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AN ENGINEERED DISULFIDE BRIDGE IN THE TRANSMEMBRANE REGION OF PHAGE M13 COAT PROTEIN STABILIZES THE α-HELICAL DIMER

Amir R. Khan# and Charles M. Deber*

Division of Biochemistry Research, Research Institute, Hospital for Sick Children, Toronto M5G 1X8, Canada

Department of Biochemistry, University of Toronto, Toronto M5S 1A8, Ontario, Canada

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Summary: A single Cys-residue (Cys^{24}) was introduced into the 50-amino acid major coat protein of M13 bacteriophage as part of a two-site substitution (Y24C-V31A) within the effective transmembrane (TM) segment (Tyr^{21} to Ile^{39}) of the coat protein. Mutant Y24C-V31A was able to complete the phage life cycle and was shown to contain free sulfhydryls in the intact virus, as evidenced by susceptibility of Y24C-V31A phage to alkylation by Cys-specific ^{14}C-iodoacetamide (^{14}C-IAN). In contrast, the protein solubilized in deoxycholate micelles was resistant to ^{14}C-IAN modification and was virtually inert to a transition from a characteristic α-helical oligomeric state to an aggregated β-sheet structure relative to WT and V31A coat proteins, as shown by circular dichroism spectroscopy and SDS-PAGE. Reduction of mainly dimeric Y24C-V31A protein using β-mercaptoethanol (β-ME) generated monomeric species and resulted in a loss of helical thermostability. The overall results indicated that solubilization of Y24C-V31A coat protein into micelles resulted in formation of thermostable disulfide-bridged helical dimers. The disulfide bridge is deduced to be positioned along the stripe of residues involved in hydrophobic packing of TM parallel helical dimers.

Most transmembrane (TM) segments of integral membrane proteins are predicted to be α-helical in conformation, as supported experimentally by the limited examples of high-resolution membrane protein structures [*e.g.*, photosynthetic reaction centre (1); and bacteriorhodopsin (2)]. Increasing evidence suggests that TM helices have functional roles beyond that of a hydrophobic membrane anchor (3) [*e.g.*, membrane insertion (4); and TM signalling (5)]. Recent studies have illustrated the capacity of the TM segment to mediate oligomerization for some membrane proteins via TM-TM association (6, 7). For example, pairwise interactions mediated by the TM region have been implicated in the assembly of the multi-subunit T-cell receptor, allowing

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

* To whom correspondence should be addressed at the Research Institute. Fax: 416-813-5005.

Abbreviations: TM, transmembrane; CD, circular dichroism; SDS-PAGE, sodium dodecylsulfate poly-acrylamide gel electrophoresis; IAN, iodoacetamide; DOC, deoxycholate; β-ME, β-mercaptoethanol; PEG, polyethylene glycol.

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subunits to be "mixed and matched" to generate receptor diversity (8). Cys residues within or adjacent to TM helices which contribute to function through disulfide bond formation have also been reported, *viz.*, the E5 oncoprotein of bovine papilloma virus forms membrane-associated disulfide-bonded dimers which transform fibroblasts *in vitro* (9).

Our laboratory has utilized the major (gene VIII) coat protein of bacteriophage M13 for identifying those elements of the primary sequence of a prototypic TM region which contribute to structural and functional properties of membrane proteins. The protein is first synthesized as a precursor - the procoat - with a 23-residue basic and apolar peptide appended to the N-terminus of the 50-residue mature sequence (10, 11). Following membrane insertion, the host *E. coli* leader peptidase excises the leader sequence (12, 13). The mature sequence (AEGDDPAKAAFNSLQAS-ATEYIGYAWAMVVVIVGATIGIKLFKKFTSKAS) consists of an N-terminal periplasmic domain, a 19-residue apolar transmembrane (TM) region (underlined); and an 11-residue C-terminal basic domain which resides in the cytosol. Using site-directed mutagenesis, we previously generated a library of *ca.* 60 single- and multiple-site viable (functional) mutants within the putative TM region (Tyr^{21}-Ile^{39}) of coat protein (14). Despite their common functionality, significant variations were found among wild-type (WT) and mutant coat proteins with respect to relative populations and thermostabilities of monomeric and dimeric helical species upon solubilization by micelles. We have now engineered a cysteine residue into the TM region of M13 coat protein. We report here the biochemical and biophysical consequences of disulfide bridge formation within the TM region and its effects on helix stability.

