Oxidation−Reduction Properties of Disulfide-Containing Proteins of the *Rhodobacter capsulatus* Cytochrome *c* Biogenesis System†

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ABSTRACT: Oxidation−reduction titrations for the active-site disulfide/dithiol couples of the *helX*- and *ccl2*-encoded proteins involved in cytochrome *c* biogenesis in the purple non-sulfur bacterium *Rhodobacter capsulatus* have been carried out. The *R. capsulatus* HelX and Ccl2 proteins are predicted to function as part of a dithiol/disulfide cascade that reduces a disulfide on the apocytochromes *c* so that two cysteine thiols are available to form thioether linkages between the heme prosthetic group and the protein. Oxidation−reduction midpoint potential (E_m) values, at pH 7.0, of -300 ± 10 and -210 ± 10 mV were measured for the HelX and Ccl2* (a soluble, truncated form of Ccl2) *R. capsulatus* proteins, respectively. Titrations of the disulfide/dithiol couple of a peptide designed to serve as a model for *R. capsulatus* apocytochrome c_2 have also been carried out, and an E_m value of -170 ± 10 mV was measured for the model peptide at pH 7.0. E_m versus pH plots for HelX, Ccl2*, and the apocytochrome c_2 model peptide were all linear over the pH range from 5.0 to 8.0, with the −59 mV/pH unit slope expected for a reaction in which two protons are taken up for each disulfide that is reduced. These results provide thermodynamic support for the proposal that HelX reduces Ccl2 and that reduced Ccl2, in turn, serves as the reductant for the production of the two thiols of the CysXxxYyyCysHis heme-binding motif of the apocytochromes.

The biogenesis of *c*-type cytochromes in the photosynthetic purple bacterium *Rhodobacter capsulatus* had been demonstrated to require at least eight genes that encode proteins specific to the biogenesis pathway (*1*). Similar genes are required for cytochrome *c* biogenesis in nonphotosynthetic Gram-negative bacteria such as *Bradyrhizobium japonicum*, *Escherichia coli*, and *Paracoccus denitrificans* (see refs *1*−*3* for reviews). The eight genes essential for the biosynthesis of all *c*-type cytochromes in *R. capsulatus* are located at three different loci on the bacterial chromosome, *helABCDX* (*4*−*6*), *ccl1-ccl2* (*4*, *7*), and *cycH* (*8*). Recently, two additional genes, *cycJ* and *ccdA*, neither of which is located adjacent to the previously characterized genes, were shown to be required for cytochrome *c* biogenesis in *R. capsulatus* (*9*).

Possible functions have been assigned to most of the *R. capsulatus* proteins known to be required for this pathway. The *cycJ* gene product may function as a periplasmic heme chaperonin (*9*). The *helA*, *helB*, *helC*, and *helD* gene products have been shown to function in *R. capsulatus* as subunits of an ATP-dependent exporter that is located in the cytoplasmic membrane and which may serve to transport ferroheme from the cytoplasm to the *ccl1* gene product at the periplasmic surface (*10*, *11*). The HelX and Ccl2 proteins, encoded by the *helX* and *ccl2* genes, respectively, both appear to be anchored in the cytoplasmic membrane by a single membrane-spanning helix, with most of both proteins exposed in the periplasm (*12*). HelX, Ccl2, and apocytochromes *c* each contain two cysteine residues separated by two intervening amino acids, with the cysteine pairs of both HelX and Ccl2 proteins likely to be located in the periplasmic space (*12*). Given that reduction of the apocytochromes is known to occur in the periplasm, after transport of the apocytochrome from the cytoplasm (*1*, *2*), the predicted location of these HelX and Ccl2 cysteines would allow them to participate in a periplasmic dithiol/disulfide cascade which ultimately reduces any disulfides on apocytochromes *c* to the dithiol form required for heme attachment. The membrane-spanning CcdA protein, encoded by the *ccdA* gene, may function to convey reducing equivalents from cytoplasmic reductants to HelX (*9*).

