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FORUM REVIEW ARTICLE

# Cytosolic Disulfide Bond Formation in Cells Infected with Large Nucleocytoplasmic DNA Viruses

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## Abstract

Proteins that have evolved to contain stabilizing disulfide bonds generally fold in a membrane-delimited compartment in the cell [*i.e.*, the endoplasmic reticulum (ER) or the mitochondrial intermembrane space (IMS)]. These compartments contain sulfhydryl oxidase enzymes that catalyze the pairing and oxidation of cysteine residues. In contrast, most proteins in a healthy cytosol are maintained in reduced form through surveillance by NADPH-dependent reductases and the lack of sulfhydryl oxidases. Nevertheless, one of the core functionalities that unify the broad and diverse set of nucleocytoplasmic large DNA viruses (NCLDVs) is the ability to catalyze disulfide formation in the cytosol. The substrates of this activity are proteins that contribute to the assembly, structure, and infectivity of the virions. If the last common ancestor of NCLDVs was present during eukaryogenesis as has been proposed, it is interesting to speculate that viral disulfide bond formation pathways may have predated oxidative protein folding in intracellular organelles. *Antioxid. Redox Signal.* 13, 1261–1271.

## Introduction

Disulfide bond formation and reduction is a powerful switch to control the structures and assembly states of proteins. Various virus families have taken advantage of disulfides to regulate steps in their cell penetration mechanisms and to control other aspects of viral replication and maturation. For example, SV40 polyoma virus is gracefully uncoated with the aid of ER oxidoreductases (55). It has been proposed that HIV entry requires reduction of gp120 disulfides catalyzed by protein disulfide isomerase or other oxidoreductases after co-receptor binding (1, 3, 11, 15). Similarly, the envelope proteins of Newcastle disease virus, an avian paramyxovirus, are reduced upon contact with the cell surface in a step required for fusion (24). Mammalian leukemia retroviruses undergo a thiol/disulfide rearrangement as part of their cell entry mechanism (29, 64).

Disulfides are common in viral structural and surface proteins, regardless of whether they undergo rearrangements or reduction during cell entry. The exterior proteins of enveloped viruses typically follow the secretory pathway to the cell surface and thus resemble cellular secreted proteins in their post-translational modifications and reliance on ER chaperones and oxidoreductases. In fact, studies of the folding and assembly of viral envelope proteins, in particular influenza hemagglutinin, contributed greatly to our understanding of the ER oxidative folding environment (7). To incorporate envelope proteins, virus capsids assemble on the cytoplasmic side of the plasma membrane and bud from the surface of the cell, becoming enveloped in sections of plasma membrane and virally encoded proteins embedded therein. In a variation on this theme, rotaviruses bud into the ER itself, acquiring disulfide-bonded surface proteins and a temporary membrane that is later shed (35). Herpesvirus acquires disulfide-bonded glycoproteins by budding into the *trans*-Golgi network (38). In all these cases, the disulfide-bonded ectodomains of the membrane-embedded virus surface proteins fold in environments topologically equivalent to the extracellular environment.

Some nonenveloped viruses also contain disulfides. For example, reoviruses, which infect the human gastrointestinal and respiratory tract but cause mild symptoms, if any, have a disulfide bond linking subunits of an outer capsid protein (43). An intermolecular disulfide is also seen in the surface proteins of foot and mouth disease virus (FMDV) (46). The capsid proteins of both these viruses fold and assemble in the cytosol, which is typically a reducing environment that does not support disulfide formation. The reovirus disulfide appears late in the viral infection cycle, and it has been proposed that breakdown of the cytosolic reduction mechanisms in dead or dying cells allows disulfides to form. For FMDV, the disulfides form only after release of the virus from the host cell (32).

The Nucleocytoplasmic Large DNA Viruses (NCLDVs) and certain other viruses [*e.g.*, baculoviruses (34)] may be unique in that a specific mechanism, rather than the failure of

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or escape from cytosolic reductants, is responsible for disulfide formation in virion proteins that fold in the cytosol. NCLDVs comprise five main families: *poxviridae, asfarviridae, iridoviridae, mimiviridae,* and *phycodnaviridae*. These viruses are known for their enormous genome sizes (100 kilobases to 1.2 megabases) and their relative independence of the host cell nucleic acid replication machinery. All NCLDVs encode DNA helicases, polymerases, and topoisomerases, as well as factors that aid in transcription initiation and elongation (23). In addition, all NCLDV families encode a catalyst of disulfide bond formation [*i.e.,* a sulfhydryl oxidase (56; see however ref. 75)]. The viral sulfhydryl oxidases are cytosolic, and in the cases that have been examined to date, they function late in the virus infection cycle and are essential for viral assembly (59).

NCLDVs are enveloped viruses, but they have a mechanism for envelopment (65) that is different from those described above and that limits their use of the ER as an oxidative folding environment. These viruses assemble in a region of the cytosol largely devoid of organelles and designated the "viral factory." An NCLDV viral factory production line can be seen in an electron micrograph of mimivirus virions in various stages of assembly and maturation (Fig. 1). The exact mechanism of envelopment, the source of the viral membranes in which the capsids are wrapped in the viral factory, and the number of lipid bilayers in mature virions is debated (66). Furthermore, there may be differences in the membrane source and mechanism of encapsulation among different NCLDVs. What appears to be general, however, is that most of the membrane and surface proteins of NCLDV virions do not traverse the secretory pathway as do the surface proteins of other enveloped viruses. Rather, being either type III transmembrane proteins or tail-anchored proteins, they expose their major portions to the cytosol. Many of these proteins nevertheless acquire disulfide bonds that are essential for folding, assembly, and function. The apparently universal need for NCLDVs to re-program sections of the cytosol for disulfide formation is striking, given the diversity in size, morphology, hosts, genome structure, and gene content of these virus families.