MATERIALS AND METHODS

Site-directed mutagenesis. Mutagenesis of the TM region to obtain viable mutant Y24C-V31A was performed using an oligonucleotide-directed mutagenesis kit [Amersham Corp., Chicago, IL] based on the Eckstein method (15) as described previously (14). A mutagenic oligonucleotide (5'-CGCCCACGCATAACCGATATAT-3'), targetted to the region Tyr^{21} to Ala^{27}, was "randomized" such that each nucleotide in the (WT) coat protein sequence was contaminated with 2.5% of each of the other three nucleotides (16). Oligonucleotides were synthesized at the Biotechnology Centre, Banting Institute, University of Toronto, and purified with oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA.). The template used for the reaction was mutant V31A M13 ssDNA, with the purpose of preserving the single V31A mutation and introducing a second-site substitution. Single mutant V31A was generated in previous work (17) in this lab. Following the mutagenesis reaction, the re-circularized double-stranded (mutant Y24C-V31A) M13 DNA was transformed into $CaCl_2$-competent JM101 (*E. coli*) cells. Following ssDNA isolation (18), mutants were identified by DNA sequencing (19).

Large-scale preparations. WT and mutant phages were amplified to milligram amounts by infecting liter cultures of JM101 cells in 2-TY media (1.6% tryptone, 1% yeast extract and 86 mM NaCl; 17). Phage was precipitated using 0.2 x volume of a polyethylene glycol/NaCl solution (PEG/NaCl; 20% PEG and 14% NaCl). Phage was purified by density ultracentrifugation (20) using a KBr gradient (4°C, SW27 rotor, 20 hours at 24000 rpm) and subsequently desalted using a Centriprep-3 filter (Amicon Co.). Coat protein was separated from DNA by gel filtration [Sephacryl S-200 HR column, equilibrated with 8 mM Na deoxycholate (DOC) and 25mM sodium borate elution buffer, pH 9.0], as described previously (21).

Circular dichroism spectra and SDS-PAGE. CD spectra were recorded on the Jasco J-720 spectropolarimeter with protein concentrations typically at 1 mg/mL (30 mM DOC, 25 mM Na borate, pH 9.0). Each curve was the average of 4 scans, recorded between 190-250 nm in a cell of

path length 0.1 mm. SDS-PAGE was performed using either 5%-stacking and 15%-separating acrylamide gels, or "pre-cast" Tris-Gly 4-20% acrylamide gradient gels (Novex, San Diego, CA.).

Iodoalkylation of sulfhydryls. Iodo[1-^{14}C]acetamide [^{14}C-IAN (Amersham Corp.); 50 μCi total activity dissolved in 500 μL of 10 mM Tris and 1 mM ethylenediaminetetraacetic acid (TE, pH 8.0)] was used to alkylate free -SH groups of cysteine residues. Standard conditions of the reaction were 37°C, pH 8.5 (22). The molar ratio of accessible sulfhydryl groups to the total concentration of protein was quantified as follows: (a) The molar concentration of Y24C-V31A coat protein in phage was determined by absorption spectroscopy at 269 nm using a molar extinction co-efficient of 457 [in units of L/(mol x cm)] (23). In addition, a Peterson-Lowry protein assay was performed (24). (b) ^{14}C-IAN stock solution (1.8 mM, 53 mCi/mmol) was mixed with an equal volume of 200 mM cold IAN (Sigma Co.). The resulting concentration of ^{14}C-IAN was 100 mM with a specific activity of 1.1 x 10^9 dpm/mmol. A minimum of 10x molar excess of this solution was used to radioalkylate a known concentration of Y24C-V31A coat protein in the intact phage using the standard conditions described above. (c) Labelled phage was separated from soluble ^{14}C-IAN by precipitation in PEG/NaCl. The precipitation was repeated several times to reduce the background from unbound ^{14}C-IAN. Subsequently, phage was placed in a vial consisting of aqueous counting scintillant (ACS, Amersham Corp.) for measurement using a Beckman LS 6000 IC counter.

