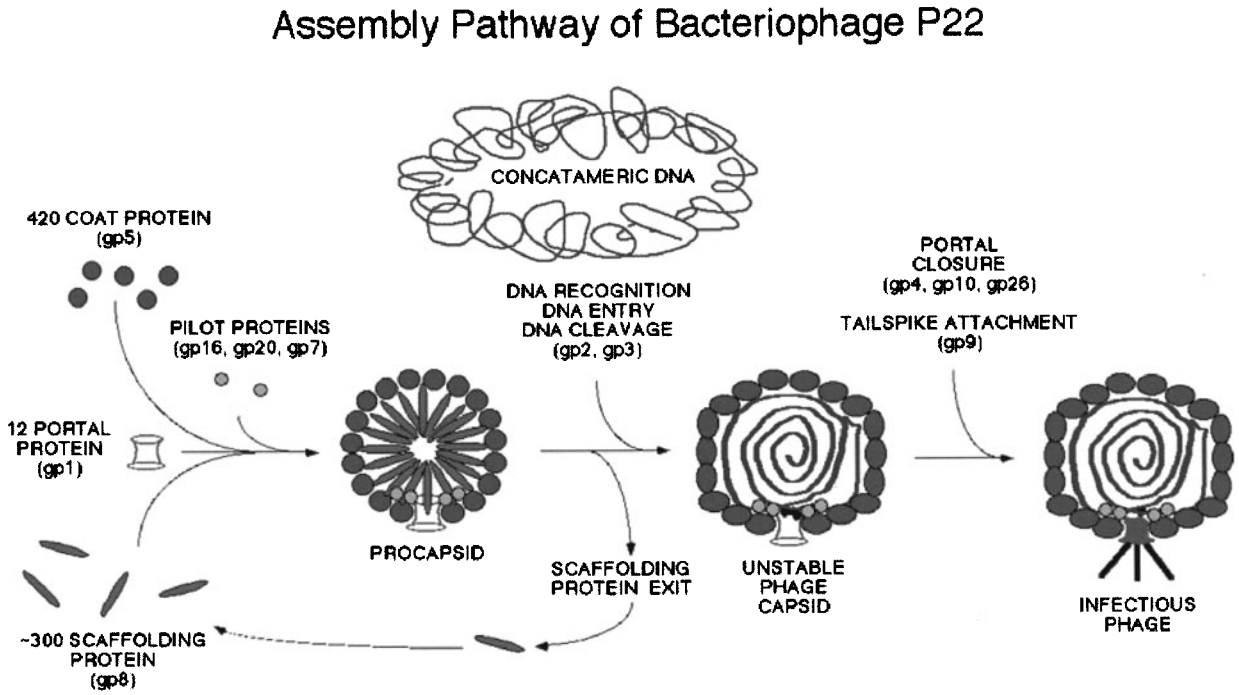

Figure 1. Bacteriophage P22 morphogenesis. Approximately 420 molecules of coat protein, ~ 300 molecules of scaffolding protein and 12 molecules of the portal protein polymerize into a double-shelled procapsid in which the scaffolding protein forms an internal core. The replicated DNA is packed by a headful mechanism into the procapsid, resulting in a release of scaffolding protein and expansion of the coat protein lattice yielding the capsid. The freed scaffolding protein can recycle and participate in further rounds of assembly.

subunit-subunit interfaces during expansion is mediated by domain interchange (Tuma *et al*., 1998), but the molecular basis for procapsid metastability remains unknown.

A series of single-site coat protein mutants with temperature-sensitive (ts) phenotype have been isolated (Gordon & King, 1994; Gordon *et al*., 1994) and described as folding-deficient (tsf, temperature-sensitive folding; Teschke & King, 1995). Unlike ts stability mutants, the tsf class is used to describe substitutions that affect stability of folding intermediates but not the native state. Accordingly, these mutations disrupt the coat protein folding pathway at non-permissive temperatures without compromising the stability of phage grown at permissive temperatures (Gordon & King, 1994). While the mutant subunit is not affected, the pressure stability of the mutant procapsids is substantially reduced, suggesting that the mutations may lie at subunit-subunit interfaces (Galisteo *et al*., 1995; Foguel *et al*., 1995). Specifically, the replacement of a non-polar by a polar amino acid (W48Q) results in a direct destabilization of the particle due to loss of entropic stabilization (Foguel *et al*., 1995).

The use of hydrostatic pressure to induce conformational changes in proteins and protein assemblages is well documented (Heremans, 1982; Silva & Weber, 1993; Jonas & Jonas, 1994; Robinson & Sligar, 1995; Silva *et al*., 1996; Foguel *et al*., 1996, 1998). In general, the polymerization of supramolecular structures such as viruses is driven by entropic interactions and is accompanied by an increase in system volume (Prevelige *et al*., 1994; Da Poian *et al*., 1995). Hydrostatic pressure induces the dissociation and denaturation of proteins because the processes of folding and association are generally accompanied by an increase in volume (Silva & Weber, 1993; Gross & Jaenicke, 1994). This positive volume change is due to the combined effects of the formation of solvent-excluding cavities in the protein interior or at the intersubunit interfaces and the release of bound solvent. Kinetic studies under pressure permit the determination of the activation volume changes for the unfolding-dissociation transition (Vidugiris *et al*., 1995). The activation volume is sensitive to changes in polypeptide and side-chain packing within the assembly and serves as a gauge of protein compactness within the assembly.

Here, we utilize high-pressure dissociation of empty procapsids and heat-expanded (mature) shells of wild-type bacteriophage P22 to compare polypeptide packing in both structures. Such comparison shows that packing defects (lower compactness) contribute to the metastability of the procapsid shell. In addition to wild-type (wt), procapsid and expanded shells of two mutant phage strains with temperature-sensitive folding (tsf) mutations in the coat protein were examined: W48Q (tryptophan 48 replaced by glutamine) and T294I (threonine 294 replaced by isoleucine). We demonstrate that these mutations introduce additional packing defects into the procapsid shell lattice even when grown and examined at permissive temperatures. These defects are absent in expanded shells. Thus, in addition to interfering with folding of coat protein monomer, the tsf mutations affect packing of subunit interfaces that are specific for the procapsid shell but absent in the mature virion. The tsf mutations also alter energetics of the expansion reaction and thus constitute a useful tool for understanding the process of capsid expansion.

