Identification of Protein-Protein and Protein-Ribosome Interacting Regions of the C-terminal Tail of Human Mitochondrial Inner Membrane Protein Oxa1L

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The mammalian mitochondrial inner membrane protein Oxa1L is involved in the insertion of a number of mitochondrial translation products into the inner membrane. During this process, the C-terminal tail of Oxa1L (Oxa1L-CTT) binds mitochondrial ribosomes and is believed to coordinate the synthesis and membrane insertion of the nascent chains into the membrane. The C-terminal tail of Oxa1L does not contain any Cys residues. Four variants of this protein with a specifically placed Cys residue at position 4, 39, 67, or 94 of Oxa1L-CTT have been prepared. These Cys residues have been derivatized with a fluorescent probe, tetramethylrhodamine-5-maleimide, for biochemical studies. Oxa1L-CTT forms oligomers cooperatively with a binding constant in the submicromolar range. Fluorescence anisotropy and fluorescence lifetime measurements indicate that contacts near a long helix close to position 39 of Oxa1L-CTT occur during oligomer formation. Fluorescence correlation spectroscopy measurements demonstrate that all of the Oxa1L-CTT derivatives bind to mammalian mitochondrial ribosomes. Steady-state fluorescence quenching and fluorescence lifetime data indicate that there are extensive contacts between Oxa1L-CTT and the ribosome-encapsulating regions around positions 39, 67, and 94. The results of this study suggest that Oxa1L-CTT undergoes conformational changes and induced oligomer formation when it binds to the ribosome.

Mammalian mitochondria synthesize 13 hydrophobic proteins that are components of the respiratory chain complexes in the inner membrane. Protein synthesis in this organelle is performed by specialized ribosomes, which are quite distinct from the ribosomes of other translational systems. In particular, they are quite rich in protein and have truncated rRNAs (1). Because of the presence of regions of high hydrophobicity, mitochondrially synthesized proteins are thought to be integrated into the membrane during or immediately following their synthesis on mitochondrial ribosomes.

Mitochondrial ribosomes are associated with the inner membrane (2, 3), and several proteins have been implicated in the binding of these ribosomes to the membrane. The best characterized of these are members of the Oxa1 family of proteins. Oxa1 is located in the inner membrane, where it acts as a component of the machinery that mediates the insertion of certain hydrophobic proteins into the membrane. It belongs to the YidC and Alb3 family of proteins found throughout prokaryotes and eukaryotes (4, 5). The human form is designated Oxa1L. Oxa1L consists of an N-terminal section located in the mitochondrial intermembrane space, five transmembrane helices, and a C-terminal tail of ~100 amino acids exposed in the mitochondrial matrix (6).

The C-terminal tail of the yeast homolog of Oxa1L (Oxa1p) has been shown to bind mitochondrial ribosomes (6). Deletion of this region of Oxa1p severely diminishes the efficiency of membrane insertion of subunit II of cytochrome oxidase, indicating that the C-terminal tail of yeast Oxa1p plays an active role in the insertion of mitochondrial translation products into the lipid bilayer (6). Cryo-EM studies of yeast Oxa1p bound to bacterial ribosomes indicate that it interacts near the exit tunnel located on the back of the large ribosomal subunit (7).

Previous studies of the C-terminal tail of human Oxa1L (Oxa1L-CTT) suggested that, in solution, the C-terminal tail exists as an equilibrium of the monomer, dimer, and tetramer forms (8). Circular dichroism studies indicated that it contains ~20% α-helical content. Despite predictions based on yeast Oxa1p, human Oxa1L-CTT does not form a coiled-coil structure. Two copies of Oxa1L-CTT bind to ribosomes with a binding constant in the range of 0.3–0.8 μM. Cross-linking studies suggested that Oxa1L-CTT contacts three proteins that have bacterial homologs (MRPL13, MRPL20, and MRPL28) and three proteins that are unique to mitochondrial ribosomes (MRPL48, MRPL49, and MRPL51). These proteins are believed to be located on the solvent side of the large ribosomal subunit (8). Despite this recent work, our understanding of the structure of human Oxa1L-CTT and its interaction with the mammalian mitochondrial ribosomes is quite limited. In this study, we have investigated the regions of Oxa1L-CTT that interact during formation of the oligomer and examined which regions of Oxa1L-CTT interact with the ribosome using static and dynamic fluorescence spectroscopy.

