Cross-linking of the cms-T Maize Mitochondrial Pore-forming Protein URF13 by \(N,N'\)-Dicyclohexylcarbodiimide and Its Effect on URF13 Sensitivity to Fungal Toxins*

(Received for publication, September 9, 1992)

Cyril I. Kaspi and James N. Siedow‡

From the Department of Botany, Duke University, Durham, North Carolina 27706

URF13 is a membrane protein unique to mitochondria from maize having the Texas male-sterile cytoplasm (cms-T), which is capable of permeabilizing biological membranes in the presence of a family of pathotoxins (T-toxins) produced by certain fungi or the insecticide methomyl. The carboxylate-specific reagent dicyclohexylcarbodiimide has been shown previously to protect URF13-containing membranes against the permeabilizing effects of added T-toxin or methomyl. Dicyclohexylcarbodiimide was found to covalently cross-link URF13 into higher order oligomers, including dimers, trimers, and tetramers, in isolated cms-T mitochondria and Escherichia coli cells expressing URF13. In intact E. coli cells and isolated spheroplasts, the observed protection against the effects of methomyl was not associated with the appearance of dimers but was correlated with the appearance of cross-linked trimers and tetramers. Following treatment of E. coli cells expressing URF13 with dicyclohexylcarbodiimide, the specific binding of tritiated T-toxin was reduced by 59% and all binding cooperativity was lost. A similar decrease in the level of T-toxin binding and loss of binding cooperativity were observed with site-directed, T-toxin-insensitive URF13 mutants at aspartate 39, the residue known to undergo reaction with dicyclohexylcarbodiimide. When coupled with a postulated three membrane-spanning helical model of URF13, these results provide initial insights into the intermolecular interactions involved in URF13 oligomer formation.

URF13 is a 13-kDa protein unique to the inner mitochondrial membrane of maize carrying the Texas male sterile (cms-T) cytoplasm (1–3). The mitochondrial gene encoding URF13 (T-urf13) arose from a series of novel rearrangements within the mitochondrial genome (4). Maize carrying the cms-T genotype is uniquely susceptible to the fungal pathogens Bipolaris maydis race T and Phylllostica maydis, which produce a family of pathotoxins (T-toxins) that specifically affect cms-T mitochondria (5–8) and Escherichia coli cells expressing the URF13 protein (9). Exposure of either cms-T mitochondria or E. coli expressing URF13 to T-toxin induces a dramatic membrane permeabilization, resulting in the leakage of small molecules (e.g. Ca\(^{2+}\) and NAD\(^{+}\)) and membrane swelling, among other effects (5–8, 10, 11). A structurally unrelated compound, the insecticide methomyl, mimics all the effects of T-toxin but at markedly higher concentrations (12, 13).

Incubation of cms-T mitochondria (14–16) or E. coli cells expressing URF13 (17) with \(N,N'\)-dicyclohexylcarbodiimide (DCCD) confers protection against the permeabilizing effects of added T-toxin or methomyl. DCCD is a reagent that attacks carboxylate residues in proteins, activating them to undergo one of several subsequent reactions (18). In a hydrophobic milieu, the resulting carboxylate-DCCD intermediate can undergo a rearrangement to form a stable, covalently bonded DCCD adduct (18). Experiments using \(\text{[^{14}C]}\text{DCCD has shown that URF13 is covalently labeled by DCCD (9, 17), and site-directed mutagenesis of URF13 has demonstrated that Asp-39 is the amino acid involved in this labeling reaction (17), suggesting that the side chain of Asp-39 is localized in a hydrophobic environment. Asp-39 is also important for T-toxin sensitivity; any mutation at Asp-39 leads to a loss of T-toxin sensitivity in the associated E. coli cells (17).}

