Accessibility and Environment Probing Using Cysteine Residues Introduced along the Putative Transmembrane Domain of the Major Coat Protein of Bacteriophage M13

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ABSTRACT: The major coat protein of the filamentous bacteriophage M13 is located in the inner membrane of host cell *Escherichia coli* prior to assembly into virions. To identify the transmembrane domain of the coat protein, we have introduced unique cysteine residues along the putative transmembrane domain at position 25, 31, 33, 36, 38, 46, 47, 49, or 50. The mutant major coat protein was solubilized by membrane-mimicking detergents or reconstituted into mixed bilayers of phospholipids. Information about the environmental polarity was deduced from the wavelength of maximum emission, using *N*-[[(iodoacetyl)-amino)ethyl]-1-sulfonaphthylamine (IAEDANS) attached to the SH groups of the cysteines as a fluorescent probe. Additional information was obtained by determining the accessibility of AEDANS for the fluorescence quencher molecules acrylamide and 5-doxylstearic acid, and the reactivity of the cysteine's sulfhydryl group toward 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Our data suggest transmembrane boundaries close to residues 25 and 46, with residue 25 inside the hydrophobic part of the membrane in very close proximity to the membrane−water interface and residue 46 located at the membrane−water interface. Domains of the mutant coat protein which are packed or coated by cholate molecules and various other detergents [except for sodium dodecyl sulfate (SDS)] are at least similarly packed by phospholipid molecules in bilayers. SDS is a good solubilizing detergent but badly mimics the typical nature of a membrane structure. The overall results are interpreted with respect to the established conformation of the coat protein and its membrane anchoring mechanism.

The filamentous bacteriophage M13 and related bacteriophages have long been studied by many groups in the fields of genetics, biochemistry, and biophysics (Sambrook et al., 1989; Russel, 1993; Hemminga et al., 1992; Glucksman et al., 1992; Deber et al., 1993; Marvin et al., 1994). They can be easily obtained in abundant quantities and can be modified and manipulated according to well-known protocols. Apart from the practical use as vector in molecular biology and genetics, the many processes involved in the bacteriophage life cycle are subject to numerous basic investigations concerning macromolecular assembly and rearrangement processes.

Many copies of the major coat protein form a helical, cylindrical coat around the circular, single-stranded DNA genome. During infection, the major coat protein (the product of gene VIII) is involved in various structural rearrangements. After phage disassembly and subsequent deposition of the parental coat protein into the *Escherichia coli* inner membrane, new coat proteins are synthesized and inserted into the membrane. Finally, new phage particles emerge from the host cell after a complex assembly and phage-extrusion process (Model & Russel, 1988; Russel, 1991; Hemminga et al., 1993). To realize these many functions of the coat protein, the primary sequence must be such to enable sufficient functioning in all processes.

In this study we have focused on the monotopic, integral membrane-bound state of the mature M13 coat protein; i.e., the state of the coat protein after membrane insertion and subsequent cleavage of the leader sequence but before participation in the combined bacteriophage assembly and extrusion process.

The primary and secondary structure of the M13 coat protein is shown in Figure 1. Much is known about the aggregational behavior and overall secondary structure of the coat protein embedded in phospholipid bilayers (Spruijt & Hemminga, 1991; Li et al., 1993). However, new insights about the structure of the coat protein originate from experiments performed on the coat protein solubilized in bilayer-mimicking detergents (McDonnell et al., 1993; Van de Ven et al., 1993; Papavoine et al., 1994). Detailed secondary structure determination as performed on the coat protein solubilized in SDS[1] clearly shows a rigid α-helix from Tyr 24 up to Phe 45, possibly extending up to Ser 50 with an increased flexibility, whereas in the N-terminal half of the coat protein, a labile, distorted α-helical or turn structure has been proposed, which clearly demonstrates an amphipathic nature (Henry & Sykes, 1992; Van de Ven et al., 1993; McDonnell et al., 1993; Wolkers et al., 1995). In addition, hydrogen/deuterium exchange experiments, per-

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[1] Abbreviations: IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-1-sulfonaphthylamine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HPSEC, high-performance size-exclusion chromatography; IPTG, isopropyl thio-β-D-galactoside; PFU, plaque-forming units; SDS, sodium dodecyl sulfate; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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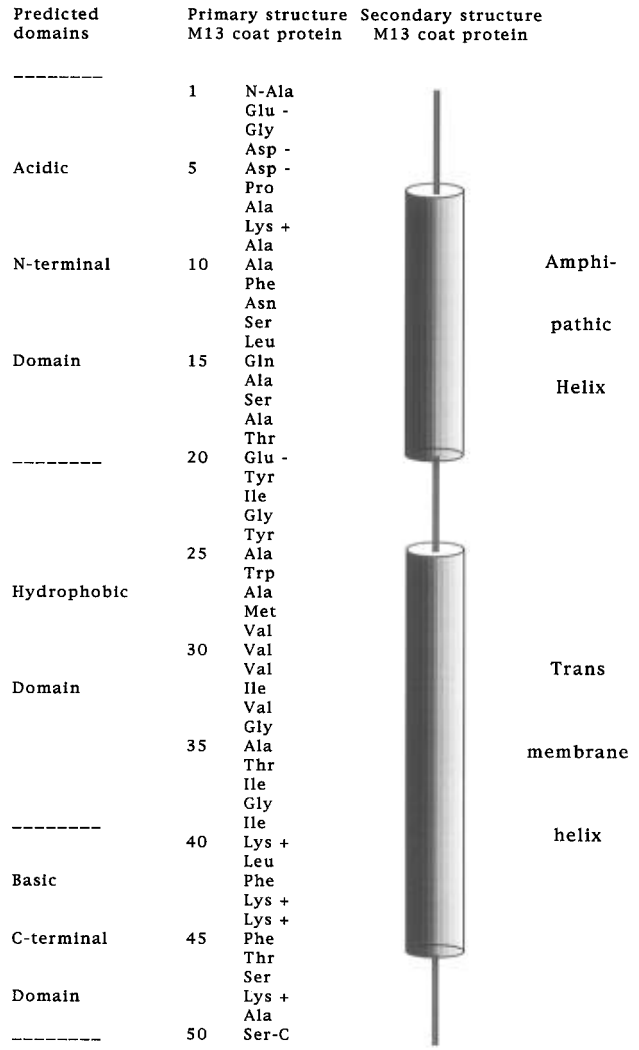

FIGURE 1: Primary structure of the major coat protein of bacteriophage M13 (Van Wezenbeek et al., 1980). The secondary (α-helical) structure, shown as cylinders, has been determined on the coat protein solubilized into SDS (Van de Ven et al., 1993; McDonnell et al., 1993). The classification of the coat protein in different domains is according to the prediction method of Kyte and Doolittle (1982).

formed again on the SDS-solubilized M13 coat protein, showed fast exchange up to residue 24 and from residue 47 to 50. Intermediate exchange rates were found from residues 25 to 27 and from residues 43 to 46 and slow rates from Met 28 to Phe 42, thereby roughly suggesting the location of the transmembrane sequence (Henry & Sykes, 1992).

