Accessibility and Dynamics of Cys Residues in Bacteriophage IKe and M13 Major Coat Protein Mutants†

Amir R. Khan,‡,§ Karen A. Williams,‡ Joan M. Boggs,‖ and Charles M. Deber*,‡

Division of Biochemistry Research, Research Institute, Hospital for Sick Children, Toronto M5G 1X8, Ontario, Canada, Department of Biochemistry, University of Toronto, Toronto M5S 1A8, Ontario, Canada, and Department of Clinical Biochemistry, University of Toronto, Toronto M5G 1L5, Ontario, Canada

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ABSTRACT: The filamentous bacteriophage major coat protein occurs as a membrane-spanning assembly intermediate prior to incorporation into the lipid-free virion. To gain insight into how this small, multifunctional protein is able to be stably incorporated into both of these distinct environments, the reactive sulfhydryl group of IKe and M13 coat protein Cys mutants was exploited to probe the mobility and environment of this residue at several loci within the hydrophobic domain of these proteins. IKe mutants P30C, G39C, and G39C-V36A and M13 mutant Y24C-V31A, each previously obtained from randomized mutagenesis, were characterized in the intact virion, the intermediate spheroidal S-form, and in membrane-mimetic sodium dodecyl sulfate (SDS) micelles. The accessibility of the Cys sulfhydryl in the virion was examined by reaction with [^{14}C]iodoacetamide (^{14}C-IAN) and other alkylating agents. The IKe mutants G39C and G39C-V36A were found to be the most reactive with ^{14}C-IAN and thus the most accessible, although this accessibility was subject to strict steric constraints since only the smallest sulfhydryl-specific alkylating agents were able to modify the Cys^{39} locus. The spin probe proxyliodoacetamide (PIAN) was used to characterize Cys side chain mobility by electron paramagnetic resonance (EPR) spectroscopy. The M13 mutant Y24C-V31A Cys side chain in the phage was observed to be the most mobile, with slightly less mobility for IKe mutant P30C and considerably less for G39C mutants. The SDS micelle-bound forms of the Cys mutants all exhibited enhanced side chain mobility compared to the virion form, with the extent of mobility dependent upon the specific location of the Cys residue. EPR and fluorescence quenching results show that the Cys side chain in the Y24C-V31A S-form is largely immobilized and inaccessible in comparison to the virion and micelle-solubilized forms. The overall results are interpreted in terms of the structural changes accompanying disassembly and insertion of the coat protein into the *Escherichia coli* inner membrane.

Filamentous bacteriophages are long flexible rods made up of α-helical protein subunits surrounding a core of circular ssDNA so that the length of the phage (9800 Å) is greater than 100 times its diameter (80 Å) [for a review, see Model and Russel (1988)]. The primary structural unit is the major coat protein (encoded by gene 8) which exists in approximately 2800 copies per virion. The architecture of bacteriophage fd/M13 has been characterized by X-ray fiber diffraction methods to ca. 7 Å resolution (Marvin et al., 1994; Glucksman et al., 1992). The virion form of the coat protein consists of a single gently curving α-helix, with the positively charged residues at the C terminus mediating electrostatic interactions with the ssDNA genome. Bacteriophage IKe and M13 belong to the class 1 phages which possess 5-fold rotational symmetry and a 2-fold screw axis. IKe and M13 share 55% overall identity with less similarity in the capsid proteins than in the proteins involved in DNA replication (Peeters et al., 1985). The primary sequences of the 50-residue M13 and 53-residue IKe coat proteins share 37% identity (Peeters et al., 1985; Nakashima et al., 1981). The two phages differ in host specificity as encoded by a conjugative plasmid; M13 is F-specific, whereas IKe is N-specific (Bradley, 1979).

The major coat protein is inserted into the inner membrane of the *Escherichia coli* host as a single-span membrane protein upon phage infection, with the concomitant release of the ssDNA genome into the cytosol (Trenkner et al., 1967; Smilowitz et al., 1972). This protein is reutilized together with newly synthesized coat protein to form the capsid of the progeny virion. The membrane-bound form of the coat protein exhibits an N-terminal amphipathic helix situated at the membrane-aqueous interface and a hydrophobic helix which spans the bilayer (van de Ven et al., 1993). When these proteins span the inner membrane of the bacterial host, they exhibit an N-terminal acidic periplasmic domain, a nonpolar transmembrane (TM) region, and a C-terminal basic domain which resides in the cytosol. In the membrane-bound form of the coat protein, the nonpolar region mediates hydrophobic interactions with the lipid, whereas in the virion, this region is responsible for maintaining the structural integrity of the capsid through hydrophobic interactions between adjacent subunits, as is evident from the resistance of phage to extremes of pH but sensitivity to organic solvents (Marvin et al., 1994). Contracted forms of the virion, termed

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* Author to whom correspondence should be addressed at the Research Institute.

‡ Research Institute and Department of Biochemistry.

§ Present address: Department of Biochemistry, University of Alberta, Edmonton T6G 2H8, Alberta, Canada.

‖ Research Institute and Department of Clinical Biochemistry.

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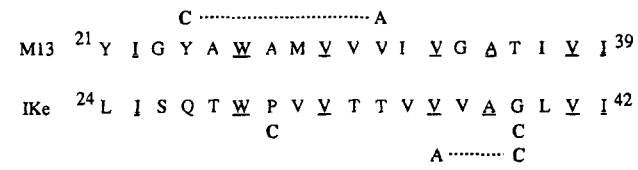


FIGURE 1: Aligned TM regions of M13 and IKe coat proteins. Identical residues are underlined. Cys mutants of M13 (Y24C-V31A) and IKe (P30C, G39C, and G39C-V36A) coat proteins are shown above and below the TM sequences, respectively. *Dashed lines indicate double mutations.* The full 50-residue sequence of M13 coat protein is AEGDDPAKAAFNSLQASATEYIGYAWAMVVVIVGATIGIKLFKKFTSKAS (Nakashima et al., 1981). The full 53-residue sequence of IKe coat protein is AEPNAATNYATEAMDSLKTQAIDLISQTWPVVTTVVVAGLVIRLFKKFSSKAV (Peeters et al., 1985).

"spheroids" or S-forms (as a consequence of their spherical appearance from electron micrographs), are formed *in vitro* upon brief exposure of the intact phages to a chloroform–water interface (Griffith et al., 1981). These particles are easily solubilized by sodium dodecyl sulfate (SDS) and may mimic an intermediate stage during phage assembly and disassembly (Dunker et al., 1991a). Phage assembly and extrusion occurs without lysis of *E. coli* cells and without incorporation of lipids into the virion.