Investigation of the topological organization of URF13 in the mitochondrial membrane indicated that the amino and carboxyl termini are localized on opposite sides of the membrane, suggesting that URF13 is disposed in the membrane with an odd number of membrane-spanning regions (19). This result is consistent with a model of URF13 that postulates the presence of three membrane-spanning \(\alpha\)-helical regions, two of which are amphipathic and are felt to participate in the formation of the hydrophilic pore (10). Because molecules as large as NAD\(^{+}\) (\(M_{r} = 663\)) can pass through the pores generated by URF13 after interaction with T-toxin, it is estimated that a minimum of six amphipathic helices are needed to form the hydrophilic channel lining (20, 21). This suggests that URF13 should exist, to at least some extent, as a trimeric structure within the membrane. The possibility that URF13 may exist as an oligomer was first suggested by the appearance of cooperativity in the binding of tritiated T-toxin from \(P. \text{maydis} \) (Pm toxin) to URF13 in E. coli (22) and by the appearance of some apparent URF13 dimeric species on SDS gels (19, 23). Subsequently, covalent cross-linking studies using the bifunctional, lysine-specific reagent, ethylene glycol bis(succinimidyl succinate) (EGS), demonstrated directly that a fraction of the URF13 in the membrane does exist in an oligomeric state, as dimers and trimers (19).

The present study examines the effect of DCCD treatment on the observed oligomerization of URF13. DCCD is found to...
covalently cross-link URF13 in both E. coli cells and cms-T mitochondria. Cross-linking of URF13 correlates with the observed protection against the effects of added methomyl. Furthermore, after DCCD treatment, E. coli cells expressing URF13 bind less [³H]Pm toxin, and all binding cooperativity is lost. Similarly, the level of binding of [³H]Pm toxin to E. coli cells expressing T-toxin-insensitive URF13 mutations at Asp-39 is markedly reduced and the binding that remains shows no cooperativity.

MATERIALS AND METHODS

Expression of URF13 in E. coli, Spheroplast Preparation, and Mitochondria Isolation from cms-T Maize—Standard URF13 and mutants at position 39 were expressed using the pLC-13T construct in E. coli as described previously (17). E. coli spheroplasts were prepared as described by Witholt et al. (24). Washed cms-T mitochondria were isolated from 6-day-old etiolated cms-T maize seedlings as described by Stegink and Siedow (25). Protein concentrations were determined by the method of Lowry et al. (26).

Immunoblot Analysis—Cells or spheroplasts at 1.0 mg of protein/ml were solubilized in SDS-PAGE loading buffer, boiled for 5 min, and then separated by 18% SDS-PAGE without urea as described previously (17). Immunoblotting was carried out according to Harlow and Lane (27). A monoclonal antibody specific for a carboxyl-terminal region of URF13 (19) was used for visualization in conjunction with the enhanced chemiluminescence (ECL) reagent system (Amersham Corp.).

ECL-visualized immunoblots were quantified by densitometry with an LKB Ultrascan XL laser scanning densitometer using the LKB 2000 GelScanXL program (version 1.2) and the procedures outlined in Amersham Tech Tip 131. An URF13 standard curve was run with each blot to determine the range of film linearity, and only those URF13 bands on the immunoblots that had values falling within the linear range of the standard curve were used for quantification.

DCCD Treatment—E. coli cells at 1.0 mg of protein/ml in 140 mM NaCl, 2 mM KCl, 10 mM NaHPO₄, and 2 mM KH₂PO₄, pH 7.2 (phosphate-buffered saline), were incubated with 1.0 mM DCCD (Sigma). Spheroplasts at 1.0 mg of protein/ml in 0.25 M sucrose and 0.1 M Tris, pH 8.0, were incubated with the indicated concentration of DCCD, after DCCD treatment. E. coli mitochondria at 1.0 mg of protein/ml in 0.3 M mannitol, 1 mM KCl, 10 mM KCl, 5 mM MgCl₂, and 10 mM HEPES, pH 7.2, were incubated with 50 μM DCCD. All reactions were carried out for 30 min at 20 °C with gentle agitation. Assays of Methionyl Sensitivity and Pm Toxin Binding—The sensitivity of respiration to methomyl in intact E. coli cells and isolated spheroplasts was measured polarographically using a Clark-type oxygen electrode as described previously (9). Spheroplast swelling was measured in a Shimadzu UV-265FW spectrophotometer as a decrease in absorbance at 530 nm, as described by Dewey et al. (9), in a buffer containing 10 mM Tris, pH 8.0, 0.25 M sucrose, and 10 mM glucose. Tritiated Pm toxin binding to E. coli cells expressing standard URF13 or Asp-39 mutants was carried out as described previously (22).

