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Electron Transfer Mechanism of the Rieske Protein from *Thermus thermophilus* from Solution NMR investigations

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Abstract

We report NMR data indicating that the Rieske protein from the cytochrome *bc* complex of *Thermus thermophilus* (*TtRp*) undergoes modest redox-state-dependent and ligand-dependent conformational changes. To test models concerning the mechanism by which *TtRp* transfers between different sites on the complex, we monitored ¹H, ¹⁵N, and ¹³C NMR signals as a function of redox state and molar ratio of added ligand. Our studies of full-length *TtRp* were carried out in the presence of dodecyl phosphocholine micelles to solvate the membrane anchor of the protein and the hydrophobic tail of the ligand (hydrubiquinone). NMR data indicated that hydrubiquinone binds to *TtRp* and stabilizes an altered protein conformation. We utilized a truncated form of the Rieske protein lacking the membrane anchor (trunc-*TtRp*) to investigate redox-state-dependent conformational changes. Local chemical shift perturbations suggested possible conformational changes at prolyl residues. Detailed investigations showed that all observable prolyl residues of oxidized trunc-*TtRp* have *trans* peptide bond configurations but that two of these peptide bonds (Cys151–Pro152 and Gly169–Pro170 located near the iron-sulfur cluster) become *cis* in the reduced protein. Changes in the chemical shifts of backbone signals provided evidence for redox-state- and ligand-dependent conformational changes localized near the iron-sulfur cluster. These structural changes may alter interactions between the Rieske protein and the cytochrome *b* and *c* sites and provide part of driving force for movement of the Rieske protein between these two sites.

Thermus thermophilus Rieske protein (*TtRp*) is a catalytic subunit of the bacterial cytochrome *bc* complex. The biological function of the cytochrome *bc*₁ complex (EC 1.10.2.2) is to transfer electrons from hydroquinone to cytochrome *c*₁, to pump protons

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Supporting Information. Information regarding strains for protein production, additional NMR assignments, and other ligands tested. Eight figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS *TtRp*, full-length Rieske protein from *Thermus thermophilus*; trunc-*TtRp*, version of *TtRp* truncated to remove the N-terminal transmembrane region (residues 1–45) and the nine C-terminal residues (202–210); in addition, it contains the point mutation W142F. This is the version of the protein used in earlier X-ray (3), NMR (30, 32) studies and other study (31). Residue names and numbers in normal font refer to the Rieske protein from *Thermus thermophilus*; residue names and numbers in italic font refer to the Rieske protein from *Bos taurus*.

across the membrane, and to store the energy as a chemiosmotic potential (1-5). The electron transfer mechanism is known as the bifurcated Q-cycle (6-10). The Rieske protein serves as an electron and proton carrier between hydroquinone and cytochrome c_1 . A homologous Rieske protein has similar functions in the cytochrome b_6f complex. The cytochrome bc_1 complex consists of three key subunits with cofactors: cytochrome b with di-heme, cytochrome c_1 with a heme, and the Rieske iron-sulfur protein with a [2Fe-2S] cluster. In *T. thermophilus*, all subunits contain at least one membrane domain anchored on the inner membrane. On the basis of X-ray structures of related proteins (1, 2), the bc_1 complex contains two primary binding sites for the Rieske protein: one, called the b -site, is close to cytochrome b , and the other, called the c -site, is close to cytochrome c_1 . Hydroquinones are oxidized in the b -site. After the proton and electron transfer at the b -site, the Rieske protein moves to the c -site, where it donates an electron to cytochrome c_1 (1, 2). Increased viscosity, which presumably slows movement of the Rieske protein between the two sites, was found to impede the rate of electron transfer and to be part of the rate-limiting process owing to the large distance between the cytochrome b and c_1 sites (11).

Several models have been advanced to explain the mechanism by which the Rieske protein (ISP) moves. Their features are summarized in Table S1 (Supporting Information). These models can be classified as being driven by a conformational change (Model 1) or being driven by diffusion (Model 2) as discussed below.

(Model 1) The multiple conformations of the Rieske protein observed in different X-ray structures suggested that large redox-state-coupled and/or ligand-coupled conformational changes promote transfer between the two sites (1, 12-14). The two putative regions undergoing conformational changes are the iron-sulfur cluster binding domain (15, 16) and the neck domain (also called the linker or tether domain) (17). Figure 1 illustrates the proposed movements/conformational changes. When the ISP is located at hydroquinone binding site (b -site) its neck domain does not form a helix (Figure 1A). When the head domain (soluble domain) of the Rieske protein moves to the intermediate site (int -site), the neck domain becomes helical (15) (Figure 1B). The neck domain remains helical when the ISP is in the c -site (15) (Figure 1C). Two types of redox-state-dependent conformational changes have been proposed: a conformational change in the neck domain and a conformational change in the head domain (not emphasized in Figure 1) of the Rieske protein.