Structural characterization of the membrane-bound form of the major coat protein by solution and solid state NMR and studies of the intact virion by X-ray fiber diffraction have been complemented with an array of other spectroscopic approaches including circular dichroism (Clack & Gray, 1989), Fourier transform–infrared spectroscopy (Azpiazu et al., 1993), and intrinsic Trp fluorescence (Roberts & Dunker, 1993). Many studies have exploited the aromatic residues of the coat protein as probes; for example, the tilt of M13 coat protein Trp^{26} indole in the virion was characterized using flow linear dichroism (Clack & Gray, 1992). The suppression of ellipticity at 208 nm in the fd phage CD spectra may be due to the stacking of Trp^{26} and Phe^{45} between adjacent subunits (Arnold et al., 1992); Raman spectroscopy confirmed that the fd Tyr residues are located in an unusual environment (Overman et al., 1994). Raman studies also suggested that the fd Trp^{26} indole may participate in H bond while in the virus (Aubrey & Thomas, 1991).

The structural properties of the TM region are critical in mediating the conformational interconversions of the coat protein during the phage life cycle. Using randomized site-directed mutagenesis, our lab has previously isolated over 100 viable mutants of M13 and IKe coat proteins with substitutions in the TM region (Li & Deber, 1991; Williams & Deber, 1993; Deber et al., 1993). Among the mutants were a subset containing Cys residues [the M13 double mutant Y24C-V31A (Khan & Deber, 1995); the IKe mutants P30C, G39C-V36A, and G39C (Williams & Deber, 1993) (Figure 1)]. The wild type sequences of IKe and M13 major coat proteins do not contain Cys (see Figure 1 legend). Cys mutants have been employed to characterize membrane protein topology (Altenbach et al., 1989), as well as the conformational changes which accompany peptide–membrane fusion (Yu et al., 1994a,b). M13 coat protein has previously been spin-labeled preferentially at the single methionine (Met^{28}) and partially at Lys residues by a nitroxide derivative of iodoacetamide under acidic conditions and reconstituted into lipid vesicles (de Jongh et al., 1990; Hemminga et al., 1992). Electron paramagnetic resonance (EPR) spectroscopy has been employed to characterize the structure of intact virions (Hemminga et al., 1977; Kruse & Hemminga, 1981).

In the present work, we have modified Cys in intact phage with a variety of sulfhydryl-specific reagents to assess the accessibility of hydrophobic regions of M13 and IKe coat proteins. One of these reagents, the sulfhydryl-specific spin-label proxyliodoacetamide (PIAN), is further utilized to probe the local mobility and solvent exposure of the accessible side chains in the intact phage, in membrane-mimetic SDS micelles, and in the S-form using fluorescence and EPR spectroscopy. These studies offer insight into the environment of several distinct loci in the hydrophobic domain of M13 and IKe coat proteins. The results are discussed in terms of the conformational changes which characterize the major coat protein during virion assembly.

METHODS

Sample Preparation. The Cys mutants were obtained from randomized mutagenesis of the TM regions of M13 and IKe coat proteins as previously described (Williams & Deber, 1993; Deber et al., 1993; Khan & Deber, 1995). WT and mutant phages were amplified by infecting liter cultures of JM101 (M13) and JM101/pCU109 (IKe) *E. coli* cells in 2-TY media (1.6% tryptone, 1% yeast extract, and 86 mM NaCl) (Li & Deber, 1991; Williams & Deber, 1993). Bacterial cultures were grown at 37 °C with shaking, except for mutant P30C phage preparations which were grown at room temperature to facilitate higher yields of the virus. Phage was precipitated using 0.2 volume of a poly(ethylene glycol)/NaCl solution (PEG/NaCl; 20% PEG and 14% NaCl), purified by density ultracentrifugation using a KBr step gradient (4 °C, Ti-28 rotor, 20 h at 24 000 rpm) (McDonnell et al., 1993), and desalted using a Centriprep-3 filter (Amicon, Inc., Beverly, MA). In each preparation, a fraction of the final purified phage solution was sequenced to verify the identity of each Cys-containing mutant protein.

Solubilization of coat protein in SDS micelles was achieved by adding an equal volume of 10% SDS and 30% v/v chloroform, with shaking, at 37 °C for 2–3 h (Henry et al., 1986) to a final SDS concentration of 174 mM. A relatively high detergent concentration was employed to minimize coat protein aggregation (van de Ven et al., 1993; Spruijt & Hemminga, 1991). Coat protein was separated from DNA by gel filtration (Sephacryl S-200 HR column, equilibrated with 10 mM SDS and 25 mM sodium borate elution buffer), in a manner similar to elution with deoxycholate (Henry et al., 1986). However, separation of the SDS–protein complex from DNA does not alter the properties of the coat proteins (Williams & Dunker, 1977), so that the gel filtration step was later omitted. The concentration of coat protein (as part of the intact phage) was obtained by absorbance measurements at 265 nm for IKe coat protein (ϵ = 3.5 for 1 mg/mL WT coat protein) and 269 nm for M13 coat protein (ϵ = 3.84 for 1 mg/mL WT protein) (Clack & Gray, 1989, and references cited therein).

S-Form Preparation. Phage in TE buffer, pH 8.0 (10 mM Tris, 0.5 mM EDTA), was mixed with an equal volume of chloroform and mildly vortexed for 5 s per minute for 5 min. The supernatant containing the S-forms was collected, and any remaining chloroform was removed under a flow of nitrogen for 1–2 min (Griffith et al., 1981; Manning et al.,

1981; Lopez & Webster, 1982). The S-form of M13 mutant Y24C-V31A exhibited a spherical morphology indistinguishable from that of the wild type on the basis of electron microscopy (not shown). The concentration of coat protein in the S-form was determined using the Peterson−Lowry assay (Peterson, 1977), and comparison with the concentration of the protein in the phage confirmed that there was no substantial loss of protein upon contraction (Lopez & Webster, 1982).