RESULTS

Immunoblot Analysis of URF13 in E. coli Cells and cms-T Mitochondria Treated with DCCD—The hydrophobic carbodiimide DCCD has been shown to protect cms-T mitochondria and E. coli cells expressing URF13 from the deleterious effects of methomyl or T-toxin (14–17). Previous studies utilized [¹⁴C]-labeled DCCD in conjunction with SDS-PAGE and autoradiography to visualize the binding of DCCD to URF13 (9, 17). These studies demonstrated that URF13, which ran at the position of a monomer on SDS gels, was covalently labeled with the [¹⁴C]DCCD. Directed mutagenesis studies have indicated that DCCD labeled primarily Asp-39, with some limited labeling of Asp-12 (17). The formation of a stable DCCD adduct at Asp-39 is consistent with the localization of the side chain of Asp-39 in a predominantly hydrophobic environment. However, if the labeled DCCD had activated a carboxylate residue that was susceptible to attack by an appropriate nucleophilic side chain on a proximally located amino acid residue, the [¹⁴C]DCCD would be displaced as a substituted urea (18, 28), rendering the DCCD-reactive carboxylate residue invisible to autoradiography. Furthermore, if the attacking nucleophile were located on an adjacent URF13 monomer within an URF13 oligomer, cross-linking of the two monomers would result. To test for this possibility, immunoblot analysis of DCCD-treated pLC-13T E. coli cells expressing URF13 was carried out with an URF13-specific antibody, to detect directly any cross-linked species. Induced pLC-13T cells treated with 1.0 mM DCCD gave rise to bands at 25 and 38 kDa, consistent with the formation of URF13 dimeric and trimeric species (Fig. 1, lane 2). Spheroplasts isolated from induced pLC-13T cells and treated with DCCD showed a similar set of bands, as well as a band having a mobility consistent with a tetrameric URF13 species and, possibly, a limited level of even higher order species (Fig. 1, lane 3). In all cases, the resulting oligomers appear to be homogeneous because of the absence of any intermediate-sized species. Quantification of the distribution of URF13 species in a representative sample of DCCD-treated spheroplasts gave the following percentages: 55% as monomer, 25% as dimer, 15% as trimer, and 4% as tetramer.

FIG. 1. SDS-PAGE immunoblots of DCCD-treated intact E. coli cells and spheroplasts expressing URF13 and isolated cms-T mitochondria probed with an URF13-specific antibody. Samples were treated with either 1.0 mM (lanes 2 and 3) or 50 μM (lane 5) DCCD as described under "Materials and Methods." Control lanes (lanes 1 and 4) were incubated in the absence of DCCD. The blots were probed with the URF13-specific monoclonal antibody, mAb-C (19). Oligomeric species of URF13 are denoted on the right.
Typical cross-linking reaction with cms-T mitochondria, only 30% of total URF13 protein remained as monomer, with 35% appearing as dimer, 28% as trimer and 9% as tetramer. The dimeric band in untreated cms-T mitochondria (Fig. 1, lane 4) corresponded to about 14% of the total URF13 protein.