(Model 2) An alternative mechanism involving passive diffusion between the two sites has been proposed (8, 18, 19). This diffusion model decouples the relationship between conformation and function and predicts that ligand binding and redox state changes are not the primary driving forces behind the movement of the Rieske protein.

We have used NMR spectroscopy to monitor redox-state-dependent and ligand-dependent conformational changes in *T*Rp. The conformational change models (1, 2, 13, 15, 16) predict a large conformational change originating from redox state change and/or ligand binding. By monitoring NMR chemical shifts and NOE values as a function of the redox and ligation states, it should be possible to identify the location and characteristics of the conformational changes. Because our preliminary studies showed large chemical shift perturbations around Gly169 and Pro170, we proposed that the conformational exchange might originate from the *cis-trans* isomerization of peptidyl-proline peptide bond. To test the hypothesis, we prepared a sample of the truncated form of the Rieske protein (trunc-*T*Rp) lacking the membrane anchor and incorporating ^{13}C and ^{15}N labeled proline and ^{13}C labeled glycine: [U - ^{13}C , ^{15}N -Pro]-[^{13}C -Gly]-trunc-*T*Rp. After collecting carbon chemical shifts and proton NOESY spectra under different conditions, we used criteria from the literature (20, 21) to determine the likelihood of the Gly169-Pro170 peptide bond being *cis* or *trans*.

(see Methods and Supporting Information for details). We detected small conformational changes, but found no evidence for the large conformational changes predicted by Model 1 (1, 12-14). Thus, our results appear to be more consistent with the diffusion model (Model 2) (8, 18, 19).

MATERIALS AND METHODS

Chemicals, Bacterial Strains, and Vectors

[99% ^{15}N] H_4Cl , [99% ^{13}C]-D-glucose, 99% D_2O , and sodium 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS) were purchased from Cambridge Isotope Laboratories (Andover, MA). Unlabeled amino acids, BME vitamins, redox reagents and corresponding antibiotics were purchased from Sigma Aldrich (St. Louis, MO). Isopropyl β -D-thiogalactoside (IPTG) was purchased from LabScientific (Livingston, NJ). Dodecyl phosphocholine (DPC) was purchased from Avanti Polar Lipids (Alabaster, Alabama). *Escherichia coli* strain BL21(DE3)pLysS, and λ -(DE3) lysogenization kit were purchased from Novagen (Madison, WI). Expression vector, pET17b carrying cDNA encoding a truncated soluble domain of *TtR*p from *T. thermophilus* strain HB8 (pET17b / trunc-*TtR*p), was provided by Dr. James A. Fee (The Scripps Research Institute, CA). Expression vector, pET22b carrying cDNA encoding a full length *TtR*p from *T. thermophilus* strain HB27 (pET22b / *TtR*p) was provided by Dr. Bernd Ludwig (Biozentrum der Universität Frankfurt, Germany). *Escherichia coli* strain CAG18447 (with proline deficiency) was purchased from the Coli Genetic Stock Center (Yale University, New Haven, CT). Differences between the two strains are summarized in the Supporting Information.

Protein Expression and Labeling

[^{15}N]-trunc-*TtR*p and [^{13}C , ^{15}N]-trunc-*TtR*p were produced from BL21(DE3)pLysS cells containing pET17b / trunc-*TtR*p grown in M9 medium containing 1 g/L $^{15}\text{NH}_4\text{Cl}$ as described previously (3) but with modifications: 50 mg/L (w/v) FeCl_3 and 1 % (v/v) BME vitamins were added to the growth media. For ^{13}C labeling [^{13}C]-glucose was added in two aliquots with 2 g/L added at the beginning and 2 g/L added when OD_{600} reached 2.5 (immediately after IPTG induction).

[^{13}C , ^{15}N -Pro]-[^{13}C -Gly]-trunc-*TtR*p, [^{13}C -Gly]-trunc-*TtR*p, and [^{15}N -Gly]-trunc-*TtR*p were produced from CAG18447(DE3) cells containing pET17b grown in a rich, synthetic medium as described previously (22), with the modification that 50 % of the amino acids, including the selectively labeled amino acids, were added initially, and the remaining 50 % of the amino acids were added with IPTG. The strain CAG18447(DE3) was derived from CAG18447 by using the standard protocol with a lysogenization kit (Novagen).

[^{15}N]-*TtR*p, and [^{13}C , ^{15}N]-*TtR*p were produced by BL21(DE3)pLysS cells containing pET22b / *TtR*p grown in a similar way to [^{15}N]-trunc-*TtR*p, except that the cells were grown initially in 2 L LB medium to a high cell density (total OD of ~6 if pooled into 1 L) before the transfer to the M9 medium. The yield from this medium was ~1 mg/L culture. 0.2%~2% DPC and 1 mM DTT were required throughout the purification process.