Alkylation of Sulfhydryls. The initial 2 mM stock solution of iodo[1-^{14}C]acetamide (^{14}C-IAN; 50 μCi/mL, Amersham Corp.) was brought to 100 mM (specific activity = 1.1 × 10^9 dpm/mmol) by mixing in a 1:1 (v/v) ratio with 200 mM cold IAN (Sigma Co.) to provide the excess IAN concentration required for quantification of free sulfhydryls. Alkylation was carried out at 37 °C in Tris buffer (10 mM) in the dark, pH 8.5 (Hollecker, 1989), with the maximum incorporation of ^{14}C obtained after ca. 90 min. Labeled phages were separated from soluble ^{14}C-IAN by precipitation in PEG/NaCl. The standard reaction conditions were also employed for coat protein modification by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co.), *N*-ethylmaleimide (NEM), maleimidoproxyl (MIP), and proxyl-2-iodoacetamido (PIAN), where proxyl = 2,2,5,5-tetramethyl-1-pyrrolidinyloxy. 5-Fluorescein-substituted iodoacetamide (FIAN, Pierce Chemical Co.) was coupled with free sulfhydryls at room temperature for 2 h in the dark (Gorman, 1987). A typical reaction involved 250 μL of 1 mg/mL protein (as phage) in 10 mM Tris buffer mixed with an equal volume of a given sulfhydryl reagent stock solution (1 mg/mL). Prior treatment with DTT (5-fold molar excess, 15 min, 37 °C) (Hollecker, 1989) did not increase modification by IAN or any other sulfydryl reagent (data not shown).

Electron Paramagnetic Resonance Spectroscopy. Intact phage consisting of spin-labeled (PIAN) coat proteins were separated from soluble spin-label by phage precipitation with PEG/NaCl. To further reduce the background, PIAN-modified phages were dialyzed three times against excess [at least 1000× (v/v)] Tris buffer, pH 8.5 (TE; 10 mM Tris, 0.2 mM EDTA). Spectra were recorded with 1 mg/mL solutions of Y24C-V31A and P30C, while higher concentrations (5−6 mg/mL) were used for G39C and G39C-V36A. As a control, PIAN-modified Y24C-V31A and P30C phages were also characterized at concentrations up to 6 mg/mL. Higher concentrations did not effect line shapes or motion parameters (not shown). EPR spectra were recorded at room temperature using a Varian E104B spectrometer, equipped with a Varian temperature control accessory and a DEC LSI-11-based microcomputer system. The microwave power used was 10 mW. The accessibility of the PIAN spin-label to the aqueous phase was monitored from its susceptibility to $NiSO_4$ (125 mM final concentration) broadening (Polnaszek et al., 1978) and ascorbate ion reduction (2 mM ascorbate, 2−4 °C; Schreier-Muccillo et al., 1976). Ascorbic acid was added at 4 °C, and the spectrum was obtained within 10 min. In all experiments, the pH of the solutions was maintained above 8.0 (Tris buffer). Ascorbate reduction of SDS-solubilized coat proteins could not be measured since SDS precipitates at low temperatures.

Analysis of EPR Spectra. For fast, nearly isotropic motion, the empirical motion parameter τ_o was obtained from spectra according to the equation $\tau_o = KW_o[(h_o/h_{-1})^{1/2} - 1]$ (Eletr & Keith, 1972), where $K = 6.5 \times 10^{-10}$ s, W_o is the width of the center line, and h_o and h_{-1} are the heights of the center and high field first derivative lines, respectively (Boggs & Moscarello, 1978). The rotational motion of the side chains of PIAN-labeled coat proteins solubilized in SDS was analyzed in this manner. However, the remaining spectra were characteristic of slower motion; as a relative measure of the immobilization of the side chains, the maximum hyperfine splitting T_{max} was obtained from the spectra (Boggs et al., 1976). For SDS-solubilized coat proteins, the hyperfine splitting value a_o was determined and compared with the value for proxyliodoacetamide solubilized in aqueous solution at room temperature (Boggs et al., 1980). Accessibility to $NiSO_4$ and ascorbate was estimated from the spectral intensity. Since the height of the derivative lines is proportional to the concentration of the spin-label, the ratio of the height of the center line in the presence *vs* the height of the center line in the absence of these solutes will approximate the degree of spin-label reduction or broadening.

Extrinsic Fluorescence Studies. Intact Y24C-V31A phage was modified by FIAN as described above, but with dialysis carried out in the dark. Fluorescence was performed using a Hitachi spectrophotometer (model F-4000), with the excitation wavelength set at 490 nm and the emission spectrum recorded between 495 and 600 nm. Aliquots of 6 M acrylamide (Bio-Rad) were added directly to the cuvet, and the quenching was analyzed according to the Stern−Volmer equation $F_o/F = 1 + Ka[q]$, where F_o = fluorescence in the absence of quencher, F = fluorescence at $[q]$ concentration of acrylamide, and Ka = Stern−Volmer constant obtained from the slope of a plot of F_o/F vs $[q]$ (Eftink & Ghiron, 1976).

SDS-PAGE Studies. In order to obtain SDS-solubilized coat protein for gel electrophoresis, 50 μL of phage (1 mg/mL protein, TE pH 8.0) was contracted to the S-form, solubilized in SDS by mixing with an equal volume of 10% SDS, and then immediately loaded on the gel. The final concentration of SDS in the micelle−protein complex was 174 mM. SDS-PAGE was performed using precast Tris-Gly 4−20% acrylamide gradient gels (Novex, San Diego, CA) and stained with Coomassie blue.

RESULTS

Accessibility of Cys Residues in IKe and M13 Phages. The Cys major coat protein mutants of IKe (P30C, G39C, and G39C-V36A) and M13 (Y24C-V31A) are shown with the aligned TM sequences of these proteins (Figure 1) (Nakashima et al., 1981). Intact phages were incubated with ^{14}C-IAN, and the percentage of SH sites modified relative to the total coat protein concentration was assessed (Figure 2). Susceptibility of the Cys residues to IAN-mediated alkylation was strongly dependent upon the specific locus, with G39C and G39C-V36A being the most accessible (70%), followed by Y24C-V31A (35%) and P30C (15%). In preliminary attempts to increase the fraction which was accessible, we titrated the intact phage with denaturants and detergents (below the critical micelle concentration), with the aim of preserving phage structure but exposing more sulfhydryls for modification. However, no change in exposure of SH groups of intact phages was observed using guanidinium hydrochloride, deoxycholate, urea, or SDS. The only exception was a 20% increase in ^{14}C-IAN uptake by P30C with 2−3 M urea (representing an increase from 15

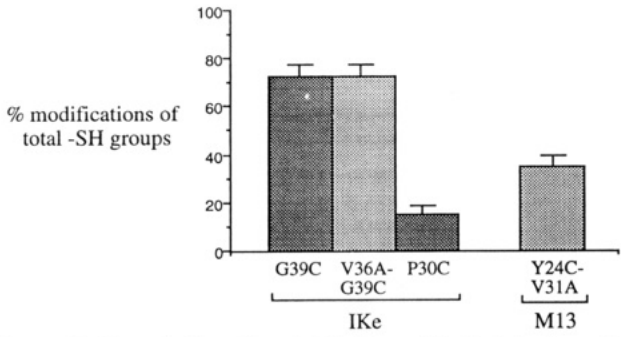


FIGURE 2: Quantitation of accessible free sulfhydryls in the major coat protein of M13 and IKe mutant phages. The intact phages were treated with [^{14}C]iodoacetamide (^{14}C-IAN) at 37 °C, in the dark, for ca. 90 min. The percentage of total SH groups alkylated was calculated as described in Methods.

to 18% of total SH groups alkylated). These observations reinforce the notion that hydrophobic interactions between adjacent subunits in the virion are not easily perturbed (Manning et al., 1981). Incubation with DTT also did not increase alkylation (*vide infra*).