**EXPERIMENTAL PROCEDURES**

**Materials**—High-purity grade chemicals were purchased from Sigma or Fisher. Tetramethylrhodamine-5-maleimide

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**SUPPLEMENTAL MATERIAL**

The on-line version of this article (available at http://www.jbc.org) contains supplemental “Methods,” Figs. S1–S3, and Table S1.

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2 The abbreviations used are: Oxa1L-CTT, Oxa1L C-terminal tail; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; FCS, fluorescence correlation spectroscopy; TCSPC, time-correlated single-photon counting; 2CFI, two-component fit improvement.
was obtained from Invitrogen. Sephadex G-15 was purchased from Sigma. Bovine mitochondria and mitochondrial ribosomes (55 S) and ribosomal subunits (28 S and 39 S) were prepared as described previously (9).

Preparation of Mutant Derivatives of Oxa1L-CTT—The cDNA clone of mature human Oxa1L was obtained from American Type Culture Collection (ATCC 10961183, IMAGE 40017377). The region encompassing the C-terminal tail was cloned, expressed, and purified as described previously (8). Four mutant derivatives were prepared in which specific residues were converted to Cys (A4C, A39C, T67C, and S94C) (shown in Fig. 1, upper). These derivatives were prepared using the QuikChange site-directed mutagenesis protocol (Stratagene) and forward primers CCGATTCCATGTGTAGCAGCATGTACTTTAACATC (for position 4), GGCTGGAAACATTGTGAAATGACG (for position 39), GGCTGGAAAAATTGTGAAATGACG (for position 67), and AGCAAAAAAGTGTACTTAAAATC (for position 49). The reverse primers used were the inverse complements of the forward primers in each case. All mutations carrying Oxa1L-CTT were transformed into Escherichia coli DH5α, and the nucleotide sequences of the inserted DNAs were confirmed. The variants are designated Oxa44-CTT, Oxa39-CTT, Oxa67-CTT, and Oxa94-CTT, indicating the position of the Cys residue. The mutant plasmids were subsequently transformed into E. coli BL21(DE3) RIL cells (Stratagene) for expression. Cells were grown to an A660 of 0.6 in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and induced by the addition of 50 μM isopropryl 1-thio-β-D-galactopyranoside for 6 h at 37 °C. After induction, cells were lysed, and Oxa1L-CTT was purified using nickel-nitrioltriacetic acid resin, followed by HPLC purification on a TSKgel SP-5PW cation exchange column as described previously (8) except that buffers contained 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as the reducing agent unless indicated otherwise. Proteins were purified with and without TCEP, keeping all other conditions the same. The mutant derivatives were tested for structural integrity by comparing the secondary structures of the Oxa1L-CTT variants did not change upon mutation at any of these positions.

Detection of Dimers by S–S Bond Formation—Oxa1L-CTT variants prepared in the absence of TCEP (5 μg, 10 μl) were kept under slow passing O2 gas for 30 min and then held at 4 °C overnight in buffer containing 20 mM HEPES-KOH (pH 7.6) and 50 mM KCl in the presence and absence of 10 mM MgCl2. An equal volume of 2X SDS loading dye without β-mercaptoethanol was added to the reaction mixtures. Samples were run on a 15% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue, and the monomer and dimer bands were quantified using UN-SCAN-IT gel software.