NMR Sample Preparation

Amicon centrifuge concentrators (Millipore, Billerica, MA) were used for protein concentration and for buffer exchanges following protein purification. The standard NMR buffers were 50 mM Tris, and 100 mM NaCl, in 9 % D_2O at pH 7.5 with 0.2% DPC for *TtR*p; 50 mM Tris and 100 mM NaCl at pH 7.4 or triple buffers of 20 mM Tris, 20 mM phosphate, 20 mM borate at pH 8.5 for trunc-*TtR*p. Tris was omitted from the buffers of samples used to collect NOE data. 2% DPC was required for the studies of ligand binding to

*T*Rp. Sodium dithionite or sodium dithiothreitol (only for *T*Rp) were used as reducing agents; ferricyanide was used as the oxidizing agent. NMR samples contained DSS as the internal chemical shift standard. Shigemi (Shigemi, Inc., Allison Park, PA) susceptibility matched NMR tubes were used for NMR samples containing oxidized Rieske protein. NMR samples containing reduced Rieske protein were prepared in an anaerobic chamber and transferred to NMR tubes sealed by a J. Young valve (Wilmad, Vineland, NJ). The pH meter was calibrated before taking readings.

NMR Spectroscopy

The temperature of samples of trunc-*T*Rp used for HSQC experiments was controlled at 298 K. The temperature of samples containing *T*Rp was controlled at 298 K or 313 K. The redox state of the Rieske protein was controlled by adding oxidizing or reducing agents, and the redox state was determined on the basis of the pattern of one-dimensional (1D) ^1H NMR hyperfine shifts.

Conventional triple-resonance data sets, including ^{15}N -HSQC, ^{13}C -HSQC, HNCACB, CBCACONH, CCONH, HAHBCONH, HCCONH, and NOESY, were collected for trunc-*T*Rp and used in determining spectral assignments at given temperatures. ^{15}N -HSQC, HNCA, CACONH data sets were collected for *T*Rp.

NMR Data Processing and Analysis

Proton chemical shifts were referenced relative to internal DSS (taken as 0 ppm); carbon and nitrogen spectra were referenced indirectly by the canonical ratios (23). All spectra were processed by XWIN-NMR or Topspin software (Bruker) with exponential line broadening. COSY spectra were apodized by applying a Gaussian window function. SPARKY software (24) was used for peak picking.

Criteria to distinguish the *cis* and *trans* configuration

Xaa_{*i*-1}-Pro_{*i*} sequences in the *cis* configuration typically have prolyl $^{13}\text{C}^\gamma$ chemical shifts at 24.4 ± 0.7 ppm and $^{13}\text{C}^\beta$ chemical shifts at 33.8 ± 1.2 ppm with a $\delta(^{13}\text{C}^\beta) - \delta(^{13}\text{C}^\gamma)$ chemical shift difference of 9.64 ± 1.27 ppm. In addition for *cis* peptide bonds, a NOESY cross peak is expected between $^1\text{H}^{\alpha_{i-1}}$ and $^1\text{H}^{\alpha_i}$. Xaa_{*i*-1}-Pro_{*i*} sequences in the *trans* configuration typically have prolyl $^{13}\text{C}^\gamma$ chemical shifts at 27.4 ± 0.9 ppm and $^{13}\text{C}^\beta$ chemical shifts at 31.8 ± 1.0 ppm with a $\delta(^{13}\text{C}^\beta) - \delta(^{13}\text{C}^\gamma)$ chemical shift difference of 4.51 ± 1.17 ppm. In addition, for *trans* peptide bonds, a NOESY cross peak is expected between $^1\text{H}^{\alpha_{i-1}}$ and $^1\text{H}^{\delta_i}$ (20, 21). We also made use of the Promega software package that provides a probabilistic analysis of the configuration of a peptidyl-prolyl peptide bond on the basis of the local sequence and experimental NMR chemical shifts (20).

Results

NMR Resonance Assignments

Of the 156 residues of trunc-*T*Rp, we were able to assign NMR signals to 134 residues in the reduced state and 130 residues in the oxidized state (Figure 2A) by standard double- and triple-resonance methods. Apart from the 18 residues belonging to the two loops (131—140 and 150—157) that surround the Fe-S cluster, which we did not expect to be able to assign because of the paramagnetism of the cluster, the assignments account for more than 94% of the residues of trunc-*T*Rp. We then extended these assignments to the full-length Rieske protein (*T*Rp) by comparison to triple-resonance data. We used residue-selective and reverse-labeling methods (see Supporting Information Figure S1) to extend and verify the assignments to a level of 55% (Figure 2B). Paramagnetic optimized NMR pulse sequences

(25, 26) were required to observe signals from residues near the Fe-S cluster in the reduced protein.