The sequence of HelX is somewhat homologous to the sequences of members of the thioredoxin family (*6*), proteins known to carry out disulfide/dithiol redox reactions (*13*). Although Ccl2 is not obviously homologous to thioredoxins (*5*), the presence of a pair of cysteines in Ccl2 with spacing identical to that found in thioredoxins suggests a possible redox function for the Ccl2 protein. Evidence has been obtained to support the hypothesis that the two cysteines at the active site of the reduced HelX protein reduce the active-site disulfide of the oxidized Ccl2 protein and then the reduced Ccl2 protein, in turn, reduces a disulfide on

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apocytochrome *c* (*12*). This final step provides the two thiols on the apocytochrome that are needed for formation of the thioether bonds that covalently link the heme to the apocytochrome (*1*). To provide thermodynamic evidence for the feasibility of the proposed dithiol/disulfide cascade in *R. capsulatus*, and to provide the first characterization of the redox properties of these proteins, we have determined the oxidation−reduction midpoint potentials (E_m) for the *R. capsulatus* HelX and Ccl2 proteins and for a model peptide for *R. capsulatus* apocytochrome c_2.

EXPERIMENTAL PROCEDURES

The properties of the plasmid and the *E. coli* strain used for the overexpression of the *helX* gene product have been described previously (*12*). The form of HelX used for all of the measurements described below was engineered to contain six histidines (hereafter called a His tag) at the C-terminus so that it could be purified by chromatography on a nickel affinity column. This His tag has previously been shown not interfere with the function of the protein in vivo (*12*). Cells of *E. coli* BL21λDE3 containing the *helX* plasmid (pRGK256) were used to overproduce HelX as described previously (*12*). HelX was released from the cells and purified, using Ni affinity chromatography, as described previously (*12*). At this stage of purification, SDS−PAGE analysis reveals a doublet (i.e., two Coomassie-stained bands located very close to each other). As has been previously reported, the upper band of the doublet contains the complete HelX signal sequence, while the lower band contains only a portion of the signal sequence, FVGAGY, at its N-terminus (*12*). The concentrated HelX protein was subjected to gel filtration chromatography on a Superose 6 column using 50 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl. The gel filtration fraction used for all of the measurements below contains only the lower band of the doublet observed after SDS−PAGE (*12*). The purified HelX protein was stored at −80 °C at a protein concentration of approximately 1 mg/mL.

The *ccl2* gene product was engineered, as described previously (*12*), so that its C-terminal transmembrane domain was removed and replaced with six histidine residues. This Ccl2* protein is produced as a soluble protein in the periplasmic space of *E. coli* and was purified on a nickel affinity column, as described previously (*12*). Isolation and purification of Ccl2* were carried out as described previously (*12*), and the resulting protein is more than 98% pure, as determined by N-terminal sequence analysis and SDS−PAGE (*12*). The purified Ccl2* was stored at −80 °C at a protein concentration of approximately 1 mg/mL.

The peptide used as a model for the mature form of *R. capsulatus* apocytochrome c_2 has the sequence VQKCAQCHTVE, identical to the sequence of the 11 amino acids at the N-terminus of the protein (*14*, *15*). The peptide was synthesized at the University of Texas Southwestern Medical Center at Dallas (Dallas, TX) on a Rainin Symphony synthesizer using standard solid-phase peptide synthesis methods. The synthesis was carried out at room temperature using fluorenylmethoxycarbonyl-protected amino acids and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronim hexafluorophosphate for carboxyl group activation. The peptide was cleaved from the support using a mixture of trifluoroacetic acid (87.5%), water (5%), thioanisole (5%), ethanedithiol (2.5%), and phenol (4.3%), with all the percentages representing weight/volume figures. The predicted molecular mass for the peptide of 1246 Da was verified by mass spectrometry on a Perseptive Biosystem Voyager DE MALDI-TOF mass spectrometer. The purity of the peptide was estimated to be 95−99% as determined by reverse-phase chromatography with a water/acetonitrile gradient and a C18 column, using a Waters HPLC system.

Oxidation−reduction equilibration, labeling of cysteine thiols with monobromobimane (mBBr),[1] separation of the protein from small molecules after mBBr treatment by trichloroacetic acid precipitation of the protein, measurement of the fluorescence of the mBBr adducts of the proteins, and fitting redox titration data to the Nernst equation for a two-electron reaction were carried out as described previously (*16*, *17*), except that glutathione redox buffers were used for the titrations of Ccl2* and the apocytochrome c_2 model peptide instead of the dithiothreitol (DTT) redox buffers that were used for the HelX titrations. DTT redox buffers were also used for control titrations of wild-type *E. coli* thioredoxin (samples of the thioredoxin were generously provided by M. Miginiac-Maslow, J.-P. Jacquot, and P. Chivers). For titrations of the model peptide, a higher concentration of TCA (20%) was needed in the wash step to eliminate the loss of peptide that was observed if the 1% TCA used for the HelX and Ccl2* titrations was used. For HelX, which is isolated with the active site disulfide reduced (*12*), oxidation of the active-site cysteines was accomplished by incubating the HelX protein, on ice, for 3 h in the dark in the presence of 2 mM diamide. Buffer exchange, using a 10 kDa cutoff Centricon ultrafiltration membrane, was employed to remove any unreacted diamide prior to redox equilibration.