Results

T294I mutation leads to destabilization of procapsid shells

Empty procapsid shells (wt and T294I mutant) at a concentration of 0.2 mg/ml were subjected to a constant pressure of 2.2 kbar at 22 °C (Figure 2) and the kinetics of dissociation were monitored by the shift of the intrinsic fluorescence band (Figure 2(a) and inset) and by the decrease in light-scattering (Figure 2(b)). The kinetics of dissociation were followed because in the absence of the scaffolding protein dissociation is irreversible, precluding equilibrium measurements (Prevelige *et al*., 1994; Foguel *et al*., 1995).

The procapsid shells composed of the T294I mutant protein were extremely labile at this pressure compared to wt shells. While wt procapsid shells did not completely dissociate at 2.2 kbar, 22 °C even after 200 minutes, T294I procapsid shells dissociated completely within less than 25 minutes. The behavior of the wt procapsid shells is consistent with previous reports that complete dissociation of wt procapsid shells is achieved only at low temperature and/or higher pressures (Prevelige *et al*., 1994; Foguel *et al*.; 1995). The dissociation of T294I procapsid shells was irreversible as expected, since scaffolding protein is necessary to promote the reassembly of coat protein subunits into procapsid (Prevelige *et al*., 1988; Figure 1). However, upon return to atmospheric pressure the dissociated subunits refolded as indicated by the return of the center of spectral mass to a value corresponding to that of the folded coat protein subunits (~28,800 cm^{-1}, previously reported by Foguel *et al*., 1995). The starting value for the shell of 29,300 cm^{-1} reflects an additional spectral blue shift because of solvent shielding of the tryptophan residues in the icosahedral lattice (Figure 2(a)).

Similar pressure-induced dissociation has been observed for another tsf mutant W48Q (Foguel *et al*., 1995), although its dissociation process was much slower than that of the T294I, requiring approximately 80 minutes to complete. These data indicate that T294I is thus far the most pressure-sensitive folding mutant characterized.

The temperature-dependence of pressure-induced dissociation offers insight into the type of dominant stabilizing force. As shown previously (Foguel *et al*., 1995), the wt procapsid shells are entropically stabilized because they dissociate

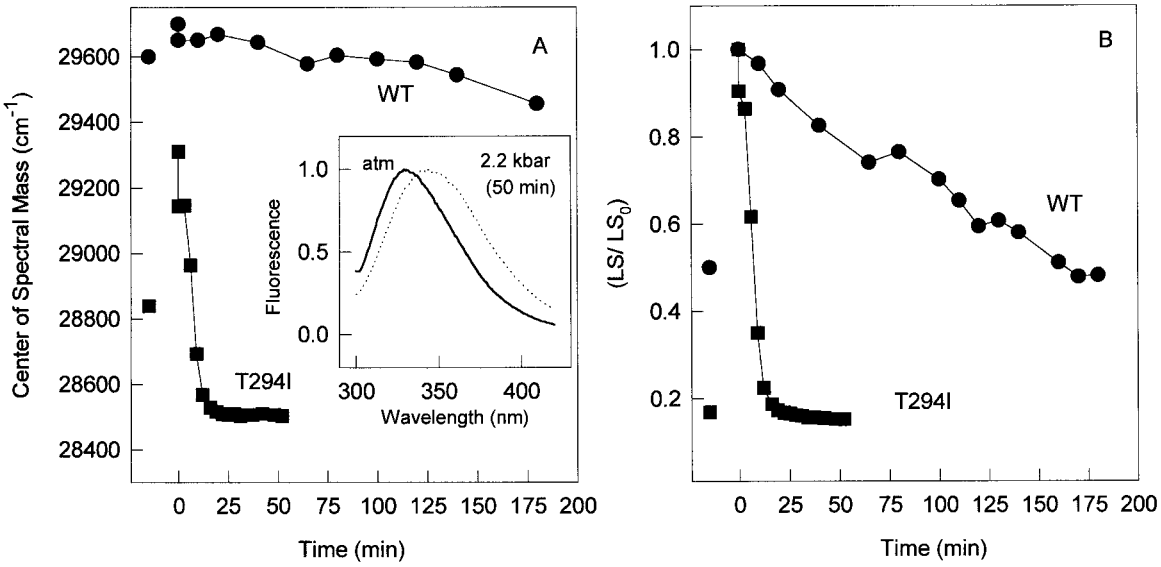

Figure 2. The kinetics of the pressure-induced dissociation of wt and T294I shells at 2.2 kbar, 22 °C. (a) Center of spectral mass, ⟨υ⟩ (for definition, see Materials and Methods), shift *versus* time for the dissociation of wt (●) and T294I mutant shells (■) (smaller values indicate dissociation and unfolding). (b) Light-scattering change *versus* time for the dissociation of wt (●) and T294I mutant shells (■). Values in (b) are plotted as fraction of light-scattering value at zero time. The isolated symbols in the left side of each panel represent the center of spectral mass and the light-scattering values achieved after decompression. Inset in (a), normalized fluorescence emission spectra of T294I before pressure treatment (—) and after 50 minutes under 2.2 kbar (· · · · · ·) at 22 °C. Protein concentrations were 0.2 mg/ml.

(more than eight times) faster at 1 °C than at 22 °C (Figures 2 and 3). In contrast, the T294I procapsid shells exhibit only 50 % increased rate of dissociation at lower temperature (Figures 2 and 3). Thus, the mutant shells have lost most of the entropic stabilization observed in the wt shells. A similar loss of entropic stabilization has been observed for the W48Q tsf mutant (Foguel *et al.*, 1995).