Fluorescence Tagging of the Oxa1L-CTT Derivatives with Rhodamine—Each HPLC-purified Oxa1L-CTT variant (78 μM in 500 μl, 1 mg/ml) was labeled with rhodamine-5-maleimide basically as described by the manufacturer, producing Oxa4-Rh, Oxa39-Rh, Oxa67-Rh, and Oxa94-Rh. The concentrated fluorescent probe (33 mM dissolved in Me2SO) was added to the protein solution in buffer containing 20 mM HEPES-KOH (pH 7.6), 50 mM KCl, and 1 mM TCEP at a molar ratio of 5:1 (probe/protein). The mixture (500-μl final volume) was incubated for 1 h at room temperature with gentle shaking in the dark. The reactions were quenched by the addition of excess β-mercaptoethanol (100 mM). Tagged proteins were separated from free dye by chromatography on a Sephadex G-15 column (0.7 × 20 cm) equilibrated in buffer containing 20 mM HEPES-KOH (pH 7.6), 50 mM KCl, 10 mM MgCl2, 10% glycerol, and 1 mM TCEP. The protein concentration was calculated using the following formula: protein concentration = (A280 − Amax,CF)/e, where Amax is the absorbance of rhodamine-5-maleimide at the wavelength maximum (550 nm), CF is the correction factor (CF = A280(free dye)/A280(free dye)), and e is the molar extinction coefficient of Oxa1L-CTT based on the amino acid composition (12,700 M−1 cm−1). The CF value is equal to 0.34 (Thermo Scientific). The degree of labeling was calculated using the following formula: % label = Amax of the labeled protein/e’ protein concentration, where e’ is the molar extinction coefficient of the fluorescent dye (65,000 M−1 cm−1). The tagging efficiency was 70–100%.

Measurement of Rhodamine Steady-state Fluorescence Intensity and Anisotropy to Detect the Oligomer-forming Segments of Oxa1L-CTT and Binding to Ribosomes—The rhodamine fluorescence anisotropy of Oxa4-Rh, Oxa39-Rh, Oxa67-Rh, and Oxa94-Rh was examined on an SLM 48000 spectrofluorometer (SLM Amino, Urbana, IL) using an excitation wavelength of 545 nm and an emission wavelength of 573 nm. The rhodamine-tagged protein (100 nm) was in 20 mM HEPES-KOH (pH 7.6), 50 mM KCl, 5 mM MgCl2, and 1 mM TCEP. Data were collected in L-format using 0.4-cm path length quartz cuvettes with 5-nm excitation and 5-nm emission slits at 25 °C in the absence and presence of the indicated concentrations of wild-type Oxa1L-CTT. Control measurements using the corresponding buffer containing wild-type Oxa1L-CTT were recorded and subtracted from the sample readings. The fluorescence anisotropy was calculated using the following equation: χ = (Ivh − 2GⅠvh)/(Ivh + 2GⅠvh), where Ivh is the polarized emission intensity with vertical excitation and vertical emission polarizers, Ivh is the polarized emission intensity with vertical excitation and horizontal emission polarizers, and G is the instrumental correction factor (G-factor = Ihh/Ivh, where Ihh is the polarized emission intensity with horizontal excitation and horizontal emission polarizers). For Oxa-Rh binding to ribosomes, rhodamine fluorescence intensity was recorded with the addition of ribosomes, keeping the same conditions as described above. Background intensity (rhodamine alone in buffer) was monitored and subtracted from the sample reading.

Measurement of Protein Dynamics Using Fluorescence Correlation Spectroscopy (FCS) and Time-correlated Single-photon Counting (TCSPC)—FCS and TCSPC measurements were performed using a home-built, two-photon optical microscope (10, 11). This instrument uses ultrashort pulses generated by a Coherent Chameleon Ultra II laser (~140-fs pulse duration, 80-MHz repetition rate), which is tuned to 825 nm for optimal excitation of rhodamine. The laser output is attenuated by a low-bandwidth electro-optic modulator and is then directed through a second higher bandwidth electro-optic modulator that permits individual pulse selection (both modulator sys-
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FIGURE 1. Upper, the primary sequence of the Oxa1L-CTT, with different colors indicating the positions where Cys was used to replace the given amino acids. The expressed protein has a methionine at the start of the sequence and LE-His6 at the C terminus from the vector (not shown in the sequence). Lower, computer-generated three-dimensional structure of Oxa1L-CTT developed using Rosetta (26) and displayed using PyMOL. The Cys residues incorporated at the indicated positions are shown space-filled and in different colors.

