Folding of the Phage P22 Coat Protein *in Vitro*†

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ABSTRACT: Within infected *Salmonella* cells, newly synthesized 47-kDa phage P22 coat polypeptides fold without covalent modifications into assembly-competent subunits. Coat protein subunits interact with scaffolding protein to form the icosahedral procapsid precursor of the mature, $T = 7$, virions. In these lattices, the coat subunits form seven classes of local bonding interactions [Prasad, B. V. V., Prevelige, P. E., Marieta, E., Chen, R. O., Thomas, D., King, J., & Chiu, W. (1993) *J. Mol. Biol. 231*, 65–74]. Coat protein denatured in guanidine hydrochloride could be refolded to soluble, monomeric subunits by rapid dilution into buffer at concentrations of protein up to 25 μg/mL. The fluorescence emission spectrum of soluble coat protein monomers was between that of the assembled shells and the denatured protein, suggesting the presence of tryptophans at the subunit interfaces in the shells. Kinetic studies of the refolding of coat protein revealed an intermediate whose continued folding could be inhibited by the hydrophobic dye bisANS. The kinetic intermediate bound 10.80 ± 1.20 bisANS molecules while the folded monomer bound 1.24 ± 0.36 bisANS molecules. When coat polypeptide chains were refolded at 50 μg/mL, aggregation competed with folding. Aggregation of the folding intermediates increased in the presence of bisANS. The kinetic folding intermediate that binds bisANS probably represents the species at the junction of the productive pathway to soluble and assembly-competent coat monomers and the off-pathway steps to inclusion bodies. The relationship between these soluble monomers and the conformations observed in the $T = 7$ lattice remains unclear.

The subunits forming the icosahedral shells of animal, plant, and bacterial viruses interact intimately and tightly to protect the viral genome. Surfaces in the soluble subunits that would be expected to be exposed to the solvent are buried in the mature capsid. The folding of structural proteins such as viral capsid subunits has not been as extensively investigated as the folding of globular proteins (Jaenicke, 1987; Kim & Baldwin, 1990; Teschke & King, 1992). In fact, some of the most common structural proteins, such as actin or tubulin, have only recently been refolded from the denatured state with the help of chaperones (Gao *et al.*, 1992; Frydman *et al.*, 1992).

In the capsids of a number of viruses, identical subunits occupy positions in the lattice that require alternative bonding interactions. For example, the X-ray structure of SV40 shows that the chemically identical capsid protein in a pentamer can fold with either two or three α-helixes depending on whether the pentamer makes 2-fold or 3-fold contacts in the capsid (Liddington *et al.*, 1991). The amino terminus of the tomato bushy stunt virus coat protein either forms a β-structure with the amino termini of adjacent subunits or is disordered, depending on the position of the subunit in the capsid (Harrison *et al.*, 1978). In both the cauliflower mosaic virus and the bacteriophage P22, each subunit in a hexamer appears to have a different conformation (Cheng *et al.*, 1992; Prasad *et al.*, 1993).

These findings raise questions concerning the folding and conformation of viral protein subunits prior to polymerization: is there a stable precursor conformation or are multiple conformations populated in solution under physiological conditions? The former model suggests that the solution state of the capsid subunit is flexible so that the protein can be switched into the conformation necessary for the position it will occupy in the capsid during assembly (Caspar, 1976; Fuller & King, 1980). For example, the major capsid protein of the polyomavirus, VP1, can associate into particles of 12, 24, or 72 capsomeres, as well as tubelike structures, depending on the buffer conditions during association (Salunke *et al.*, 1989). The ability to switch into the conformations necessary for the different structures must lie in the VP1 subunit itself, since no additional protein components were added to these reactions. The flexibility of capsid proteins can be accomplished by tertiary and quaternary changes through order/disorder transitions, hinge movement, conformational changes, or alterations in interacting surfaces (Rossman & Erickson, 1985).

In the dsDNA viruses there is an additional conformational diversity. The direct product of the subunit polymerization is not the mature capsid but a procapsid that is the precursor in the DNA packaging reactions (Casjens & King, 1975). Procapsid stages are found in the assembly of animal viruses such as herpesvirus and adenovirus (D'Halluin *et al.*, 1978; Morin & Boulanger, 1984; O'Callaghan *et al.*, 1977; Preston *et al.*, 1983; Newcomb & Brown, 1991).

Procapsids have been best characterized for the dsDNA bacteriophages. The assembly of these capsids requires not only coat protein subunits but many molecules of a scaffolding protein. The procapsid of P22, a dsDNA phage of *Salmonella*, consists of 420 copies of the coat protein and ~300 copies of the scaffolding protein, as well as the minor components—the portal and pilot proteins. The scaffolding protein exits prior to DNA packaging with a concomitant transformation of the coat protein lattice to the mature lattice by an expansion of the capsid by 15% in diameter. Expansion is accompanied by a change in the shape of the particle from spherical to icosahedral (Earnshaw & Casjens, 1980; Casjens & Hendrix, 1988; Prasad *et al.*, 1993).

Coat protein is encoded by gene 5 of P22 and is 430 amino acids in length (Botstein *et al.*, 1973; Eppler *et al.*, 1991).

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[1] Abbreviations: bisANS, 1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid, dipotassium salt; GuHCl, guanidine hydrochloride.

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Coat protein has only one cysteine and no disulfide bonds or covalent modifications have been found in any of its forms (Fuller & King, 1981). Hence, conformational transitions must be responsible for the transformations of the newly synthesized polypeptide to folded subunit, polymerization of folded subunits into the procapsid lattice, and expansion of the procapsid lattice into the mature structure.

Low-resolution structures of the P22 procapsid and mature capsid have been determined by both low-angle X-ray scattering and by image reconstruction of cryoelectron micrographs (Earnshaw *et al.*, 1976, 1978; Prasad *et al.*, 1993). Image reconstruction of cryoelectron micrographs shows that the subunits in the procapsid are in seven different bonding environments, six in each hexamer and one in each pentamer. With the expansion, each of these subunits changes conformation so that each coat protein subunit could possess the potential for 14 different positionally-dependent conformations (Prasad *et al.*, 1993). The secondary structure changes only 2% with capsid expansion but a number of side-chain environments are altered (Prevelige *et al.*, 1993a). Therefore, it appears that capsid expansion is accomplished through motions of the coat subunit domains rather than through secondary structure changes.

The assembly of the P22 procapsid from coat and scaffolding subunits has been studied in considerable detail *in vitro*. Dissociation of the procapsid in 3 M GuHCl, followed by dialysis away from GuHCl, yielded soluble coat and scaffolding subunits (Fuller & King, 1981). These subunits were monomeric by sucrose gradient centrifugation and size-exclusion column chromatography (Fuller & King, 1982; Prevelige *et al.*, 1988). Upon mixing coat subunits with scaffolding subunits, closed double shells assembled efficiently *in vitro* (Prevelige *et al.*, 1988). The reaction showed a dependence on coat protein concentration, as well as a critical concentration, below which the assembly reaction did not nucleate (Prevelige *et al.*, 1993b).