## Viral Sulfhydryl Oxidases

There are two main sulfhydryl oxidase families in eukaryotic cells. One family functions in the ER and is responsible for oxidative protein folding and for maintaining the proper redox poise in the secretory pathway (63). The second family consists primarily of members that function in the mitochondrial IMS (18). Viral catalysts of disulfide formation are related to the mitochondrial family, which are known as the "Erv" sulfhydryl oxidases. Erv stands for "essential for respiration and viability," and the name reflects the important role of the mitochondrial enzyme in biogenesis of the respiratory chain (27, 30).

Historically, building on the finding that poxvirus proteins contain disulfides though they are synthesized in the cytosol (21, 31, 78), Moss and colleagues set out to characterize the redox biology behind poxvirus structure and assembly. Inspection of poxvirus sequences revealed that the E10R protein contains a Cys-X-X-Cys sequence (57), a motif common in proteins that undergo dithiol/disulfide exchange reactions. This motif is found in redox-active proteins of the thioredoxin fold family (Fig. 2A), including protein disulfide isomerase and related factors that function in oxidative protein folding in the ER. However, the secondary structure prediction for E10R (58) was inconsistent with the thioredoxin fold. Instead, E10R was placed into the newly identified Erv enzyme family, members of which were shown during this period to be flavoenzymes that catalyze net disulfide bond formation (19, 28)

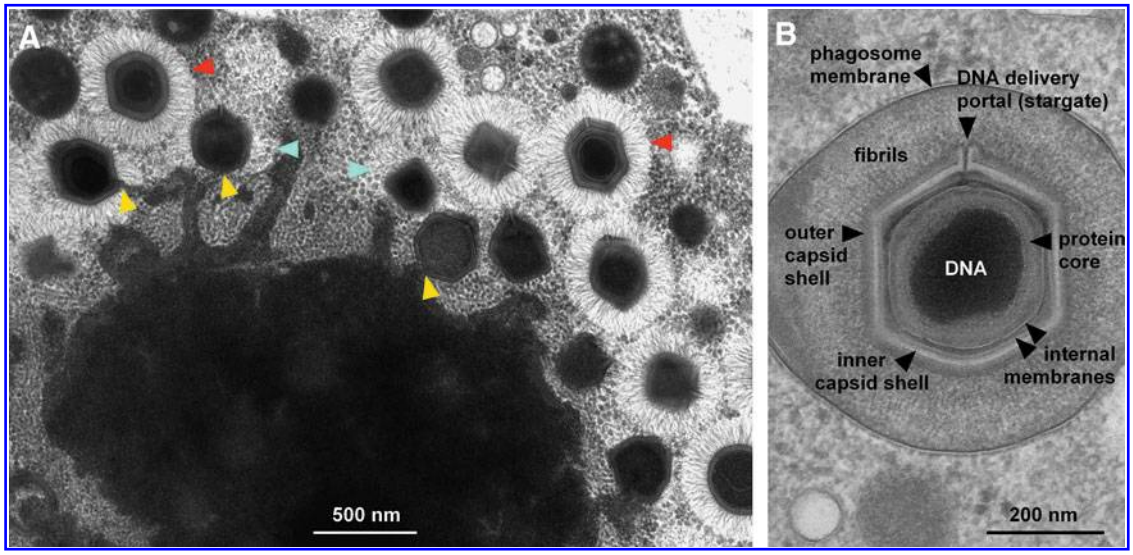


**FIG. 1. Mimivirus viral factory and virion structure.** Images were generously provided by Nathan Zauberman and Abraham Minsky, Weizmann Institute of Science. *Acanthamoeba polyphaga* infected with mimivirus were cryo-immobilized, stained, sectioned, and imaged by transmission electron microscopy as described (80). (**A**) The large dark region toward the *lower left* contains viral DNA, which is seen being packaged into pre-assembled virus capsids (*yellow arrowheads*). The *cyan arrowheads* indicate virions in the process of acquiring fibrils. *Red arrowheads* indicate virions that have completed the assembly and DNA packaging processes. (**B**) Features of the mimivirus virion structure are labeled on a virus internalized in an *A. polyphaga* phagosome. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

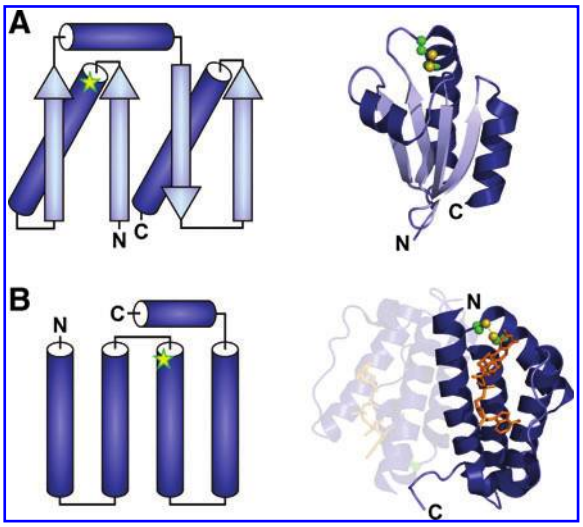


**FIG. 2. Comparison of thioredoxin and Erv enzyme folds.** Topology diagrams are shown on the *left*, with *stars* indicating the active-site Cys-X-X-Cys disulfides. Ribbon diagrams of representative structures are on the *right*. (**A**) Thioredoxin contains a central $\beta$-sheet surrounded by helices, with the active-site disulfide at the amino-terminus of the helix following the first $\beta$-strand in the sheet. (**B**) In the Erv enzymes, the active-site disulfide is at the amino-terminus of the third helix in the fold, next to the isoalloxazine of the bound FAD cofactor. The second subunit in the Erv enzyme dimer is shown semitransparent. The ribbon diagrams in (**A**) and (**B**) were constructed from the protein data bank files 3F3Q and 1JR8, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