RESULTS

Oxa1L-CTT is a matrix-exposed domain that binds to mitochondrial ribosomes and plays a role in the insertion of mitochondrially synthesized proteins into the inner membrane. There is no direct structural information on this protein. However, a three-dimensional model has been developed using Rosetta (8). In this model (Fig. 1), Oxa1L-CTT contains a long α-helical segment along with several shorter helical regions (Fig. 1). Four mutants were prepared to place Cys residues at specific positions of Oxa1L-CTT. The first mutation converted Ala\(^{39}\) to Cys (the numbers refer to amino acid residues in the C-terminal tail, which encompasses residues 334–436 of the full-length protein) and was expected to be positioned close to the membrane. The second mutation converted Ala\(^{39}\) to Cys and was located at the base of the long helical segment. The third mutation (Thr\(^{67}\) to Cys) was in a loop region predicted to lie close to the long helix. Finally, the fourth mutation (Ser\(^{94}\) to Cys) was found close to the C terminus (Fig. 1). CD results indicated that the secondary structures of all Oxa1L-CTT derivatives were the same as that of wild-type Oxa1L-CTT under several buffer conditions, including the presence and absence of Mg\(^{2+}\), indicating that the secondary structures of the variants are the same as that of the wild-type protein.

Oxa1L-CTT forms oligomers, predominantly dimers, in solution (8). To assess the regions of Oxa1L-CTT that might be in close contact in the dimer, S–S bond formation in the unlabeled proteins was forced by passing \(\text{O}_2\) through a solution of each derivative, followed by SDS-PAGE analysis to separate monomers and dimers. The amount of dimer formed with each derivative was quantified by measuring the band intensity. In the presence of Mg\(^{2+}\), almost 100% of Oxa39 was converted to dimer, whereas Oxa4, Oxa67, and Oxa95 formed 25, 60, and 65% dimers, respectively (Fig. 2). The easy formation of a disulfide bond at position 39 of Oxa1L-CTT suggests that this region of the protein is in contact in the dimer. Other regions have a lower tendency to form dimers, suggesting less direct contact between them in the dimer. In the absence of Mg\(^{2+}\), significantly different results were observed, with Oxa94 forming the most dimers and a decrease in dimer formation at position 39. There was a limited change at positions 4 and 67 in the absence of Mg\(^{2+}\). The different patterns obtained in the presence and absence of Mg\(^{2+}\) indicate that this cation induces local conformational changes in Oxa1L-CTT. The effect of Mg\(^{2+}\) could arise from the binding of this cation to acidic residues in Oxa1L-CTT, such as the RERE sequence in the long helical
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FIGURE 2. Efficiency of disulfide bond formation of Oxa1L-CTT mutants. Samples were treated as described under “Experimental Procedures” and analyzed by SDS-PAGE. Bands were quantified using UN-SCAN-IT gel software and are plotted as the percent S–S with respect to different mutants.

Mitochondrial protein synthesis requires Mg\(^{2+}\), suggesting that results obtained with Mg\(^{2+}\) are the more physiologically relevant. The concentration of monovalent salt (50–300 mM KCl) and the pH (5.5–8.5) had no effect on S–S bond formation (data not shown).

Identification of the Interacting Regions in the Oxa1L-CTT Oligomer during Formation Using Fluorescence Anisotropy Measurements—The disulfide cross-linking results suggested that the residues near position 39 are in contact in the dimer. However, random S–S bonds can form even from monomeric proteins due to random collisions. To obtain further insights into interactions that could occur in the dimer, the Cys residues in each mutant protein were derivatized with rhodamine, leading to Oxa4-Rh, Oxa39-Rh, Oxa67-Rh, and Oxa94-Rh, respectively. Each of these proteins could be fluorescently tagged with rhodamine at position 39 is hindered (restricted rotation) in the presence of wild-type Oxa1L-CTT due to oligomerization. The data for Oxa4-Rh were fit to a single-exponential model to obtain the lifetime of rhodamine at position 39. Another possibility is the interaction of Mg\(^{2+}\) with the Glu residues at positions 26 and 30, perhaps stabilizing the short helical segment in which these residues are present.