As part of the process of understanding how the amino acid sequences of structural proteins determine both their folding and subunit interactions, we have characterized the folding of the P22 coat protein from the unfolded state *in vitro*. This is one of the few structural proteins to be successfully folded *in vitro* in the absence of cellular chaperones.

MATERIALS AND METHODS

Chemicals. BisANS was purchased from Molecular Probes, ultrapure guanidine hydrochloride from Pierce, and acrylamide from Bio-Rad. All other chemicals were reagent grade from common sources.

Buffer. Unless otherwise noted, the buffer was 50 mM Tris, 25 mM NaCl, and 2 mM EDTA, pH 7.6.

Preparation of Coat Protein. Empty procapsid shells were purified by the procedure of Prevelige *et al.* (1988). Briefly, *Salmonella typhimurium* cells were infected with a P22 phage defective in the production of the DNA packaging protein, gp2. After 3 h of infection, the cells were harvested by centrifugation, lysed by repeated freezing and thawing, and treated with DNase and RNase. A low-speed centrifugation was used to remove debris, and the procapsids were collected from the supernatant by a high-speed centrifugation. The procapsids were extracted 3 times with 0.5 M GuHCl to release the scaffolding protein and finally chromatographed on a Bio-Gel-A 0.5-m column in 0.5 M GuHCl. This yielded empty procapsid shells that were less than ~5% scaffolding protein.

Folded coat protein monomers were prepared by dissociation of the empty procapsid shells as described in Prevelige *et al.* (1988). Empty shells were brought to 3 M GuHCl and chromatographed through a Bio-Gel-A 5-m column in 2 M GuHCl to remove any residual phage. The peak fractions of coat protein were pooled and dialyzed against two changes of cold buffer so that the residual GuHCl was less than 1 mM. The protein was filtered through a Millex-GV 0.22-μM syringe filter to remove any aggregated protein, and the protein concentration was determined by the absorbance at 280 nm using an extinction coefficient of 1 for a 1 mg/mL solution (Fuller & King, 1981). On average, the filtration step gave a yield of ~90% and had no effect on the activity of the coat protein in an assembly reaction which yielded ~70% active protein.

Unfolded coat protein, for studies of the unfolded coat protein or for refolding by rapid dilution, was prepared by adding 6 M GuHCl to a concentrated stock of shells for a final concentration of 4.1–5.2 M.

Sucrose Gradient Sedimentation. Isokinetic 5–20% sucrose gradients were run in a Beckman L8-M ultracentrifuge at 20 °C using a SW 50.1 rotor for 14 h at 41 500 rpm to determine the distribution of folded coat protein. To determine the distribution of aggregates, 5–20% sucrose gradients were run in the SW 50.1 at 20 °C for 35 min at 35 000 rpm. Fractions were collected from the bottom of the gradient, and the protein was precipitated by the addition of trichloroacetic acid to 5%. The fractions were spun in a microfuge for 10 min and the pellets were suspended in sample buffer containing 0.67 M Trizma base to neutralize any residual acid. The samples were run on a 10% sodium dodecyl sulfate–polyacrylamide gel and visualized by silver stain (Rabilloud *et al.*, 1988). The amount of protein in the samples was quantified using an LKB gel scanner.

Fluorescence Measurements. Fluorescence experiments were performed using a Hitachi F-4500 spectrofluorometer interfaced with a personal computer. The excitation bandwidth was set to 2.5 nm and the emission bandwidth to 5 nm. The temperature of the samples were thermostated at 25 °C unless otherwise indicated. Any inner filter effect was corrected by the formula $F_{corrected} = F_{observed}$ antilog $[(OD_{ex} + OD_{em})/2]$ where the OD_{ex} and OD_{em} are the optical densities at the excitation and emission wavelengths (Lakowicz, 1983).

Unfolding of Empty Procapsid Shells to an Apparent Equilibrium. Shells were added at a concentration of 20 or 50 μg/mL to increasing concentrations of buffered GuHCl and incubated overnight at room temperature, when no further changes in the spectral properties of the samples were observed. The fluorescence of each sample was monitored, as well as the light scattering at 340 nm. The values were corrected for baseline fluorescence or scattering. The fraction unfolded was determined by the formula $(X_n - X_{obs}/X_n - X_u)$ where X_n is the fluorescence or light scattering of the native shells, X_{obs} is the observed value, and X_u is the fully denatured value. X_n and X_u were determined by linear extrapolation of the folded and unfolded plateaus into the region of the transition at each GuHCl concentration (Creighton, 1987).

Refolding of Coat Protein to an Apparent Equilibrium. Shells were dissociated and unfolded in 5.2 M GuHCl at 8 mg/mL. The unfolded coat protein was added to tubes such that the final protein concentration would be 20 μg/mL. Buffered GuHCl was added to the tubes at concentration from 0 to 5 M and allowed to incubate for 6–7 h at room temperature, when no further change in fluorescence with time was observed. The fluorescence of each sample was monitored and the values were corrected for baseline fluorescence. The fraction unfolded was determined with the formula described above. Since even low concentrations of

GuHCl caused unfolding of coat protein, the fluorescence of coat protein in the absence of GuHCl was used as X_n. The fluorescence of the unfolded plateau, which was consistent in slope in all experiments, was extrapolated into the transition region to determine X_u.

Quenching Experiments. KI quenching experiments were performed in buffer or buffer containing 6 M GuHCl. To keep the iodide reduced, 10 mM $Na_2S_2O_3$ was added to all the solutions (Lakowicz, 1983). The ionic strength was maintained by the addition of KCl, so that the total concentration of KI and KCl was 0.25 M, to avoid any changes in the quenching due to alterations in the ionic strength. The samples were excited at 295 nm and the emission was monitored at 335 nm for shells, 340 nm for folded coat protein monomers, and 350 nm for unfolded coat protein. There was no inner filter effect at the concentrations of KI used. Acrylamide quenching experiments were performed in either buffer or buffer containing 6 M GuHCl at the same excitation and emission wavelengths and were corrected for the inner filter effect. The quenching data were analyzed with the Stern–Volmer equation using a plot of F_0/F vs Q, where F_0 is the fluorescence of the sample in the absence of quenching agent, Q, and F is the fluorescence in the presence of quenching agent. (Lakowicz, 1983; Lehrer, 1971). The slope was determined by least-squares analysis.