RESULTS AND DISCUSSION

The mutant Y24C-V31A (14) introduced into M13 coat protein the first Cys residue into the major coat proteins of the entire filamentous bacteriophage family, which are otherwise devoid of cysteine [*e.g.*, f1, Pf1, Pf3, and IKe (25-31)]. In previous mutagenesis studies in this lab, Gly^{23} and Tyr^{24} loci were found to be susceptible to polar mutations (*e.g.*, G23R and Y24D). Accordingly, it was proposed that Gly^{23} and Tyr^{24} probably occur at the membrane-aqueous interface, where they constitute the entry point of the hydrophobic TM helix of membrane-bound coat protein (17). Thus, Cys^{24} (as part of mutant Y24C-V31A) would likely reside at the hydrophobic-aqueous interface in micelles.

In order to determine whether Y24C-V31A coat protein - which coats the DNA of the intact virus - consisted of exposed -SH groups, intact Y24C-V31A phage was subjected to radioalkylation with cysteine-specific ^{14}C-iodoacetamide (^{14}C-IAN). The phage was precipitated (PEG/NaCl) after 90 min to determine the extent of alkylation (Fig. 1). Y24C-V31A incorporated ^{14}C, while V31A exhibited little reactivity with ^{14}C-IAN. Minor components of the intact phage (*viz.*, coat proteins corresponding to genes VII and IX at one end of the virion) contain Cys residues, but there are few copy numbers of these proteins in the intact virus (32). Further experiments showed that Y24C-V31A incorporation of ^{14}C-IAN could be eliminated by pre-incubation with excess cold IAN (Fig. 1). Thus, Y24C-V31A phage contains free and accessible sulfhydryls at the 24 locus. In contrast, purified Y24C-V31A coat protein reconstituted into DOC micelles was not derivatized by ^{14}C-IAN (data not shown). This result suggested initially that, upon solubilization of coat protein into micelles, either Cys^{24} -SH groups were sterically inaccessible to modification by IAN, or cysteine residues were disulfide-bonded.

The molar ratio of accessible sulfhydryls to the total concentration of coat protein was quantified. A typical experiment using 25 μg of Y24C-V31A coat protein (as part of the intact phage) resulted in the incorporation of 1000 dpm with a background (V31A phage) of 12 dpm. The

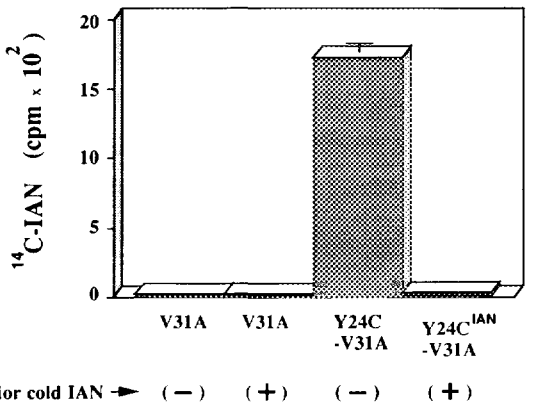

Figure 1. Free -SH groups in intact M13 bacteriophage coat protein double-site mutant Y24C-V31A, modified by iodo[1-^{14}C]acetamide. Reactions were performed at 37°C for 90 min., pH 8.5, using 50 μg of phage at a concentration of 1 mg/mL. Phages were either untreated (-) or pre-treated (+) with cold iodoacetamide (to give Y24C^{IAN}-V31A) as indicated. Non-specific radioactivity - monitored with V31A phage and Y24C^{IAN}-V31A phage in parallel reactions - was negligible relative to ^{14}C-incorporation into Y24C-V31A.

maximum percentage of modified -SH groups determined from several experiments was 35% (+/- 5%). Prior treatment with reducing agent (dithiothreitol) failed to increase the ratio of modified -SH groups. These results suggest that the 24 locus may be partially buried in the protein coat, and is consistent with mutagenesis and model building studies in conjunction with x-ray diffraction models of the intact phage (33). The Y24 locus has been shown to be at least partially susceptible to lactoperoxidase-mediated iodination (34).