Pressure stability of T294I monomer

The T294I mutant is a folding mutant. In order to establish whether the pressure sensitivity of the T294I shells arises from pressure sensitivity within the folded state of the subunit, we have compared the reversible pressure denaturation of isolated subunits (Figure 4). The T294I subunit is more stable than wt coat protein subunit, as shown in Figure 4. The greater stability of the mutant could be due to dimerization or to a slightly different folded conformation (Galisteo *et al.*, 1995; Teschke & King, 1995). In either case, subunit stability cannot be the cause of the decreased procapsid shell stability. It is worth noting that other tsf subunits exhibit slightly greater pressure stability (Foguel *et al.*, 1995).

Dissociation and unfolding of T294I mutant coat are uncoupled

In order to compare the stability of wild-type and T294I procapsid shells at atmospheric pressure, the stability of these shells was probed by urea dissociation. The dissociation was followed by the center of spectral mass shift (Figure 5(a)) and light-scattering (Figure 5(b)). The T294I procapsid shells dissociated and the subunits unfolded more readily (midpoint of denaturation $C_{1/2} \approx 2.5$ M urea) than the wt protein, which unfolded with $C_{1/2} \approx 4.5$ M urea. As measured by light-scattering (Figure 5(b)), the T294I procapsid shells dissociated at a lower urea concentration ($C_{1/2} \approx 1.5$ M) than was required to denature the subunits. This is in contrast to the wt procapsid shells, for which the urea-induced dissociation released completely unfolded subunits (Figure 5).

To verify this observation, we have measured the bis-ANS binding to the procapsid shells as a function of urea concentration. It has previously been shown that procapsid shells and denatured coat protein cannot bind bis-ANS, while folded and partially folded coat protein monomers can (Teschke *et al.*, 1993). As expected, the wt coat protein did not display any bis-ANS binding over the entire range of urea concentration, confirming that the procapsid shell dissociation and subunit denaturation are coupled (Figure 5(c)). In contrast, the T294I procapsid shells exhibited a peak of bis-ANS binding at about 2.5 M urea, at which concentration the shells have already dissociated (see light-scattering results in Figure 5(b)). Because of the lower stability of the T294I procapsid shells, the dissociated subunits retain residual structure that enable the observed binding of bis-ANS.

Procapsid shells of tsf mutants exhibit larger cavity defects

A measure of the relative size of cavity defects in different procapsid shells is the activation volume for pressure dissociation. In order to obtain these activation volumes, we performed kinetic experiments at different pressures at 1 °C (Figure 6). The ranges of pressure were chosen for each sample such that the rate of dissociation could be measured on a convenient time-scale. In addition to wt (Figure 6(a)) and T294I procapsid shells (Figure 6(b)), the activation volume for another mutant procapsid shell, W48Q, was also determined (Figure 6(c)).

A logarithm plot of the data presented in Figure 6 furnished the values of the rate constant (k_p) at each pressure. The volumes of activation were obtained from the slope of a plot of ln (k_p) *versus* pressure according to equation (5) (Figure 7). The calculated parameters are expressed in Table 1. The absolute values of activation volumes for the dissociation of mutant procapsids were almost twice as large as that for the wt shells. This explains the enhanced pressure sensitivity of the mutants. Such an increase in the activation volume can be explained by less efficient packing of polypeptides in the procapsid lattice of the tsf mutants.

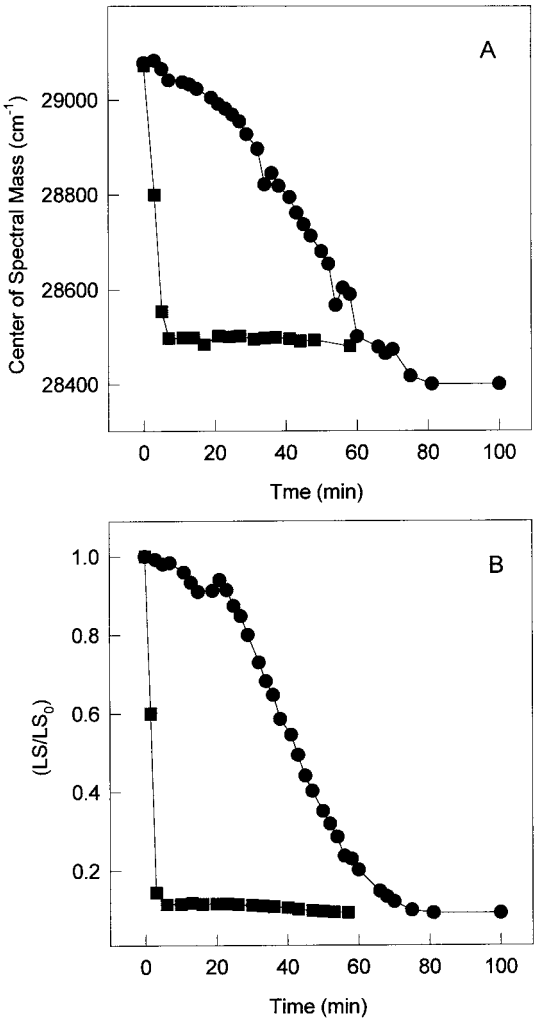

Figure 3. The kinetics of the pressure-induced dissociation of wt and T294I shells at 2.2 kbar, 1 °C. (a) Center of spectral mass shift *versus* time for the dissociation of wt (●) and T294I mutant shells (■). (b) Light-scattering change *versus* time for the dissociation of wt (●) and T294I mutant shells (■). Values in (b) are plotted as fraction of light-scattering value at zero time. Protein concentrations were 0.2 mg/ml.