Binding Studies with BisANS. A concentrated solution of bisANS was prepared and the concentration was determined by the absorbance at 385 nm using an extinction coefficient of 16 790 L mol^{-1} cm^{-1} (Farris *et al.*, 1978). Increasing concentrations of bisANS were added to coat protein that was folded by dialysis and incubated for at least 30 min. The dissociation constant was calculated by the quenching of tryptophans (excitation λ = 280 nm, emission λ = 340 nm) and by direct enhancement of the fluorescence due to the binding of bisANS (excitation λ = 280 or 400 nm, emission λ = 495 nm). The fluorescence was corrected for the inner filter effect. The binding of bisANS as determined by quenching of tryptophan was analyzed with the modified Stern–Volmer equation (Eftink & Ghiron, 1981; Prasad *et al.*, 1986). The binding of bisANS analyzed by enhancement of fluorescence at 495 nm was determined by the method of Webb (1963) as described in Bagshaw and Harris (1987) where a plot of [bisANS]/α vs $1/\{1-(1/\alpha)\}$ was made from the saturation data. The slope of this plot is the K_d and the y-intercept is the total number of binding sites in solution. α is F/F_{max}, where F is the fluorescence intensity of the sample in a particular concentration of bisANS and F_{max} is the maximum fluorescence. The maximum fluorescence enhancement when the binding of the bisANS to coat protein was totally saturated was determined graphically by plotting the inverse of the fluorescence intensity against the inverse of the bisANS concentration. The y-intercept was the maximal fluorescence (Bagshaw & Harris, 1987). The binding of bisANS to the kinetic intermediate was determined by extrapolating the kinetic data to the initial fluorescence of the reaction. The excitation wavelength was 280 or 400 nm and the emission wavelength was 495 nm. The fluorescence was corrected for the inner filter effect and analyzed as described by Bagshaw and Harris (1987). All of the linear binding data were analyzed using least-squares analysis.

Kinetics of Refolding. Coat protein that had been unfolded in 5.2 M GuHCl was rapidly diluted 200-fold to a final concentration of 5 μg/mL into buffer alone or with increasing concentrations of bisANS. The cuvettes were pretreated with a solution of 5% Tween-20 and rinsed when folding into buffer to prevent nonspecific binding to the cuvette. The presence of bisANS in the buffer prevented the nonspecific binding; therefore the pretreatment of the cuvettes was eliminated. The folding reaction was monitored by fluorescence. Fluorescence excitation was at 280 nm. The fluorescence emission at 340 or 495 nm was monitored with time. The kinetic data was analyzed by computer and fit to a single exponential by the formula for a first-order reaction, $F_t = F_\infty - \Delta F(e^{-kt})$. The relaxation time, τ, was k^{-1}.

Kinetics of Aggregation. Shells were unfolded at 2 mg/mL in a final concentration of 4.4 M GuHCl. Unfolded coat protein was rapidly diluted to a concentration of 50 μg/mL into buffer or buffered bisANS at 15 μM. The kinetics were monitored by light scattering with a Hitachi F-4500 fluorescence spectrophotometer with the excitation and emission wavelengths set to 500 nm. The excitation and emission bandwidths were 5 and 10 nm, respectively, and the photomultiplier tube voltage was set to 400 V.

RESULTS

Unfolding and Refolding of Coat Subunits. In the previous work on the control of subunit polymerization, soluble coat subunits were prepared by dissociation of empty procapsid shells. These are shells from which the scaffolding has been removed by repeated extraction with 0.5 M GuHCl, leaving the coat protein lattice intact (Prevelige *et al.*, 1988). The shells were dissociated to subunits by incubation with 3 M GuHCl, and the GuHCl was removed by dialysis. It was unclear in these experiments if the dissociated subunits remained partially folded (Prevelige *et al.*, 1988).

Coat protein has 430 amino acids with six tryptophanyl residues evenly distributed throughout the linear amino acid sequence (Eppler *et al.*, 1991). The 600-Å-diameter shell scatters light significantly (Prevelige *et al.*, 1988). To examine the relationship between shell dissociation and subunit denaturation, empty procapsid shells were incubated with varying concentrations of GuHCl and the tryptophan fluorescence and light scattering were monitored until the signals were constant with time (Figure 1A). The fluorescence and the light scattering curves were coincident over most of the transition, indicating that dissociation and denaturation were coupled. Both displayed a high degree of cooperativity, as would be expected for a lattice whose stability depends on the lattice interactions. The small shoulder on the transition curve detected by fluorescence probably represents residual structure in the dissociated protein subunits at intermediate GuHCl concentrations. The shape of the transition curves was not sensitive to protein concentration.

Under the dilute conditions of the dissociation reaction, coat subunits cannot initiate shell formation. Scaffolding subunits are required for assembly as well as coat subunit concentrations above 0.3 mg/mL (Prevelige et al., 1988, 1993b). Thus, in the transition region shells are dissociating, but it is unlikely that assembly could be reinitiated by nucleation. However, the edges of partially dissociated shells could serve as substrates for the polymerization of dissociated subunits, generating an apparent equilibrium. We refer to these results as apparent equilibrium curves to operationally distinguish them from the kinetic experiments described below.

The fluorescence emission maximum shifted from 335 to 350 nm when the particles were dissociated and denatured, consistent with increased exposure of coat protein tryptophans to solvent (Figure 2). No change in the fluorescence of the dissociated shells occurred above 2.4 M GuHCl, suggesting that the protein was fully denatured. Nonetheless, it remained

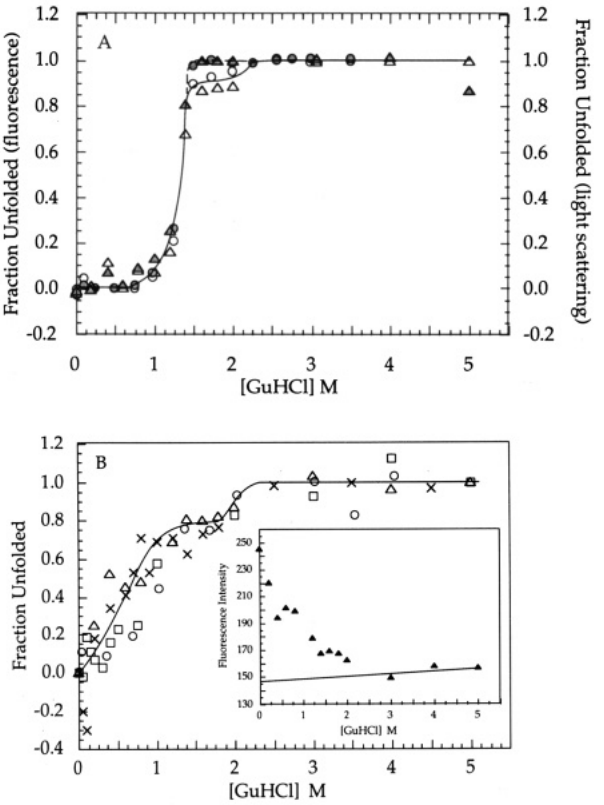

FIGURE 1: Unfolding of empty procapsid shells and refolding of denatured coat protein to apparent equilibriums. Panel A: Shells, 20 or 50 μg/mL, were added to increasing concentrations of buffered GuHCl. When equilibrium was reached after incubation overnight, the fraction unfolded was determined as described in Materials and Methods from the fluorescence emission at 340 nm (open circles and triangles, solid line) when excited at 280 nm and the light scattering at 340 nm (shaded circles and triangles, dashed line). The circles and triangles represent two separate experiments. Panel B: Coat protein, unfolded in 5.2 M GuHCl, was added to decreasing concentrations of buffered GuHCl at 20 μg/mL. When equilibrium was reached at 6–7 h, the fraction unfolded was determined from the fluorescence emission at 340 nm when excited at 280 nm (○, □, △, ×, —). The different symbols represent separate determinations. The inset shows a representative experiment indicating the unfolded plateau used in the normalization of the refolding data. This baseline was characteristic of all the determinations. The curves are added to aid the eye and are not meant to represent the fit to any model.