Summarizing, most data about the presence of a stable α-helical structure and the possible location of the transmembrane domain of the coat protein are obtained from experiments performed on the SDS-solubilized coat protein. However, there is little experimental evidence about the detailed localization of the transmembrane domain in membranes. Due to the different properties of SDS and phospholipid molecules, different parts of the coat protein may be bound to SDS and phospholipid molecules. It should be emphasized that experimental data elucidating the transmembrane domain are most reliable when the coat protein is embedded in a membrane environment.

In the absence of reliable data about the localization of the transmembrane domain, various transmembrane prediction methods have been employed (Von Heijne, 1992; Turner & Weiner, 1993; Milik & Skolnick, 1995). Depending on different hydropathy scales and subsequent calculational strategies, the predicted transmembrane domain ranges between residues 18 and 46. In contrast, only about 21 amino acid residues of the α-helical coat protein, which is oriented preferentially parallel to the membrane normal (Thiaudière et al., 1993), are required to traverse the phospholipid bilayer. The variety in predictions is due to the presence of four polar and charged lysines at positions 40, 43, 44, and 48, which are recognized to be outside the hydrophobic part of the membrane, as well as the presence of apolar residues which are in fact part of the adjacent N-terminal amphipathic helix of the coat protein. However, it is very well possible for the lysine α-carbon to be within the hydrophobic part of the membrane while the ϵ-amine group can interact with the polar headgroups of the lipids (Tanford & Reynolds, 1976; Ballesteros & Weinstein, 1992; Hemminga et al., 1993). The presence of aromatic residues (Tyr 21, Tyr 24, Trp 26, Phe 42, and Phe 45) might also give information about the location of the α-helix, since aromatic residues are often found near the membrane−water interface (Deisenhofer et al., 1986; Henderson et al., 1990; Weiss et al., 1991; Cowan et al., 1995). In this view, both the aromatic and the basic amino acid residues are expected to be important in membrane anchoring.

In the present study we follow a site-specific probing approach as has been described for bacteriorhodopsin and colicin A (Flitsch & Khorana, 1989; Altenbach et al., 1989; Lakey et al., 1991). We prepared a number of coat protein mutants containing unique cysteine residues at defined positions along the putative transmembrane amino acid sequence. The cysteine residues were specifically labeled with the fluorescent probe AEDANS that has been shown to be very sensitive to the polarity of the environment (Hudson & Weber, 1973). Further, the accessibilities for the polar quencher acrylamide and the hydrophobic quencher 5-doxylstearic acid were determined. In addition, the reactivity of the sulfhydryl groups of the cysteines toward the negatively charged DTNB has been measured. For these purposes, the coat protein mutants have been reconstituted into mixed phospholipid bilayers of DOPC and DOPG and have been solubilized in sodium cholate and SDS, enabling comparison with the many data in the literature about detergent-solubilized coat protein. Our data suggest a localization of the transmembrane boundaries close to residues 25 and 46, with residue 25 inside the hydrophobic part of the membrane in very close proximity to the membrane−water interface and residue 46 located at the membrane−water interface.

MATERIALS AND METHODS

Preparation of Cysteine-Containing Major Coat Protein Mutants. Cysteine-containing major coat protein mutants were prepared using oligonucleotide-mediated site-directed mutagenesis by selection against template DNA strands that contain uracil, as originally developed by Kunkel (1985). Uracil-containing M13 mp18 DNA was synthesized in *E. coli* CJ236 (*dut*$^-$ *ung*$^-$ F′). Mutagenic oligonucleotides (Pharmacia Biotech) were designed to introduce unique cysteine residues at various positions along the primary structure of the coat protein. The mutagenesis procedure was performed as described by Sambrook et al. (1989). Transfected cells were incubated overnight in the presence of IPTG and the chromogenic substrate X-gal enabling

α-complementation (Sambrook et al., 1989). The resultant blue color strongly enhanced the visualization of very small plaques. Plaques were taken out and incubated in 2× YT medium [1.6% (w/v) bactotryptone, 1% (w/v) yeast extract and 0.5% (w/v) NaCl]. Bacteriophages were isolated by a double cycle of precipitation as induced by poly(ethylene glycol) 6000 and NaCl, as described previously (Spruijt et al., 1989), and screened for the presence of cysteine residues in the SDS-disrupted bacteriophage, using DTNB (obtained from Sigma) as described by Riddles et al. (1983). Cysteine-containing mutant bacteriophages were grown to milligram quantities in M9 medium supplemented with 0.33% (w/v) casamino acids, 0.5% (w/v) glucose, and 0.05% (w/v) yeast extract and purified as decribed (Spruijt et al., 1989). The presence of only 1 cysteine residue/coat protein molecule was established using DTNB and a quantified amount of bacteriophage. The weight fraction of coat protein in bacteriophage particles was assumed to be 0.85. The concentration of bacteriophage was measured spectrophotometrically using $A_{260nm} = 3.80$ for a 0.1% (w/v) suspension at 1 cm path length (Nozaki et al., 1976; Berkowitz & Day, 1976). The primary structure of the mutant coat protein was deduced from the DNA sequence as obtained from automated sequencing.

Sample Preparation: Coat Protein Labeling and Reconstitution. Each of the available cysteine-containing coat protein mutants was labeled with IAEDANS (purchased from Molecular Probes) directly after bacteriophage disruption. Bacteriophage disruption was performed by suspending 2 mg of bacteriophage into 1 mL of 80 mM sodium cholate (Sigma) in 10 mM Tris-HCl, 0.2 mM EDTA, and 150 mM NaCl buffer, pH 8.0 (called TEN8 buffer), and subsequent addition of chloroform up to 2.5% (w/v). The mixture was incubated at 37 °C under occasional mixing until a nonopalescent suspension was obtained. At this point a 5-fold molar excess of IAEDANS was added and the mixture was stirred at room temperature in the dark for 2 h. The reaction was stopped by addition of excess cysteine. The reaction mixture was then applied to a Superose 12 prep-grade HR 16/50 column (Pharmacia) and eluted with 25 mM sodium cholate in TEN8 buffer to separate the labeled coat protein from the viral DNA and excess AEDANS. The labeling efficiency was calculated using the molar extinction coefficients of AEDANS ($\epsilon_{340nm} = 6 \times 10^3\ M^{-1}\ cm^{-1}$ and $\epsilon_{280nm} = 1 \times 10^3\ M^{-1}\ cm^{-1}$ (Hudson & Weber, 1973) and the molar extinction coefficient of the M13 coat protein ($\epsilon_{280nm} = 8.4 \times 10^3\ M^{-1}\ cm^{-1}$). The labeled coat protein was kept in the dark as much as possible to prevent photodegradation of the AEDANS probe.