E_m values obtained for HelX, Ccl2*, and the model peptide were independent of the time used for redox equilibration, for incubation times centered around the 30 min incubation time used for all of the titrations shown below. The E_m value for *E. coli* thioredoxin was independent of the time used for redox equilibration, for incubation times centered around the 2 h incubation time used for these titrations. The E_m values obtained for HelX and *E. coli* thioredoxin were independent of the total DTT concentration, in the concentration range centered around the 10 mM (*E. coli* thioredoxin) and 20 mM (HelX) values used for the titrations described below. Similarly, the E_m values obtained for Ccl2* and the model apocytochrome c_2 peptide were independent of total glutathione concentration, in the concentration ranges centered around the 20 mM (Ccl2*) and 5 mM (the model peptide) values used for all of the titrations shown below. E_m values that are independent of time and buffer concentration are expected for true equilibrium titrations.

Titrations were performed under both anaerobic and aerobic conditions. Oxygen-free conditions were established by purging the reaction mixtures with argon prior to the addition of the protein, and by subsequently supplying a constant flow of argon over the surface of the reaction mixtures during the course of the experiments, as described

[1] Abbreviations: DTT, dithiothreitol; mBBr, monobromobimane; MES, 2-(*N*-morphilino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane.

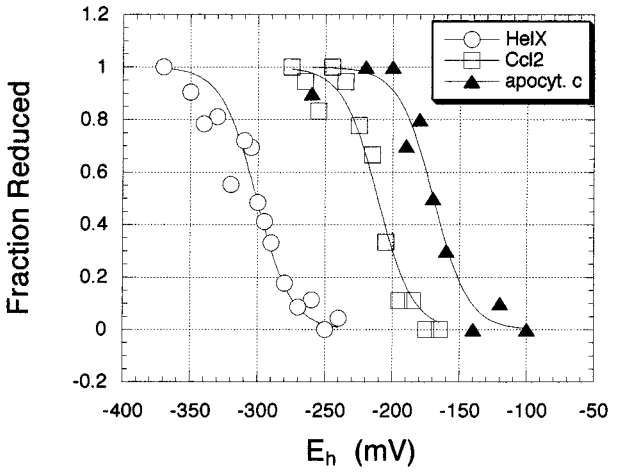

FIGURE 1: Oxidation−reduction titrations of *R. capsulatus* HelX, Ccl2*, and the apocytochrome c_2 model peptide at pH 7.0. The reaction mixture contained either HelX (○), Ccl2* (□), or the apocytochrome *c* peptide (▲) at a concentration of 200 μg/mL in 100 mM MOPS buffer (pH 7.0) and either 20 mM oxidized and reduced DTT (the HelX titration), 20 mM oxidized and reduced glutathione (the Ccl2* titration), or 5 mM oxidized and reduced glutathione (the apocytochrome c_2 model peptide). Oxidation−reduction equilibration was carried out for 30 min prior to addition of mBBr. The solid lines represent the best-fit, $n = 2$ Nernst curves with E_m values of −302 (HelX), −211 (Ccl2*), and −170 mV (the apocytochrome *c* model peptide).

by Hutchison and Ort (*18*). We had previously demonstrated, using titrations of the thiol groups of reduced DTT with the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid), that reduced DTT is completely stable against oxidation for periods up to 3 h without any precautions to remove dissolved oxygen from the buffers (*19*). Similar measurements with 5,5′-dithiobis(2-nitrobenzoic acid), carried out as part of the study presented here, indicated that reduced glutathione was completely stable against oxidation for at least 1 h without any precautions to remove dissolved oxygen from the buffers.

E_m values, at pH 7.0, of −330 mV for DTT and −245 mV for glutathione were used for the calculation of the E_m values for HelX, Ccl2*, and the apocytochrome c_2 model peptide. The E_m values used for DTT (*18*) and glutathione (*20*) are the averages of closely agreeing values from the literature. An E_m versus pH relationship of −59 mV/pH unit (*21*) was used to calculate E_m values for glutathione and DTT at other pH values. Fitting the E_m versus pH relationship for HelX, Ccl2*, and the apocytochrome c_2 model peptide was carried out as described previously (*16*, *17*, *19*).