Redox-State-Dependent NMR Spectral Changes

We collected ^1H - ^{15}N HSQC NMR spectra of oxidized and reduced trunc-*TtRp*. (Figure 3A), and compared the chemical shift differences of the ^1H and ^{15}N signals as a function of residue number (Figure 3B). The largest chemical shift changes mapped onto residues 76–78 (strand), 149–150 (strand), and 169–173 (loop). We carried out a similar analysis of full-length Rieske protein (*TtRp*) in the presence of 0.2% DPC micelles (Figure 4). In this case, the largest redox-state-dependent chemical shifts mapped to residues 130, 140, 168, 170, and 178. Residues Gly169, Pro170, Pro171, Pro172, and R173 exhibited large redox-state-dependent chemical shift differences in both *TtRp* and trunc-*TtRp*. We refer to these residues as the proline loop. Based on the X-ray structure (see Figure 2C), these residues have an average distance from the Fe-S of ~6 Å.

Configuration of the Gly169–Pro170 Peptide Bond in Reduced Rieske Protein. We collected data from multiple spectra of reduced trunc-*TtRp* and focused on signals from residues around Gly169 and Pro170 (Figure 5A). A set of weak NOESY cross peaks (Figure 5B–D) confirmed the assignments. We collected NOESY data at different mixing times (τ_m), temperature and pH: τ_m = 23 ms, 298 K, pH 7.5 (Figure 5C); τ_m = 45 ms, 298 K, pH 7.5 (Figure 5D); τ_m = 45 ms, 298 K, pH 8.0 (Figure 5E); and τ_m = 45 ms, pH 8.0, 300 K (data not shown). Cross peaks were observed under all these conditions indicative of a *cis* configuration for the G169–P170 peptide bond.

Analysis of Prolyl Residue Chemical Shifts

In addition to NOEs, prolyl $^{13}\text{C}^\beta$ and $^{13}\text{C}^\gamma$ chemical shifts are helpful for determining the configuration of the peptidyl-prolyl peptide bond, and the chemical shift difference $\delta(^{13}\text{C}^\beta) - \delta(^{13}\text{C}^\gamma)$ provides an even better diagnostic (21). Trunc-*TtRp* contains 19 prolyl residues. With oxidized *TtRp* (Figure 6A), we determined both the $^{13}\text{C}^\beta$ and $^{13}\text{C}^\gamma$ chemical shifts for 12 prolyl residues (P47, P51, P54, P67, P69, P77, P86, P89, P110, P115, P152, and P176) and only $^{13}\text{C}^\beta$ for one (P171). With reduced *TtRp* (Figure 6B), we determined both $^{13}\text{C}^\beta$ and $^{13}\text{C}^\gamma$ chemical shifts for 14 prolyl residues (P47, P51, P54, P67, P69, P77, P86, P89, P110, P115, P152, P172, P176, and P196) and only $^{13}\text{C}^\beta$ for two (P170, P171). In the oxidized state, the prolyl $^{13}\text{C}^\beta$ and $^{13}\text{C}^\gamma$ chemical shifts of trunc-*TtRp* (Figure 6A) are all in the range expected for the *trans* configuration as are the $\delta(^{13}\text{C}^\beta) - \delta(^{13}\text{C}^\gamma)$ values (Figure 6C). In the reduced state, the $^{13}\text{C}^\beta$ chemical shifts of P152 and P170 (Figure 6B) are indicative of the *cis* configuration, and the $\delta(^{13}\text{C}^\beta) - \delta(^{13}\text{C}^\gamma)$ value for P152 confirms the *cis* configuration. Although we were unable to determine the chemical shift difference for P170 in reduced trunc-*TtRp*, because the signal from its $^{13}\text{C}^\gamma$ was not observed, Promega (20) analysis of the observed chemical shifts yielded a high (normalized) probability (94%) for a *cis* G169-P170 peptide bond. Moreover, the NOE results (Figure 5) provided unequivocal evidence for a *cis* peptide bond. Further information on the redox-state-dependence of prolyl chemical shifts is in Figure S2 of the Supporting Information.

Hyperfine Signals from Residues in the Proline Loop

Several atoms from residues in the proline loop are close enough to the iron-sulfur cluster to experience severe broadening. We used NMR pulse sequences optimized for hyperfine signals to determine their chemical shifts in fully reduced and fully oxidized Rieske protein. To determine the fraction reduced, we measured the intensity of the hyperfine ^1H NMR signals arising from the histidine residues that ligate the cluster, which are observed only when the Rieske protein is reduced (Figure 7A). We then correlated the fraction reduced with spectral changes in signals assigned to the carbonyl carbons of G169, P170, P171, and

G195 (Figure 7B). G195, which is not part of the proline loop, exhibited no chemical shift change with redox state. The chemical shift of G169 $^{13}\text{C}'$ changed by only ~ 0.1 ppm between the oxidized and reduced states, with the signal changing continuously as indicative of fast exchange on the chemical shift time scale. By contrast, the $^{13}\text{C}'$ signals from P170 and P171 signals changed by ~ 1.0 ppm between oxidation states and exhibited slow exchange.