Solubilization of the labeled coat protein into sodium cholate and into SDS micelles was achieved by adjusting the concentration of sodium cholate up to 80 mM and by the addition of SDS (Merck; biochemical grade, and Bio-Rad, highly purified electrophoresis grade) to a final concentration of 150 mM. M13 coat protein labeled with AEDANS at position 31 or 36 was solubilized by various nonionic, cationic, and anionic detergents by adding these detergents to a final concentration of 100 mM in TEN8 buffer. The detergents used were octyl β-glucoside (Sigma), dodecyl β-maltoside (Sigma), Triton X-100 (Pharmacia), lauryldimethylamine oxide (Fluka), cetyltrimethylammonium bromide (Serva), and sodium deoxycholate (Sigma).

Reconstitution of the labeled coat protein into DOPC (obtained from Sigma) and DOPG (Sigma) in a 80%/20% (w/w) ratio, at final molar lipid to protein (L/P) ratios of 50 and 250, was performed in the dark using the cholate dialysis procedure as described earlier (Spruijt et al., 1989). The resultant proteoliposomes (in TEN8 buffer) were checked to exclude the presence of the β-polymeric form of the coat protein using SDS-HPSEC as described (Spruijt & Hemminga, 1991).

Steady-State Fluorescence Measurements. The fluorescent properties of the AEDANS-labeled coat protein, reconstituted either into phospholipid bilayers or detergent micelles, were recorded at room temperature on a Perkin-Elmer LS-5 luminescence spectrophotometer. The excitation wavelength was 340 nm and emission scans were recorded from 400 to 550 nm. Excitation and emission slits were set at 5 nm. The concentration of labeled coat protein was 5 μM. The optical density at the excitation wavelength never exceeded 0.1. The fluorescent quantum yield was normalized to a fixed scale of 1.0, an AEDANS labeling efficiency of 1.0, and corrected for the phospholipid or detergent background contribution.

Steady-state quenching studies were performed by addition of acrylamide (Bio-Rad) to a final concentration of 125 mM and by addition of 5-doxylstearic acid (Aldrich) to a final overall concentration of 0.39 mM. Emission spectra were recorded 2 min after each addition and the fluorescent quantum yields were corrected for dilution.

Determinations of Reaction Rates of DTNB to Free Sulfhydryl Groups. Due to the different amphiphilic media used, different bacteriophage disruption and reconstitution procedures had to be applied. To perform the measurements on the cholate-solubilized coat protein, bacteriophage were disrupted using the cholate/chloroform procedure as described before. As a consequence, the coat protein was solubilized into 80 mM sodium cholate in TEN8 buffer. Disruption and solubilization into 150 mM SDS in TEN8 buffer was achieved by dissolving the desired amount of bacteriophage in this buffer. Bacteriophage particles were found to be disrupted instantaneously and completely. To reconstitute the coat protein into phospholipid bilayers of DOPC/DOPG 80%/20% (w/w) at L/P 50, a suitable procedure was found in the direct sonication protocol (Fodor et al., 1981). Phospholipids were dried under a flow of nitrogen and subsequently under high vacuum. Then, a bacteriophage suspension in TEN8 buffer was added and the lipid−bacteriophage mixture was sonicated using a Branson sonifier B15 for 5 min (duty cycle 50%, output 60 W) while keeping the suspension on ice.

The reaction rates of DTNB to the free sulfhydryl groups of the mutant coat protein were determined directly after disruption and subsequent fast reconstitution or solubilization. Typically, 0.15 mg bacteriophage (corresponding to about 0.13 mg coat protein) suspended in 900 μL TE 8 buffer has been applied in each measurement. The reaction was started by the addition of 100 μL of a freshly prepared solution of DTNB (10 mM DTNB in 50 mM sodium phosphate buffer pH 8.0). The increase in absorbance at 412 nm was recorded at room temperature on a Kontron Uvikon 810 spectrophotometer.

Table 1: Positions Tested as Possible Sites for Introduction of a Cysteine Residue along the M13 Coat Protein's Primary Structure[a]

amino acid position coat protein	coat protein mutant	bacteriophage yield (mg/L)	plaque-forming ability (PFU/mL)
3	NV		
7	NV		
9	NV		
10	NV		
13	NV		
17	NV		
22	NV		
23	NV		
25	A25C/A27S	51	1×10^{12}
27	NV		
28	NV		
31	V31C	36	2×10^{10}
33	V33C/A27S	12	2×10^{11}
34	NV		
35	NV		
36	T36C/A27S	10	2×10^{11}
38	G38C/A27S	17	6×10^{10}
39	NV		
40	NV		
41	NV		
42	NV		
43	NV		
44	NV		
46	T46C/A27S	2	1×10^{10}
47	S47C/A27S	3	6×10^{8}
49	A49C/A27S	25	6×10^{10}
50	S50C/N12D	5	6×10^{10}
	wild type	150	5×10^{12}

[a] In the case of successful X-Cys substitutions, the mutations as deduced from DNA sequencing are shown. The yields obtained after preparative growing of mutant and wild-type bacteriophage M13 were found in enriched M9 medium as described in Materials and Methods. Bacteriophage yields may vary up to 30% depending on the conditions. NV, not viable.

RESULTS

Mutagenesis on the Major Coat Protein and Screening for Functional State. To obtain single cysteine residues all along the primary sequence of the major coat protein of bacteriophage M13, 28 out of a total of 50 amino acid positions were systematically tested as possible sites for X-Cys substitutions, using oligonucleotide-directed mutagenesis. A list of all positions tested is given in Table 1. Only in the case of X-Cys substitutions at amino acid positions 25, 31, 33, 36, 38, 46, 47, 49, and 50 could viable bacteriophages be harvested. All these sites are in the hydrophobic domain and the C-terminal end. It is remarkable that all X-Cys substitutions at positions in the N-terminal end, the N-terminal amphipathic α-helix, and the interhelical kink (see Figure 1) as well around the predicted C-terminal membrane−water interface (residues 39−44) were lethal. Apparently, the success of the mutagenesis procedure appears to be fully dependent on the screening and selection method applied as well as the nature of the amino acid to be replaced. In this respect, it should be noted that the blue colored plaques, as a result of α-complementation, strongly enhanced the chance to observe very small plaques.