as opposed to the dithiol/disulfide exchange reactions of thioredoxin-like oxidoreductases. Indeed, X-ray crystallographic studies of cellular Erv family sulfhydryl oxidases subsequently revealed a completely different structure from thioredoxin (16, 74, 79) (Fig. 2B). Erv enzymes are found as dimers of a compact five-helix bundle fold that encloses the flavin adenine dinucleotide (FAD) cofactor and positions a pair of cysteines next to the isoalloxazine ring system, the redox-active part of the FAD. The juxtaposition of the cysteines with the FAD allows oxidation of these cysteines to a disulfide by transfer of electrons to the FAD, a feature not shared by the thioredoxin family.

After their initial characterization in poxviruses, the ease with which Erv family proteins can be recognized even with very low sequence identity (10) facilitated their identification across the NCLDVs. Comparisons can then be made between sulfhydryl oxidases from the various virus families (Fig. 3). For example, poxvirus sulfhydryl oxidases tend to be highly compact, typically comprising only the helices of the fold in a mere $\sim$95 amino acids. In contrast, the ascovirus and mimivirus sulfhydryl oxidases have carboxy-terminal extensions of approximately 80 and 160 amino acids, respectively, downstream of the FAD-binding domain. The baculovirus sulfhydryl oxidase (34) has a region of $\sim$100 amino acid residues predicted to form four helices amino-terminal to the FAD-binding domain. The additional sequences present in some of these viral sulfhydryl oxidases contain multiple cysteine residues (Fig. 3), and it remains to be determined if these cysteines form structurally or mechanistically important disulfides.

Though not immediately evident from sequence alignments, a particularly striking difference between sulfhydryl oxidases from different virus families is seen in the structures of the African swine fever virus (ASFV) and mimivirus enzymes determined by X-ray crystallography (17) (Fig. 4). The mimivirus enzyme R596, like the cellular Erv enzymes and by prediction most other viral sulfhydryl oxidases as well, forms a dimer using an interface consisting of the first and second helices of the fold. In contrast, the ASFV enzyme, B119L, dimerizes using an orthogonal protein face, yielding a dramatic difference in the relative positions of the two active sites in the dimer. The functional implications of the different dimerization modes of the ASFV and mimivirus sulfhydryl oxidases are not known, and neither is the extent or nature of the selective pressure under which the change occurred. The change in oligomerization mode was perhaps possible at all because the fundamental biochemical activity of Erv enzymes appears to be independent of dimerization. In support of this asser-

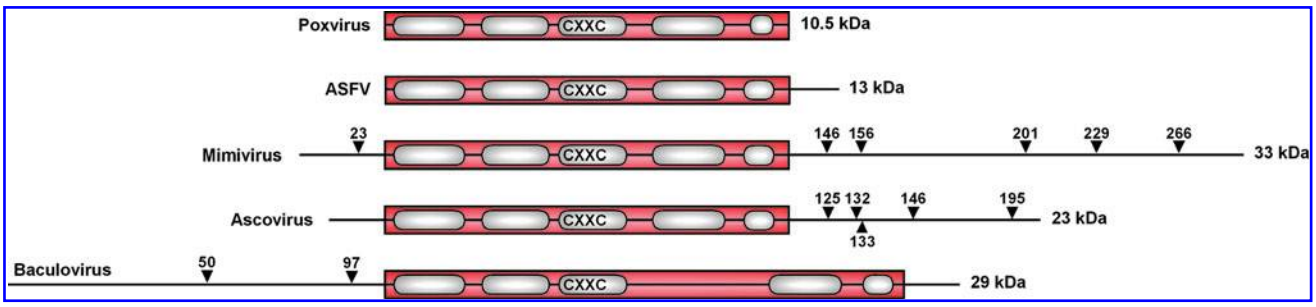


**FIG. 3. NCLDV sulfhydryl oxidases.** Maps of the primary structures of various NCLDV sulfhydryl oxidases demonstrate the amino- and carboxy-terminal extensions present in some of the enzymes. The Erv domain is shown as an *orange rectangle*, and experimentally observed or predicted helices within this domain are shown as *gray oblongs*. The position of the active-site disulfide at the beginning of the third helix in the fold is indicated by CXXC, and the approximate positions of additional cysteines in each protein are shown by *black arrowheads* and labeled according to amino acid residue number. The molecular weights predicted from amino acid sequence are shown to the *right*. The following proteins are represented: poxvirus = E10R, ASFV = pB119L, mimivirus = R596, ascovirus = HVAV3e_gp073, and baculovirus = Ac92 (p33). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

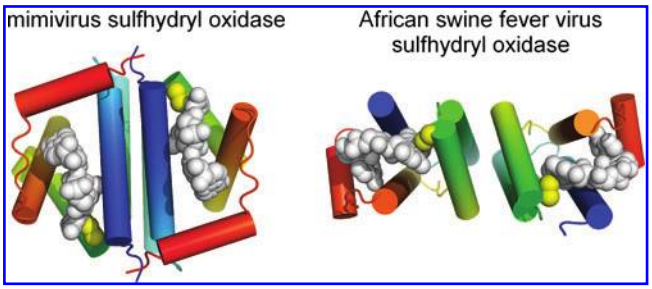


**FIG. 4. Comparison of the African swine fever virus and mimivirus sulfhydryl oxidase structures.** The figure was prepared using the coordinate files with protein data bank codes 3GWL (ASFV) and 3GWN (mimivirus). The α-helices in each structure are represented as *cylinders*. The polypeptide chain is colored in a *rainbow* with *blue* corresponding to the amino terminus and *red* to the carboxy terminus of each subunit. The ASFV sulfydryl oxidase is pB119L residues 1 to 103. The mimivirus structure is of the isolated Erv-like domain of R596, with the amino- and carboxy-terminal extensions shown in Figure 2 removed. The FAD cofactor bound to each subunit is shown in *white space-filling* representation. *Yellow spheres* are sulfur atoms of cysteine side chains. The view of each dimer is down the two-fold rotational symmetry axis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

tion, monomeric mutants of viral and cellular Erv enzymes retain sulfhydryl oxidase activity (17), though they may have altered stability and substrate selectivity (73). The quaternary structural states of viral sulfhydryl oxidases may be significant in the context of protein-membrane and protein-protein interactions within the viral factory and may have evolved to guide protein localization and substrate recognition.