Increasing the DCCD concentration or the time of incubation with DCCD resulted in a only slightly greater accumulation of higher order species, but the monomeric band could not be eliminated under any set of conditions (data not shown), suggesting that a distribution of monomeric, dimeric, trimeric, and tetrameric URF13 species exists within the membrane. Previous demonstration of cross-linking of URF13 oligomers using the lysine-specific cross-linker, EGS, also suggested the presence of a distribution of species within the membrane, but considerably less trimer, and essentially no tetramer formation, was observed with that reagent (19). Preincubation of cms-T mitochondria or E. coli cells expressing URF13 with either T-toxin or methomyl did not alter the observed pattern of cross-linking obtained with DCCD (data not shown), similar to the results seen with EGS (19).

Correlation of DCCD Cross-linking of URF13 and Inhibition of Methomyl Sensitivity—Addition of 1.0 mM DCCD to intact E. coli cells expressing URF13 occasionally resulted in complete methomyl insensitivity, but in the majority of cases, limited sensitivity to methomyl remained. This is in contrast to cms-T mitochondria where markedly lower concentrations of DCCD (50 μM) consistently result in completely T-toxin- or methomyl-resistant mitochondria (Refs. 14-16 and data not shown). However, spheroplasts derived from induced pLC-13T cells could routinely be rendered either fully or almost completely methomyl-insensitive by 1.0 mM DCCD as determined by respiration associated oxygen uptake (Fig. 2) or a spheroplast swelling assay (Fig. 3) that measures the rate of decrease in absorbance at 520 nm after methomyl addition (9). When the extent of DCCD-induced cross-linking of URF13 was compared between partially methomyl-sensitive pLC-13T cells (Fig. 2B) and almost fully methomyl-resistant spheroplasts (Fig. 2C), similar amounts of URF13 dimer were seen but the spheroplasts showed much higher levels of the trimeric and tetrameric species.

To characterize better the relationship between the formation of covalently cross-linked, higher order URF13 oligomers and methomyl insensitivity, a DCCD concentration series was utilized. Treatment of pLC-13T spheroplasts from induced cells with different DCCD concentrations yielded species having a range of methomyl sensitivities. After treatment with 10 μM DCCD only a slight accumulation of dimer was noticed and no accumulation of trimer or tetramer was apparent (Fig. 3B). The resulting spheroplasts were completely methomyl sensitive, as determined by the decrease in absorbance at 520 nm. After treatment with 250 μM DCCD some trimer was detected, as well as an increase in the amount of dimer (Fig. 3C). This correlated with a significant decrease in the rate of the absorbance change at 520 nm, indicating partial methomyl insensitivity. After incubation with 1.0 mM DCCD, strong trimeric and tetrameric bands were observed (Fig. 3D) and essentially no spheroplast swelling could be detected upon methomyl addition, suggesting the cells were completely methomyl-resistant. Taken together, these results suggest that covalent cross-linking of URF13 into trimeric (and possibly tetrameric) species is necessary to achieve the loss of methomyl sensitivity observed in DCCD-treated cells.

[^H]Pm Toxin Binding to E. coli Cells Treated with DCCD—It had been shown previously that E. coli cells expressing URF13 specifically bind[^H]Pm toxin in a cooperative manner with a Hill coefficient of 1.3-1.7, suggesting some form of interaction between individual toxin binding sites (22). Because DCCD treatment yields a toxin-insensitive state of URF13, it was of interest to determine the nature of[^H]Pm toxin binding to DCCD-treated, induced pLC-13T cells. DCCD-treated cells bound almost 50% less[^H]Pm toxin (per mg of E. coli protein) than did untreated cells (Fig. 4). Furthermore, the labeled T-toxin that did specifically associate with DCCD-treated cells bound in a strictly hyperbolic manner, indicating that cooperativity was completely abolished by DCCD treatment. This is clearly illustrated by the linear nature of the Scatchard transformation of the binding data, in contrast to the non-linear behavior of the untreated control.