In general, all substitutions result in a dramatic decrease in phage yield and plaque-forming ability (Table 1) as compared to the wild-type phage. In contrast to the relatively harmless modifications of the DNA in the intergenic region, frequently employed in genetics (Sambrook et al., 1989), in our case the modifications are performed on the functional machinery of the bacteriophage itself (Russel, 1993; Williams et al., 1995).

No attempts were made to search for the reason why some positions did not yield viable bacteriophages, but it is known from the literature that the coat protein is involved in several processes during the bacteriophage life cycle (Kuhn & Wickner, 1985; Model & Russel, 1988). For the same reason it is not possible to explain simply the reduced phage yield (Table 1) and the nonlinear relationship with its plaque-forming ability. However, bacteriophage DNA size determination using nondenaturing agarose gel electrophoresis clearly demonstrated the presence of incomplete genomes packaged into, thus noninfectious, phage particles (data not shown).

Bacteriophages consisting of the cysteine-containing coat protein were propagated to milligram quantities and checked for the DNA sequence of gene VIII. Except for the single modification in the V31C mutant, other mutants appeared to have a second, probably compensating, modification: A27S (Table 1). Also the S50C substitution was concomitant with a second N12D mutation, which in fact changed the M13 coat protein into the coat protein of the closely related bacteriophage fd and f1 (Van Wezenbeek et al., 1980; Hill & Petersen, 1982). Additional checks on the free sulfhydryl content confirmed the presence of only one, thus accessible, cysteine residue per coat protein molecule. Mutant coat proteins were found to be stable for prolonged times when stored as bacteriophage particles. However, the infective ability of mutant bacteriophage showed an enhanced decrease upon storage as compared to wild-type bacteriophage (data not shown).

AEDANS Labeling of Coat Protein Mutants. AEDANS was site-specifically and covalently bound to cysteine residues directly after phage disruption and subsequent uptake of the coat protein into sodium cholate. Background labeling on wild-type coat protein, which does not contain cysteine, could not be demonstrated using absorbance measurements. During the labeling and coat protein purification as performed by preparative HPSEC, a normal aggregational and conformational behavior was observed for all mutants, except in the case of the V33C mutant. AEDANS-labeled V33C coat protein showed a strongly enhanced reversible aggregation state, as observed during preparative cholate-HPSEC and subsequent analysis using SDS-HPSEC. Purification was only possible with a low efficiency despite the use of an increased concentration of sodium cholate in the elution buffer.

The labeling efficiency, as calculated from the molar extinction coefficients, varied between 0.3 and 0.9 AEDANS molecule/molecule of coat protein. Labeled coat protein stocks were subjected to analysis by SDS-HPSEC (Spruijt & Hemminga, 1991), showing coat protein monomers and a population of coat protein dimers that could be roughly quantified to the amount of unlabeled coat protein present. This indicates that dimerization of the coat protein is effectively prevented by attachment of the AEDANS reagent to the thiol group, as was shown earlier by Khan et al. (1995).

Environmental Probing Using AEDANS Fluorescence. The spectral properties of AEDANS provide local environmental information concerning the extent of membrane penetration. The AEDANS fluorescence maximum, as reported, varies between 520 nm in an aqueous environment and 448 nm in dimethylformamide (Hudson & Weber, 1973;

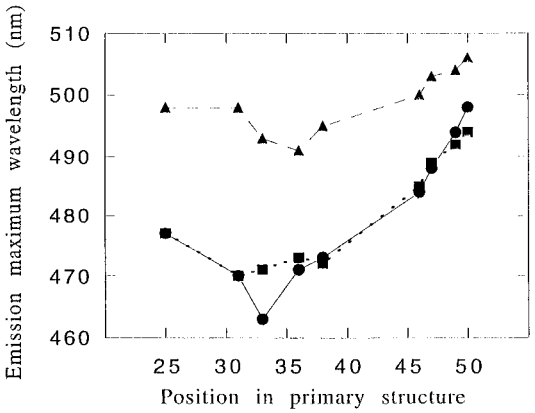

FIGURE 2: Wavelength of maximum emission of AEDANS attached to cysteines at different positions along the primary structure of the M13 coat protein. The coat protein was reconstituted into DOPC/DOPG phospholipid bilayers at L/P 250 (circles; solid line), solubilized by sodium cholate at 80 mM final concentration (squares; dotted line), and solubilized by SDS at 150 mM final concentration (triangles; dashed line).

LaPorte et al., 1981). For AEDANS-labeled cysteine, when introduced into phospholipid bilayers, a fluorescence maximum at 460 nm was found (Merrill et al., 1990). The wavelength of maximum emission of the attached AEDANS probes at different positions in the mutant coat protein's primary structure is shown in Figure 2. For the coat protein solubilized into sodium cholate and reconstituted into DOPC/DOPG bilayers, the lowest values of emission maximum, indicative for a hydrophobic environment, are found for AEDANS attached to the residues 31, 33, 36, and 38. Higher wavelength values are observed at position 25 and at the C-terminal end (positions 46−50). It should be noted that the maximum emission wavelengths in the C-terminal region increased continuously with the position in the primary structure and that typical wavelength values as obtained for AEDANS in an aqueous environment, which is about 520 nm (Hudson & Weber, 1973), are never found.

Surprisingly, upon solubilization in SDS, all wavelengths of maximum emission of the AEDANS-labeled coat protein mutants observed are 8−30 nm higher, depending on the position of AEDANS attachment. This is indicative of a more polar and hydrophilic environment as compared to the sodium cholate and phospholipid systems. As can be observed in Figure 2, the difference between the highest and lowest wavelength of maximum emission among all positions tested of the SDS-solubilized coat protein is only 15 nm, suggesting only minor changes in polarity of the local environment along the coat protein backbone. This is remarkable since the difference between the highest and lowest wavelength of maximum emission as obtained for the labeled coat proteins in sodium cholate is about 24 nm and in DOPC/DOPG even 35 nm.

We have employed additional nonionic, cationic, and other anionic detergents to solubilize the coat protein labeled with AEDANS at transmembrane positions 31 and 36. Except for SDS, the wavelengths of maximum emission, shown in Table 2, vary between 467 and 473 nm, similar to those observed in sodium cholate and in DOPC/DOPG. This finding emphasizes the exceptional property of SDS in solubilizing M13 coat protein.