To assess the steric requirements for accessibility, mutant phages were incubated with a variety of sulfhydryl-specific reagents (Table 1) prior to reaction with ^{14}C-IAN so that the extent to which the incorporation of ^{14}C was inhibited could be determined. The populations of P30C and Y24C-V31A accessible to IAN were also susceptible to derivatization by a variety of bulky reagents. In contrast, the much larger population of Cys residues in the mutants G39C and G39C-V36A which was susceptible to labeling by IAN was fully accessible to maleimide (MAL), but only partially accessible to proxyliodoacetamide (PIAN) and maleimidoproxyl (MIP). The more bulky reagents dithionitrobenzene (DTNB) and fluorescein−iodoacetamide (FIAN) were virtually unreactive with phage G39C and G39C-V36A. These results suggest that the 39-locus of phage IKe coat protein, although solvent-exposed, is accessible only to sterically "small" alkylating agents. In contrast, the majority of SH side chains in the P30C and Y24C-V31A mutants are inaccessible even to small reagents, although the fraction which is accessible is not subject to steric restrictions.

Mobility of Cys Residues in Phage, S-Form, and SDS-Solubilized Coat Protein. Intact mutant phages were modified at their accessible Cys residues by the spin-label reagent PIAN. Even though only a fraction of the coat protein sulfhydryls were spin-labeled, the effects of different environments and forms of the protein could be compared from the behavior of the spin-labeled fraction. Typical EPR spectra of the intact phage and SDS-solubilized PIAN-labeled P30C (A and B) and G39C (C and D) coat proteins are shown in Figure 3. The EPR spectra of phage P30C indicate increased side chain mobility in the SDS-solubilized form of the protein (sharp line spectra, Figure 3B) relative to the protein in the intact phage (broad line spectra, Figure 3A). This trend was observed for each of the Cys mutants in this study, although the extent of immobilization in the phage was dependent upon the locus. The G39C and G39C-V36A EPR spectra were indistinguishable, and therefore, only the G39C spectra are shown (Figure 3C,D). The spectrum of the side chain in phage G39C (Figure 3C) consisted primarily of a component exhibiting significantly more restricted motion in comparison to the side chain in bacteriophage P30C (Figure 3A). Solubilization of G39C in SDS (Figure 3D) resulted in increased side chain motion, as was also observed with P30C. The minor mobile species in G39C phage (Figure 3C) was present in IKe wild type phage (which lack Cys) at the identical concentration of protein (not shown) and is thus attributed to background labeling. Minor coat proteins corresponding to genes III and VI—located at one end of the intact phage particle—which are involved in host recognition and binding, contain Cys residues (Peeters et al., 1985). However, it has been shown that the gene III minor coat protein of fd phage (which, like IKe, contains six Cys residues) is not susceptible to alkylation with or without prior DTT reduction in the intact virus (Kremser & Rasched, 1994). Alternatively, the single Met^{14} of IKe or an N-terminal primary amino group may be partially modified. The sharp line spectrum of this minor component is indicative of a mobile side chain(s).

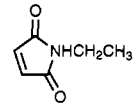
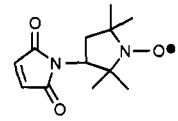
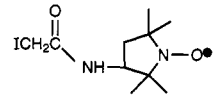
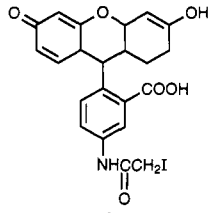
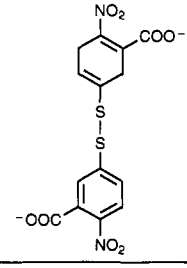
The EPR spectra of PIAN-labeled Y24C-V31A phage, S-form, and SDS-solubilized form are shown in Figure 4. The labeled side chain is more mobile in the Y24C-V31A phage (Figure 4A) than in the IKe mutants. Contraction of the filament to the S-form resulted in a significant immobilization of the Cys side chain, although a minor mobile component was also evident (Figure 4B). The S-form spectrum was somewhat exchange-broadened, suggestive of spin−spin interactions due to spin exchange and dipolar interactions between nitroxide radicals. A qualitative estimate of the degree of broadening can be made from the ratio of the height of the center peak to the value of the double integral of the spectrum, provided that the motional characteristics are comparable. This ratio was 80−89 for other spin-labeled samples of phage and S-forms for which the spin-label was similarly immobilized, in contrast to a value of 66 for the Y24C-V31A S-form. Furthermore, a less labeled sample of Y24C-V31A S-form, whose spectrum did not appear broadened (not shown), had a value of 82.

Conversion of the P30C coat protein to the S-form also resulted in immobilization to a similar degree as for Y24C-V31A (not shown). G39C exhibited a small further decrease in mobility in the S-form, but it was already relatively immobilized in the intact phage (Figure 3C). Thus, the spin-label on all mutants was immobilized to a similar extent in the S-form. There was no evidence of spin−spin interactions for the S-forms of P30C and G39C. This may have been due to the lower amount of label bound to these mutants compared to Y24C-V31A or to the different location of the Cys residue.

Upon solubilization of the coat protein into SDS micelles (Figure 4C), the EPR spectrum was indicative of a single mobile component for the side chain motion (Figure 4C), as was observed for the IKe mutants. The T_{max} values of the spectra of spin-labeled IKe and M13 coat proteins in intact phage and S-form are shown with the corresponding spectrally derived empirical motion parameter τ_0 and hyperfine splitting a_o for SDS-solubilized coat proteins in Table 2.