RESULTS

Figure 1 shows the results of oxidation−reduction titrations of *R. capsulatus* HelX, Ccl2*, and the model peptide at pH 7.0, in which the fluorescence of the mBBr adducts of the proteins and the peptide is used to monitor the extent of reduction of the disulfide/dithiol couple. Monobromobimane (mBBr) is a reagent that is nonfluorescent and which reacts specifically with thiols to form a fluorescent adduct (*22*). As mBBr does not react with protein disulfides (*22*, *23*) and the protocol used completely separates unreacted mBBr and any mBBr adducts of the reduced form of DTT or glutathione from the protein (*16*, *17*), this method is well-suited for monitoring the appearance of protein thiol groups during redox titrations. In a previous "proof of method" experiment, we demonstrated that the mBBr-labeling method gave an E_m value for *Chlamydomonas reinhardtii* thioredoxin *h* that was identical to one obtained via enzyme-equilibrated $NADP^+$/NADPH redox poising (*16*). As a further test of the method, we have performed a series of titrations of *E. coli* thioredoxin and obtained an average E_m value, from four replicate titrations at pH 7.0, of −280 ± 10 mV (data not shown). This value agrees, within the experimental uncertainties of the measurements, with the E_m values centered around −270 mV that have been obtained by other methods and reported in the literature (*20*, *24*−*26*).

The results of the three titrations shown in Figure 1 give good fits to the Nernst equation for a single two-electron redox component, with E_m values of −302 mV for HelX, −211 mV for Ccl2*, and −169 mV for the apocytochrome c_2 model peptide. A series of three independent titrations for both proteins and for the model peptide gave average E_m values (at pH 7.0) of −300 ± 10 mV for HelX, −210 ± 10 mV for Ccl2*, and −170 ± 10 mV for the model peptide. Attempts to fit the data to two $n = 2$ components did not improve the quality of the fit. For HelX, it was also shown that the E_m value that was obtained did not depend on whether one started with oxidized or reduced HelX, as would be expected for an equilibrium titration. The relatively positive E_m value obtained for Ccl2* is consistent with our observation that the disulfide of Ccl2* was fully reduced in DTT redox buffers with the highest ratio of oxidized to reduced DTT that could be accurately obtained. The active-site disulfide of HelX, in contrast, was fully oxidized in such a DTT redox buffer. These results, which are consistent with a large difference in E_m values between the two proteins, necessitated the use of different redox-buffering reagents for titrations of HelX (DTT) and Ccl2* (glutathione). For the same reason, it was also necessary to use glutathione redox buffers for the titration of the model peptide.

For both the HelX and apocytochrome c_2 model peptide, identical E_m values were obtained regardless of whether the titrations were carried out anaerobically or aerobically. In contrast, for Ccl2* the E_m values obtained from anaerobic titrations were approximately 80 mV more positive than those obtained from aerobic titrations at all the pH values that were tested (data not shown). For example, the E_m value obtained under anaerobic conditions at pH 7.0 for Ccl2* is −130 ± 10 mV, compared to the value of −210 mV shown in Figure 1 for an aerobic titration. The reason for this difference is not clear (see the Discussion below). However, the fact that identical E_m values were obtained for the model peptide from titrations with glutathione redox buffers, regardless of the presence or absence of dissolved oxygen, suggests that the difference does not arise from a systematic error in the experimental design. Furthermore, the difference cannot be attributed to oxidation of the reduced form of glutathione by oxygen in the aerobic titrations during the 30 min redox equilibration stage of the titrations, because oxygen-containing solutions of reduced glutathione were shown by titrations with the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) to be completely stable against oxidation, under conditions identical to those employed during the titrations, for periods of at least 60 min (see Experimental Procedures above).