Accessibility of Site-Specific AEDANS-Labeled Coat Protein Toward Acrylamide and 5-Doxylstearic Acid. Because

Table 2: Wavelengths of Maximum Emission of AEDANS, Attached to M13 Coat Protein at Positions 31 and 36, When Solubilized by Various Nonionic, Cationic, and Anionic Detergents[a]

detergent	emission maximum at position 31 (nm)	emission maximum at position 36 (nm)
octyl β-glucoside	473	473
dodecyl β-maltoside	468	471
Triton X-100	467	468
lauryldimethylamine oxide	467	468
cetyltrimethylammonium bromide	468	468
sodium deoxycholate	469	473
sodium cholate	470	473
SDS (biochemical grade)	497	491
SDS (electrophoresis grade)	498	491

[a] The final concentration of detergents was 100 mM in TEN8 buffer, except for sodium cholate (80 mM in TEN8 buffer) and SDS (150 mM in TEN8 buffer).

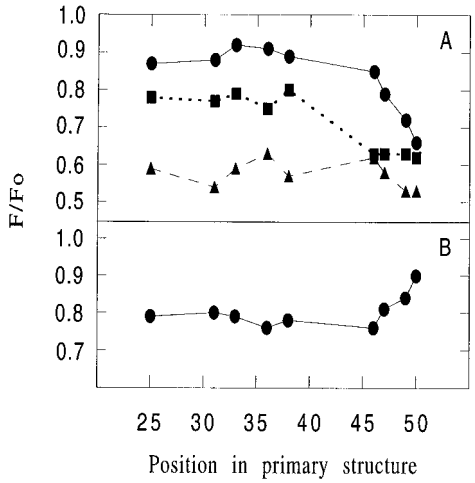

FIGURE 3: Reduction of fluorescent quantum yield of AEDANS, attached to cysteines at different positions along the primary structure of the M13 coat protein, (A) after addition of 125 mM acrylamide and (B) after addition of the apolar 5-doxylstearic acid up to 0.39 mM final concentration. The coat protein was reconstituted into DOPC/DOPG phospholipid bilayers L/P 250 (circles; solid line), solubilized by sodium cholate at 80 mM final concentration (squares; dotted line), and solubilized by SDS at 150 mM final concentration (triangles; dashed line).

the accessibility of a fluorophore to a quencher depends upon the polarity and steric effects (Lehrer & Leavis, 1978; Eftink & Ghiron, 1981; Mandal & Chakrabarti, 1988), important information can be obtained to elucidate the putative transmembrane domain of the AEDANS-labeled mutant coat protein when embedded in amphiphilic media. Both a polar, but uncharged, quencher molecule (acrylamide) and a hydrophobic quencher molecule (5-doxylstearic acid) were used. The reduced fluorescent intensity in the presence of 125 mM acrylamide as a function of different AEDANS attachment sites on the coat protein and its amphiphilic surroundings is given in Figure 3A.

For the AEDANS-labeled coat protein reconstituted into DOPC/PG at L/P 250, it should be noted that because of the proposed membrane impermeability of acrylamide (Eftink & Ghiron, 1981), acrylamide is only effective in quenching the AEDANS fluorescence from the outside of the unilamellar vesicles. Assuming a randomly oriented state of the coat protein, with only 50% of the N-termini and 50% of the C-termini of this monotopic protein sticking out on the outside of the vesicle, the quenching efficiency can be at

maximum half of that obtained for the AEDANS-labeled coat protein solubilized by detergents, where all termini are reachable. As can be seen clearly in Figure 3A, a relatively low quenching effect is found for the AEDANS attached to cysteines at positions 25 up to 46, whereas in the case of labeling sites 47, 49, and 50 a strongly increased accessibility can be observed.

Apart from the about 2-fold difference in reduced fluorescent intensity, a comparable quenching efficiency curve is obtained when the coat protein is solubilized in sodium cholate. However, the accessibility at position 46 is now strongly enhanced, and no further increased quenching efficiency can be observed toward the C-terminal part of the coat protein. As a result, two clearly distinct domains can be observed for the cholate-solubilized coat proteins, with positions 25−38 showing little exposure and positions 46−50 showing high accessibility for acrylamide.

Most striking is the accessibility effect of acrylamide, which is almost independent of the site of AEDANS attachment, when the coat protein has been solubilized in SDS. Moreover, a very efficient quenching (50−60% reduction of fluorescent quantum yield) is observed in all cases.

The influence of the hydrophobic quencher molecule 5-doxylstearic acid on the fluorescent quantum yield is given in Figure 3B. A remarkable resemblance, although opposite to that of acrylamide, can be observed for the AEDANS-labeled coat protein reconstituted into DOPC/DOPG. Clearly, the accessibility of this quencher is almost equal for positions 25−46 and decreases for sites 47−50.

Similar measurements were performed on the coat protein solubilized in sodium cholate and SDS, but only very small reductions in fluorescent intensities could be determined. This is due to the fact that the hydrophobic quencher molecule is strongly diluted as indicated by the very large detergent to protein molar ratios in cholate and SDS, 16 000 and 30 000, respectively, in sharp contrast with the lipid to protein molar ratio of 250 in the DOPC/DOPG system.

Reactivity of Site-Specific Free Sulfhydryl Groups of Cysteine to DTNB. As reported in the literature, the reactivity of the negatively charged DTNB toward the free sulfhydryl groups of cysteines is dependent on steric and electrostatic effects (Riddles et al., 1983; Takeda et al., 1992). Therefore, additional experiments were carried out with DTNB to determine the reactivity of sulfhydryl groups along the polypeptide chain.

At first, the reaction with DTNB on the cysteine-containing coat protein, reconstituted into the DOPC/DOPG phospholipid system, was not successful. An explanation was found using SDS-HPSEC, showing the presence of disulfide-bonded coat protein dimers, lacking the free sulfhydryl group. After addition of β-mercaptoethanol, the coat protein completely returned to its monomeric state (data not shown). To prevent premature oxidation of the unlabeled, and thus unprotected, thiol groups of the cysteine (Khan & Deber, 1995), protective reducing agents could not be employed due to their interference in the DTNB assay. Therefore, the various cysteine-containing coat protein mutants were reconstituted into amphiphilic media using a fast procedure, as described in Materials and Methods. The measurements were performed directly after bacteriophage disruption and subsequent fast reconstitution.

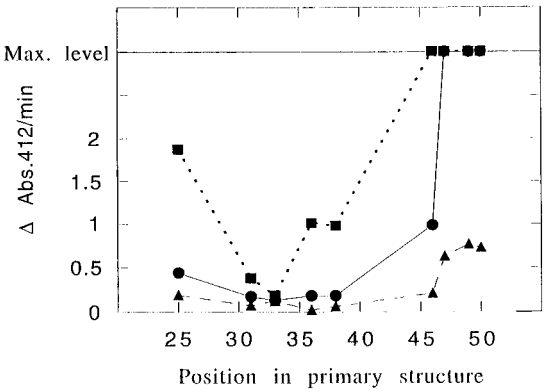

FIGURE 4: Reactivity of cysteines' free sulfhydryl groups at different positions along the primary structure of the M13 coat protein toward DTNB reagent (1 mM final concentration). The reactivity was expressed as initial increase in absorbance at 412 nm. The coat protein was reconstituted into DOPC/DOPG at L/P 50 (circles; solid line), solubilized by sodium cholate at 80 mM final concentration (squares; dotted line), and solubilized by SDS at 150 mM final concentration (triangles; dashed line). The maximum level indicates an almost instantaneous and complete reaction within seconds.