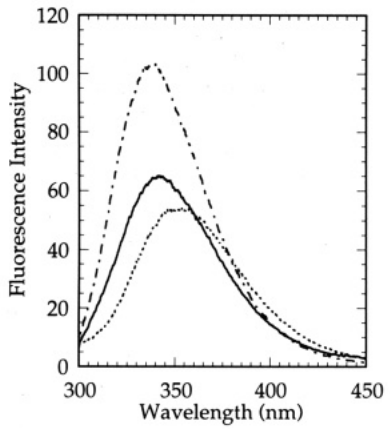

FIGURE 2: Fluorescence emission spectra of unfolded coat protein in 6 M GuHCl (···), coat protein refolded by rapid dilution (—), and empty procapsid shells (–•–). The spectra were all corrected for baseline fluorescence. The protein concentrations were 5 μg/mL. The excitation wavelength was 280 nm.

possible that there was residual structure at such intermediate GuHCl concentrations.

To examine the refolding of completely unfolded coat protein, shells were made 5.2 M in buffered GuHCl. The unfolded coat protein samples were rapidly diluted from 5.2 M GuHCl into buffer so that the protein concentration was 25 μg/mL or less and the final GuHCl concentration was 64 mM or less. Under these conditions the polypeptides remained soluble and displayed the fluorescence spectrum shown by the solid line in Figure 2. Samples diluted to concentrations higher than 50 μg/mL generated aggregates, detectable by an increase in light scattering. Samples dialyzed from 2 M GuHCl following the protocol of Prevelige *et al.* (1988) that produces assembly-competent coat protein yielded fluorescence spectra very similar to those of the coat subunits folded by rapid dilution.

To examine the refolding of coat polypeptides more carefully, unfolded coat protein was added to decreasing concentrations of buffered GuHCl and the fluorescence was recorded after incubation for 6–7 h, when no spectral changes were occurring (Figure 1B). The fluorescence refolding curve indicates that the solution conformation of coat subunits was destabilized at very low GuHCl concentrations. Although the data in this curve were scattered, most likely due to the propensity of coat protein folding intermediates to aggregate, there appeared to be two transitions in the refolding curve. A species was populated around 1 M GuHCl with 20% of the fluorescence change of the whole protein. This is presumably the same species observed in Figure 1A during the unfolding transition of the shells. Two transitions have also been observed in the pressure-induced unfolding of coat protein (P. Prevelige, J. King, and J. Silva, manuscript in preparation). These data suggest the presence of a relatively stable domain in the coat protein subunits.

Fluorescence of Coat Protein Species. As shown in Figure 2, coat protein denatured in 5.2 M GuHCl had a fluorescence intensity of 54 units and an emission maximum of 352 nm. Coat protein monomers refolded by rapid dilution had a higher fluorescence intensity of 65 units and an emission maximum that was shifted to 342 nm, indicating that some of the tryptophans became inaccessible to the solvent during folding. Shells had the highest fluorescence intensity, 102 units, and an emission maximum that was shifted to 335 nm. The tryptophans that were accessible to the solvent in the soluble coat subunits are probably buried at subunit interfaces in the shell structure, leading to the increased fluorescence intensity.

Sucrose Gradient Sedimentation of Refolded Coat Protein. Given the polymeric state of coat protein in virions, it was important to determine the association state of the molecules refolded from GuHCl. Denatured coat subunits were refolded by rapid dilution into buffer at 12.5 μg/mL and 32 mM residual GuHCl at 25 °C. As a control, coat protein was prepared by the dissociation of shells and dialysis protocol of Prevelige *et al.* (1988) that yielded coat protein monomers that were assembly-competent. Equal amounts of protein were loaded onto 5–20% sucrose gradients as described in Materials and Methods. Figure 3 shows the sedimentation profiles of refolded coat protein and bovine serum albumin used as a sedimentation marker. The coat protein prepared by dialysis or by rapid dilution gave the same sedimentation profile. The 47-kDa coat protein sedimented as expected of monomers, with no evidence of higher order oligomers with the exception of a small amount of rapidly sedimenting material in the protein produced by dialysis. This large material was absent in the coat protein prepared by rapid dilution. These data established

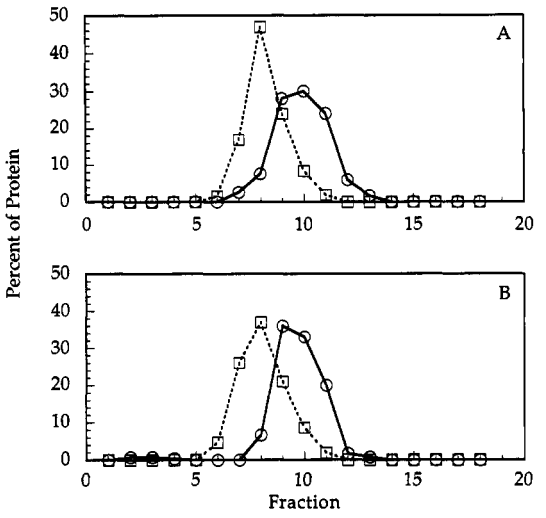

FIGURE 3: Refolded coat protein was monomeric. The distribution of coat protein (O) and bovine serum albumin (□) in an isokinetic sucrose gradient is shown. Fraction 1 is the bottom of the gradient. Panel A: Distribution of coat protein refolded at 12.5 μg/mL by rapid dilution into buffer at 25 °C. Panel B: Distribution of coat protein refolded by dialysis at 1 mg/mL from 2 M GuHCl at 4 °C.

Table I: Stern–Volmer Constants for the Quenching by Acrylamide and Iodide[a]

	K_{SV} (M^{-1})	
	iodide	acrylamide
shells	0.32	1.12
folded coat protein	1.33	3.34
unfolded coat protein	3.30	6.16
kinetic folding intermediate	nd	4.34

[a] The Stern–Volmer constants were determined from the slope of Stern–Volmer plots. nd, not determined.

that the coat protein produced by rapid dilution was monomeric.

We were particularly interested in obtaining data that could be extrapolated to the intracellular cytoplasmic environment. Therefore, we concentrated on the refolding reaction after dilution into physiological buffer, rather than pursuing the unfolding of the soluble subunits in the presence of denaturants.

Quenching of Fluorescence. To examine more closely the solvent accessibility of the tryptophans, collisional quenching of fluorescence by iodide ion and acrylamide was examined (Lakowicz, 1983). Iodide is a charged and hydrated quencher, while acrylamide is a neutral and polar quencher (Eftink & Ghiron, 1981).

Stern–Volmer plots of the quenching by acrylamide or iodide ion of coat protein unfolded with 6 M GuHCl, folded by dialysis from GuHCl, or shells were analyzed. The slope of the line in a Stern–Volmer plot is the Stern–Volmer constant, K_{SV} (Eftink & Ghiron, 1981). A steep slope indicates that the tryptophans are exposed to the quenching agent, whereas a shallow slope indicates that the tryptophans are protected from the quencher.