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Microenvironment of Oxa1L-CTT Variants in Solution—We further investigated the local environment of rhodamine in each labeled position of Oxa1L-CTT by measuring the time-dependent fluorescence of each derivative under a variety of conditions. Fig. 4a compares the fluorescence decay of free rhodamine with that of Oxa1L-CTT labeled with rhodamine at positions 4 and 94 (each at 100 nM). The fluorescence lifetime clearly changed when rhodamine was attached to the protein, and it depended on the attachment position. Previous studies have found that the fluorescence lifetime of rhodamine B, which is structurally similar to the tetramethylrhodamine derivative used to label Oxa1L-CTT, varies greatly according to the solvent in which it is dissolved. Its lifetime is roughly correlated with the solvent polarity: 1.7 ns in water, 2.5 ns in methanol, 2.9 ns in ethanol, and 3.3 ns in isopropyl alcohol. On the basis of this information, we assume that the fluorescence lifetime of rhodamine is a good indicator of its local environment. When the samples in Fig. 4a were fit to a single-exponential

FIGURE 3. Detection of regions of Oxa1L-CTT in close proximity using steady-state fluorescence anisotropy measurements. Proteins tagged with rhodamine were examined using fluorescence anisotropy in the absence and presence of wild-type Oxa1L-CTT. The data are plotted as the change in anisotropy (\(A_0\) - wild-type Oxa1L-CTT) as a function of wild-type Oxa1L-CTT concentration. The rhodamine-labeled protein concentration was 100 nM, and the experimental details are described under “Experimental Procedures.”
decay, the fluorescence lifetimes were 1.61, 2.38, and 3.18 ns for free rhodamine, Ox44-Rh, and Ox94-Rh, respectively (Table 1). It is not surprising that the local environment of rhodamine attached to the protein is less polar than in solution, but the lifetime data further indicate that rhodamine attached in position 94 is in a significantly less polar environment than rhodamine in position 4. The fluorescence lifetimes of the other Oxa1L-CTT derivatives at 100 nm indicate that the environment of rhodamine in position 39 is similar to that in position 4, whereas rhodamine in position 67 experiences an intermediate polarity. The steady-state anisotropy values were consistent with these results (0.1 for Ox44-Rh and Ox39-Rh, 0.147 for Ox67-Rh, and 0.156 for Ox94-Rh).

**Interaction of Oxa1L-CTT with Ribosomes and Identification of Interacting Regions**—It has been shown previously that Oxa1L-CTT binds to ribosomes with a $K_d$ of 0.3–0.8 μM and that two Oxa1L-CTTs bind to one ribosome (8). We used two-photon excited FCS to test the ability of the Cys mutants of Oxa1L-CTT to bind ribosomes. FCS measures time-dependent fluctuations in the amount of fluorescence arising from a spatially limited observation volume within a sample (10, 11). Such fluctuations are most typically due to fluorophore diffusion through the observation volume; in this case, the characteristic time scale over which the fluorescence autocorrelation function decays (referred to as the correlation time) is inversely related to the rate of molecular diffusion. Thus, FCS can detect the association of molecules in solution by observing changes in their diffusion and is particularly useful for studying the association of large species with smaller fluorescent molecules.

Normalized fluorescence correlation functions of rhodamine, rhodamine-labeled Oxa1L-CTT derivatives alone, and rhodamine-labeled Oxa1L-CTT derivatives in the presence of ribosomes are plotted in Fig. 5. The correlation time of each of the rhodamine-labeled Oxa1L-CTT derivatives was longer than that of free rhodamine due to the increased molecular size. The correlation functions of rhodamine and all of the free protein derivatives could be fit by a model for a single diffusing species (see supplemental “Methods”).

The curves for each protein in the presence of ribosomes contain a pronounced shoulder at longer lag times. These data can be fit using a two-component model in which the correlation time of the more quickly diffusing species matches that of the free Oxa1L-CTT derivative; we attribute the slowly diffusing species to the bound protein-ribosome complexes. All of the rhodamine-labeled Oxa1L-CTT derivatives bound ribosomes similarly. We performed control experiments to demonstrate that the background fluorescence in the ribosomal samples was insignificant and that free rhodamine did not interact with the ribosome or wild-type Oxa1L-CTT.