Spectral Changes upon Binding Ubiquinone

Members of the Rieske protein family bind different quinones: eukaryotic Rieske proteins bind ubiquinone- n , and bacterial Rieske proteins bind menaquinone- n (Figure S3). Quinones consist of various lengths of n isoprene units as membrane anchors. Our attempts at observing interactions between trunc-*T*Rp and quinones were unsuccessful; however, we found evidence from NMR that the full-length protein *T*Rp in the presence of 2% DPC bound hydroubiquinone-2 (UQ-2). The longer chain hydromenaquinone-4 was not soluble under the same conditions (see Supporting Information for details).

We collected NMR spectra of mixtures of 0.1 mM reduced *T*Rp and 1.5 mM UQ-2 at different molar ratios. NMR signals from more than five residues showed perturbations as a function of the *T*Rp:UQ-2 molar ratio (1:0, 1:0.33, 1:1, and 1:2) (Figure S4A). At molar ratio 1:0, *T*Rp was ligand free. At ratios 1:1 and 1:2, the major population likely was the *T*Rp UQ-2 complex. And at molar ratio 1:0.33, separate signals were observed from free *T*Rp and *T*Rp UQ-2 complex. Interestingly, the UQ-2 linewidth did not seem to change upon binding.

In order to determine whether UQ-2 was binding near the iron-sulfur cluster, we monitored hyperfine ^1H and ^{13}C signals from the two histidine ligands of reduced *T*Rp in the presence of 2% DPC as a function of added UQ-2 (Figure 8). The hyperfine ^1H signal at 24.2 ppm decreased in intensity with increasing UQ-2 concentration, whereas the hyperfine signal at 22.5 ppm remained the same (Figure 8A). The hyperfine ^{13}C signal at -24.5 ppm assigned to a histidine side chain carbon lost intensity with increasing UQ-2 and a new signal grew in at ~ -27 ppm (Figure 8B). ^1H - ^{15}N HSQC spectra acquired as a function of UQ-2 concentration (data not shown) exhibited changes in signals from residues around the iron-sulfur cluster (V131, Y158, G169, R173). Signals from UQ-2 also were found to change upon complex formation. The 2', 3', and 5' methyl ^{13}C signals exhibited changes attributed to hyperfine interaction with the iron-sulfur cluster (Figure S5). The model depicted in Figure 9 summarizes information from NMR on the ubiquinone-Rieske protein interaction.

Discussion

Redox-State-Dependent Peptide Bond Isomerizations

Data presented here provide information on the configuration of 13 of the 19 peptidyl-prolyl peptide bonds as a function of the oxidation state. All 13 were found to be *trans* in oxidized trunc-*T*Rp, and two of these (P152 and P170) became *cis* in reduced trunc-*T*Rp. P170 is in the proline loop that lies close to the iron-sulfur cluster and Pro152 also is close enough to the cluster to experience hyperfine effects. The position of the Pro152 $^{13}\text{C}'$ resonance exhibited a temperature dependent shift between 298 K and 313 K indicative of a Fermi-contact-contribution (data not shown). Thus the likely mechanism for both isomerizations is differential interaction of the *cis* and *trans* configurations with the oxidized and reduced cluster. Because of the close agreement between the chemical shifts of these regions in spectra of trunc-*T*Rp and *T*Rp (Figure 3B, 4B), these redox-state-dependent changes in the configuration of the P152 and P170 peptide bonds also appear to occur in full-length Rieske protein bound to micelles. Because the proline loop is highly conserved (Figure S6), it may

play an important role in the differential interaction of the Rieske protein with its two protein partners.

Iwata *et al.* have published low-resolution (3.0 Å) X-ray structures of oxidized bovine cytochrome *bc*₁ complex with and without bound inhibitors (15); their structures indicated redox-state-dependent structural changes in the Rieske protein. Their structural models showed *P175* (corresponding to *P170* in *TtRp*) in the *trans*-configuration in *c*-site and in the *cis*-configuration in the *int*-site (See Figure S6 for sequence alignment between bovine Rieske protein and *TtRp*, the blue arrow points to *P170* (*P175*)). This result is in agreement with the NMR evidence presented here for the redox-state-dependent configuration of *P170*.