Inspection of an individual Erv sulfhydryl oxidase subunit reveals an exceedingly simple and perhaps primitive fold. The fold of the Erv protomer shares the same helical topology as soluble cytochrome b562, the c′ cytochromes, and hemerythrins (Fig. 5A) as listed in the Structural Classification of Proteins (SCOP) database (41). Interestingly, these other small helical bundle proteins function by carrying electrons or oxygen. Though possession of the same fold is certainly not evidence of a common evolutionary origin, it is interesting to compare the dimeric heme-binding cytochrome c′ with the dimeric FAD-binding Erv enzymes (Fig. 5B). Cytochrome c′, like many proteins participating in respiratory redox reactions, is present in the bacterial periplasm, whereas Erv enzymes are found in the functionally comparable mitochondrial IMS, where they promote folding of proteins involved in respiration. Hemerythrin and myohemerythrin, in turn, are primitive oxygen-binding proteins found in marine invertebrates (2) and perhaps in bacteria as well (14). These proteins contain a bi-nuclear iron center rather than the heme found in oxygen-carrying globins. The Erv structure is necessarily expanded somewhat compared to hemerythrin to accommodate the FAD (Fig. 5A). This helical fold and other simple helical bundles are particularly versatile at incorporating a variety of cofactors in nature and have been used as scaffolds for protein design, due to their simplicity and robustness (25, 47). Though beyond the focus of this review, considerations of the possible ancient evolutionary origins of NCLDVs and their contribution to the dawn of the eukaryotic cell (5, 13) motivate speculations as to a possible evolutionary connection between the Erv sulfhydryl oxidase family and primordial redox-active and oxygen transport proteins.

### Disulfide Bond Formation Pathways in NCLDVs

Cellular sulfhydryl oxidases typically function in multiprotein pathways that transfer electrons from proteins undergoing oxidative folding to terminal electron acceptors. In such pathways, electrons are relayed from substrate to one or more intermediary oxidoreductases, then *via* the "shuttle disulfide" of the sulfhydryl oxidase to the active-site disulfide. The reduced active-site cysteines of the sulfhydryl oxidase are reoxidized by transfer of electrons to the bound flavin, from where they are passed to the electron acceptor, which is often but not always oxygen. Although the players differ, this scheme describes disulfide formation in both the ER and mitochondrial IMS (51). This electron transfer path-

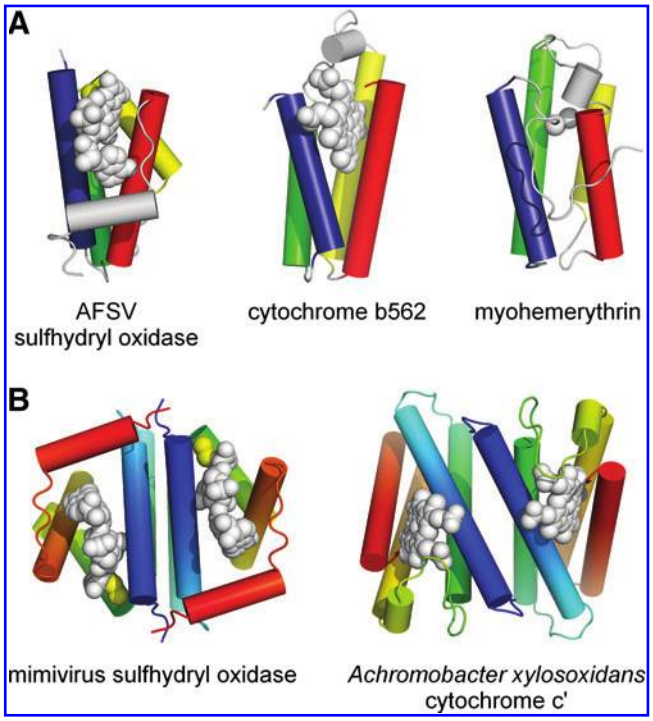


**FIG. 5. Comparison of the Erv sulfhydryl oxidase fold with other proteins of similar helical topology.** (**A**) The structure of a viral sulfhydryl oxidase subunit is compared to the fold of cytochrome b562 (protein data bank code 256B), myohemerythrin (code 2MHR). Cofactors and bound metal ions are shown in *white space filling* representation. The four main helices in the bundle are colored according to their order in the protein primary structure: *blue, green, yellow, red*. (**B**) The structure of mimivirus, represented as in Figure 4, is shown next to *A. xylosoxidans* cytochrome c′. The heme of the cytochrome, like the FAD of the sulfhydryl oxidase, is shown in *white space-filling* representation. Although there are differences in the helix packing angles, these two structures share the same helix topology and bind their cofactors in similar positions within each subunit. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