The FCS results clearly indicate that the fluorescently tagged Oxa1L-CTT derivatives are all active in binding mitochondrial ribosomes. However, these data cannot provide information on which part of the protein contacts the ribosome or on how the two Oxa1L-CTT oligomers are organized upon ribosome binding. To obtain a more detailed picture of these interactions, we measured the fluorescence intensity and fluorescence lifetime of the rhodamine-labeled Oxa1L-CTT derivatives in the absence and presence of ribosomes.

The self-quenching behavior of rhodamine was used to examine regions of the two Oxa1L-CTT monomers that are in close proximity, which could not be detected by FCS. Time-dependent fluorescence optimized fitting parameters are listed in Table 1. Each time-dependent fluorescence decay curve was fit to both a one-component (see Eq. (1)) and a two-component (see Eq. (2)) model. The models were compared by calculating the 2CFI as the fractional decrease in the residual sum of squares for the biexponential fit expressed as a percentage, as defined under “Results.” The two-component fit was used for samples that exhibited 2CFI values larger than 35%, but otherwise a one-component fit was considered adequate. Optimized models were compared by calculating the 2CFI as the fractional decrease in the residual sum of squares for the biexponential fit expressed as a percentage, as defined under “Methods.”

![FIGURE 4.](image)

**FIGURE 4.** Time-dependent fluorescence of rhodamine-labeled Oxa1L-CTT mutants as an indication of the local environment in various regions of the protein. Samples were excited using two-photon absorption, and emission was detected at the magic angle to remove anisotropic contributions. a, normalized (norm) time-dependent fluorescence decays of free rhodamine B, Ox44-Rh, and Ox94-Rh. The other derivatives were omitted for clarity. b, comparison of Ox94-Rh in the absence and presence of ribosomes. The protein concentration was 100 nm, and the ribosome concentration was 200 nm.

**TABLE 1**

| 2CFI value | One-component fit ($\alpha$) | Two-Component fit ($\alpha_1$, $\tau_1$, $\alpha_2$, $\tau_2$) |
|------------|-----------------------------|-------------------------------------------------------------|
|            | %                           | $\tau$ (ns)                   | $\tau$ (ns)                   | $\alpha_1$, $\tau_1$ (ns) | $\alpha_2$, $\tau_2$ (ns) |
| Rhodamine (100 nm) | 3 | 1.61 ± 0.06 | 0.25 ± 0.09 | 0.51 ± 0.10 | 2.46 ± 0.20 |
| Rhodamine (500 nm) | 29 | 1.61 ± 0.04 | 0.44 ± 0.03 | 0.82 ± 0.16 | 2.89 ± 0.20 |
| Ox44-Rh (100 nm) | 1 | 2.38 ± 0.16 | 0.20 | 0.20 | 0.20 |
| Ox44-Rh (500 nm) | 18 | 2.37 ± 0.03 | 0.20 | 0.20 | 0.20 |
| Ox44-Rh + ribosome | 28 | 2.50 ± 0.22 | 0.40 ± 0.05 | 1.00 ± 0.25 | 3.06 ± 0.20 |
| Ox39-Rh (100 nm) | 34 | 2.35 ± 0.14 | 0.20 | 0.20 | 0.20 |
| Ox39-Rh (500 nm) | 83 | 0.05 ± 0.13 | 0.20 | 0.20 | 0.20 |
| Ox39-Rh + ribosome | 17 | 2.65 ± 0.10 | 0.50 ± 0.07 | 0.76 ± 0.25 | 3.42 ± 0.22 |
| Ox67-Rh (100 nm) | 125 | 2.64 ± 0.36 | 0.20 | 0.20 | 0.20 |
| Ox67-Rh (500 nm) | 31 | 2.97 ± 0.38 | 0.20 | 0.20 | 0.20 |
| Ox94-Rh + ribosome | 158 | 0.04 | 0.04 | 0.04 | 0.04 |
close proximity when bound to the ribosome. As indicated in Fig. 6, very little decrease in fluorescence intensity (self-quenching) was observed with Oxa4-Rh, indicating that this region of the protein is not in close proximity when Oxa1L-CTT is bound to the ribosome. However, changes in fluorescence intensity were observed with Oxa1L-CTT derivatives at position 39, 67, or 94. This observation indicates that the rhodamine probes at these positions have come closer together. Of these three positions, only position 39 appeared to be close in Oxa1L-CTT when the dimer (oligomer) was formed in solution. This close proximity is reflected in the interaction of the two copies of Oxa1L-CTT bound to the ribosome as observed previously (8). Furthermore, these data indicate that the regions of the protein near positions 67 and 94, which were not close together in the dimer in solution, underwent conformational changes upon the binding of Oxa1L-CTT to the ribosome, bringing them into close proximity. The data were fit using a simple binding model; the apparent \( K_d \) is 25 nM. The \( K_d \) in this study is lower than the value obtained with isothermal titration calorimetry and surface plasmon resonance used in a previous study (8). The difference in the \( K_d \) could be due to differences in the percentage of active ribosomes in various preparations or to inactivation of a portion of the ribosomes in some of the previous measurements.