Coat protein in micelles. WT coat protein conformation in micelles is largely α-helical, as shown by CD spectroscopy in 30 mM deoxycholate (DOC) at room temperature (Fig. 2a). Upon gradual heating to 95°C, WT is irreversibly converted to a β-sheet aggregate (35). Similarly, spectra of the V31A mutant show an irreversible α-helix to β-sheet transition upon heating (Fig. 2b). However, as shown in Fig. 2c, Y24C-V31A was resistant to the transition, exhibiting only a moderate (and reversible) decrease in α-helicity. SDS-PAGE studies were performed independently of CD experiments (Fig. 2, insets). WT coat protein occurs as a diffuse monomer-dimer mixture at room temperature (Fig. 2a, inset). Upon heating at 95°C briefly, high molecular weight β-sheet aggregates were irreversibly formed and visible in the stacking gel and the stacking/separating gel interface. Distinct from WT, V31A coat protein exists as a discrete dimer at room temperature and is only partially sensitive to β-sheet aggregation upon brief heating at 95°C (Fig. 2b, inset). Prolonged exposure (> 5 min) to high temperatures results in fully aggregated V31A coat protein (data not shown). In contrast with WT and V31A, Y24C-V31A showed no evidence of β-sheet aggregation (Fig. 2c, inset). Instead, a "ladder" of species, beginning with the prominent dimer, were obtained and remained stable upon heating. A Western blot using (WT) anti-M13 phage antibody confirmed that these bands were oligomers of coat protein (data not shown).