Environment of Cys Residues in Phage, S-Forms, and SDS-Solubilized Protein. The hyperfine splitting a_o for the SDS-solubilized coat protein (Table 2) indicates that the spin-labeled Cys residue in each of the proteins is in a relatively polar environment. IKe coat protein Cys^{30} is in the most polar environment, suggesting that it is closest to the apolar/aqueous interface of the SDS micelle. Cys^{39} is in the least polar environment.

Table 1: Modification of IAN-Accessible SH Groups of Mutant Phages by Sulfhydryl-Specific Reagents[a]

reagent		% inhibition			
		M13	IKe		
		Y24C-V31A	P30C	G39C	G39C-V36A
N-ethylmaleimide (NEM)		100	100	100	100
maleimidoproxyl (MIP)		100	100	5	5
proxyliodoacetamide (PIAN)		100	100	5	5
fluorescein–iodoacetamide (FIAN)		100	100	~0	0
Ellman's reagent: [5′,5′-dithiobis(2-nitrobenzoic acid)] (DTNB)		100	100	0	0

[a] The IAN-accessible SH groups were 70% (G39C and G39C-V36A), 35% (Y24C-V31A), and 15% (P30C) of the total number of SH groups present in the virion (Figure 2). Reactivities were obtained by incubating phage with a given reagent, followed by incubation with [^{14}C]iodoacetamide (^{14}C-IAN). By observing the extent to which [^{14}C]iodoacetamide incorporation was reduced vs expected values (Figure 2), the % incorporation of a given (cold) sulfhydryl reagent could be inferred. As an example, alkylation of Y24C-V31A phage with *N*-ethylmaleimide resulted in 100% inhibition of ^{14}C-IAN incorporation (which corresponds to modification of 35% of total SH groups by *N*-ethylmaleimide). The maximum error is ±10% of the values shown.

The environment of the spin-label in phage, S-forms, and SDS was further studied using ascorbate and $NiSO_4$ to reduce and broaden the spectra, respectively. Each of the PIAN-modified intact phages showed essentially complete accessibility of the spin-label to Ni^{2+} (Table 2). However, ascorbate-mediated reduction of the spin-label was incomplete for those intact phages which were studied [e.g., for Y24C-V31A phage, compare 47% (ascorbate) vs 90% (Ni^{2+}), Table 2]. One explanation is that the surfaces of IKe and M13 phages are relatively acidic. The N-terminal region of IKe (Ala^1 to Asp^{23}) and M13 (Ala^1 to Glu^{20}) coat proteins each contain a net −3 charge. Thus, negatively charged ascorbate is probably less accessible to the environment of the spin-label relative to positively charged Ni^{2+}. Charges on lipid head groups can attract or repel negatively charged ascorbate ions, altering their partitioning properties into the membrane–aqueous interface (Schreier-Mucillo et al., 1976). However, since all the mutant proteins have similar charge, attraction of Ni^{2+} and repulsion of ascorbate should not affect the relative differences in reduction of broadening observed for the different mutants and dependence on the form for Y24C-V31A. This is supported by the fact that conversion of Y24C-V31A to the S-form reduced the effect of both ascorbate and Ni^{2+} on its spectrum (Table 2). Thus, in the S-form, most of the labeled population becomes less accessible to the aqueous phase than in intact phage.

Coat Protein Interactions in the Phage and S-Form. The fluorescence spectra of FIAN-modified Y24C-V31A coat protein in the intact phage and in the S-form are shown in Figure 5. The fluorescence has been corrected for changes in absorption at the excitation wavelength (490 nm) to allow for direct comparisons. There is a significant decrease in fluorescence intensity and a moderate red shift upon contraction of the filament to the S-form. To further investigate these phenomena, the acrylamide (ACR) quenching of intact phage and S-form fluorescence was examined (Figure 6 and Table 3). ACR is an aqueous-accessible molecule which is excluded from hydrophobic environments (Eftink & Ghiron, 1976; Moro et al., 1993). Significant quenching of fluorescein in the phage was not observed until ca. 15 mM ACR (Figure 6). One explanation is that there is a complex interaction between the quencher and the phage upon initial addition of ACR to the surface of the intact virus. Small additions of NaCl, even as little as 1 mM, resulted in significant increases in the fluorescence intensity of FIAN-modified phage (not shown), so that all experiments were performed at 50 mM NaCl. It is possible that, upon addition of ACR (an uncharged but polar molecule) at low concentrations, the quenching ability is initially balanced by structural

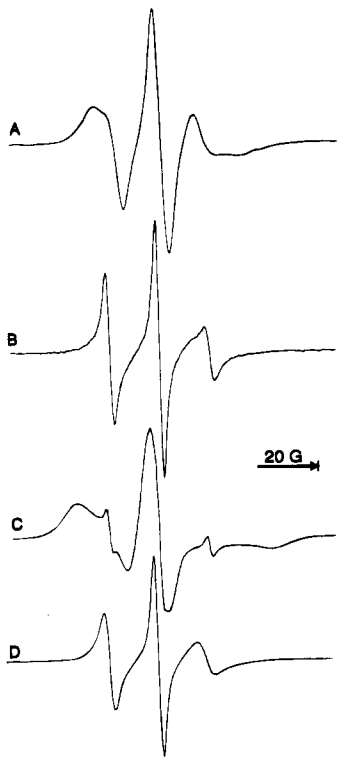


FIGURE 3: EPR spectra of PIAN-alkylated mutant IKe coat proteins as part of the intact phage and upon coat protein solubilization in SDS: (A) P30C, intact phage; (B) P30C, SDS; (C) G39C, intact phage; and (D) G39C, SDS. Coat protein concentrations were 1 mg/mL for P30C and 5 mg/mL for G39C (10 mM Tris, pH 8.5, 0.2 mM EDTA). The EPR spectra of double mutant G39C-V36A were indistinguishable from those of G39C. Addition of up to 75 mM NaCl had no effect on line shapes. Spectral line heights are shown normalized to each other.

changes at the surface of the virus which result in moderate increases in FIAN fluorescence. In control experiments, there was no lag in the quenching ability of ACR upon addition to FIAN in aqueous solution (not shown). Much of the structural flexibility of filamentous phages arises from charged groups toward the N terminus, and significant changes in protein−protein motions for fd phage (at the 24-locus) have been reported without changes in the hydrophobic packing toward the C terminus (Bhattacharjee et al., 1992).