With the exception of Oxa4-Rh, in the presence of ribosomes, the time-dependent fluorescence of the rhodamine-labeled Oxa1L-CTT derivatives did not adhere to a single-exponential decay (Table 1). This behavior is exemplified by comparing Oxa4-Rh (500 nM) fit by a single-exponential model. Upper right, Oxa4-Rh (100 nM) + ribosome (200 nM) fit by a single-exponential model and a biexponential model. Both fits track the data reasonably well, but the single model was deemed adequate according to the criteria outlined under “Results.”

![FIGURE 5. Binding of rhodamine-labeled Oxa1L-CTT mutants to ribosomes using two-photon excited FCS. Shown are normalized autocorrelation curves of rhodamine (blue), Oxa1L-CTT derivatives alone (green), and Oxa1L-CTT derivatives in the presence of ribosomes (red). FCS curves for rhodamine and the protein-only samples were fit to a one-component model; samples containing protein and ribosomes were fit to a two-component model. The protein concentration was 100 nM, and the ribosome concentration was 200 nM. Supplemental “Methods” contains more information about the models, best fit curves, and optimized fitting parameters.](image1)

![FIGURE 6. Interaction of Oxa1L-CTT derivatives with ribosomes using rhodamine fluorescence intensity quenching. The fluorescence (Fluo.) intensity of rhodamine-labeled Oxa1L-CTT mutants (100 nM) was measured in buffer and in the presence of different concentrations of ribosomes, and the normalized intensity is plotted as a function of the ribosome concentration.](image2)

![FIGURE 7. Data-fitting strategy for time-dependent fluorescence decays of rhodamine-labeled Oxa1L-CTT mutants. Each dataset was separately fit to a one-component (single-exponential) model and to a two-component (biexponential) model. The residual sum of squares for the two models was compared to determine the most appropriate model for each sample, as described under “Results” and in Table 1. Upper left, Oxa4-Rh (500 nM) fit by a single-exponential model. Upper right, Oxa4-Rh (100 nM) + ribosome (200 nM) fit by a single-exponential model and a biexponential model. Both fits track the data reasonably well, but the single model was deemed adequate according to the criteria outlined under “Results.” Lower left, Oxa94-Rh (500 nM) fit by a single-exponential model. Lower right, Oxa94-Rh (100 nM) + ribosome (200 nM) fit to a single-exponential and a biexponential model. The biexponential model is required to adequately fit the data.](image3)
We defined the two-component fit improvement (2CFI) as the fractional decrease in the residual sum of squares (RSS) for the biexponential fit expressed as a percentage: 

\[ \text{2CFI} = \frac{100 \times (\text{RSS}_{1\text{-component}} - \text{RSS}_{2\text{-component}})}{\text{RSS}_{2\text{-component}}} \]

Because of experimental noise, the biexponential model improved the fit for nearly all experimentally measured decay curves (2CFI > 0), so we further required a threshold value to use in assigning the most appropriate model for each decay curve. Because free rhodamine is expected to exhibit single-exponential fluorescence decay kinetics at submicromolar concentrations, we set the threshold at the 2CFI value of the 500 nM rhodamine sample. A two-component fit was used for samples that exhibited improvements significantly larger than free rhodamine, but otherwise a one-component fit was considered adequate.