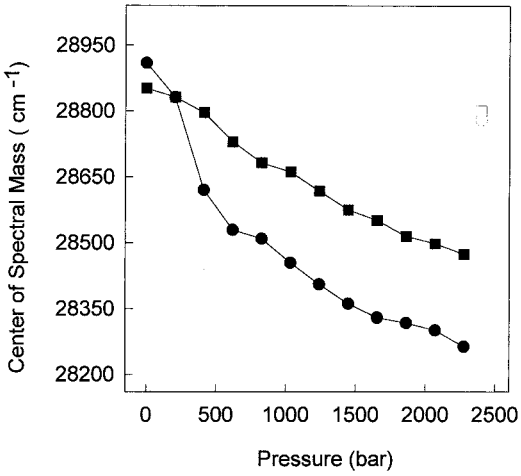

Figure 4. Pressure-induced denaturation of the coat proteins at 22 °C as followed by the center of spectral mass shift. Isolated coat proteins were prepared from wt (●) or T294I (■) shells and subjected to increasing pressures. The spectra were collected after five minutes at each pressure applied. No further change in the center of spectral mass was observed. The isolated symbols on the right represent the center of spectral mass after returning to atmospheric pressure. Protein concentrations were 50 μg/ml.

When the values of Gibbs free energy of activation (1 bar, 1 °C) are compared, the data show that the wt procapsid shells are only slightly more stable than the mutant shells (Table 1). Thus, the rate of dissociation reflects primarily activation volume changes. In order to explain the large effect of the volume changes to the rate of a reaction, equation (4) from Materials and Methods can be broken down into four terms:

$$\ln(k) = \ln(\kappa_b T/h) - \Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R - p\Delta V^{\ddagger}/RT \quad (1)$$

where k is the rate constant of the process; κ_b is the Boltzman constant; h is Planck's constant; R is the gas constant and T is the temperature at which the experiment was performed. Generally, the free energy of the transition state is far above that of the reactants (as observed for the dissociation of the procapsid shells), resulting in a small rate constant. In reactions involving a net change in covalent bonds, $\Delta G^{\ddagger}$ is positive primarily because $\Delta H^{\ddagger}$ is always positive and large. In such cases, increases in the rate constant are usually accom-

plished by decreasing the positive value of $\Delta H^{\ddagger}$. The thermal unfolding of proteins and nucleic acids, which involves the breakage of a large number of hydrogen bonds, is also accompanied by positive enthalpies of activation. In contrast, the dissociation of viral capsids does not involve the breaking of covalent bonds or the disruption of large numbers of hydrogen bonds. Therefore, during dissociation the enthalpic contribution plays a smaller role, and changes in entropy and volume play a more important role. The dissociation of protein-protein interactions followed by solvation of the newly exposed surface area is probably the major contributing factor for the large negative volume observed. A major role of solvation is corroborated by the entropy decrease upon the dissociation of viruses (Foguel *et al.*, 1995; Da Poian *et al.*, 1995) and other macromolecular assemblies (Foguel & Silva, 1994; Silva *et al.*, 1996).

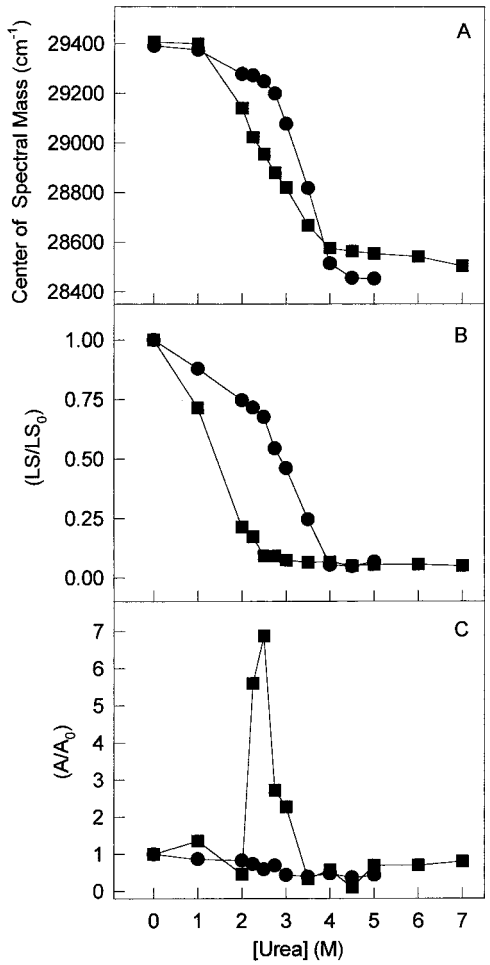

Figure 5. Binding of bis-ANS to the urea-induced denaturation of wt and T294I mutant shells. Denaturation of wt (●) and T294I (■) shells with increasing concentrations of urea as followed by (a) the center of spectral mass change; (b) by the light-scattering change; and (c) by the bis-ANS binding. The extent of bis-ANS binding was expressed as the ratio between the area of the fluorescence emission spectrum of bis-ANS at any given urea concentration and the area in the absence of urea. Other conditions were as described in Materials and Methods.

Cavity defects are reduced by *in vitro* expansion

In the previous section we determined that both wt and mutant procapsid shells contain sizable packing defects. Because the procapsid shell constitutes a metastable intermediate on the assembly pathway, it is conceivable that maturation leads to improved packing in the expanded shell. We have utilized *in vitro* expansion of procapsid shells by heat treatment at 65 °C for 20 minutes to generate expanded lattices of wt and mutant coat protein.