![Fig. 2. Protective effect of DCCD on the methomyl sensitivity of respiration in intact E. coli cells and isolated spheroplasts expressing URF13. Intact E. coli cells (B) and isolated spheroplasts (C) were treated with 1.0 mM DCCD as described under "Materials and Methods." Untreated spheroplasts (A) served as the control. Associated with each trace is the corresponding SDS-PAGE immunoblot of the sample after methomyl addition. The sensitivity of respiration-stimulated oxygen uptake to the addition of methomyl was measured with a Clark-type oxygen electrode (9). Respiration of 150-300 μg of total protein in a volume of 2.0 ml was measured at 25 °C in the presence of M-9 salts plus 10 mM glucose. Methomyl (8 mM) was added at the times indicated by the arrows. Respiration rates are listed below each trace as nanomoles of oxygen consumed/min/mg of protein. The oligomeric state of URF13 is denoted on the right.](image-url)
translated spheroplasts from on the methomyl sensitivity of iso-
tivity was measured as the rate of de-
crease in absorbance at 520 nm associ-
mM gomeric species (dimers and above) of
DCCD. Methomyl (8 mM) was added at
the times indicated by the
URF13 are denoted on the
trace
URF13 complex to undergo the
dynamic transition from a
mediated cross-linking limits the ability of the oligo-
meric species (dimers and above) of
DCCD. Methomyl (8 mM) was added at
the times indicated by the

 acquitted S.

Fig. 3. Protective effect of DCCD on the methomyl sensitivity of iso-
lated spheroplasts from E. coli cells expressing URF13. Methomyl sensi-
tivity was measured as the rate of de-
crease in absorbance at 520 nm associ-
ated with spheroplast swelling (9). Each
trace is accompanied by the correspond-
ing SDS-PAGE immunoblot of the sam-
ple following DCCD cross-linking at
25°C for 20 min using 0 mM (A), 0.01
mM (B), 0.25 mM (C), or 1.0 mM (D)
DCCD. Methomyl (8 mM) was added at
the times indicated by the arrows. Oligo-
gemeric species (dimers and above) of
URF13 are denoted on the right.

Fig. 4. Scatchard plot of the specific binding of [3H]Pm
Toxin to DCCD treated (C) and untreated (O) E. coli cells
expressing URF13. The solid lines represent theoretical curves
generated using the following parameters: untreated cells, Kd = 95
nM, P1 (maximum Pm toxin binding) = 288 pmol of Pm toxin bound/mg
of E. coli protein and Hill coefficient (n) = 1.3; DCCD-treated
cells, Kd = 90 nM, P1 = 155 pmol/mg of E. coli protein and n = 1.0.

Pm toxin binding was carried out as described under "Materials and
Methods." (Fig. 4). Interestingly, the intrinsic dissociation constant (80-
90 nM) for binding of the Pm toxin was the same for DCCD-
treated and untreated cells. These results suggest that DCCD
mediated cross-linking limits the ability of the oligo-
meric species (dimers and above) of

DCCD. Methomyl (8 mM) was added at
the times indicated by the

Fig. 5. Scatchard plot of the specific binding of [3H]Pm
Toxin to E. coli cells expressing standard URF13 (O) or URF13
having the mutations D39E (V) and D39V (V). The solid lines
represent theoretical curves generated using the following parameters:
URF13, Kd = 96 nM, P1 = 475 pmol/mg of E. coli protein and n = 1.5;
D39E, Kd = 145 nM, P1 = 198 pmol/mg of E. coli protein and n = 1.0;
D39V, Kd = 230 nM, P1 = 130 pmol/mg of E. coli protein and n = 1.0.

Pm toxin binding was carried out as described under "Materials and
Methods."

equal to 230 nM for D39V and 145 nM for D39E, compared to
a value of 80 nM for the untreated pLC-13T cells. Likewise,
the relative decrease in total Pm toxin binding in the two
mutants was well below the 50% of control level seen with
DCCD-treated pLC-13T cells (Fig. 4), declining roughly 4-
fold to 130 pmol/mg protein for D39V and 3-fold to 198 pmol/
mg protein for D39E (Fig. 5). Quantitative analysis of the
amount of URF13 protein expressed in the two Asp-39 mu-
tants relative to that in standard pLC-13T cells showed only
minor differences, with D39E and D39V containing 8 and 9%
more immunoreactive URF13 protein, respectively, than the
amount seen in pLC-13T cells. This indicates that the ob-
served reduction in Pm toxin binding by the two Asp-39
mutants was not due to reduced levels of URF13 expression
in these mutants.