On the basis of a complete accessibility of the cysteine's thiol group for DTNB in the case of all coat protein mutants (as mentioned in the Materials and Methods and Results sections), we wanted to express that some mutants need more time to complete the reaction. For this purpose the reaction rates toward the highly polar and negatively charged DTNB reagent are expressed as initial increases in absorbance at 412 nm. The determination of the reaction rate appeared to be a very sensitive method to detect the extent of accessibility of the cysteine's thiol group for DTNB. Figure 4 shows the reaction rate for the different sites of cysteine in the coat protein mutants when the coat protein is reconstituted into DOPC/DOPG at L/P 50 and when solubilized in 80 mM sodium cholate and in 150 mM SDS.

As has been explained before, only half of the coat protein termini can be involved in the initial reaction with DTNB due to the assumed random protein orientation in the phospholipid bilayer (termini inside or outside). Furthermore, the membrane is assumed to be impermeable by the effect of the highly polar and charged properties of the DTNB reagent. This assumption is confirmed by the observation that the maximal level of absorbance was about half that when the reaction was carried out on the coat protein solubilized by detergents. For the coat protein in the DOPC/DOPG system the apparent reaction rate of the cysteine residues at positions 31, 33, 36, and 38 was low, suggesting steric hindrance and unfavorable conditions for reaction for the cysteines. A moderate increase could be observed for the cysteine at position 25. Toward the C-terminal end of the coat protein, a strongly increased reactivity was seen at position 46, and an almost instantaneous and complete reaction was achieved for the cysteines at position 47, 49, and 50.

The reaction rates observed for the coat protein solubilized in SDS are very low, showing an overall low accessibility for DTNB. This is probably caused by repulsive electrostatic interactions between the negatively charged DTNB and the negatively charged SDS molecules surrounding the coat protein. On the other hand, the reaction rates follow a similar tendency as found for the coat protein in phospholipid bilayers. It should be mentioned that the most C-terminal

residues, which were suspected to be outside the micelle, show only a moderate rate of reaction, indicating that these residues are also coated by SDS. In contrast, high reaction rates were recorded for the coat protein solubilized in the also negatively charged cholate. Again the reaction rates observed are low for cysteines introduced to the coat protein at positions 31−38, higher at position 25, and maximal at the C-terminal positions 46−50.

DISCUSSION

Introduction of Cysteine Residues into the Major Coat Protein and Screening for Functional State. Cysteine-containing coat protein mutants were generated using oligonucleotide directed mutagenesis according to Kunkel (1985), allowing only the appearance of viable bacteriophage particles. Thus, only mutant bacteriophages with the coat protein in a functional state during all processes in the bacteriophage life cycle could be detected. This ensures that the mutation causes a minimal perturbation of the native conformation and functioning of the coat protein.

In the case of introduction of cysteine residues into the coat protein as selected by phage viability, the success reported is very scarce (Williams & Deber, 1993; Khan & Deber, 1995; Iannolo et al., 1995; Williams et al., 1995). Phage viability, however, is an excellent biological control of proper functionality of the coat protein, even in cases when strongly decreased phage growth is observed. Moreover, obtaining mutant coat protein assembled properly in phage particles has the advantage that mutant bacteriophage isolation from the growing medium is simple and fast. Also the stability of the coat protein (including the reduced state of the cysteine's thiol group) when part of the bacteriophage is warranted for prolonged times of storage. In contrast, other expression methods or chemical synthesis of the coat protein require excessive and complicated purification protocols (Thiaudière et al., 1993; Shon et al., 1990) and lack a biological control of the coat protein but enable unlimited modification of the coat protein.

Establishment of the Transmembrane Domain. From the environmental probing of various sites on the coat protein by AEDANS, as obtained from its fluorescent properties, information can be deduced to establish the transmembrane domain. This information is supplemented with accessibility measurements using acrylamide, 5-doxylstearic acid, and DTNB. The results show a remarkable difference between the coat protein embedded in either phospholipids or sodium cholate and the coat protein solubilized by SDS.

The emission maximum wavelength (Figure 2) observed for the coat protein reconstituted into mixed phospholipid bilayers, composed of 80% zwitterionic DOPC and 20% negatively charged DOPG, and into sodium cholate are almost identical. Since the extreme values of emission maximum wavelengths as found for free AEDANS molecules in an entirely aqueous environment or in an organic solvent are never found, it is concluded that the AEDANS probe also senses the heterogeneous surrounding provided by the various amino acid residues of the coat protein itself. However, it can be seen clearly that the AEDANS bound to residues 46−50 is in a more polar environment than when it is attached to positions 31, 33, 36, and 38. Also, the fluorescent properties of the AEDANS at position 25 are indicative of a somewhat more polar environment. Similar conclusions can be drawn from the accessibility measurements (Figures 3 and 4), underlining the transmembrane boundary positions for the residues 25 and 46 of the coat protein; residue 25 is located just inside the hydrophobic part of the membrane, probably in close proximity to the membrane−water interface, whereas residue 46 is proposed to be just at the outside of the headgroup region of the membrane. This result is in agreement with the findings of Li and Deber (1991), who place residues 23 and 24 at the membrane aqueous interface as the entry point of the hydrophobic transmembrane domain of the membrane-bound coat protein. Also, the observation of tryptophan at position 26 being in a hydrophobic surrounding, as concluded from steady-state fluorescence spectroscopy (Spruijt & Hemminga, 1991), agrees with this conclusion. However, it should be noted also that the residues 47, 49, and 50 in the C-terminal part cannot be assigned as typical "aqueous" amino acid residues and are thus in close proximity to the membrane surface.

When sodium cholate is used to solubilize the major coat protein, effects are found that are in reasonably good agreement with the results obtained for the coat protein in phospholipid bilayers. There is, however, a difference in accessibility toward acrylamide and DTNB, where the coat protein residues 46−50 are well accessible in sodium cholate and less so, although with a gradually increased accessibility from residue 46 to residue 50, in DOPC/DOPG. This observation underlines again the close proximity and influence of the membrane surface to the most C-terminal amino acid residues of the coat protein reconstituted into DOPC/DOPG, an effect that could be related to the typical headgroup nature of the phospholipids. Apparently, domains of the coat protein which are packed or coated by cholate molecules and probably all other detergents (except for SDS) listed in Table 2, are at least similarly packed by phospholipid molecules in bilayers. Only 8−9 molecules of cholate are reported to surround the coat protein monomer on the average (Makino et al., 1975; Cavalieri et al., 1976). This finding suggests a quite open detergent−protein structure, in which the hydrophobic sites of the coat protein are only slightly covered by cholate.