Figure 2 summarizes the results of extending these E_m determinations to pH values other than 7.0. A linear E_m versus pH relationship was obtained for HelX, Ccl2*, and

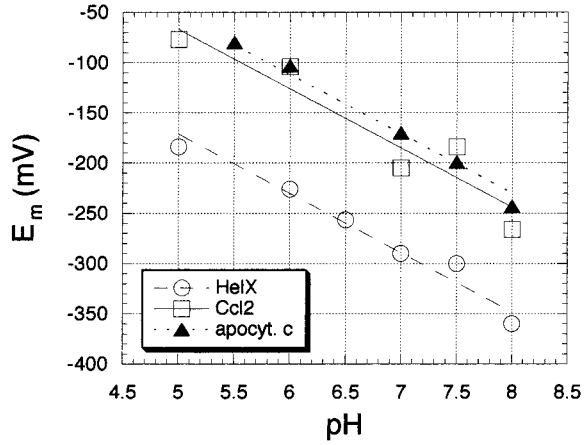

FIGURE 2: pH dependence of E_m for HelX, Ccl2*, and the apocytochrome c_2 peptide. Reaction conditions were as described in the legend of Figure 1 except that 100 mM sodium acetate buffer was used at pH 5.0, 100 mM MES buffer was used at pH 5.5−6.5, and 100 mM Tricine buffer was used at pH 7.5−8.0. The lines represent best fits to the data for HelX (○, dashed line), Ccl2* (□, solid line), and the apocytochrome c_2 model peptide (▲, dotted line), all with slopes of −59 mV/pH.

the apocytochrome c_2 model peptide. The best-fit slope for the E_m versus pH plot for the HelX line is −56 mV/pH unit, in good agreement with the −59 mV/pH unit slope predicted for a reaction in which two protons are taken up for each disulfide that is reduced (*27*). The slopes of E_m versus pH plots for Ccl2* and the model peptide, −60 and −65 mV/pH unit, respectively, are also in agreement (within the experimental uncertainties in the measurements) with this value of −59 mV/pH unit, indicating that two protons are taken up for each disulfide that is reduced for all three tested components. The simplest interpretation (*27*) of such an E_m versus pH relationship is that the pK_a values for the more acidic cysteine in HelX, Ccl2*, and the apocytochrome c_2 model peptide are >8.0 and thus both cysteines are in the thiol, rather than in the thiolate anion form, over the pH range examined in this study. If this is indeed the case, the reduction of each disulfide will result in the uptake of two protons, forming thiols at both of the sulfurs liberated when the disulfide is reductively cleaved.

DISCUSSION

The E_m values at pH 7.0 reported above for *R. capsulatus* HelX, Ccl2*, and the apocytochrome c_2 model peptide of −300, −210, and −170 mV, respectively, represent the first values reported for these components in any photosynthetic bacterium. The E_m value reported for Ccl2* represents the first such determination for any protein of this type. Furthermore, the data presented above provide the first opportunity to estimate the thermodynamic driving force for periplasmic disulfide/dithiol exchange reactions in the cytochrome *c* biogenesis pathway in any organism.

An E_m value of −217 mV at pH 7.0 had been previously measured, using redox poising with glutathione and changes in intrinsic tryptophan fluorescence to monitor redox state, for the disulfide/dithiol couple of the *B. japonicum cycY* gene product, the protein that corresponds to HelX in that bacterium (*28*). The 83 mV difference in the E_m values reported for *R. capsulatus* HelX and *B. japonicum* CycY is considerably larger than the ±20 mV uncertainty that is likely to exist for a comparison of these two E_m values. As the *B. japonicum* CycY and *R. capsulatus* HelX proteins are significantly similar to each other (*28*), the relatively large difference in E_m values is somewhat unexpected, given that the E_m values for chloroplast thioredoxins *m* from three different species are identical (*16*, *29*) and the E_m values for chloroplast thioredoxins *f* from two different species are also identical (*16*). It might also be mentioned that although the redox equilibrium between *R. capsulatus* HelX and DTT redox buffers appears to be established within 30 min, equilibration of the *B. japonicum* CycY protein with glutathione redox buffers appears to require much longer times (the legend of Figure 5 of ref *28* indicates that oxidized CycY was incubated with the redox buffers for 3 days). Perhaps these differences in redox equilibration times reflect significant differences in the accessibility of the active-site disulfides of the two proteins to small molecules.