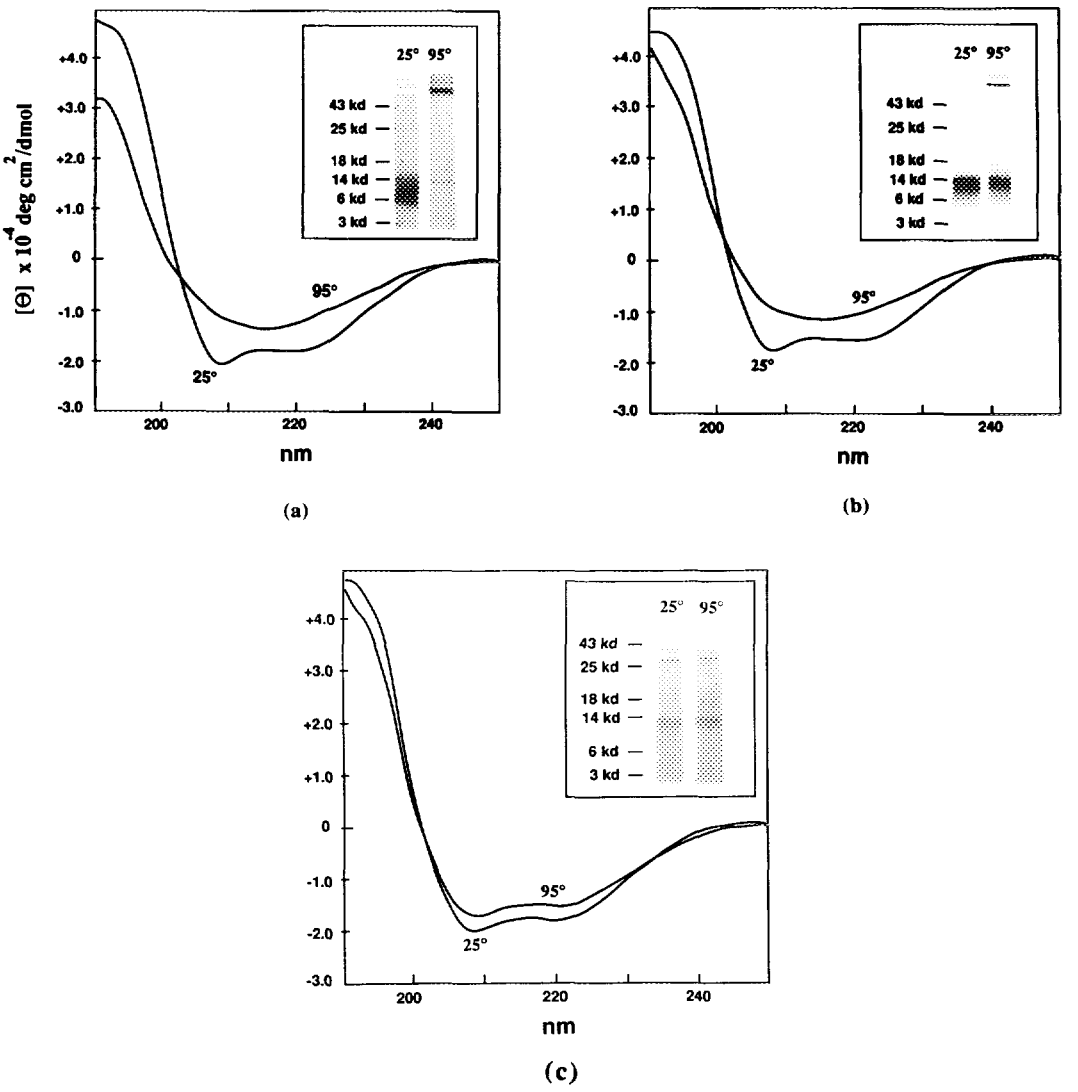

Figure 2. Circular dichroism and SDS-PAGE species of (a) wild type; (b) mutant V31A; and (c) mutant Y24C-V31A M13 coat proteins. Protein samples were 1 mg/mL in 30 mM deoxycholate and 25 mM sodium borate buffer (pH 9.0). In CD experiments, samples were heated at 5° increments from 25° to 95°; intermediate spectra are not shown. SDS-PAGE analyses of the coat protein samples used in CD experiments were performed by loading on gels an unheated sample (*i.e.*, 25°) and a sample after heating at 95° for 5 min.

The observed thermostable dimer and enhanced α-helical stability of mutant Y24C-V31A suggested a "spontaneous" disulfide bond had formed upon solubilization of Y24C-V31A coat proteins into deoxycholate. Ostensibly, the conditions under which coat protein is solubilized in micelles (shaking at 37°C, pH 9.0) are amenable to oxidation of free sulfhydryls. In order to test this hypothesis, WT and mutant coat proteins (solubilized in DOC and purified by gel filtration) were

subjected to β-ME-mediated reduction, after which their respective thermostabilities were evaluated by heating and SDS-PAGE analysis. β-ME had no effect on the properties of control proteins (Fig. 3a,b). However, treatment of Y24C-V31A protein with β-ME (25°C) and subsequent SDS-PAGE analysis showed a new population of monomeric species along with dimer (Fig. 3c). Upon heating briefly to 95°C, non-reduced Y24C-V31A protein remained stable. However, Y24C-V31A coat protein in the presence of β-ME became partially aggregated.

The *in vitro* β-polymerization process has been proposed to be initiated by head-to-tail binding of coat protein units in which electrostatic interactions play a crucial role (35). In membrane-solubilized coat protein TM mutants, formation of these aggregates may involve at least a two-step process (36) in which intramembranous helical dimers/oligomers first dissociate to monomers, followed by escape from the micelle, and ultimately water-based re-association of the monomers into high molecular weight β-sheets. The fact that (non-covalent) helical dimeric species (*e.g.*, V31A) display such greatly enhanced stability in membrane-mimetic environments *vs.* their corresponding helical monomeric forms, suggests that residue-specific packing interactions within the protein-protein interfacial region drive helix stability (37, 38). In support of this model, disulfide bond formation of mutant Y24C-V31A, which further stabilizes the dimer through a covalent S-S bond, apparently prevents protein escape from the micelle - even at elevated temperatures - and thereby inhibits β-oligomerization.