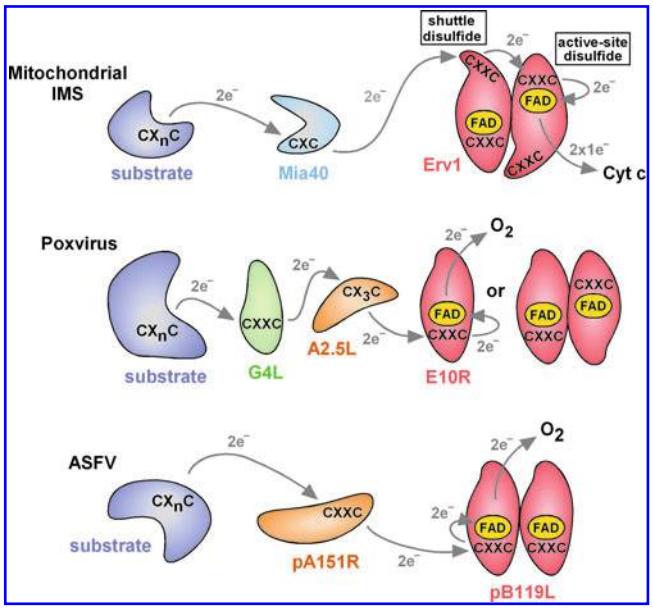


**FIG. 6. Electron transfer within and between proteins in disulfide formation pathways.** The proteins and redox-active sites involved in disulfide formation in the mitochondrial IMS (37) are compared with the cytosolic disulfide formation pathways of poxviruses and African swine fever virus (ASFV). Each two-electron transfer step is indicated by an *arrow*. The quaternary structure of the poxvirus sulfhydryl oxidase is unknown. In addition, the structures and oligomerization states of the intermediary oxidoreductases A2.5L and A151R, as well as the manner in which they may associate with their corresponding sulfhydryl oxidases, remain to be determined. "Cyt c" refers to cytochrome c, which serves as the primary electron acceptor from yeast and animal Erv1 (18). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Table 1. Catalog of Verified and Putative Redox-Active Proteins Encoded by NCLDVs

| *NCLDV* | *Erv* | *Trx* | *Grx* | *Other CXX(X)C* |
|---|---|---|---|---|
| Ascoviridae | +F | + | | |
| Asfarviridae | + | | | + |
| Iridoviridae | | | | |
| Chloriridovirus | +M | +, + | | |
| Iridovirus | + | +, + | | |
| Megalocytivirus | + | | | +M |
| Mimiviridae | +M, +F | +S, +F, + | | |
| Nudivirus | +F | | | + |
| Phycodnaviridae | | | | |
| Chlorovirus | + | + | + | |
| Coccolithovirus | +M | +S, +S | | |
| Phaeovirus | +M | | | |
| Poxviridae | + | | +, + | + |

A plus symbol indicates the presence of the protein family in the viral lineage. Where more than one representative of the protein family is encoded in the same virus, the plus symbols are separated by commas. A plus modified with the letter M means that the protein has a transmembrane region. The letter S indicates a signal sequence anchor at the amino terminus, and the letter F indicates that the representative of the protein family is fused to another predicted domain.

way appears to apply roughly to viral protein oxidation pathways as well, with the exception that most NCLDV sulfhydryl oxidases lack a shuttle disulfide and contain only the FAD-proximal disulfide (Fig. 6).

Most viruses that encode sulfhydryl oxidases also encode other proteins with putative dithiol/disulfide exchange capabilities (Table 1). Some of these have been shown experimentally to associate with the oxidases and may participate directly in disulfide formation pathways. Interestingly, a number of these proteins bear no resemblance to the intermediary oxidoreductases found in cellular pathways, even those involving Erv family enzymes. In fact, they bear no sequence similarity to any other known proteins or to one another. NCDLVs are thus a rich source of potentially novel redox-active protein folds.

The best characterized but nevertheless still puzzling example of a viral intermediary oxidoreductase is the vaccinia virus A2.5L protein, which appears to form a complex with the sulfhydryl oxidase E10R and is required for disulfide formation in viral substrates (60). The 76 amino-acid A2.5L protein contains a $CX_3C$ ($CX_2C$ in some poxviruses) motif at the amino terminus of a predicted helix as in thioredoxin. However, as originally observed for E10R, the overall high helix content predicted for A2.5L similarly rules out the

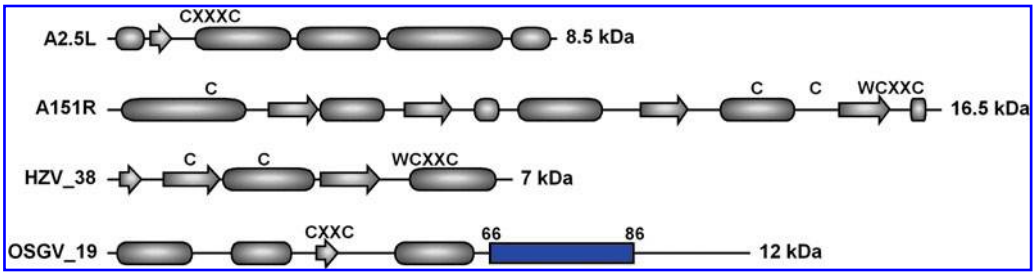


**FIG. 7. Examples of orphan intermediary oxidoreductases and putative redox-active proteins in NCLDVs.** Primary structural maps with predicted secondary structure elements indicated as *oblongs* ($\alpha$-helices) and *arrows* ($\beta$-strands). Putative redox-active di-cysteine motifs are indicated, and residue numbers at the boundaries of the OSGV_19 transmembrane region (*blue*) are shown. The accession codes for these proteins are as follows: A2.5L, poxvirus AAM49617.1; A151R, ASFV NP_042731.1; HZV_38, nudivirus NP_690457.1; OSGV_19, iridovirus AAX82328.1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

thioredoxin fold (Fig. 7). Oddly, in the absence of A2.5L, the E10R active site remains reduced (60), an unexpected effect if A2.5L serves merely as the reductant of E10R on the pathway (Fig. 6). Since E10R was found correctly oxidized in the presence of A2.5L mutants lacking cysteines, A2.5L appears to be structurally important for E10R folding or cofactor binding. The poxvirus sulfhydryl oxidase may be unique in this regard, as other NCLDV sulfhydryl oxidases fold and acquire activity, at least in recombinant systems, without the aid of other viral proteins (17, 34, 52). The additional role of A2.5L in mediating electron transfer from substrate to E10R does require the A2.5L cysteines (60).