Comparison of Solution and Crystal Structures—We measured residual dipolar couplings (RDCs) as a means for comparing the solution structure with the X-ray structure of oxidized trunc-*TtRp* (3). Our RDCs from oxidized trunc-*TtRp* are consistent with the X-ray structure, whereas those of reduced trunc-*TtRp* are not (Figure S7). Solmaz *et al.* (27) did not observe *cis* peptidyl prolyl peptide bonds in the residues homologous to Rieske *P152* and *P170* in the 1.9 Å X-ray structure of the reduced cytochrome *bc*₁ complex in the presence of the inhibitor stigmatellin. The reason for this discrepancy may be that stigmatellin, which is classified as a *P_f* inhibitor (28), “fixes” the conformation of the *b* site, causing it to lose its mobility. Other reasons could be the low sequence similarity to trunc-*TtRp*, or contributions from the other protein subunits. In an earlier study (29), Konkle *et al.* found that the X-ray structure of oxidized trunc-*TtRp* crystallized at low pH (6.0) does not differ much from that crystallized at pH 8.5. We collected ¹H-¹⁵N HSQC spectra of reduced trunc-*TtRp* at seven pH values ranging from 5.2 to 8.4 and found only minor chemical shift changes (data not shown) implying that Rieske protein does not exhibit pH-dependent conformational changes over that range.

Evidence for a Local, Rather than Global Redox-State-Dependent Conformational Change—Our ¹H-¹⁵N-HSQC results (Figures 2 and 3) suggest that the conformational change is local and minor. This is in agreement with an earlier analysis from ¹³C^α and C^β chemical shifts (30), which indicated no change in secondary structure with redox state. The neck domain of the Rieske proteins has been reported to undergo a redox-state-dependent conformational change in studies of the cytochrome *bc*₁ complex (17). However, our studies of isolated *TtRp* gave no indication of such a conformational change. The presence of other subunits may be required for this effect. In summary, we found no evidence supporting large redox-state-dependent conformational changes in the Rieske protein; however, we found evidence that the configurations of the peptidyl-prolyl peptide bonds of *P152* and *P170* change from *trans* to *cis* upon reducing the protein.

Ubiquinone Binding—Our studies show that reduced *TtRp* in the presence of DPC micelles binds UQ-2. At least six *TtRp* residues and three sites on UQ-2 showed sizable chemical shift perturbations resulting from the interaction. On the basis of observed hyperfine shifts of UQ-2 signals, and the spatial distribution of the *TtRp* residues involved, the binding is likely specific. We propose a model for the interaction with a possible hydrogen bond that would provide a pathway for the hyperfine interaction (Figure 9). Because we failed to see an interaction between benzoquinone and trunc-*TtRp*, we conclude that the membrane anchors in both the protein and quinone contribute to the binding affinity. UQ-2 binding led to changes in the ¹H^N and ¹⁵N chemical shifts of residues *V131*, *Y158*, *G169*, and *R173*; these changes may indicate that quinone binding induces a local conformational change. These residues are strictly conserved (*Y158*, *G169*), highly conserved (*V131*), or similar (*R173*) in sequence alignments (Figure S6). *Y158* may be strictly conserved because it hydrogen bonds to the iron-sulfur cluster. The two histidines (*H134* and *H154*) are conserved as cluster ligands. Glycines are known for their flexibility

and allowance for a greater range of backbone torsion angles. Prolines are known for their propensity to form loops. The loop containing P152 and P170 appears to form a conserved region for redox-state-dependent and quinone-dependent conformational changes. The redox-state-dependent and ligand-dependent chemical shift changes affecting residues V131, Y158, G169 and R173 are similar (Figure 3B, 4B, S4B). The native reduced state is in the UQ-2-free conformation, and the oxidized state conformation resembles the UQ-2 bound form. This appears to imply that a favored interaction between oxidized protein and reduced quinone (Figure 4B and Figure S4B).

Electron Transfer Mechanism

Our results show preferential binding between hydroquinone and the active site of reduced *TfRp* (Figure 9). Hydroquinone binding results in local changes in residues around the proline loop but no large conformational change. The local environment of the iron sulfur cluster is fairly rigid (Figure 2C), with the iron-sulfur cluster fixed by its cysteine and histidine ligands, its hydrogen bond contributors, and a disulfide bridge. The only flexibility seems to be in the proline loop, which undergoes a redox-state-dependent conformational change involving *cis/trans* isomerization of the G169-P170 peptide bond. This conformational change and the local flexibility around the proline loop may help the Rieske protein to adapt to different binding pockets in the *b*- and *c*-sites and to facilitate electron transfer to the quinone.

Our results do not support a large ligand-dependent or redox-state-dependent conformational change in the isolated Rieske protein. The large conformational change in the Rieske protein observed by X-ray crystallography (15, 16) appears to occur only when cytochrome *b* and/or *c* are present. Our results with *TfRp* do not support a large conformational change in the Rieske protein as the driving force. The contribution made by the proline loop to interactions at the *b*- and *c*-sites is currently unknown, but it appears likely that changes in this loop affect the interactions. Although, our results do not exclude the possibility of this conformational change as the driving force in the whole complex, they appear more consistent with diffusion driven movement (8, 18, 19) of the Rieske protein between the *b*- and *c*-sites.