While the fluorescence of the intact phage was eventually reduced by 28% in the presence of acrylamide, the fluorescence in the contracted S-form remained unchanged. Since FIAN is water-soluble, the maximum amount of quenching by ACR of fully aqueous-exposed FIAN (in solution) was evaluated. It was determined that, upon 30% reduction of the initial fluorescence value, there is no further decrease. Thus, 28% reduction of the fluorescence in intact phage represents essentially complete (>90%) quenching of the extrinsic fluorescence.

The fluorescence properties of the Y24C-V31A S-form are interpreted as resulting from an aggregation-related conformational change allowing fluorescein moieties on the Cys^{24} side chain to be in sufficiently close proximity for self-quenching. Self-quenching of fluorescein has been observed in other work [e.g., Sikkema et al. (1994)]. These results are consistent with the ESR data which imply spin−spin interactions in the S-form of spin-labeled Y24C-V31A phage (Figure 4B). They are also in agreement with reduction or

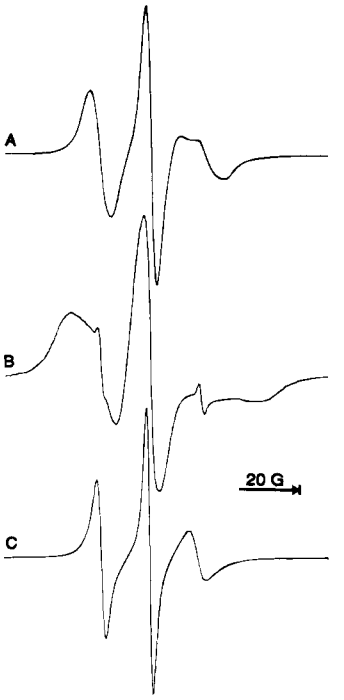


FIGURE 4: EPR spectra of PIAN-modified Y24C-V31A M13 mutant as (A) intact phage, (B) S-form, and (C) after solubilization in SDS micelles. The concentration of coat protein was 1 mg/mL (10 mM Tris, pH 8.5, 0.2 mM EDTA). As with IKe coat proteins, addition of NaCl did not affect line shapes. Spectral line heights are normalized to each other.

broadening of the spin-label of PIAN-modified Y24C-V31A phage and S-forms using Ni^{2+} and ascorbate and the lower degree of accessibility to these reagents in the S-form (Table 2). Solubilization of the S-form by SDS results in dequenching of the fluorescence, although only to ca. 90% of the original fluorescence intensity of the intact virus (not shown).

To further study the state of aggregation of the protein in the S-form, the Y24C-V31A mutant was incubated in [^{14}C]-iodoacetamide (^{14}C-IAN; 90 min at 37 °C, pH 8.5) with and without prior reduction using dithiothreitol (DTT; 15 min at 37 °C). The extent of alkylation is expressed as a percentage of total SH content and is compared with the intact phage (Figure 7). There was a significant decrease in the percentage of SH groups modified in the S-form, implying a decreased accessibility of the Cys^{24} locus in the S-form relative to intact phage. Upon DTT reduction prior to alkylation, there was little change in modification of intact phage, but a moderate (ca. 30%) increase in [^{14}C]iodoacetamide modification of the S-form. These results suggest that at least some disulfide bond formation is occurring between Cys^{24} side chains in the S-form, consistent with the spin−spin interactions and fluorescence self-quenching at this locus.

The S-form of Y24C-V31A phage was solubilized in SDS and immediately analyzed by SDS-PAGE (Figure 8). Unmodified coat protein occurs as a 5.3 kDa monomer and diffuse dimer mixture, with minor oligomeric components at 20, 30, and 43 kDa (Figure 8). The amount of dimerization increases over extended time periods (not shown). Blocking of the SH group by PIAN resulted in an increased monomer population, with reduction in the intensity of the dimer and the 20 kDa oligomer. Similar results were obtained using other SH modifying agents (e.g., DTNB and

Table 2: Spectral Parameters from the EPR Spectra of Spin-Labeled IKe and M13 Coat Proteins in Various Environments[a]

	a_o (G) (±0.2)	T_{max} (G) (±0.5)	τ_o (ns) (±0.1)	% reduction (ascorbate) (±5)	% broadening ($NiSO_4$) (±5)
P30C					
phage	–	21.5	–	ND	97
S-form	–	30.5	–	ND	ND
SDS	15.6	–	1.6	–	100
G39C					
phage	–	32.0	–	43	95
S-form	–	30.9	–	ND	ND
SDS	15.3	–	2.1	–	100
Y24C-V31A					
phage	–	20.5	–	47	90
S-form	–	29.9	–	26	34
SDS	15.4	–	1.9	–	100
free PIAN	15.8		0.03		

[a] Estimated errors are given in parentheses. Parameters were obtained as described in the Methods. ND = not determined.

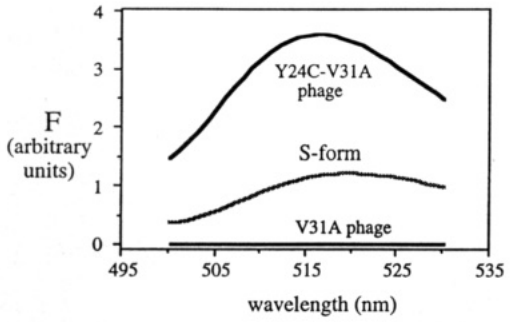


FIGURE 5: Fluorescence spectra of fluorescein−iodoacetamide (FIAN) modified Y24C-V31A coat protein (λ_{exc} = 490 nm) as part of the intact phage and upon contraction to the S-form. Protein concentrations are 10 μg/mL. Fluorescence emission was scanned from 495 to 600 nm; the 500−530 nm region is shown. Fluorescence has been corrected for minor changes in absorbance at 495 nm to allow for direct comparisons of intensities. Control spectra of M13 mutant V31A phage (which does not contain Cys) after incubation with FIAN demonstrate the absence of fluorescence emission.

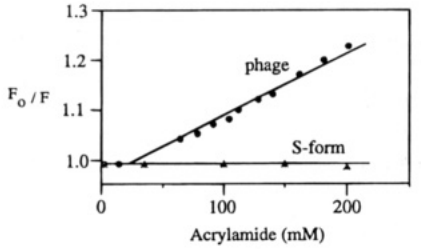


FIGURE 6: Stern−Volmer acrylamide quenching plots of FIAN-modified Y24C-V31A phage and S-form (each 10 μg/mL). Fluorescence spectra were recorded at room temperature in 10 mM Tris (pH 8.5), 0.2 mM EDTA, and 50 mM NaCl.