DISCUSSION
Although the ability of DCCD treatment to protect cms-T
mitochondria from the effects of added T-toxin has been
known for some time (14-16), the molecular mechanism be-
hind DCCD protection was not well characterized. The
URF13 polypeptide was found to be susceptible to formation of a
covalent DCCD adduct, both in cms-T mitochondria (9)

* C. J. Braun, unpublished observation.
and in E. coli cells expressing URF13 (17). One residue, Asp-39, was found to be capable of stably reacting with [14C]DCCD and was also necessary for toxin sensitivity as shown by site-directed mutagenesis (17). A second residue, Asp-12, was also shown to produce a labeled DCCD adduct, but to a considerably lesser extent than Asp-39, and site-directed modification of Asp-12 had no effect on the sensitivity of URF13 to added T-toxin or methomyl (17). A three-membrane-spanning α-helical model for the disposition of the URF13 polypeptide in the membrane has been postulated (10). In this model, both of the DCCD-reactive aspartate residues are postulated to be in hydrophobic environments; Asp-12 is located in helix-1, which is completely hydrophobic, and Asp-39 is situated on the hydrophobic face of helix-2, which is highly amphipathic.

DCCD binding to URF13 was determined by visualizing the covalent linkage of [14C]DCCD to monomeric URF13 on SDS gels using autoradiography (9, 17). When a carboxylic residue reacts with DCCD, the resulting intermediate can undergo a rearrangement to produce a covalently linked DCCD adduct (18, 28). However, the activated intermediate is also susceptible to attack by a suitably positioned nucleophile (generally an ε amino group on a proximally located lysine residue; Ref. 28) that can displace the DCCD and result in either inter- or intramolecular cross-linking. Following nucleophilic attack, the [14C]DCCD is displaced as dicyclohexylurea, rendering the cross-linked molecule invisible to autoradiography when [14C]DCCD is used in the reaction (18, 28). This potential problem was circumvented in the present study by using an URF13-specific antibody, allowing unlabeled, cross-linked URF13 oligomers to be detected directly. In fact, when DCCD-treated pLC-13T membranes were analyzed by immunoblotting, oligomers, including dimers, trimers, and tetramers, were detected (Fig. 1).

It had previously been felt that DCCD simply formed a stable adduct at Asp-39, and that this modification abolished T-toxin sensitivity, because no [14C]-containing oligomeric species were detected (17). An URF13 dimer has two reactive Asp-39 sites, only one of which needs to participate in cross-linking to produce a covalently linked dimer, leaving the second aspartate available to form a rearranged adduct which would retain the [14C]DCCD label. The absence of significant incorporation of [14C]DCCD into multimeric hands suggests that almost all the Asp-39 residues within any given URF13 oligomeric complex, although ostensibly free to undergo the DCCD rearrangement reaction, are more susceptible to nucleophilic attack, presumably by a lysine residue on the reciprocal monomer, and that this represents the favored reaction course.

The DCCD cross-linked URF13 species detected here appear similar to those reported earlier using the lysine-specific reagent EGS (18). The oligomeric products observed from the cross-linking reaction with DCCD migrate identically to those seen with EGS on SDS-PAGE, without any apparent bands that are not discrete molecular weight multiples of the monomeric URF13. However, no cross-linked species larger than a trimer are seen with EGS cross-linking (19), whereas DCCD cross-linking gives rise to both a greater amount of trimer and the appearance of some tetramer. The reason for this distinction between DCCD and EGS cross-linking is not fully understood and is currently under investigation. E. coli spheroplasts seem to be more reactive toward DCCD than intact E. coli cells, showing a greater accumulation of multimers and a consistently higher level of subsequent T-toxin resistance. The presence of the outer membrane and cell wall in intact E. coli cells may affect the accessibility of URF13 localized in the plasma membrane to DCCD, given the large number of potentially reactive carboxylate groups that DCCD would likely encounter in this region before it reaches URF13.