When measurements were taken under anaerobic conditions, the E_m values for HelX and the apocytochrome *c* peptide were identical to those obtained under aerobic conditions. In contrast, the E_m values for Ccl2* were 80 mV more positive under anaerobic than under aerobic conditions over the pH range from 6.0 to 8.0. Previously, it had been shown that the in vivo levels of *R. capsulatus* Ccl2 under anaerobic conditions are at least 20-fold lower than the levels observed under aerobic conditions (*7*). Recently, this difference in protein abundance levels has been shown to be due to a post-translational event that produces a half-life for Ccl2 that is considerably shorter under anaerobic conditions than in the presence of oxygen (K. Karberg, J. Loughman, and R. G. Kranz, unpublished observations). A possible explanation for this observation is that Ccl2 assumes different conformations under aerobic and anaerobic conditions, with the anaerobic form being more susceptible to proteolysis than the aerobic form. The difference in E_m values observed for Ccl2* under aerobic and anaerobic conditions in this study is consistent with the hypothesis that the protein adopts different conformations in the presence and absence of oxygen and provides an avenue toward further investigating possible conformational differences.

E_m versus pH profiles are useful for providing information about the pK_a values of the active-site cysteines in thioredoxin-like proteins (*27*) and also allow one to predict, by extrapolation, E_m values out of the pH range used for the redox titrations. Such predictions can be made with considerable confidence in the acidic direction (barring some pH-linked conformational change that produces a significant alteration in the microenvironment at the active site), where thermodynamic considerations dictate that the −59 mV/pH unit slope for the E_m versus pH plot will not change (*27*). Extrapolations to pH values more alkaline than those covered by the actual redox titrations are less reliable, because one cannot predict, a priori, the pK_a values of the active-site cysteines (*27*). The data depicted in Figure 2 show that HelX, Ccl2*, and a model peptide for apocytochrome c_2 exhibit identical E_m versus pH dependencies out to pH 8.0. It is thus unlikely that the in vivo thermodynamic driving force for the flow of reducing equivalents from HelX to Ccl2 to an apocytochrome in the *R. capsulatus* periplasmic space will differ substantially from that measured in vitro at pH 7.0, regardless of the pH value of the growth medium or at the periplasmic surface of the cytoplasmic membrane, where the

proteins are located. However, the possibility that the driving force for reduction of either Ccl2 by HelX or an apocytochrome *c* by Ccl2 might change at pH values significantly more alkaline than 8.0 cannot be excluded on the basis of the data presented above.

The observation that the E_m for HelX is 85 mV more negative than that for Ccl2* is consistent with a recently proposed scheme in which reduced HelX serves as the reductant for the oxidized form of Ccl2 during the terminal stage of cytochrome *c* biogenesis in *R. capsulatus* (*12*). The 40 mV difference between the E_m values of Ccl2* and the apocytochrome c_2 model peptide, while significantly smaller than the difference between the E_m values of HelX and Ccl2*, is nevertheless sufficient to make reduction of the peptide by Ccl2* thermodynamically favorable. It is possible that deleting the transmembrane domain of the Ccl2 protein, to produce the Ccl2* form of the protein used in these measurements, may have some effect on its redox properties. It would not have been possible to make the measurements described above on intact membranes containing Ccl2, with its single transmembrane domain, so the effect of deleting this transmembrane domain on the E_m value of the protein could not be checked directly. However, the fact that the site of truncation is quite distant (52 amino acid residues) from the location of the cysteines in question suggests that the E_m value obtained for Ccl2* is likely to provide a reasonable measure of the E_m value of Ccl2 in situ. An additional question concerns how faithfully the redox properties of the model peptide used reflect those of the full-length mature form of apocytochrome c_2. In this regard, the fact that apocytochromes *c* appear to be unfolded during heme attachment (*1*−*3*) suggests that possible effects of protein microenvironment on the E_m values of disulfides are not likely to make the E_m value of an apocytochrome *c* differ substantially from that of the model peptide in this case.

The 85 mV difference in the HelX and Ccl2* E_m values, combined with the "steepness" of the Nernst equation for a two-electron process, is sufficiently large so that if the two proteins are in redox equilibrium, Ccl2 could be maintained in a predominantly reduced state even if HelX were largely oxidized. Thus, for example, at an ambient potential of −270 mV where Ccl2 would be ca. 99% reduced (at pH 7.0), HelX would be only 9% reduced. However, observations on the redox state of the two proteins in situ indicate that HelX is present in a considerably more reduced state than Ccl2* (*12*). Such a situation could represent a steady-state kinetic phenomenon and not represent a condition where thermodynamic equilibrium had been established. The in situ situation is what would be expected if the rate of oxidation of HelX in vivo by Ccl2 is slow compared to that for the reduction of HelX, possibly by CcdA (*9*) or by DipZ (*1*, *30*), and the oxidation of Ccl2 by the oxidized apocytochrome is significantly more rapid than Ccl2 reduction by HelX.

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