Our findings are compatible with the following model. In the *b*-site, where the Rieske protein is oxidized and histidine 154 is deprotonated, P152 and P170 adopt the *trans* configuration. After the concerted proton and electron transfer from hydroquinone (30, 31), the Rieske protein is reduced, histidine 154 is protonated and P152 and 170 adopt the *cis* configuration. The conversion to the *cis* configuration might help the Rieske protein dissociate from the *b* site so as to reach the *int*- and/ or *c*-site. After reaching the *c*-site, the electron is transferred to cytochrome *c*, and *TfRp* reverts to the *trans* state and histidine 154 becomes deprotonated (before binding hydroquinone), releasing the proton to the environment to resume the next cycle. The redox-state-dependent and ligand-dependent conformational changes may facilitate binding events in both *b*- and *c*-site pockets to accelerate electron transfer and also to prevent short-circuiting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Summary of multiple conformations and sites of the Rieske protein (ISP) in the cytochrome *bc*₁ complex as reviewed in (16). (A) The ISP with flexible neck (red) and an oxidized cluster (orange diamond) binds at the *b*-site near cytochrome *b* and hydroquinone (QH₂ in blue), poised to donate an electron and proton to the ISP (12). (B) Following the transfer of an electron and proton to the ISP (reduced cluster shown in gray), the neck adopts a helical conformation (*int*-site) (15). (C) ISP moves to the *c*-site ready to donate an electron to cytochrome *c*₁ and a proton to the extracellular space (15).

Figure 2.

The Rieske protein from *Thermus thermophilus* studied here. (A) Primary sequence of the truncated, water-soluble fragment of the Rieske protein (trunc-*Tirp*) used here and in previous crystallographic (3) and NMR studies. The construct (30, 32) was truncated to remove the N-terminal transmembrane region (residues 1–45) and nine C-terminal residues (202–210); in addition, it contained the point mutation W142F. The proline loop is highlighted in yellow as is the second GP sequence. Residues highlighted in green indicate other residues whose NMR assignments were determined in both the oxidized and reduced forms of the protein. (B) Primary sequence of the full-length version of the Rieske protein (*Tirp*); see Supporting Information for details. Highlighted in purple are residues with assigned NMR signals. The protein contained a C-terminal His₆ tag used for protein purification shown in light gray. (C) Wall-eyed stereoscopic view of the Fe-S cluster of trunc-*Tirp* and the surrounding amino acid residues from the X-ray structure (3). The proline loop is highlighted. Prolines are depicted in dark green, G169 is shown in blue, and A168 and R173 are in light green. Blue dashed lines indicate hydrogen bonds to the cluster. The G169-P170 peptide bond is modeled as *trans*.

Figure 3.

Redox-state-dependent chemical shifts of trunc-*TTRp*. (A) Overlaid ^1H - ^{15}N HSQC spectra of (red) oxidized and (blue) reduced [U - ^{13}C , ^{15}N]-trunc-*TTRp*. The sample conditions were ~ 8 mM protein at pH 8.5 and 298 K. (B) Differences in the chemical shifts of backbone amide groups of oxidized and reduced trunc-*TTRp* plotted as a function of residue number: (orange) $\Delta\delta^1\text{H} = \delta^1\text{H}_{\text{red}} - \delta^1\text{H}_{\text{oxid}}$; (blue) $\Delta\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{red}} - \delta^{15}\text{N}_{\text{oxid}} \cdot 0.2$. Very large values were truncated. Secondary structural elements derived from the X-ray structure (3) are indicated at the bottom of the figure.

Figure 4.

Redox-state-dependent chemical shifts of *Trp*. (A) Overlaid ^1H - ^{15}N HSQC spectra of (red) oxidized and (blue) reduced [U - ^{13}C , ^{15}N]- *Trp*. The sample conditions were 0.1 mM protein at pH 7.5 in 0.2% DPC micelles. NMR spectra were acquired at 313 K. (B) Differences in the chemical shifts of backbone amide groups of oxidized and reduced *Trp* plotted as a function of residue number: (orange) $\Delta\delta^1\text{H} = \delta^1\text{H}_{\text{red}} - \delta^1\text{H}_{\text{oxid}}$; (blue) $\Delta\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{red}} - \delta^{15}\text{N}_{\text{oxid}}$. Secondary structural elements derived from the X-ray structure (3) are indicated at the bottom of the figure.

Figure 5.