Table 1 lists the 2CFI values and the optimized fitting parameters of the most appropriate model for each sample. At a sufficiently low concentration (100 nM), all four rhodamine-labeled Oxa1L-CTT derivatives were adequately fit by a single-exponential model. The 2CFI values for the 500 nM samples indicate that the fluorescence decay of rhodamine at positions 4, 67, and 94 is single-exponential, whereas rhodamine at position 39 requires a biexponential fit. The longer time constant for the Oxa39-Rh variant at 500 nM matches the corresponding time constant to 100 nM within experimental error. We attribute the shorter biexponential time constant to quenching upon oligomer formation at the higher concentrations. These data are consistent with the disulfide cross-linking and fluorescence anisotropy results indicating that position 39 is in closest contact when Oxa1L forms dimers in solution, as discussed above.

The FCS results demonstrate that all four Oxa1L-CTT variants bind ribosomes, but under the same conditions, TCSPC shows that the fluorescence of rhodamine at position 4 was unaffected, whereas rhodamine at positions 39, 67, and 94 was significantly quenched. These data also confirm the steady-state quenching presented in Fig. 6. They are consistent with the model in which Oxa1L-CTT binds to ribosomes as a dimer (or oligomer) because rhodamine at position 39 was quenched at higher concentrations in the protein-only samples. Rhodamine at positions 67 and 94 exhibited more quenching than expected based on the protein-only data, which could be due to a conformational change upon binding. Therefore, we conclude from the lifetime and steady-state fluorescence data that Oxa1L-CTT binds to ribosomes as an oligomer and may undergo a conformational change.

**DISCUSSION**

Oxa1L plays an important role in the insertion of proteins into the inner membrane of mitochondria through an interaction of the matrix-localized C-terminal tail with the large ribosomal subunit. Fig. 8 shows the schematic model for the organization of Oxa1L in the membrane and when it binds to ribosomes. Our results indicate that human Oxa1L-CTT can dimerize and that the formation of the dimer depends on contacts around position 39 of the tail. Additional contacts are likely to occur in the transmembrane helices (20). The interaction near position 39 remains strong as the two Oxa1L-CTT monomers bind to the ribosome. However, there are substantial conformational changes in other regions of the C-terminal tail when the protein binds to ribosomes, affecting especially regions close to residues 67 and 94 (Fig. 8). These interactions indicate a significant rearrangement of residues in the tail during the formation of the functional Oxa1L-ribosome interaction. The data also indicate that there is no detectable interaction between the ribosome and regions of Oxa1L-CTT near position 4. This residue is near the portion of the C-terminal tail that emerges from the membrane, suggesting that contacts between the ribosome and Oxa1L are mediated by residues that may be spatially separated from direct contact with the membrane.

This work represents the first application of FCS and TCSPC to the study of the binding of a ligand to mitochondrial ribosomes. FCS and TCSPC have previously been used widely for monitoring biological events (21–25). For example, FCS was used to study ligand binding to *E. coli* ribosomes (23) and to monitor both *in vivo* and *in vitro* translation (24). In this work, the use of static and dynamic fluorescence spectroscopy has allowed us to monitor the segmental interactions within protein molecules and to map the interacting regions between Oxa1L-CTT and mammalian mitochondrial ribosomes. We have specifically introduced the use of tetramethylrhodamine labeling and TCSPC as a means to assess the local polarity in various regions of a protein by correlating the fluorescence lifetime enhancement with solvent-dependent lifetime data. This approach could be applied to investigate the surface environment of other proteins. Finally, the combination of FCS and fluorescence quenching was essential to confirm the association of labeled proteins with mitochondrial ribosomes in solution and to provide spatial information about their interaction. FCS is ideally suited to monitor the binding of individual proteins to relatively massive ribosomes, and therefore, it could become a valuable tool to study the interaction of other ligands with ribosomes.

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