The tryptophans within the shells were the least accessible to the quenching agents, while the tryptophans of unfolded coat protein were most accessible. The folded coat protein monomers showed intermediate levels of quenching as compared to the shells and unfolded coat protein. The K_{SV} values for the quenching are shown in Table I. These data indicated that the folded coat protein had solvent-exposed tryptophans and that the tryptophans of shells were buried within the shell lattice since their fluorescence was virtually unaffected by the quenchers.

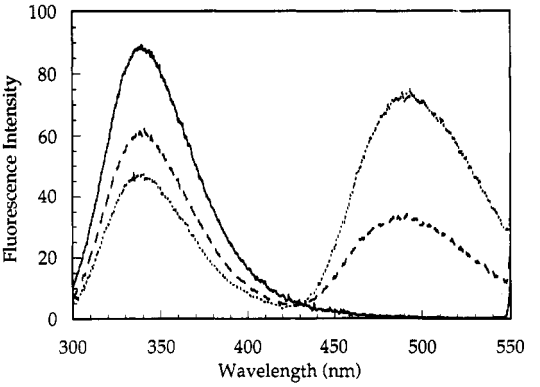

FIGURE 4: Nonradiative energy transfer between tryptophans of the coat protein and bound bisANS. The excitation wavelength was 280 nm and the spectra were corrected for the baseline fluorescence. The scans were done at the following bisANS concentrations: (—) 0 μM, (- - -) 2.5 μM, and (· · ·) 5 μM. The protein concentration was 5 μg/mL (100 nM).

Binding of BisANS to Folded Coat Protein. To examine if the tryptophans that were accessible to the solvent were localized on the surface of the folded coat subunits in hydrophobic regions, an environmentally sensitive, hydrophobic fluorescence probe, bisANS, was used. BisANS has low fluorescence in polar solvents but high fluorescence when in a nonpolar environment (Farris *et al.*, 1978). The energy of excited tryptophans can be transferred nonradiatively to bisANS (Prasad *et al.*, 1986; Secnik *et al.*, 1990). The transfer of energy causes a decrease in the fluorescence emission of the tryptophans and an increase in the fluorescence emission of the bisANS if the tryptophan and the bisANS are in close proximity, ≤20–50 Å (Lakowicz, 1983).

Figure 4 shows the transfer of the fluorescence energy from tryptophans of folded coat protein to bisANS. When coat protein was excited at 280 nm there was emission at 340 nm. The addition of low concentrations of bisANS to the protein solution caused a decrease in the emission at 340 nm and an increase in the bisANS emission at 495 nm due to nonradiative energy transfer. This indicated that a bisANS bound near a tryptophan. BisANS is probably too bulky to intercalate into the protein itself, suggesting that a bisANS molecule is binding on a hydrophobic patch on the surface of the protein. Shells have been shown to bind bisANS with at least an order of magnitude less affinity than coat subunits (Teschke *et al.*, 1993).

The binding of the bisANS molecules to coat protein can be determined from either the quenching of the tryptophan fluorescence or the increase in fluorescence of the bisANS (Prasad *et al.*, 1988; Bagshaw & Harris, 1987). The fluorescence of the tryptophans was quenched ~3-fold and the fluorescence of bisANS increased ~600-fold. Both the quenching of the fluorescence of the coat protein tryptophans (three separate determinations) and the enhancement of the bisANS fluorescence (three separate determinations) indicated that 1.24 ± 0.36 bisANS molecules bound to each coat protein subunit with a K_d of 7.23 ± 1.37 μM (data not shown). If all six tryptophans in coat protein have the same quantum yield, then approximately four were quenched by the bisANS.

Kinetic Analysis of the Refolding of Coat Protein. Intrinsic fluorescence of tryptophans was utilized to follow the kinetics of refolding of coat protein upon dilution from denaturants into buffer. Unfolded coat protein was rapidly diluted 200-fold into either buffer (Figure 5A) or buffer containing bisANS (Figure 5B) and monitored with time. When unfolded coat protein was rapidly diluted into buffer, there was an initial jump in the fluorescence that corresponded to the addition of

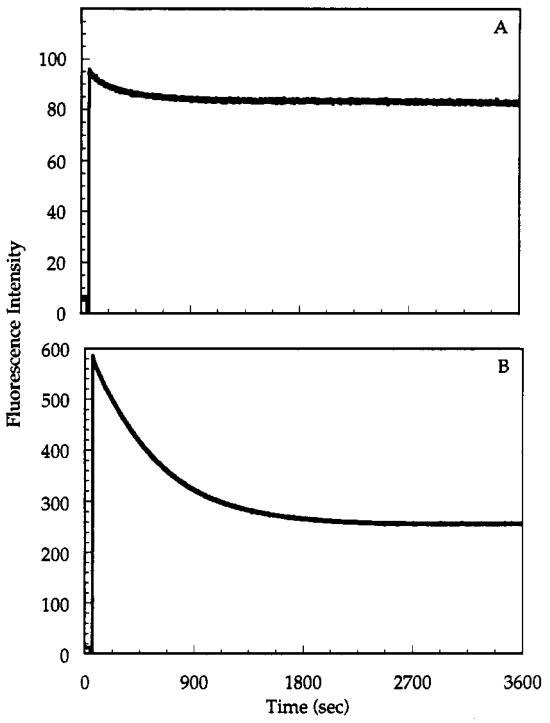

FIGURE 5: Refolding of coat protein after rapid dilution from denaturant. The coat protein, 5 μg/mL, was rapidly diluted into either buffer (panel A) or buffer plus 2.5 μM bisANS (panel B) 1 min after the scan was initiated. The excitation wavelength was 280 nm and the emission wavelength was 340 nm (panel A) or 495 nm (panel B).

protein as well as additional fluorescence. The fluorescence decreased ~15% with time until a plateau was reached. The fluorescence value of the plateau corresponded to that of the coat protein folded by dialysis. The relaxation time for the reaction was ~300 s and fit well to a single exponential. The relaxation time was independent of concentration over a 5-fold concentration range and the 90° light scattering at 340 nm measured in the fluorometer was linear over that range. By analytical ultracentrifugation, folded coat protein does not display oligomers in solution at concentrations 100-fold greater than those used here (P. E. Prevelige, Jr., and T. Laue, personal communication). Thus, it is unlikely that the decrease in fluorescence was due to aggregation or polymerization.

To determine the extent of the tertiary structure in the kinetic intermediate observed upon rapid dilution from denaturant, an emission scan was done immediately after the dilution (Figure 6). Within the 38 s necessary for the scan to be completed, the emission maximum was 340 nm, rather than the 350-nm maximum of the unfolded coat protein, indicating that tryptophans were already buried in the kinetic intermediate of coat protein. The secondary structure, as monitored by circular dichroism, did not change with time after rapid dilution from denaturant, indicating that the fluorescence change was not due to alterations in the secondary structure (data not shown).