FIAN). In addition, there were subtle variations in the mobilities of the monomers, likely reflecting the polarity and/or steric effects of coupling the various alkylating agents to the coat protein. The dimer (CON lane, Figure 8) could also be converted to a monomer by reduction using β-mercaptoethanol (Khan & Deber, 1995) and thus was shown to be disulfide-bonded. The migrations of the SDS-PAGE species of WT and V31A coat proteins (Deber et al., 1993) were not affected by reduction and/or alkylation (Khan & Deber, 1995). These results demonstrate that, during the transition of Y24C-V31A coat protein from intact phage to membrane-mimetic environments, Cys side chains are in sufficient proximity to allow the formation of disulfide-linked dimers.

Table 3: Fluorescence Studies of FIAN-Modified Y24C-V31A[a]

	λ_{max} fluorescence emission (nm) (±1)	relative fluorescence[b]	K_{sv} (M^{-1})	maximum quenching of fluorescence[c] (%) (±2)
Y24C-V31A				
phage	516	1.0	1.4	28
S-form	519	0.3	0	0
SDS	518	0.9	0	0
free FIAN	517	–	ND	30

[a] Coat protein concentration at each stage (phage, S-form, and SDS) was 10 μg/mL (10 mM Tris, pH 8.5; 0.2 mM EDTA). The final SDS concentration was 174 mM. λ_{exc} = 490 nm. ND = not determined. [b] The fluorescence of the coat protein in the intact phage was assigned a value of 1.0 to facilitate comparisons of the relative changes in fluorescence in S-forms and in SDS. [c] The value of fluorescence used to calculate the maximum quenching by acrylamide, as well as relative fluorescence changes, was obtained from the λ_{max} (emission) in the S-form (519 nm) and SDS (518 nm).

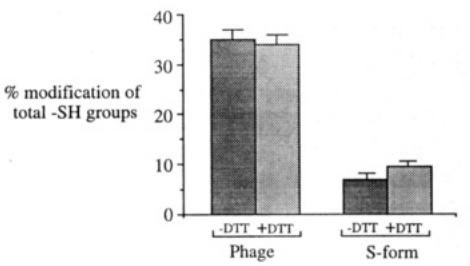


FIGURE 7: Alkylation of mutant Y24C-V31A phage and S-form to determine whether disulfide bonds form in the S-form of Y24C-V31A. Y24C-V31A S-form was incubated with (+) and without (−) dithiothreitol (DTT) for 15 min at 37 °C prior to alkylation with ^{14}C-IAN using the standard reaction conditions (see Methods). Results are compared with those of mutant Y24C-V31A intact phage under the same two conditions. An increase in ^{14}C incorporation of ca. 30% was observed for the S-form upon exposure to DTT.

DISCUSSION

Filamentous Bacteriophage Structure. The Cys-containing IKe and M13 coat proteins obtained through randomized mutagenesis afford an excellent opportunity to probe the environment of specific resiudes within the hydrophobic segment of the coat protein in the intact virion. To our knowledge, these mutants are the only viable Cys-containing coat proteins of filamentous phage reported to date. The Cys mutants were found to display distinct steric requirements for alkylation by sulfhydryl-specific reagents. IAN

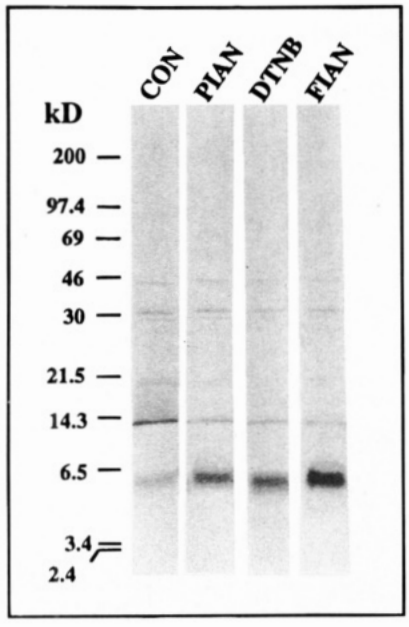


FIGURE 8: SDS-PAGE species analyses of unmodified and alkylated mutant Y24C-V31A coat proteins. CON = control, with no prior modification of the coat protein SH groups. PIAN = proxyliodoacetamide-treated coat protein. DTNB = 5′,5′-dithiobis(2-nitrobenzoic acid). FIAN = fluorescein−iodoacetamide. The faint 43 kDa band corresponds to gene III minor coat protein (Kremser & Rasched, 1994).

readily modified the Cys residues of IKe mutants G39C and G39C-V36A, whereas the majority of Y24C-V31A (M13) and P30C (IKe) Cys sulfhydryls were inaccessible (Table 1). The accessibility of Cys^{39} to IAN is somewhat surprising since this residue is expected to be sequestered well within the capsid of the virion, in comparison to the more N-terminally situated residues Pro^{30} (IKe) and Tyr^{24} (M13). The G39C locus was subject to steric restrictions, however, since large reagents were unable to modify Cys (Table 1).

The inaccessibility of the Tyr^{24} locus is supported by model-building studies of the intact phage based on fiber diffraction data (Marvin et al., 1994). Tyr^{24} is predicted to reside within a hydrophobic environment comprised of aromatic residues Tyr^{21} and Trp^{26} (interactions within a given subunit designated subunit "0"), Phe^{11} (axial contact between subunits "0" and "6"), and Phe^{42} and Phe^{45} (layer contacts between subunits "0" and "11") (Marvin et al., 1994; Williams et al., 1995).

The importance of the IKe Pro^{30} locus in coat protein packing in the virion is supported by the observation that saturation mutagenesis of this residue resulted in the isolation of mutants in which Pro could be replaced by only the four smallest amino acids (Ala, Gly, Ser, and Cys) (Williams & Deber, 1993). Indeed, large scale P30C phage preparations were typically carried out at room temperature (vs 37 °C for WT) in an attempt to enhance yields, although even with this method, yields were typically ca. 1 mg/L P30C vs >40 mg/L for WT IKe. The coat proteins which resist labeling may in fact represent the population which is important in mediating interactions of the phage, although the strategy employed herein sheds light only on the fraction which is accessible.

PIAN-labeled Cys mutant bacteriophage exhibited relatively restricted mobility compared to the SDS-solubilized coat protein, with the sulfhydryl of Y24C-V31A being the most mobile, followed by the IKe mutant P30C, and with the Gly^{39} mutants experiencing the most restricted motion (Figures 3 and 4). This suggests that, when modification by the relatively large reagent PIAN occurs, its mobility is directly correlated with the exposure of the residue toward the surface of the virus. The M13 Tyr^{24} locus is clearly more peripherally located than Gly^{39} in IKe (Marvin et al., 1994).