A less well-characterized orphan protein that may be part of a viral disulfide formation pathway is ASFV A151R (52). A151R is a protein of 151 amino acids containing five cysteines, two of which are found in a WCTKC motif. As surmised for poxvirus A2.5L, the ASFV A151R protein does not appear to be a thioredoxin fold family member; the putative redox-active motif is near the carboxy terminus rather than the amino terminus of the protein, and the secondary structure prediction does not correspond to the thioredoxin fold in either classical or permuted form (Fig. 7). Inspection of protein sequences from other NCLDVs reveals more potential participants in dithiol/disulfide exchange reactions. For example, *Heliothis zea* virus 1 contains a 63 amino acid protein (HZV_38) with four cysteines, two of which are in a WCTHC motif predicted to sit at the amino-terminus of a helix. This protein is too small to be a thioredoxin. The genome of the Orange spotted grouper virus encodes a 111 amino acid protein with two cysteines in a CVDC arrangement. Again, the protein cannot contain a full thioredoxin domain since it has a predicted transmembrane region between residues 66 and 86. These last two proteins are highlighted here based on interesting sequence features alone and have not been characterized or shown to function in oxidative folding of viral components.

In addition to these orphan proteins with potential redox activity, NCLDVs also encode other proteins that do belong to the thioredoxin superfamily. The vaccinia virus disulfide bond formation pathway contains an example of such a protein: the glutaredoxin-like G4L. G4L is the direct oxidant of the terminal viral substrates and is presumably returned to its oxidized state by the poxvirus sulfhydryl oxidase E10R, perhaps *via* A2.5L (60). Thioredoxin fold proteins are encoded by many other NCLDV families (Table 1), but it is not known whether they are involved in disulfide bond formation pathways, or alternatively function as protein reductants like the cellular cytosolic thioredoxins. In support of the latter, one of the mimivirus thioredoxin family proteins has the active-site sequence WCGPC, which is identical to that found in cellular thioredoxins from bacteria to humans. Furthermore, NCLDVs encode ribonucleotide reductases, and a major function of thioredoxins in the cell is in maintaining ribonucleotide reductases in reduced and functional form. A thioredoxin-fold protein in poxviruses has been assigned such a role in relation to virally encoded ribonucleotide reductase (48). Aside from the mimivirus thioredoxin, the CXXC motifs of the various thioredoxin fold proteins in NCLDVs are diverse and differ from those found in cellular proteins that have been studied to date. Therefore, conclusions about their reduction/oxidation potentials and likely functions cannot be gleaned from sequence alone and requires experimental study.

In cellular disulfide bond formation pathways, intermediary oxidoreductases do not transfer electrons directly to the active sites of sulfhydryl oxidases, but rather first to an additional disulfide, the shuttle disulfide mentioned above, present on a flexible segment of polypeptide just outside the helical domain (16). After the shuttle disulfide is reduced by dithiol/disulfide exchange with the substrate, it is then reoxidized at the enzyme active site. Due to the head-to-tail arrangement of the two subunits in the dimer of cellular Erv enzymes, the shuttle disulfide of one subunit interacts with the active site of the second subunit in the dimer (Fig. 6).

Viral sulfhydryl oxidases differ from their cellular counterparts in that many of them lack cysteine residues that could serve as disulfide shuttles. Some viral sulfhydryl oxidases, such as the baculovirus, mimivirus, and ascovirus enzymes with amino- or carboxy-terminal extensions mentioned above, do have additional cysteines outside the active sites, but these are not present in the typical patterns CXC, $CX_2C$, or $CX_4C$ that constitute the shuttle disulfides of the eukaryotic enzymes. Why the shuttle disulfide appears to be important for the function of cellular enzymes but dispensable for the viral enzymes is not known. One possibility is that the shuttle confers substrate selectivity on the cellular enzymes, whereas selectivity is achieved by other means in virus-infected cells, for example, by co-localization of enzyme and substrate or through association with other redox-active proteins that function in place of the shuttle disulfide, as perhaps poxvirus A2.5L does.

A number of the proteins involved in NCLDV disulfide formation pathways in the various viruses are membrane-targeted. Some of the sulfhydryl oxidases themselves have carboxy-terminal transmembrane regions. These include the enzyme from chloriridovirus, one of the two sulfhydryl oxidases from mimivirus, and the sulfhydryl oxidases from some of the phycodnavirus subfamilies (Table 1). Even the poxviral sulfhydryl oxidase E10R, which does not have a transmembrane region, appears on the basis of immunogold labeling and electron microscopy to be present on membranes in viral factories. Furthermore, the enzyme is present in detergent-extractable form in mature virions (59). The G4L oxidoreductase is similarly membrane-associated and attached to virions (76). Thus, the entire poxvirus disulfide formation pathway may occur on a special membrane-surface microenvironment. The ASFV sulfhydryl oxidase has also been localized using fluorescence microscopy to viral factories (52), but it is not known whether the enzyme adheres to membrane surfaces within the factories. One obvious benefit of membrane localization for the disulfide formation pathway is that many of the substrates of this pathway are also membrane-associated.