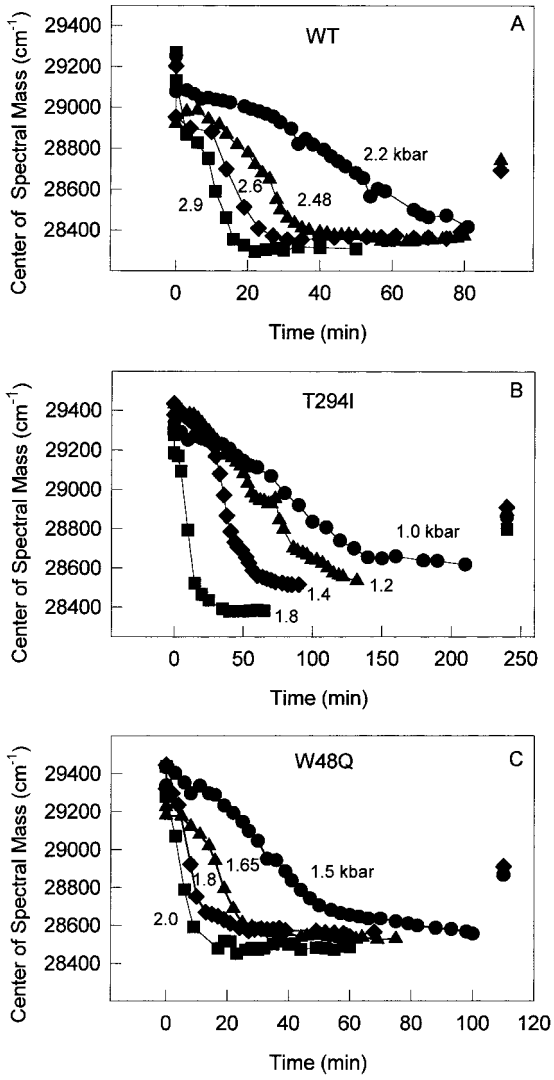

Figure 6. Kinetics of pressure induced dissociation of the wt, T294I and W48Q shells at 1 °C as followed by the center of spectral mass change. (a) Dissociation of wt shells at 2.2 (●); 2.48 (▲); 2.6 (◆); and 2.9 kbar (■). (b) Dissociation of T294I at 1.0 (●); 1.2 (▲); 1.4 (◆); and 1.8 kbar (■). (c) Dissociation of W48Q at 1.5 (●); 1.65 (▲); 1.8 (◆); and 2.0 kbar (■). The isolated symbols at the right of each curve represent the center of spectral mass after decompression. The protein concentration was 0.2 mg/ml in all experiments.

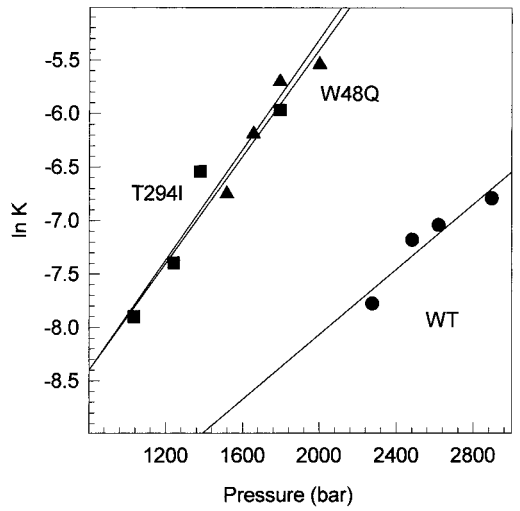

Figure 7. Logarithm of the rate constant *versus* pressure for the experiments presented in Figure 6. wt (●); T294I (■); and W48Q (▲) shells. From these curves it was possible to calculate the volume of activation for the dissociation of P22 bacteriophage (Table 1).

Shells expanded by this protocol are as stable as mature shells (Galisteo & King, 1993), and exhibit the same protease resistance, peptide conformation and amide hydrogen-deuterium exchange as mature phage (Prevelige *et al.*, 1990; Prevelige & King, 1993; Tuma *et al.*, 1996, 1998; R.T. & P.E.P., unpublished results) and therefore represent a suitable model of the mature phage icosahedral lattice. Figure 8 shows a comparison of pressure-induced dissociation of procapsid and *in vitro* expanded shells. It is evident that expanded shells of both wt and T294I mutant are greatly stabilized against pressure dissociation either by following the center of spectral mass change (Figure 8(a) and (c)) or the light-scattering (Figure 8(b) and (d)). The wt shells were examined at 1 °C to achieve dissociation at a pressure identical with that applied to the much less stable T294I procapsid shells. Expanded wt shells show considerably slower dissociation than the procapsid shell when the light-scattering was analyzed (Figure 8(a) and (b)). The T294I expanded shells were completely resistant to pressure dissociation at 22 °C (Figure 8(c) and (d)). Therefore, the stability of the mature shell of this mutant is similar to that of the wt at the same temperature and the packing defects introduced by this mutation are diminished or completely eliminated by the thermal-induced expansion. It is interesting to note that upon expansion the center of spectral mass values of the tryptophan emission undergo a blue shift, suggesting an additional shielding of these residues in the lattice (from ~29,100 to 29,370 cm^{-1} in T294I and from 29,350 to 29,600 cm^{-1} in wt shells).

Table 1. Volume and energy change of activation for the dissociation of wt and mutant procapsid shells of bacteriophage P22 at 1 °C

	$\Delta V^{\ddagger}$(ml/mol)[a]	$\Delta G^{\ddagger}$ (kcal/mol) (1 atm, 1 °C)[a]
wt	−34.7 (±6.9)	22.03 (±0.51)
T294I	−59.0 (±12.0)	21.68 (±0.41)
W48Q	−57.1 (±13.7)	21.99 (±0.57)

[a] Activation volumes and energies are expressed per mol of monomeric subunit.

Cavity defects affect shell expansion *in vitro*

Because the T294I substitution greatly affects polypeptide packing in the procapsid shell but not the mature shell, it is likely that this mutation has a profound effect on the dynamics of the shell expansion process. Therefore, we have compared expansion rates of the wt and T294I procapsid shells *in vitro* at 50, 55 and 59 °C. Shell expansion was monitored by native agarose gel electrophoresis as described (Tuma *et al.*, 1998; and see Figure 9(a)). The rates of expansion were extracted from the time-dependent band intensities and are presented in an Arrhenius plot (Figure 9(b)). In comparison with wt shells, the T294I shells expand more readily, especially at higher temperature. The apparent activation energy, obtained from the slope, is about threefold higher for the T294I mutant ($E_a = 130$ kcal/mol) than for the wt ($E_a = 40$ kcal/mol). The higher overall rate of T294I shell expansion is consistent with lower entropic stabilization of the metastable procapsid subunit interfaces. The more pronounced temperature dependence (higher E_a) most likely results from changes in the enthalpic contribution to the activation energy.