Evidence showing that the Gly169–Pro170 peptide bond is *cis* when the protein is reduced. (A) Diagram showing a *cis* Gly-Pro dipeptide. Indicated in gray are the experiments used identifying signals from key atoms. Indicated in green are the nuclear Overhauser effect (NOE) experiments used to identify short distances between ¹H atoms. (B–D) Planes from 3D proton-carbon-proton NOESY-HSQC spectra of freshly-reduced 3 mM [*U*-¹³C, ¹⁵N]Pro-[*U*-¹³C]-Gly-trunc-*T*Rp. acquired at pH 7.5 without Tris buffer for higher sensitivity, 298 K with NOESY mixing times of 23 ms and 45 ms and repeated at pH 8.0 with a NOESY mixing time of 45 ms. (B) A plane from an experiment collected with $\tau_m = 23$ ms; the sample was at pH 7.5 and 298 K. Starting from the Gly169 ¹H^N, we extended the assignments (by following the dashed lines between B and C2) to Gly169 ¹H^α. (C1) Plane containing the signal from Pro170 ¹³C^α (at 63.0 ppm). Two NOE cross peaks were observed: Pro170 ¹H^α to Gly169 ¹H^α2 and Pro170 ¹H^α to Gly169 ¹H^α3. The mixing time was 23 ms, and the sample was at pH 7.5 and 298 K. (C2) Plane containing the signal from Gly169 ¹³C^α (at 44.8 ppm). Again, two NOEs were observed: Gly169 ¹H^α2 to Pro170 ¹H^α and Gly169 ¹H^α3 to Pro170 ¹H^α. The mixing time was 23 ms, and the sample was at pH 7.5 and 298 K. (D1) Same sample and spectral plane as shown in C1 except that the mixing time was 45 ms. (D2) Same sample and spectral plane as shown in C2 except that the mixing time was 45 ms. (E1) Same spectral plane as in C1 and D2; the mixing time was 45 ms, and the sample pH was 8.0. (E2) Same spectral plane as in C1 and D2; the mixing time was 45 ms, and the sample pH was 8.0.

Figure 6.

Redox state dependent proline chemical shift changes of trunc-*Trp*. (A) Proline $\text{C}\beta$ and $\text{C}\gamma$ chemical shifts of 3 mM [$U\text{-}^{13}\text{C}$, ^{15}N -Pro]-[^{13}C -Gly]-trunc-*Trp* at pH 7.4 and 298 K in the oxidized state. (Brown bars) $^{13}\text{C}\gamma$; (orange bars) $^{13}\text{C}\beta$. Signals from three prolyl residues (P80, P174, P179) were not assigned in both redox states and were omitted (C) and (D) for clarity; these three are far from the iron-sulfur center. Three prolyl residues that are close to the iron-sulfur center exhibited paramagnetic broadening (P152, P170, and P171). Gray arrows denote atoms whose signals were missing or unassigned. (B) Carbon chemical shifts in the reduced state. (C) Chemical shift differences ($\delta^{13}\text{C}\beta - \delta^{13}\text{C}\gamma$) (blue) in the reduced state and (orange) in the oxidized state. The dashed lines marked *trans* and *cis* indicate average chemical differences for these respective peptide bond configurations.(21) (D) Difference between the chemical shift differences [$(\delta^{13}\text{C}\beta - \delta^{13}\text{C}\gamma)_{\text{reduced}} - (\delta^{13}\text{C}\beta - \delta^{13}\text{C}\gamma)_{\text{oxidized}}$] in reduced and oxidized trunc-*Trp*.

Figure 7.

Redox-state-dependent conformational changes observed in NMR spectra of trunc-*TIRp*. (A) Proton-SuperWEFT and (B) ^{13}C - ^{15}N difference decoupling spectra of a 6.5 mM [U - ^{13}C , ^{15}N -Pro]-[^{13}C -Gly]-trunc-*TIRp* collected at pH 7.4, 298 K. The ^1H hyperfine shifted signals around 22–23 ppm in A, which are only observed in the reduced protein and serve as a monitor of the redox state, were assigned to His134/154 $\text{H}^\epsilon 1$. The ^{13}C signals assigned to P171 and P170 in A exhibited large, redox-state-dependent chemical shift changes; the ^{13}C signal assigned to G169 exhibited a smaller change; and the ^{13}C signal assigned to G195 exhibited no change.

Figure 8.

Effect of added UQ-2 on hyperfine signals from reduced *Trp* in the presence of 2% DPC micelles. The NMR sample contained 0.9 mM [*U*-¹³C, ¹⁵N]-*Trp* at pH 7.5; data were collected at 313 K. (A) His ¹H^ε1 signals from the two iron-ligating histidines. (B) Side chain carbon signals from the iron-ligating histidines.

Figure 9.

Model depicting the putative interaction between oxidized *TtRp* and reduced quinone (UQ-2). The protein structure shown is adapted from X-ray structure of trunc-*TtRp*.(3) The N-terminal residue of that structure (T46) is depicted in orange. The neck domain and the membrane anchor domain (not modeled in the X-ray structure) are in gray. Residues that exhibited sizable chemical shift changes upon the addition of UQ-2 are in red. Residues of the Rieske protein whose hyperfine signals exhibited changes upon the addition of UQ-2 are shown in magenta. NMR signals from the terminal methyl protons of UQ-2 (shown in red) exhibited changes in the presence of the protein. The model proposes specific binding with a possible hydrogen bond between the hydroxyl group of UQ-2 and the deprotonated ring nitrogen of H154.