In discussing the results of the SDS-solubilized coat protein, it should be emphasized that only minor environmental differences are found for all sites tested along the coat protein backbone. Typical differences in polarity and accessibility that could be expected based on various assumptions in literature with respect to hydrophobic and hydrophilic domains on the coat protein (Turner & Weiner, 1993; Pebay-Peyroula et al., 1995) were not found. Therefore, it is concluded that the part of the coat protein investigated is not embedded in a micellar structure of SDS molecules, as proposed by Makino et al. (1975) and Papavoine et al. (1994). It is more likely that the coat protein is only coated by SDS molecules over the full length, thereby covering and shielding the protein backbone almost independently of the nature of the amino acid side chains involved. In this view, it is interesting that SDS creates a more polar environment around the residues within the transmembrane domain, but as well a more apolar environment around the residues that were expected to be outside the "micelle". This observation is in agreement with the findings of polar local environments for spin-labeled-cysteine containing coat proteins of bacteriophages M13 and IKe in

the presence of SDS (Khan et al., 1995). The SDS-solubilized coat protein, which is found to be monomeric (Spruijt & Hemminga, 1991; McDonnell et al., 1993; Van de Ven et al., 1993), is on average scale covered by 30 SDS molecules (Makino et al., 1975), which is only slightly more than the generally assumed binding ratio of about 1 molecule of SDS/2 amino acid residues (Reynolds & Tanford, 1970). In this respect, the location of amphiphiles in the coat protein−amphiphile complex needs to be established, for example, as has been reported by Pebay-Peyroula et al. (1995) for OmpF porin solubilized by the detergent octyl β-glucoside.

The polar local environment as concluded from the fluorescent maximum (Figure 2) is provided by the sulfate headgroups of the SDS molecules, whereas the absence of a hydrophobic barrier, as expected in case of micellar structured SDS molecules, allows acrylamide to be a very effective quencher. The overall distribution of negatively charged sulfate headgroups strongly obstructs the reaction with DTNB. Therefore, it must be concluded that SDS is a good solubilizing agent but badly mimics the typical nature of a membrane structure.

Biological Implications. On the basis of our findings above, the transmembrane boundaries of the membrane-bound coat protein are close to residues 25 and 46. This localization of the transmembrane domain is in good agreement with the presence of a stable α-helical structure of the coat protein as performed on the coat protein solubilized by SDS (McDonnell et al., 1993; Van de Ven et al., 1993). As a consequence, at least three of the four C-terminal lysine residues must have their Cα moiety within the membrane interior. It has been described already that, due to its long side chain, the positively charged ϵ-amino group can interact with negatively charged moieties as provided by the phosphate headgroups of the phospholipids (Ballesteros & Weinstein, 1992; Hemminga et al., 1992). A special case is lysine 40, since this residue is buried deeply in the membrane. However, molecular modeling by Stopar (personal communication) indicates that the ϵ-amino group of lysine 40 is close enough to the headgroup region to enable charge neutralization. Because of the large excess of negative charges provided by the phosphate groups of the lipid molecules, it is likely also that the ϵ-amino groups of lysines 43, 44, and even 48 (whose Cα moiety is assumed to be in the aqueous phase according to our model) are in close proximity to the headgroup region and will electrostatically interact with the phosphate groups. This mechanism could as well provide a protection of the coat protein, since it prevents the sticky lysines from undesired interactions. Also, the unexpected incomplete cleavage by several proteases at this C-terminal part of the coat protein in phospholipids, as reported before (Chamberlain et al., 1978; Bayer & Feigenson, 1985), is in agreement with this phenomenon. Apart from this, the positive-inside rule as proposed by Von Heijne (1992), can be extended for the membrane-bound M13 coat protein in a literal way.

As a consequence of the proposed transmembrane domain, tyrosine 24 and tryptophan 26, as well as the two phenylalanines at positions 42 and 45, are close to the membrane surface. This agrees well with the finding that aromatic amino acid residues are often found at the membrane−aqueous interface, where they are thought to play a key role in anchoring the protein in the membrane (Deisenhofer et al., 1986; Henderson et al., 1990; Weiss et al., 1991; Cowan et al., 1995).

The major coat protein of bacteriophage M13 is, thus, very well embedded in the membrane by (I) the presence of mainly hydrophobic amino acid residues, (II) the presence of anchoring aromatic amino acid residues on both ends of the transmembrane domain, and (III) the presence of several basic lysine residues, which provide an anchoring by interacting with phospholipid phosphates in an electrostatical way. It should be realized, however, that anchoring does not mean that the coat protein is fixed firmly in the membrane. The coat protein will almost certainly float in the membrane in a dynamical way, thereby sometimes exposing side chains to an unfavorable environment. With respect to the comprehensiveness of macromolecular interacions during bacteriophage infection, this dynamic behavior offers an interesting view on the transient stay of the coat protein in the membrane. This mechanism provides a protection of the coat protein in one way, but enables on the other hand the release of the coat protein from its membrane environment during the phage assembly and extrusion process, which is obviously mediated by the proteins constituting the membrane-bound viral assembly site (Webster & Lopez, 1985; Russel, 1994).

REFERENCES

Altenbach, C., Flitsch, S. L., Khorana, H. G., & Hubbell, W. L. (1989) *Biochemistry 28*, 7806−7812.

Ballesteros, J. A., & Weinstein, H. (1992) *Biophys. J. 62*, 107−109.

Bayer, R., & Feigenson, G. W. (1985) *Biochim. Biophys. Acta 815*, 369−379.

Berkowitz, S. A., & Day, L. A. (1976) *J. Mol. Biol. 102*, 531−547.

Cavalieri, S. J., Goldthwait, D. A., & Neet, K. E. (1976) *J. Mol. Biol. 102*, 713−722.

Chamberlain, B. K., Nozaki, Y., Tanford, C., & Webster, R. E. (1978) *Biochim. Biophys. Acta 510*, 18−37.

Cowan, S. W., Garavito, R. M., Jansonius, J. N., Jenkins, J. A., Karlsson, R., König, N., Pai, E. F., Pauptit, R. A., Rizkallah, P. J., Rosenbusch, J. P., Rummel, G., & Schirmer, T. (1995) *Structure 3*, 1041−1050.