URF13 expressed in E. coli and cms-T mitochondria shows a somewhat different distribution of oligomeric species after cross-linking with DCCD. In E. coli, the monomeric band is the most prevalent one, representing 55% of the total URF13 protein. By contrast, in cms-T mitochondria only 30% of the immunoreactive URF13 protein remains as monomer after DCCD cross-linking. Hence, the fraction of total URF13 protein seen in any given oligomer is considerably greater in cms-T mitochondria than in E. coli. Interestingly, when pLC-13T E. coli cells were cross-linked with DCCD at various times following induction of URF13 expression, times corresponding to the earliest appearance of URF13 protein and the concomitant onset of methomyl sensitivity, only the dimeric and trimeric species appeared on immunoblots.3 As the induction period increased, the amount of dimer and trimer remained relatively constant, with almost all additional URF13 protein appearing in the monomeric band. Given this observation, the lower relative amount of URF13 monomer seen in DCCD cross-linked cms-T mitochondria could reflect a tighter level of regulation of expression of URF13 in its native system.

The correlation seen between the formation of trimeric and tetrameric cross-linked URF13 species and the onset of DCCD protection against the effects of T-toxin or methomyl in E. coli cells and spheroplasts is intriguing. The inability of either EGS or DCCD to completely chase monomeric (or even dimeric) URF13 into the higher order oligomers, even under conditions of high cross-linker concentration and/or extended reaction times, suggests that the distribution of monomers and oligomers observed after cross-linking reflects the in situ distribution of URF13 oligomeric species in the membrane. However, the exact nature of the DCCD cross-linked URF13 species is open to interpretation. The dimeric species seen in SDS-PAGE upon treatment of E. coli spheroplasts with low concentrations of DCCD (Fig. 3B) could reflect either the covalent linking of true dimeric units in the membrane or the partial covalent linking of two URF13 subunits participating in trimer or tetramer formation. In either case, cross-linking that leads solely to dimer formation (Fig. 3B) does not seem to be sufficient to eliminate T-toxin-induced pore formation. Only when trimers and tetramers have been covalently linked can pore formation be completely prevented and protection against the effects of T-toxin observed. It is important to emphasize that a single functional URF13 pore is likely to be sufficient to inhibit respiration in an E. coli cell (29). Therefore, a protective reaction such as DCCD cross-linking may not show complete inhibition of T-toxin- or methomyl-mediated toxicity until every potential pore-forming multimer within a given cell (or mitochondrion) has been cross-linked.

It has been proposed that URF13 contains three membrane-spanning α-helices, with the two COOH-terminal helices being amphipathic in nature and capable of participating in hydrophilic pore formation (10). Six to eight membrane-spanning helices are theoretically needed to generate a cylindrical pore consistent with the size of the molecules known to pass through the inner membrane in T-toxin-permeabilized cms-T mitochondria (M, < 650; Ref. 6) (20, 21). The observation that URF13 trimers and tetramers represent the species that need to be covalently cross-linked to achieve insensitivity to T-toxin is consistent with the proposed thre-helical model, given that trimers and tetramers would give rise to six and eight amphipathic helices lining the resulting URF13 pore, respectively.