The quenching of the tryptophans of the kinetic intermediate was determined by extrapolation to the initial fluorescence after rapid dilution of unfolded coat protein into increasing concentrations of buffered acrylamide, as a measure of the solvent accessibility of the tryptophans. If the kinetic intermediate was in a conformation similar to the unfolded polypeptide in secondary and tertiary structure, the quenching of the tryptophans would be expected to be large. However, the tryptophans were only slightly more exposed to solvent in the kinetic intermediate than in the folded coat subunits,

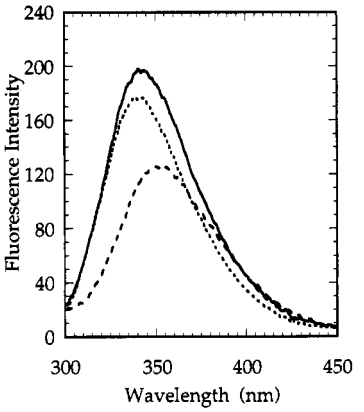

FIGURE 6: Fluorescence emission spectra of unfolded coat protein in 6 M GuHCl (- - -), the kinetic intermediate (—), and folded coat protein (···). The emission scan of the kinetic intermediate was initiated within 10 s after rapid dilution of unfolded coat protein from GuHCl into buffer, and the scan took 38 s to complete. The spectra of the folded coat protein was taken 30 min after rapid dilution. The excitation wavelength was 280 nm. The protein concentration was 10 μg/mL.

indicating that the tryptophans were already protected from solvent (Table I). Accordingly, the kinetic intermediate appears to be collapsed, not random coil, and the decrease in fluorescence is most likely due to a rearrangement of the hydrophobic environment surrounding some of the tryptophans from this collapsed state.

To determine if there was a change in the hydrophobicity during the folding reaction, unfolded coat protein was rapidly diluted into bisANS. When unfolded coat protein was rapidly diluted into buffer containing bisANS and monitored at the emission maximum of the bisANS, there was a large increase in the fluorescence followed by a decrease in fluorescence to a plateau (Figure 5B). The fluorescence due to the binding of bisANS indicates that there are hydrophobic sites on the kinetic intermediate. The bisANS fluorescence decreased with time, suggesting that the hydrophobic sites decrease in either number or affinity.

Binding of BisANS to the Kinetic Intermediate. To investigate if the change in bisANS fluorescence with folding was due to a change in the affinity for bisANS by coat protein or a decrease in the number of sites on coat protein, the binding of the bisANS to the kinetic intermediate was measured. Coat protein was refolded into increasing concentrations of bisANS and the fluorescence was extrapolated to the initial fluorescence at time zero. The binding was determined as described in Materials and Methods by direct enhancement of bisANS fluorescence in three separate determinations. The kinetic intermediate was found to have 10.80 ± 1.20 binding sites for the bisANS with a K_d of 2.11 ± 1.10 μM. Thus, it is not the affinity of the kinetic intermediate for bisANS that changes with time but the number of sites.

Kinetics of Folding of the Coat Protein in BisANS. To determine if bisANS had an effect on the folding of the coat protein, the relaxation time of folding was determined in increasing concentrations of bisANS. The relaxation time increased 86 s with each 1 μM increase of bisANS, indicating that the bisANS slowed the folding of the coat protein (data not shown). The decrease in the rate of folding with increasing bisANS concentrations probably indicates that the bisANS stabilizes the kinetic folding intermediate.

Kinetics of Aggregation. To determine if bisANS had an effect on aggregation of the coat polypeptides, coat protein was refolded at high concentrations. Coat protein was rapidly

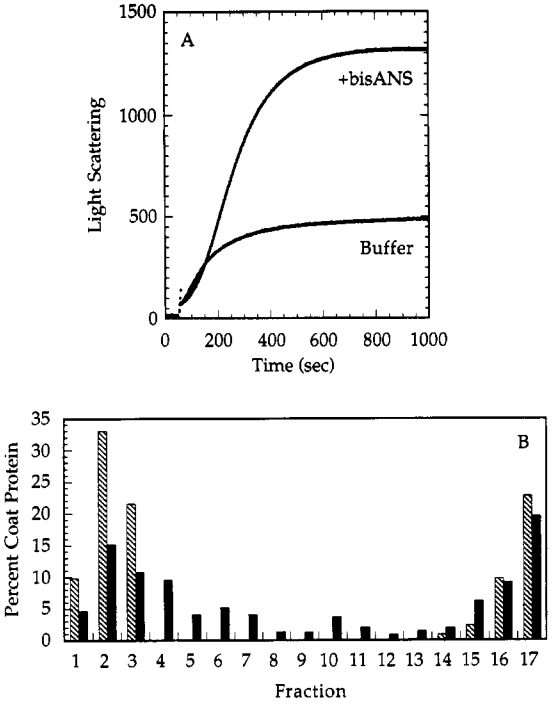

FIGURE 7: Aggregation of folding intermediates. Panel A: Light scattering at 500 nm monitored with time. Unfolded coat protein was rapidly diluted into buffer or buffer with 15 μM bisANS at 25 °C at 50 μg/mL. Panel B: Distribution of coat protein folded as in panel A in buffer (hatched bars) or buffer supplemented with 15 μM bisANS (solid bars) by sucrose gradient sedimentation.

diluted to 50 μg/mL in either buffer or buffer supplemented with bisANS and the 90° light scattering at 500 nm was monitored with time (Figure 7A). Coat protein refolded into buffer exhibited an increase in light scattering. Analysis of the products of the refolding reaction in buffer by sucrose gradient centrifugation demonstrated that 65% of the coat protein was soluble (Figure 7B). When coat protein was refolded into buffered bisANS, the light scattering increased beyond that of the coat protein refolded into buffer. Analysis of the products of the refolding in bisANS by sucrose gradient centrifugation showed that the distribution of coat protein had altered such that nearly 70% of the protein was shifted from the position of soluble protein at the top of the gradient.

The aggregates formed in the refolding reactions could be similar to inclusion bodies formed of partially folded polypeptides lacking biological activity (Rudolf *et al.*, 1979; Mitraki & King, 1989; Gordon & King, 1993). Another possibility is that the aggregates could be the product of an aberrant polymerization of nativelike subunits, seen in cells infected with mutants defective in scaffolding protein (Lenk *et al.*, 1975; Earnshaw & King, 1978). To distinguish between these possibilities, the aggregates formed in the refolding experiments described above were examined by negative stain electron microscopy. The samples appeared to have the heterogeneous morphology associated with inclusion bodies and not the distinctive morphology of the aberrant polymerization reaction (data not shown).

DISCUSSION

Newly synthesized coat polypeptide chains pass through a number of conformational states in the course of their cytoplasmic assembly into infectious virions. These include folding intermediates; soluble subunits; the procapsid, where coat protein subunits are interacting with other coat protein subunits and scaffolding protein; and the mature capsid, where the coat protein subunits associate with each other in the absence of scaffolding protein. Within both the procapsid and mature phage, multiple conformations can be distinguished at the different positions within the $T = 7$ icosahedral lattices (Prasad *et al.*, 1993).