Discussion

The destabilization of tsf procapsid shells shows the importance of residues 48 and 294 for the formation of the procapsid lattice. These residues are not critical for the stability of the monomeric coat protein, since both W48Q (Foguel *et al.*, 1995) and T294I (Figure 4) monomers were as stable as the purified wt protein. It was shown by Galisteo *et al.* (1995) that the chemical and thermal stability of these monomeric mutant subunits is comparable to that of the wt. Similarly, although the mutants were isolated as folding mutants, the *in vitro* folding of these two mutants does not seem to differ significantly from that of the wt monomer (Teschke & King, 1993, 1995). Interestingly, these two mutants have been shown to oligomerize at low temperature, a feature that is different from the wt protein (Galisteo *et al.*, 1995) and suggests a difference in association properties.

These two mutants were characterized as tsf because of their recessive nature, selective rescue by GroEL/ES and the thermostability of the mature phage or subunit (Gordon & King, 1994; Gordon *et al.*, 1994; Galisteo *et al.*, 1995). It was proposed that these tsf mutations lead to the

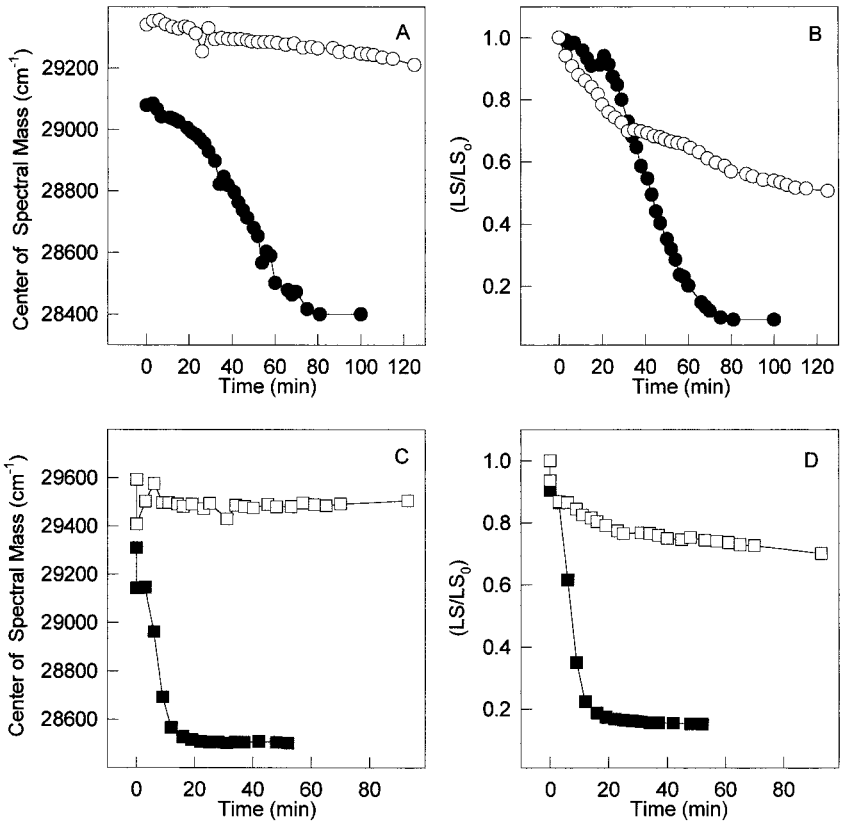

Figure 8. The kinetics of the pressure-induced dissociation of procapsid and mature (heat-expanded) shells of P22. (a) and (c) Center of spectral mass shift *versus* time for the dissociation of wt (●, ○) and T294I mutant shells (■, □). (b) and (d) Light-scattering change *versus* time for the dissociation of wt (●, ○) and T294I mutant shells (■, □). In all cases, the filled symbols represent the changes related to the procapsids, while the open symbols represent the changes observed with mature shells. The expansion was achieved by placing the shells under 65 °C for 20 minutes. Other conditions as in Material and Methods.

accumulation of a coat protein folding intermediate, which subsequently aggregates. The present results clearly demonstrate that these mutations greatly destabilize the procapsid shell *via* introduction of packing defects, and that these defects are eliminated during expansion, yielding a stable mature phage. Because the mutant procapsid shells lose the entropic stabilization observed for the wt shells, it is conceivable that their assembly process is compromised at higher temperature, where the entropic contribution to stability is more important. As a consequence, assembly at the restrictive temperature may be blocked due to low stability of certain assembly intermediates and, subsequently, the unassembled coat protein could accumulate and aggregate into the inclusion bodies as observed previously (Gordon *et al.*, 1994). One likely candidate for such assembly intermediate is the complex of coat protein with the scaffolding protein. This complex, which is required for assembly, is stabilized by ionic interactions that may be weakened at higher temperatures (Parker & Prevelige, 1998).

In vitro expansion, which represents a good model of P22 capsid maturation, effectively diminished packing defects introduced by the T294I mutation. Thus, the mature shell lattice of the mutant is similar to that of the wt virion, which is consistent with the previously described stability of the mature T294I virions at non-permissive temperatures (Gordon & King, 1994). The W48Q shells are destabilized at high temperature to the point that efficient *in vitro* expansion becomes impossible due to dissociation (data not shown). Thus, it is impossible to determine if cavities are eliminated during expansion of this mutant. As mature virions of this mutant are also stable at non-permissive temperatures, it is likely that the effect of the cavity introduced by the W48Q mutation is reduced during maturation.