The causal role of the cross-linking reaction in the gener-

---

3 P. Campbell, unpublished observation.
ation of the T-toxin-resistant state remains unclear. In this regard it should be noted that D39E and D39V both form covalently linked oligomers when treated with EGS. The mutation D39E is also cross-linked by DCCD, but D39V, lacking a carboxylate residue at position 39, does not cross-link with DCCD (data not shown). As the observed URF13 cross-linking pattern does not change in the presence of T-toxin or methomyl (19), the specific residues involved in both the DCCD and EGS cross-linking reactions may be localized in a region of URF13 in which the monomer:monomer interactions are not significantly altered by the structural changes that take place when the URF13 oligomer achieves a pore-forming configuration in response to T-toxin.

The ability of DCCD to bring about the formation of intermolecular cross-links in URF13 should prove useful in helping to shed light on the nature of the URF13 side chains that stabilize oligomer formation. Asp-39 must be situated in close proximity to the displacing nucleophile, presumably a lysine, on the adjacent URF13 molecule with which it reacts in the DCCD-mediated cross-linking. Lys-32, in the interhelical region between helices 1 and 2, and Lys-97, in the amphipathic helix 2, are both candidates for reaction with DCCD-activated Asp-39. The ability of ε-amino and carboxylate groups to bond directly either via ionic forces or through hydrogen bonding (30) suggests that Asp-39 and its DCCD reactive partner may interact in the native URF13 oligomer and contribute to stabilization of the URF13 oligomeric structure. Future analysis of site-directed mutants of URF13 at these lysine residues should help to clarify their roles in oligomerization.

DCCD treatment affects Pm toxin binding in two ways: 1) the level of specific T-toxin binding is decreased by a factor of roughly 2, and 2) cooperative binding is lost (Fig. 4). Analogous results were obtained with the T-toxin-insensitive mutations at Asp-39 (Fig. 5). Since URF13 seems to exist in the E. coli membrane as a mixed distribution of monomers and higher order oligomers, it is possible that the cooperativity of T-toxin binding observed in untreated cells (Hill coefficient = 1.3-1.7; Ref. 22) represents the weighted sum of hyperbolic binding to monomers and cooperative binding to dimers, trimers, and tetramers. Analysis of previous binding data suggests that a model for T-toxin binding with a fraction of the URF13 species showing strictly hyperbolic binding and the rest showing cooperative binding with a Hill coefficient around 2 yields a better fit to the data than does a model having a single binding species showing lower cooperativity. Additional T-toxin binding studies will need to be carried out to better substantiate this hypothesis.

The absence of cooperative T-toxin binding in both DCCD-treated cells and the Asp-39 mutants is intriguing. In DCCD-treated cells, the cross-linked oligomeric complexes are apparently unable to make the transition to the "open" conformation. This could have two origins. DCCD cross-linked URF13 oligomers may not bind T-toxin at all. In that case, the residual, hyperbolic binding would be due to T-toxin associating with the population of URF13 monomers present in the sample. Alternatively, DCCD cross-linked URF-13 oligomers may bind T-toxin no differently than do isolated monomers, either because of the covalent cross-linking per se, or because of the perturbation of specific monomer:monomer interactions after modification at Asp-39. Similarly, the Asp-39 mutants may not be capable of forming the monomer:monomer interactions necessary for pore formation. It is notable that the Asp-39 mutants do not differ from the DCCD-treated cells in that DCCD treatment led to no apparent change in the intrinsic Kₘ for Pm toxin binding, in spite of the loss of cooperativity, while the Asp-39 mutants both showed significant (2-3-fold) increases in the value of the intrinsic Kₘ, suggesting that a small decrease in the interaction energy (=0.4-0.65 kcal/mol) between URF13 and Pm toxin is lost as a result of the changes in the Asp-39 mutants.

Studies are currently under way to investigate both the Pm toxin binding and cross-linking properties of other toxin-insensitive mutants of URF13, both in the region of helix-2 examined in this study as well as in other domains of the protein, to better elucidate the nature of the interactions underlying URF13 oligomerization and subsequent T-toxin-induced pore formation.

Acknowledgments—We thank Sam Leavings and Ken Korth for helpful discussions related to this work.

REFERENCES


4. C. I. Kaspi and J. N. Siedow, personal observation.