Deber, C. M., Khan, A. R., Li, Z. M., Joensson, C., Glibowicka, M., & Wang, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11648−11652.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1986) *Nature 318*, 618−624.

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Chem. 114*, 199−227.

Flitsch, S. L., & Khorana, H. G. (1989) *Biochemistry 28*, 7800−7805.

Fodor, S. P. A., Dunker, A. K., Ng, Y. C., Carsten, D., & Williams, R. W. (1981) *Prog. Clin. Biol. Res. 64*, 441−455.

Glucksman, M. J., Bhattacharjee, S., & Makowski, L. (1992) *J. Mol. Biol. 226*, 455−470.

Hemminga, M. A., Sanders, J. C., & Spruijt, R. B. (1992) *Prog. Lipid Res. 31*, 301−333.

Hemminga, M. A., Sanders, J. C., Wolfs, C. J. A. M., & Spruijt, R. B. (1993) in *Protein−Lipid Interactions* (Watts, A., Ed.) *New Comprehensive Biochemistry*, Vol. 25, pp 191−212, Elsevier, Amsterdam.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol 213*, 899−929.

Henry, G. D., & Sykes, B. D. (1992) *Biochemistry 31*, 5284−5297.

Hill, D. F., & Petersen, G. B. (1982) *J. Virol. 44*, 32−46.

Hudson, E. N., & Weber, G. (1973) *Biochemistry 12*, 4154−4161.

Iannolo, G., Minenkova, O., Petruzzelli, R., & Cesareni, G. (1995) *J. Mol. Biol. 248*, 835−844.

Khan, A. R., & Deber, C. M. (1995) *Biochem. Biophys. Res. Commun. 206*, 230−237.

Khan, A. R., Williams, K. A., Boggs, J. M., & Deber, C. M. (1995) *Biochemistry 34*, 12388−12397.
Kuhn, A., & Wickner, W. (1985) *J. Biol. Chem. 260*, 15907−15913.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A 82*, 488−492.
Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol 157*, 105−132.
Lakey, J. H., Baty, D., & Pattus, F. (1991) *J. Mol. Biol. 218*, 639−653.
LaPorte, D. C., Keller, C. H., Olwin, B. B., & Storm, D. R. (1981) *Biochemistry 20*, 3965−3972.
Lehrer, S. S., & Leavis, P. C. (1978) *Methods Enzymol. 49*, 222−236.
Li, Z. M., & Deber, C. M. (1991) *Biochem. Biophys. Res. Commun. 180*, 687−693.
Li, Z. M., Glibowicka, M., Joensson, C., & Deber, C. M. (1993) *J. Biol. Chem. 268*, 4584−4587.
Makino, S., Woolford, J. L., Jr., Tanford, C., & Webster, R. E. (1975) *J. Biol. Chem. 250*, 4327−4332.
Mandal, K., & Chakrabarti, B. (1988) *Biochemistry 27*, 4564−4571.
Marvin, D. A., Hale, R. D., Nave, C., & Citterich, M. H. (1994) *J. Mol. Biol. 235*, 260−286.
McDonnell, P. A., Shon, K., Kim, Y., & Opella, S. J. (1993) *J. Mol. Biol. 233*, 447−463.
Merril, A. R., Cohen, F. S., & Cramer, W. A. (1990) *Biochemistry 29*, 5829−5836.
Milik, M., & Skolnick, J. (1995) *Biophys. J. 69*, 1382−1386.
Model, P., & Russel, M. (1988) *Filamentous Bacteriophage*, Plenum Press, New York.
Nozaki, Y., Chamberlain, B. K., Webster, R. E., & Tanford, C. (1976) *Nature 259*, 335−337.
Papavoine, C. H. M., Konings, R. N. H., Hilbers, C. W., & van de Ven, F. J. M. (1994) *Biochemistry 33*, 12990−12997.
Pebay-Peyroula, E., Garavito, R. M., Rosenbusch, J. P., Zulauf, M., & Timmins, P. A. (1995) *Structure 3*, 1051−1059.
Reynolds, J. A., & Tanford, C. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1002−1007.
Riddles, P. W., Blakeley, R. L., & Zerner, B. (1983) *Methods Enzymol. 91*, 49−60.
Russel, M. (1991) *Mol. Microbiol 5*, 1607−1613.
Russel, M. (1993) *J. Mol. Biol. 231*, 689−697.
Russel, M. (1994) *Science 265*, 612−614.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Shon, K., Schrader, P., Opella, S., Richards, J., & Tomich, J. (1990) *UCLA Symp. Mol. Cell. Biol., New Ser. 109*, 109−118.
Spruijt, R. B., & Hemminga, M. A. (1991) *Biochemistry 30*, 11147−11154.
Spruijt, R. B., Wolfs, C. J. A. M., & Hemminga, M. A. (1989) *Biochemistry 28*, 9158−9165.
Takeda, K., Shigemura, A., Hamada, S., Gu, W., Fang, D., Sasa, K., & Hachiya, K. (1992) *J. Protein Chem. 11*, 187−192.
Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457*, 133−170.
Thiaudière, E., Soekarjo, M., Kuchinka, E., Kuhn, A., & Vogel, H. (1993) *Biochemistry 32*, 12186−12196.
Turner, R. J., & Weiner, J. H. (1993) *Biochim. Biophys. Acta 1202*, 161−168.
Van de Ven, F. J. M., Van Os, J. W. M., Aelen, J. M. A., Wymenga, S. S., Remerowski, M. L., Konings, R. N. H., & Hilbers, C. W. (1993) *Biochemistry 32*, 8322−8328.
Van Wezenbeek, P. M. G. F., Hulsebos, T. J. M., & Schoenmakers, J. G. G. (1980) *Gene 11*, 129−148.
Von Heijne, G. (1992) *J. Mol. Biol. 225*, 487−494.
Webster, R. E., & Lopez, J. (1985) in *Structure and assembly of the class I filamentous bacteriophage* (Casjens, S., Ed.) pp 235−267, Jones and Bartelett Publishers, Inc., Boston, MA.
Weiss, M. S., Abele, U., Weckesser, J., Welte, W., Schiltz, E., & Schulz, G. E. (1991) *Science 254*, 1627−1630.
Williams, K. A., & Deber, C. M. (1993) *Biochem. Biophys. Res. Commun. 196*, 1−6.
Williams, K. A., Glibowicka, M., Li, Z., Li, H., Khan, A. R., Chen, Y. M. Y., Wang, J., Marvin, D. A., & Deber, C. M. (1995) *J. Mol. Biol. 252*, 6−14.
Wolkers, W. F., Haris, P. I., Pistorius, A. M. A., Chapman, D., & Hemminga, M. A. (1995) *Biochemistry 34*, 7825−7833.

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