## Substrates of NCLDV Disulfide Formation Pathways

The majority of genes shared among all NCLDV families encode functions related to polynucleic acids. These protein products include DNA and RNA polymerases, helicases, ribonucleotide reductases, and transcription factors. A small number of core genes encode structural components of the virions. Since the sulfhydryl oxidase is so highly conserved among NCLDVs, it is likely to be functionally important due to its activity on conserved virion components. Two virion proteins with homologs in all NCLDV families are the major

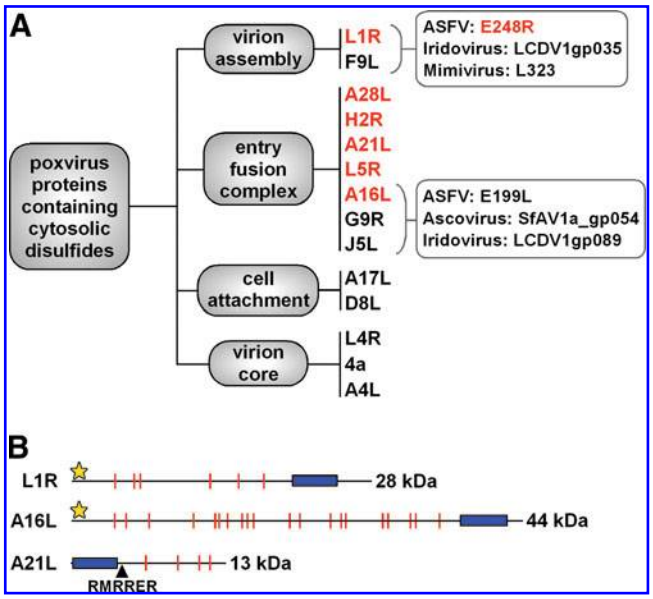


**FIG. 8. Poxvirus proteins containing cytoplasmic disulfide bonds.** (**A**) Proteins experimentally shown to contain inter- or intramolecular disulfides that are predicted or verified to be cytosolic are classified roughly according to function in the virus. Protein names indicated in *red* have been shown experimentally to be substrates of the poxvirus sulfhydryl oxidase pathway. Homologs in other viruses for some of the proteins are listed to the *right*. (**B**) Primary structural maps of several sulfhydryl oxidase pathway substrates indicate the locations of cysteines (*red bars*) in the protein sequences. *Blue boxes* represent transmembrane regions, and *yellow stars* highlight myristoylation sites. A basic stretch of amino acid residues putatively contributing to type III topology in A21L is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

capsid protein and a myristoylated virion protein with a carboxy-terminal transmembrane region (56). In addition, another virion-associated protein that is also myristoylated and contains a carboxy-terminal transmembrane region, but is otherwise unrelated to the first, is found in most NCLDVs (56).

There is no evidence that the major capsid protein of NCLDVs contains disulfide bonds or is a substrate of the viral sufhydryl oxidases. However, the two myristoylated membrane proteins are indeed targets of the disulfide bond formation pathway (Fig. 8). One of these, known as L1R in vaccinia virus, is recognized by neutralizing antibodies and is a component of smallpox vaccines (12, 20). L1R and the related F9L are virion structural proteins important for assembly, maturation, and release of virus particles (50). Both contain six conserved cysteines, but F9L lacks the amino-terminal myristoylation site. The L1R sequence and topology suggest that it does not enter the secretory pathway but rather inserts post-translationally into intracellular membranes with its major portion, containing the cysteines, facing the cytosol. In the absence of E10R *in vivo*, L1R failed to acquire disulfides between these cysteines (58). The L1R family member in ASFV, pE248R, has also shown to interact with components of its corresponding viral disulfide formation pathway (52).

One question is how substrates of cytosolic sulfhydryl oxidases remain oxidized in the presence of competing systems for disulfide reduction. This problem can in principle be solved by physical segregation on two levels. On the first level, burial of disulfides in the cores of viral proteins would protect them from cytosolic reductants. On the second level, segregation of viral protein production and folding from bulk cytosol may help to exclude proteins that catalyze disulfide reduction (58). The flux of cellular proteins and metabolites into and out of the viral factory is a subject for future study. Furthermore, what delimits the viral factory is still unknown.

L1R is the only viral sulfhydryl oxidase pathway substrate with known high-resolution structure (67) and can be used to address the question of how its disulfides avoid reduction. Indeed, the structure of a soluble recombinant version of L1R revealed that the three disulfide bonds are buried in the protein core, where they are expected to be resistant to reduction. For L1R to acquire disulfides, however, G4L must be oxidized, and the redox-active di-cysteine motifs of single-domain thioredoxin fold proteins are surface exposed. The X-ray crystal structure of G4L shows an atypical thioredoxin fold structure, with some of the canonical secondary structure elements projecting outward from the globular domain and making crystal contacts with neighboring molecules (68). On one hand, this projection partially shields the active-site cysteines from solvent and would perhaps protect them from reduction. On the other hand, the projection would also shield the cysteines from the substrate to be oxidized. It remains to be determined if the partially unraveled structure observed for G4L is an artifact or rather represents a means of potentially regulating the protein. Furthermore, how the distribution of G4L conformers and the potential for off-pathway reduction are affected by membrane association and co-localization of disulfide formation pathway components and substrates *in vivo* are poorly understood.