Coat Protein Refolding in Vitro. Within the cell, newly synthesized coat protein polypeptides are released from the ribosomes and fold into the assembly-competent state. Here we have shown that the refolding of assembly-competent subunits *in vitro* in the absence of additional factors can be achieved at low protein concentrations from coat protein fully denatured by GuHCl. These refolded subunits were not distinguishable from the monomeric subunits characterized by Fuller and King (1981, 1982) and Prevelige *et al.* (1988, 1993b) that are the kinetic precursors in the procapsid assembly reaction.

The kinetic intermediate that was observed during refolding of coat protein rapidly diluted from denaturant could be distinguished from the native state by both its increased intrinsic fluorescence and number of binding sites for bisANS. The difference in the number of bisANS binding sites between the intermediate and folded conformations could be accounted for by an intermediate with a molten globule conformation, or one domain folded and the other significantly unfolded, or with two (or more) domains folded but not yet docked against each other.

The intermediate observed in the apparent equilibrium refolding experiments had only 20% of the fluorescence of the folded protein and a shifted emission maximum of 348 nm that falls between the emission maxima of the folded and unfolded proteins. Coat protein may contain a hinge that allows movement during expansion between domains that are involved in lattice contacts, since the procapsid shell expands in radius without any covalent modification (Earnshaw & King, 1978; Casjens, 1979; C. M. Teschke, unpublished results). It is possible that the equilibrium folding intermediate observed represents a species that has one folded domain and one unfolded domain.

The following pathway of folding can account for the results presented here:

$$U \longleftrightarrow [I] \longleftrightarrow [I'] \longleftrightarrow N_S$$
$$\downarrow$$
$$\text{aggregates}$$

In this pathway U represents the coat polypeptide denatured in 5.2 M GuHCl, [I] is the intermediate detected in the apparent equilibrium refolding experiments, [I′] is the intermediate with multiple bisANS binding sites identified in the kinetic experiments, and N_S represents the folded, assembly-competent subunit. Although we have not determined its kinetic relationship to [I′], we have placed [I] as preceding [I′] in the pathway above, on the basis of its fluorescence spectrum, which is less nativelike than that of [I′]. The solution conformation of N_S probably differs from the conformation of coat subunits within the lattice of the assembled shells.

The refolded coat protein has a bisANS binding site that is absent in shells and procapsids (Teschke *et al.*, 1993). The tryptophans of the shells are protected from quenching agents, while the tryptophans of the folded coat subunits are not. The hydrophobic site bound by bisANS is presumably involved in the subunit interfaces in the shells.

Studies of Coat Protein with Altered Amino Acid Sequences. Seventeen sites of temperature-sensitive folding (*tsf*)

mutants have been defined in the coat protein (Gordon & King, 1993). Coat polypeptides with these amino acid substitutions fold and assemble into procapsids and mature virions at low temperature. At high temperature, the *tsf* polypeptides are unable to fold into assembly-competent subunits but instead form inclusion bodies. Gordon and King (1993) interpreted these results as identifying an intermediate *in vivo* that was blocked from proceeding down the productive folding pathway and formed inclusion bodies. We are particularly interested in the possibility that the intermediates described in the folding experiments *in vitro* are related to the intermediates populated in cells infected with *tsf* mutants. Experiments on the refolding of coat protein with *tsf* amino acid substitutions are underway.

Differences in the Stability of Free or Assembled Coat Protein Subunits. Just as native proteins do not significantly unfold under physiological conditions, mature viral capsids do not detectably dissociate into their component subunits. Yet, the properties of the soluble coat protein subunit suggests that it is not particularly stable. The thermal denaturation of procapsid shells has been studied by differential scanning calorimetry. The shells dissociate and denature at temperatures above 80 °C (Galisteo & King, 1993). The thermal denaturation of the free coat protein subunits occurs around 40 °C (M. L. Galisteo and J. King, unpublished results). These results suggest that the stability of the mature virion is associated with lattice stabilization at the interfaces between subunits.

During the guanidine-induced dissociation and unfolding of the shells, an apparent equilibrium was established between shells and dissociated, denatured subunits. However, coat subunits at these protein concentrations cannot initiate shell assembly since scaffolding subunits are lacking and the coat subunits are below the critical concentration. Electron microscopy of samples in the transition region of the curve after the apparent equilibrium was established revealed large numbers of partial shells. Such structures could grow by addition of subunits to the shell edge, bypassing the need for the nucleation step with its fifth-order dependence on coat concentration (Prevelige *et al.*, 1993b). An equilibrium condition probably represents subunits dissociating from the shell edge and denaturing and subunits refolding and binding back to the shell edge, thus stabilizing the subunit conformation.

Some quantitative assessments of viral capsid subunit interactions have been made using crystallographic data that suggest there can be as much as a 100 kcal/mol difference in the free energies of association between the most stable putative intermediates in assembly. Even when assembly intermediates are not considered, each subunit has a free energy of association of 10–20 kcal/mol (Horton & Lewis, 1992).

Aggregation versus Polymerization. At higher polypeptide concentrations, aggregation competed with productive folding. Aggregation of folding intermediates frequently competes with refolding, particularly in the folding of multimeric proteins (Jaenicke, 1987; Mitraki & King, 1989). Two aggregated states have been identified for coat protein. In the absence of scaffolding protein, a fraction of coat protein polymerizes into aberrant spiral structures as well as shells of incorrect dimensions (Earnshaw *et al.*, 1978). These structures can also be formed *in vitro* from assembly-competent coat protein subunits in the absence of scaffolding protein (Prevelige *et al.*, 1990). These aggregates are incorrectly polymerized shells and are composed of nativelike subunits. BisANS inhibits this form of uncontrolled polymerization (Teschke *et al.*, 1993).

The second of the aggregated states is the inclusion bodies found in cells infected with temperature-sensitive mutants in coat protein (Gordon & King, 1993). The inclusion bodies are probably derived from the polymerization of intracellular folding intermediates (Mitraki & King, 1989; Mitraki *et al.*, 1991). The aggregates formed *in vitro* most likely represent this class. Since bisANS both slows the folding of the coat protein and causes an increase in the propensity of the coat protein to aggregate, the kinetic intermediate may be the precursor to both the folded soluble subunit and the aggregated protein (Rudolf *et al.*, 1979; Mitraki & King, 1989). BisANS may promote aggregation by stabilizing the conformation of the intermediate at the junction between the pathway to aggregation and productive folding.

When *in vitro* refolding experiments were carried out at the protein concentrations required for the capsid polymerization reaction, above 0.3 mg/mL, the unfolded polypeptides failed to reach the native state but instead accumulated as inactive aggregates. The formation of the aggregated, inclusion body state from folding intermediates is a general feature of both *in vivo* and *in vitro* folding reactions, particularly for multimeric proteins (Rudolph *et al.*, 1979; Mitraki & King, 1989; Teschke & King, 1992). Within infected cells, coat polypeptides are able to fold into functional subunits at high protein concentrations. We are especially interested in the mechanisms by which the folding intermediates avoid the kinetically trapped aggregated state within the cell. In fact, coat protein *tsf* mutants are rescued *in vivo* by overproduction of the GroEL and GroES chaperonins, indicating a role for host chaperones (C. Gordon, S. Sather, and J. King, unpublished results; Ellis & van der Vies, 1991; Van Dyk *et al.*, 1989).