Characterization of S-Form. A Molten Globule? Exposure of intact M13 phage to a chloroform−aqueous interface at room temperature results in a transition to the S-form which is characterized by a reduction in ellipticity at 222 nm and a moderate decrease in overall helicity by CD spectroscopy (Dunker et al., 1991a,b; Roberts & Dunker, 1993). A contracted rodlike intermediate (I-form) of the virion can be isolated at 2 °C and exhibits α-helicity intermediate between that of the phage and S-form (Roberts & Dunker, 1993; Manning et al., 1981). It has been speculated that the I-form and S-form have nonrigidly packed side chains resembling a molten globule assembly (Roberts & Dunker, 1993). In contrast, EPR spectra described herein of the spin-labeled Y24C-V31A and P30C demonstrate that those side chains which are mobile in the phage experience an immobilization upon contraction to the S-form (Figure 4). This is not unexpected considering that the filament decreases in size from an extended structure (1 μm) to a small sphere (0.04 μm) (Griffith et al., 1981). However, since the spin-label is bulkier than the WT side chain, it will necessarily be present in a more crowded local environment and so Tyr^{24} and Pro^{30} of the WT coat protein may not experience immobilization in the S-form. Nevertheless, the results indicate that the spin-labels bound to Cys^{24} and Cys^{30} have less freedom of motion in the S-form than in the intact phage, indicating greater steric hindrance in the S-form. The spin-label bound to Cys^{39} showed little change in side chain mobility upon contraction to the S-form, but this is due to the fact that it was already immobilized in the intact phage.

The EPR spectra of spin-labeled Y24C-V31A S-form suggested the occurrence of spin−spin interactions between nitroxides, although those of the other mutants did not. In addition, the S-form of FIAN-conjugated Y24C-V31A showed a 66% reduction in fluorescence intensity relative to the intact virus without drastic changes in the λ_{max} (emission), suggesting aggregation-related self-quenching of fluorescein molecules (Figure 5). Intrinsic Trp^{26} fluorescence was also shown to be quenched without a significant change in λ_{max} (emission) upon contraction of fd phage (Roberts & Dunker, 1993). The dramatic reduction in ACR accessibility to Trp^{26} in the S-form (Roberts & Dunker, 1993) parallels the inaccessibility of ACR to FIAN-modified Y24C-V31A S-form as described herein. It was further demonstrated that the S-form of spin-labeled Y24C-V31A phage is resistant to signal reduction or broadening by both ascorbate and $NiSO_4$ relative to the intact virus (Table 2). These results are consistent with an aggregation-related conformational change upon contraction of the filaments so that Cys^{24} becomes embedded within a hydrophobic interior. As a consequence, although this residue is out of register between adjacent subunits in the phage, the side chains appear to be in close proximity (aligned roughly parallel) in the S-form. The in-register alignment of coat proteins in the S-form is supported by SDS-PAGE analyses of the mutant Y24C-V31A. Upon solubilization of Y24C-V31A

coat protein into micelles, disulfide-bonded dimers were formed (Figure 7).

SDS Micelle-Bound Coat Protein. Solution and solid state NMR studies indicate that fd/M13 coat protein consists of a short N-terminal amphipathic helix which lies at the membrane interface and a longer hydrophobic helix which spans the membrane (Papavoine et al., 1994; McDonnell et al., 1993). The most slowly exchanging backbone protons are found between Met^{28} and Phe^{42} (Henry & Sykes, 1992; van de Ven et al., 1993); this stretch of residues likely comprises the hydrophobic helix residing within the core of the micelle. Thus, the Cys^{24} side chain of mutant Y24C-V31A coat protein likely resides near the micelle−aqueous interface.

Solubilization of spin-labeled coat proteins (Y24C-V31A, P30C, G39C, and G39C-V36A) resulted in an increase in mobility of the spin-label, compared to that observed in the phage. The main aggregation unit was the monomer for Y24C-V31A coat protein (Figure 8) as well as for IKe coat proteins (Williams & Deber, 1993). The hyperfine splitting values a_o for each of these spin-labeled coat proteins, together with complete accessibility to $NiSO_4$, are indicative of side chains exposed to the aqueous environment. The hyperfine splitting value increases with increasing polarity of the environment of the spin-label (Briere et al., 1965). For PIAN solubilized in aqueous buffer at room temperature, a_o was determined to be 15.8 (±0.1) G. The values for M13 and IKe coat proteins in SDS (Table 2) varied between 15.3 and 15.6 G, a relatively small deviation from fully solvent-exposed spin-label. Pro^{30} in SDS micelles exhibits a mobile side chain (τ_c = 1.6 ns) and is likely situated at the micellar interface. The side chains of spin-labeled mutants G39C and G39C-V36A (IKe phage) in micelles were also observed to be relatively mobile (τ_c = 2 ns) and susceptible to $NiSO_4$-mediated broadening of the EPR spectrum. The overall results from this work suggest that, while the Cys^{39} side chain in mutant G39C is aqueous-exposed in the intact phage as well as in SDS micelles, the environment around the Gly^{39} locus is more spatially restricted in the phage, as shown by the large decrease in Cys side chain mobility.

SUMMARY

The reactive Cys side chains in the hydrophobic regions of M13 and IKe coat protein mutants were modified by a number of sulfhydryl-specific reagents, including IAN and its proxyl derivative. The accessibility of the side chain at distinct loci was evaluated, and the dynamics of spin-labeled coat proteins in forms which mimic assembly intermediates (the spheroidal and SDS-solubilized coat proteins) were characterized by EPR. This study has shown that the Gly^{39}→Cys locus in IKe phage is relatively susceptible to sulfhydryl modification by IAN in comparison to the more N-terminally situated loci, Pro^{30}→Cys and Tyr^{24}→Cys, although the G39 locus is subject to steric constraints. The spin-labeled side chains exhibited varying degrees of mobility with more restricted motion observed for P30C and G39C compared to the Y24 locus. Side chain mobility was enhanced in the SDS-solubilized form, which is consistent with the transition from the virion—a highly ordered macromolecular assembly—to individually solubilized coat protein subunits, and reduced in the S-form. The reactivity of the Cys sulfhydryl makes the phage coat protein Cys mutants versatile tools with which to probe virion structure and monitor characteristic conformational changes that occur during the phage life cycle.

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