Although an effect of the T294I and W48Q tsf mutations on certain folding intermediates cannot be completely ruled out, most likely these mutations affect primarily the procapsid state of the coat protein *via* intersubunit packing defects. As discussed above, the procapsid state of the coat protein indeed retains some characteristics of a folding intermediate and thus the previous tsf designation of these mutants parallels similar classification of the tailspike mutants (Gordon *et al.*, 1994).

The results of these studies on subunit packing in the procapsid and expanded shells are consistent with the suggestion that the P22 procapsid resembles a late protein folding intermediate. The decreased stability of the procapsid shell relative to that of the expanded virion arises, in part, due to looser packing and point mutations can further destabilize the procapsid by exacerbating the packing defects. However, the adjustments that take place in the icosahedral lattice during the expansion remove the polypeptide packing defects that are responsible for the pressure sensitivity of the procapsid shell. It is conceivable that at least some

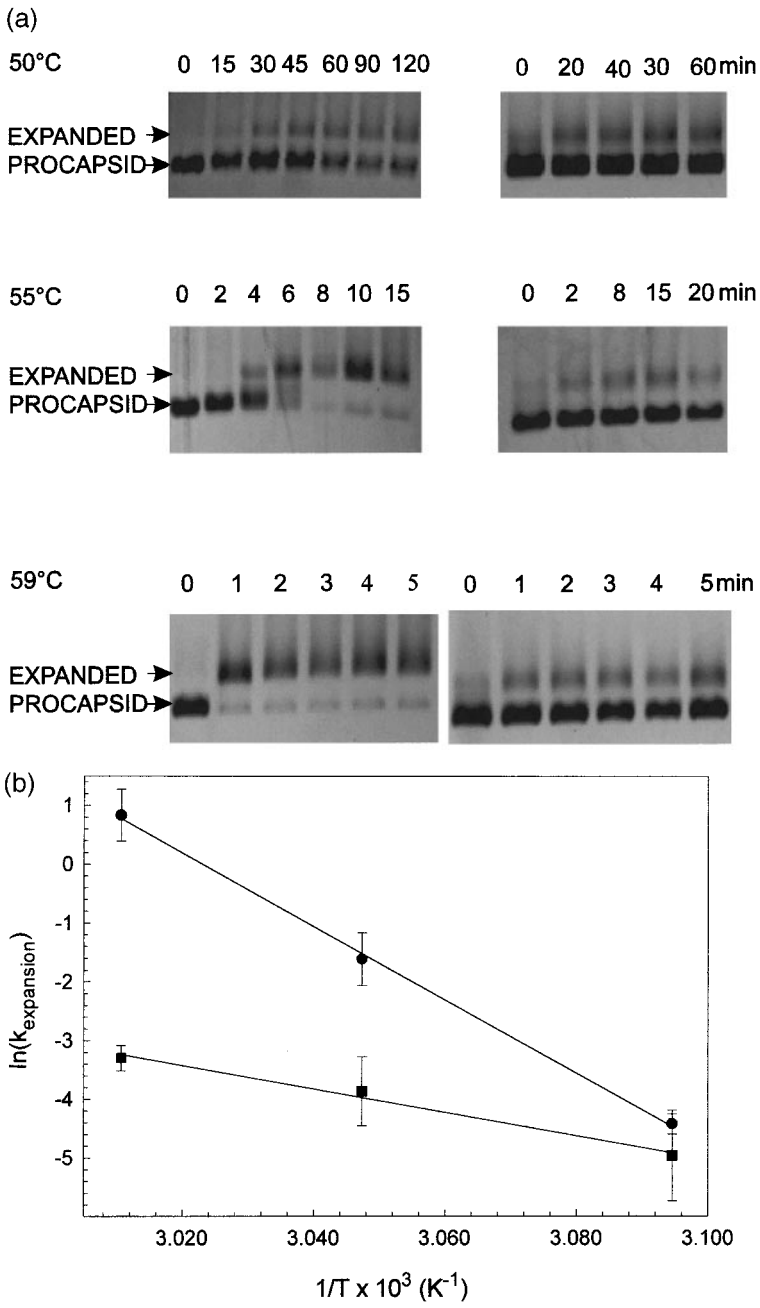

Figure 9. (a) The rate of thermal expansion of wt and T294I shells monitored by gel electrophoresis. T294I (left) and wt (right) shells were heated at 50, 55, and 59 °C for the indicated time. Samples were removed and analyzed for the extent of expansion by native gel electrophoresis. (b) Arrhenius plot for the expansion of T294I and wt shells. The rate of expansion was calculated from the data in (a). The data were digitized, and the rate of expansion determined by fitting the data with an exponential fit. The ln of the rate of expansion was plotted *versus* the temperature for both the T294I (●) and wt (■) shells.

of the intersubunit contacts within the procapsid shell are temporary, and are replaced by stable contacts during maturation. Recently, by measuring hydrogen/deuterium exchange rates, it has also been shown that the procapsid lattice undergoes more dynamic fluctuations than the mature capsid lattice (Tuma *et al.*, 1998). Interestingly, compact protein folding intermediates usually exhibit packing defects due to imperfect side-chain packing (Vidugiris *et al.*, 1995). Such intermediates also possess very little hydrogen/deuterium exchange protection (Kamtekar *et al.*, 1993).

The packing defects introduced by T294I mutation are eliminated during maturation. Thus, the region affected by T294I mutation corresponds to the domain that contributes to the metastable intersubunit contacts and participates in expansion. Such metastable contacts would correspond to non-native interactions within protein folding intermediates that result in significant energy barriers during the folding process (Sosnick *et al.*, 1994).

Present work shows that imperfect polypeptide packing may serve as a means to construct metastable assembly intermediates that undergo substantial rearrangement of subunits during maturation. Subunits comprising such metastable intermediates would also constitute folding intermediates with lower side-chain packing efficiency and higher dynamic flexibility that is required for the conformational changes during maturation.