A helical wheel representation of the segment Tyr^{21} to Ile^{39} is shown in Fig. 4. The disulfide bridge in Y24C-V31A coat protein is seen to occur two helical turns up the major helical

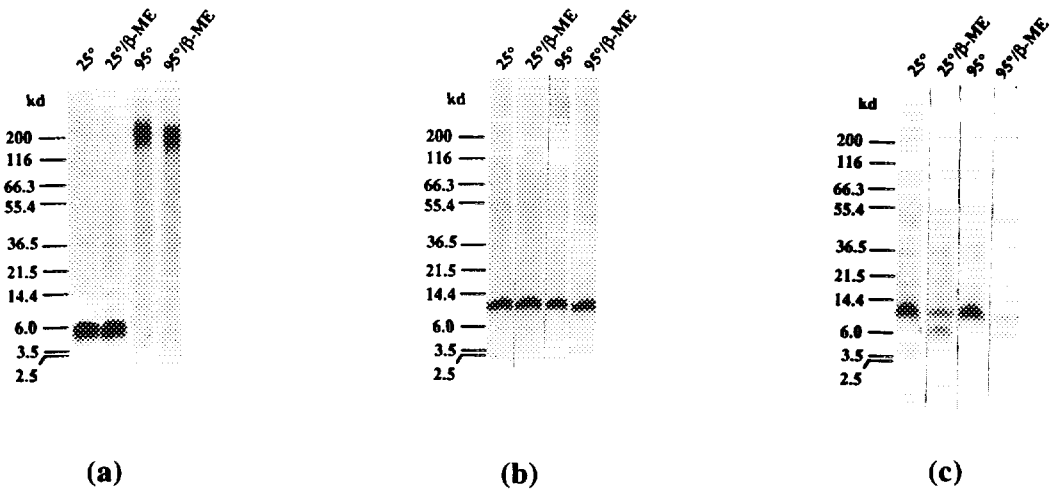

Figure 3. SDS-PAGE analysis of (a) wild type; (b) V31A; and (c) Y24C-V31A M13 coat proteins as a function of β-mercaptoethanol treatment. Each protein (1 mg/mL) solubilized in 30 mM deoxycholate and 25 mM sodium borate buffer, pH 9.0) was either untreated, or incubated in excess β-mercaptoethanol at room temperature for 10 min as indicated in the diagram. Protein samples were run at 25° and after heating at 95° for 5 min.

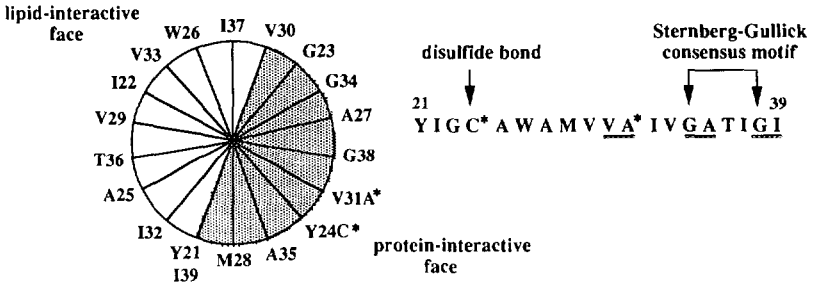

Figure 4. Helical wheel representation of M13 coat protein residues Tyr^{21} to Ile^{39}. Mutational and conformational analysis has suggested the involvement of the underlined residues - which constitute a protein-interactive face (shaded) - in mediating helical dimer formation in membrane-mimetic environments (14). The opposing face would form largely non-specific contacts with lipids. Both mutant residues in Y24C-V31A coat protein are aligned within the protein-interactive face. See text for a further discussion.

axis - but along the same face - relative to the Val^{31} (and mutant Ala^{31}) side chain. The disulfide bridge is also along the same face as the Gly^{34} and Gly^{38} residues, which comprise a portion of the 'Sternberg-Gullick' motif (GATIG) present in this region of the M13 coat protein TM helix. Such 5-residue motifs, which require position 0 to have a small side chain, position 3 an aliphatic side chain, and position 4 only the smallest (Gly or Ala) residue, have been implicated in mediating the homo- or heterodimerization of many receptor tyrosine kinases (39). The diversity in the biochemical properties of WT and TM mutant coat proteins reveals that the stability of helical dimers [*i.e.*, WT < V31A <<< Y24C-V31A] can be drastically altered by mutations in TM helices. With the availability of sulfhydryl-specific fluorescent and spin-label probes, the introduction of a Cys residues into the TM segment of M13 coat protein, and into the major coat protein of IKe bacteriophage (40), becomes a useful probe for further structural investigations.

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