Another major class of sulfhydryl oxidase substrates identified in vaccinia comprises the components of the entry-fusion complex (Fig. 8A). This complex, found on the surface of mature vaccinia virions, consists of the products of the genes A28, H2, A21, L5, G3, A16, G9, J5, O3, and I2 (42, 54, 62). The first five proteins in this list are type III transmembrane proteins containing membrane-spanning regions near the amino-termini that are inserted co-translationally into the ER membrane, and the rest of each protein is exposed to the cytosol. Within this group, H2, A21, and L5 all have clusters of basic residues immediately downstream of the transmembrane region (Fig. 8B), a feature that promotes this topology. With the exception of G3, which does not contain cysteines, the other type III membrane proteins (A28, H2, A21, and L5) each contain at least two cysteines and have been shown experimentally to be substrates of the viral disulfide bond formation pathway (61, 71, 72). The final three proteins in the entry-fusion complex, A16, G9, and J5, have carboxy-terminal transmembrane regions. These proteins, which share homology and conserved cysteines, are expected to be tail-anchored proteins that are inserted into membranes post-translationally like L1R described above. In addition, A16 and G9 contain amino-terminal myristoylation sites. A16, which contains 20 cysteines, depends on E10R for oxidation (45). Other NCLDVs contain cysteine-rich proteins with recognizable homology to this group of poxvirus tail-anchored proteins (22) (Fig. 8A), and a requirement for oxi-

dizing these proteins may explain the conservation of the NCLDV sulfhydryl oxidases.

The vaccinia gene products of A14L, A17L, D8L, A10L (4a), L4R, and A4L were also found to contain inter- and intramolecular disulfide bonds (31) (Fig. 8A). A14L, A17L, and D8L are membrane proteins, whereas A10L (4a), L4R, and A4L are virus core proteins. The A14L cysteine participating in covalent dimerization of this protein is exposed to the lumen of the ER and is thus presumably oxidized by the oxidative folding machinery of the cell secretory pathway (36). A17L contains two ER luminal cysteines and one cytoplasmic cysteine, the latter linking subunits into dimers (6). D8L, which binds to cell-surface glycosaminoglycans during virus entry, contains a carboxy-terminal transmembrane region that may be inserted into the membrane post-translationally such that its single cysteine faces the cytoplasm. The covalent dimers observed for D8L are thus formed *via* a cytosolic disulfide, but it is not known if formation of this disulfide, or that of A17L, is catalyzed by the viral sulfhydryl oxidase pathway.

As mentioned in the introduction, there have been accounts of disulfides that were acquired by viral proteins as they transit the secretory pathway of infected cells becoming reduced when the mature virus is exposed to a fresh target cell. Reduction contributes to uncoating and infectivity. Is disulfide formation in NCLDVs necessary for protein stability and assembly only, or is it a reversible post-translational modification important for cell entry? A clear picture on this issue has not yet emerged. On one hand, the viral core proteins 4a and L4R, which are found in the oxidized state in mature virions before infection, become reduced within the first hour after entry into fresh host cells (31). On the other hand, *in vitro* dithiothreitol treatment of mature virions causes membrane detachment but not uncoating (9, 53), and the particles retain full infectivity (31). The latter observation indicates that a reducing environment is not sufficient for core uncoating, but it may nevertheless be necessary or at least helpful at some stage of disassembly.

## NCLDVs from the Cellular to the Global Scale

More than two centuries ago, the NCLDVs rose to prominence when one of its member viruses became the first target of intentional vaccination. Despite the eradication of smallpox, NCLDVs have not diminished in importance as potential threats to human health. Both bioterrorism and the possibility of NCLDVs that infect other organisms hopping the species barrier to humans remain real dangers. However, there has been an explosion of interest in NCLDVs in recent years for a number of entirely different reasons. First, the suggestions that NCLDVs may have been involved in the emergence of eukaryotic cells, and that a primordial NCLDV may have been, for example, the origin of the nucleus (4, 69), has put a spotlight on NCLDVs in theoretical biology. How to place NCLDVs within the context of our understanding of the diversity of life has led to heated debates and some questioning of basic assumptions about living organisms (8, 26, 40).

Another reason for interest in NCLDVs is their sheer numbers in the biosphere as pathogens of unicellular marine eukaryotes. NCLDVs infect protozoans (by mimivirus) and photosynthetic single-celled organisms such as algae and *Ostreococcus* (by phycodnaviruses). The latter tiny organisms are increasingly appreciated for their enormous importance in global carbon fixation and as potential producers of biofuels, and interest in the ecology of these organisms is expanding. Remarkably, the mimivirus genome was published little more than 5 years ago (49), but analysis of ocean sampling sequences showed that the second largest viral group, after bacteriophages, consisted of sequences related to mimivirus (39, 77). Approximately 85 distinct strains were represented. In addition, ASFV-like sequences also turned up in marine sampling, a surprising finding considering that the only known hosts for this virus family had been swine and their tick vectors. A relative of ASFV has now been specifically identified as a parasite of dinoflagellates (44), and such viruses may be key determinants of the size and dynamics of dinoflagellate blooms (70).

In addition to the identification of ASFV-like sequences in a marine microorganism, ASFV-like viruses have recently been discovered in humans as well. Polynucleotide sequences found in a sample of human serum from Middle Eastern subjects did not correspond to any known virus but were most similar to ASFV (33). ASFV itself is not known to infect humans. The discovery of a possible *Asfaviridae* member in humans raises the possibility that this virus family is more widespread than originally thought and perhaps of concern to human health. These many motivations will fuel continued investigations into the replication mechanisms of NCLDVs. As the disulfide bond formation pathway is a key feature of the infection cycle of all NCLDVs, an understanding of this pathway is essential for understanding the unique biology of these giant viruses.

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**Abbreviations Used**

ASFV = African swine fever virus
DNA = deoxyribonucleic acid
ER = endoplasmic reticulum
FAD = flavin adenine dinucleotide
FMDV = foot and mouth disease virus
HIV = human immunodeficiency virus
IMS = intermembrane space
NADPH = nicotinamide adenine dinucleotide phosphate
NCLDV = nucleocytoplasmic large DNA viruses
RNA = ribonucleic acid
SCOP = structural classification of proteins
SV40 = simian virus 40

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