Materials and Methods

Preparation of protein samples

The preparation of empty shells of coat protein was as described (Prevelige *et al*, 1988). The procapsids employed were purified from *Salmonella typhimurium* strain DB7136, which had been infected with P22 phage carrying 2^{-}am/13^{-}am mutations. The 2^{-} mutation blocks DNA packaging, resulting in the accumulation of procapsids, while the 13^{-} mutation delays lysis, thereby increasing the yield. The properties of the coat protein mutants W48Q and T294I have been described by Gordon & King (1994). Most procapsids retained their portal complex.

For the urea-induced denaturation of the shell, the samples were incubated in the presence of urea overnight to allow completion of the equilibrium. The coat proteins were prepared by incubating the shells in 8 M urea for three hours. After this time, the urea was diluted out with buffer B (50 mM Tris-HCl (pH 7.6), 25 mM NaCl, 1 mM EDTA) keeping the sample on ice. The final urea concentration was 0.1 M. The sample was kept overnight in the refrigerator for complete refolding.

For the fluorescence experiments, the expanded shells (mature shells) were prepared by incubating the shells at 65 °C for 20 minutes.

Optical methods

The high-pressure bomb, purchased from ISS (Champaign, IL) has been described (Silva *et al*., 1992). Fluorescence spectra were recorded on an ISS K2 computer-controlled spectrofluorometer (Champaign, IL).

The coat protein of bacteriophage P22 contains six tryptophan residues distributed more or less uniformly through the primary sequence (Eppler *et al*., 1991). Intrinsic fluorescence of the coat protein subunit exhibits a blue shift upon association into coat protein shell, indicating that the tryptophan residues are less solvent-exposed in the polymerized form (Teschke & King, 1993; Prevelige *et al*., 1994). Similarly, fluorescence of coat protein monomer undergoes a blue shift upon folding (Teschke & King, 1993). Thus, the center of spectral mass of the fluorescence emission band (described below) is a sensitive probe of both virus integrity and the extent of folding of the released coat protein. Tryptophan emission was collecting from 300-420 nm with excitation set at 280 nm. Fluorescence spectra at pressure p were quantified by specifying the center of spectral mass $\langle\upsilon\rangle$:

$$\langle\upsilon\rangle = \Sigma\upsilon_i F_i / \Sigma F_i \qquad (2)$$

where F_i stands for the fluorescence emitted at wavenumber υ_i and the summation is carried out over the range of appreciable values of F (Silva *et al*., 1992).

Light-scattering measurements were made in an ISS K2 spectrofluorometer (Foguel *et al*., 1995). Scattered light (320 nm) was collected at an angle of 90° of the incident light by integrating the intensity in the 315-325 nm window.

The binding of bis-ANS (bis (8-anilinonaphthalene-1-sulfonate) was monitored by exciting the samples at 360 nm and collecting the emission in the range 400-600 nm (Silva *et al*., 1992). The fluorescence spectrum areas were used to evaluate the extent of bis-ANS binding and it was expressed by dividing the spectral area at any given condition by the initial spectral area (absence of urea). The concentration of bis-ANS used in all experiments was 2 μM.

All reported experiments were recorded in 50 mM Tris-HCl (pH 7.2), 100 mM NaCl, 1 mM EDTA.

Thermodynamic parameters of activation

In order to calculate the activation parameters for the dissociation of bacteriophage P22, the center of spectral mass ($\langle\upsilon\rangle$) change was converted into degree of dissociation (α) by the equation:

$$\alpha = (\langle\upsilon\rangle_{p;\,T} - \upsilon\rangle_{atm})/(\langle\upsilon\rangle_{p;\,T\,end} - \langle\upsilon\rangle_{atm}) \qquad (3)$$

Where $\langle\upsilon\rangle_{p;\,T}$ is the center of spectral mass at any given time after pressurization; $<\upsilon>_{atm}$ is the center of spectral mass at atmospheric pressure (native shell) and $\langle\upsilon\rangle_{p;\,T\,end}$ is the center of spectral mass after complete dissociation of the shell.

A semilogarithm plot of ln α *versus* time gives a straight line in the region where dissociation of the particles takes place (see Figure 7). The rate constant k is calculated from the slope of this plot.

The $\Delta G^{\ddagger}$ (standard free energy of activation) values were calculated according to the literature (Guggenheim, 1937; Glasstone *et al*., 1941; Denbigh, 1981) from the equation:

$$k = \kappa_b T/h \exp(-\Delta G^{\ddagger}/RT) \qquad (4)$$

where k is the rate constant of the process; κ_b is the Boltzman constant; h is Planck's constant; R is the gas constant and T is the temperature where the experiment was performed.

The volumes of activation ($\Delta V^{\ddagger}$) were calculated by performing kinetic experiments at different pressure at constant temperature (1 °C). The rate of dissociation at a given pressure p, k_p, can be expressed in terms of the rate at atmospheric pressure, k_o, and the activation volume $\Delta V^{\ddagger}$ for the formation of transition state:

$$\log(k_p/k_o) = -p(\Delta V^{\ddagger})/RT \qquad (5)$$

The logarithm of the rate constants obtained from these experiments was plotted against pressure and the slopes of the curves furnish the volumes of activation. The volumes of activation were calculated from kinetics experiments performed at 1 °C, since the wt shells undergo dissociation in a reasonable times only at this low temperature (Foguel *et al*., 1995).

Rate of expansion *in vitro*

In vitro expansion was performed by incubation at temperatures of 50-60 °C in 50 mM sodium phosphate buffer (pH 7.5), 25 mM NaCl using protein concentration of 0.5 mg/ml and separating the products on 1.2 % agarose gel (separation buffer; 40 mM Tris (pH 8.0), 10 mM CH_3COONa, 2 mM EDTA). The extent of procapsid shell expansion was obtained by densitometry of Coomassie-stained bands corresponding to procapsid and expanded species.

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