What Is the "Native" Conformation of a Structural Protein? Native conformations of proteins are usually defined by their mature structure. Though the 3D structures of many viral proteins have been determined by X-ray crystallography, in no case has the structure of an unpolymerized capsid subunit been solved. It seems likely that a coat subunit which has not interacted with scaffolding protein and has not passed through the polymerization reaction might represent a different conformation than its own assembled state. Soluble coat protein subunits might have a unique conformation or a flexible domain whose structure is defined only upon polymerization.

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REFERENCES

Bagshaw, C. R., & Harris, D. A. (1987) in *Spectrophotometry & spectrofluorometry, a practical approach.* (Harris, D. A., & Bashford, C. L., Eds.) pp 91–114, IRL Press, Eynsham, England.

Botstein, D., Waddell, C. H., & King, J. (1973) *J. Mol. Biol. 80*, 669–695.

Casjens, S. (1979) *J. Mol. Biol. 131*, 1–13.

Casjens, S., & King, J. (1975) *Annu. Rev. Biochem. 44*, 555–611.

Casjens, S., & Hendrix, R. (1988) in *The Bacteriophages* (Calender, R., Ed.) Vol. 1, pp 15–91, Plenum Publishing, New York.
Caspar, D. L. D. (1976) in *Structure–function relationships of proteins*, Proceedings of the third John Innes symposium (Markham, R., & Horne, R. W., Eds.) pp 85–99, Elsevier, New York.
Cheng, R. H., Olson, N. H., & Baker, T. S. (1992) *Virology 186*, 655–668.
Creighton, T. E. (1987) in *Protein Engineering* (Oxender, D. L., & Fox, C. F., Eds.) pp 83–90, Alan R. Liss, Inc., New York.
D'Halluin, J. C., Milleville, M., Boulanger, P., & Martin, G. R. (1978) *J. Virol. 26*, 344–356.
Earnshaw, W., & King, J. (1978) *J. Mol. Biol. 126*, 721–747.
Earnshaw, W., & Casjens, S. (1980) *Cell 21*, 319–331.
Earnshaw, W., Casjens, S., & Harrison, S. C. (1976) *J. Mol. Biol. 104*, 387–410.
Earnshaw, W., King, J., Harrison, S. C., & Eiserling, F. A. (1978) *Cell 14*, 559–568.
Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.
Ellis, R. J., & van der Vies, S. M. (1991) *Annu. Rev. Biochem. 60*, 321–347.
Eppler, K., Wykoff, E., Goates, J., Parr, R., & Casjens, S. (1991) *Virology 183*, 519–538.
Farris, F. J., Weber, G., Chiang, C. C., & Paul, I. C. (1978) *J. Am. Chem. Soc. 100*, 4469–4474.
Frydman, J., Nimmesgern, E., Erdjument-Bromage, H., Wall, J. S., Tempst, P., & Hartl, F. U. (1992) *EMBO J. 11*, 4767–4778.
Fuller, M. T., & King, J. (1980) *Biophys. J. 32*, 381–401.
Fuller, M. T., & King, J. (1981) *Virology 112*, 529–547.
Fuller, M. T., & King, J. (1982) *J. Mol. Biol. 156*, 633–665.
Galisteo, M. L., & King, J. (1993) *Biophys. J. 65*, 227–235.
Gao, Y., Thomas, J. O., Chow, R. L., Lee, G. H., & Cowan, N. J. (1992) *Cell 69*, 1043–1050.
Gordon, C. L., & King, J. (1993) *J. Biol. Chem. 268*, 9358–9368.
Harrison, S. C., Olson, A. J., Schutt, C. E., Winkler, F. K., & Bricogne, G. (1978) *Nature 276*, 368–373.
Horton, N., & Lewis, M. (1992) *Protein Sci. 1*, 169–181.
Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117–237.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631–60.
Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum, New York.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.
Lenk, E., Casjens, S., Weeks, J., & King, J. (1975) *Virology 68*, 182–199.
Liddington, R. C., Yan, Y., Moulsi, J., Sahli, R., Benjamin, T. L., & Harrison, S. C. (1992) *Science 354*, 278–284.
Mitraki, A., & King, J. (1989) *Bio/Technology 7*, 690–697.
Mitraki, A., Haase-Pettingell, C., & King, J. (1991) in *Protein refolding* (Georgiou, G., & deBernardez, E., Eds.) American Chemical Society, Washington, DC.
Morin, N., & Boulanger, P. (1984) *Virology 136*, 153–167.
Newcomb, W. W., & Brown, J. C. (1991) *J. Virol. 65*, 613–620.
O'Callaghan, D. J., Kemp, M. C., & Randall, C. C. (1977) *J. Gen Virol. 37*, 585–594.
Prasad, A. R. S., Luduena, R. F., & Horowitz, P. M. (1986) *Biochemistry 25*, 3536–3540.
Prasad, B. V. V., Prevelige, P. E., Marieta, E., Chen, R. O., Thomas, D., King, J., & Chiu, W. (1993) *J. Mol. Biol. 231*, 65–74.
Preston, V. G., Coates, J. A. V., & Rixon, F. J. (1983) *J. Virol. 45*, 1056–1064.
Prevelige, P., Thomas, D., & King, J. (1988) *J. Mol. Biol. 202*, 743–757.
Prevelige, P. E., Thomas, D., King, J., Towse, S. A., & Thomas, G. J. (1990) *Biochemistry 29*, 5626–5633.
Prevelige, P. E., Thomas, D. H., Aubrey, K. L., Towse, S. A., & Thomas, G. J. (1993a) *Biochemistry 32*, 337–343.
Prevelige, P. E., Thomas, D., & King, J. (1993b) *Biophys. J. 64*, 824–835.
Rabilloud, T., Carpentier, G., & Tarroux, P. (1988) *Electrophoresis 9*, 288–291.
Rossman, M. G., & Erickson, J. W. (1985) in *Virus Structure and Assembly* (Casjens, S., Ed.) pp 29–74, Jones & Barlett Publishers, Inc., Boston, MA.
Rudolph, R., Zettlmeissl, G., & Jaenicke, R. (1979) *Biochemistry 18*, 5572–5575.
Salunke, D. M., Caspar, D. L. D., & Garcea, R. L. (1989) *Biophys. J. 56*, 887–900.
Secnik, J., Wang, Q., Chang, C.-M., & Jentoff, J. E. (1990) *Biochemistry 29*, 7991–7997.
Teschke, C. M., & King, J. (1992) *Curr. Opin. Biotechnol. 3*, 468–473.
Teschke, C. M., & King, J., & Prevelige, P. E. (1993) *Biochemistry* (in press).
Van Dyk, T. K., Gatenby, A. A., & LaRossa, T. A. (1989) *Nature 342*, 451–453.
Webb, J. L. (1963) in *Enzyme and Metabolic Inhibitors*, Vol. I, p 71, Academic Press, New York.