Complex I plays a central role in cellular energy production, coupling electron transfer between NADH and quinone to proton translocation. The mechanism of this highly efficient enzyme is currently unknown. Mitochondrial complex I is a major source of reactive oxygen species, which may be one of the causes of aging. Dysfunction of complex I is implicated in many human neurodegenerative diseases. We have determined several x-ray structures of the oxidized and reduced hydrophilic domain of complex I from *Thermus thermophilus* at up to 3.1 Å resolution. The structures reveal the mode of interaction of complex I with NADH, explaining known kinetic data and providing implications for the mechanism of reactive oxygen species production at the flavin site of complex I. Bound metals were identified in the channel at the interface with the frataxin-like subunit Nqo15, indicating possible iron-binding sites. Conformational changes upon reduction of the complex involve adjustments in the nucleotide-binding pocket, as well as small but significant shifts of several α-helices at the interface with the membrane domain. These shifts are likely to be driven by the reduction of nearby iron-sulfur clusters N2 and N6α/b. Cluster N2 is the electron donor to quinone and is coordinated by unique motif involving two consecutive (tandem) cysteines. An unprecedented “on/off switch” (disconnection) of coordinating bonds between the tandem cysteines and this cluster was observed upon reduction. Comparison of the structures suggests a novel mechanism of coupling between electron transfer and proton translocation, combining conformational changes and protonation/deprotonation of tandem cysteines.

Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) is the first enzyme of the mitochondrial and bacterial respiratory chains. It catalyzes the transfer of two electrons from NADH to quinone, coupled to the translocation of approximately four protons across the membrane, contributing to the proton-motive force required for the synthesis of ATP (1, 2). The mitochondrial enzyme consists of 45 subunits (3) with a combined mass of ~980 kDa. The prokaryotic enzyme is simpler, consisting of ~14 subunits conserved from bacteria to humans, and has a total mass of ~550 kDa (2). The mitochondrial drial and bacterial enzymes contain equivalent redox components and have a similar L-shaped structure, with the hydrophobic arm embedded in the membrane and the hydrophilic peripheral arm protruding into the mitochondrial matrix or the bacterial cytoplasm (2, 4). Thus, the bacterial enzyme represents a “minimal” model of complex I. Because of the central role of complex I in respiration, mutations in individual subunits can lead to many human neurodegenerative diseases (5). Complex I, along with complex III (bc1), has been suggested to be a major source of reactive oxygen species (ROS) in mitochondria, which can damage mitochondrial DNA and may be one of the causes of aging (6). Parkinson disease, at least in its sporadic form (which represents ~95% of cases), may be caused by increased ROS production from malfunctioning complex I (7).

We have previously determined the crystal structure of the hydrophilic domain (eight different subunits of 280 kDa total mass) of complex I from *Thermus thermophilus*, establishing the electron transfer pathway from the primary electron acceptor flavin mononucleotide (FMN) through seven conserved iron-sulfur clusters to the quinone-binding site (Q-site) at the interface with the membrane domain (8). Two additional iron-sulfur clusters, which are not part of the main redox chain, may represent an evolutionary remnant (cluster N7) and a possible anti-oxidant (cluster N1a; cluster names are assigned to structural motifs as in Ref. 8). The membrane-spanning part of the enzyme lacks covalently bound prosthetic groups (9) but must contain essential components of the proton translocating machinery. Its atomic structure is currently unknown.

The mechanism of the highly efficient coupling between electron transfer and proton pumping, conserving nearly 100% of the available energy, remains a mystery. Two models are being discussed: direct (redox-driven through chemical intermediates, usually employing modifications of the Q cycle, with quinol as a mobile proton/electron carrier) (10) and indirect or conformation-driven coupling (2, 4, 11, 12). Sequence comparisons indicate that the three largest hydrophobic subunits of complex I, Nqo12, 13, and 14 (*Thermus* nomenclature), are homologous to each other and to the antiporter family (Mrp) (13, 14) and so are likely to participate in proton translocation. Two of these subunits, Nqo12 and Nqo13, are located ~100 Å away from the Q-site (15), which implies the need for conformational coupling as at least a part of the mechanism. We have
now determined several structures of the oxidized and reduced hydrophilic domain of complex I from *T. thermophilus*, which show how NADH interacts with the complex and provide novel insights into the coupling mechanism.

**EXPERIMENTAL PROCEDURES**

**Crystallization**—The hydrophilic domain of complex I from *T. thermophilus* was purified and crystallized as described previously (8, 16). To improve the diffraction properties of the crystals, a wide range of additives were tested. The most useful was the replacement of CaCl₂ for either MgCl₂ or MnCl₂, as well as the addition of low amounts of polyethylene glycol 400 in some cases. To obtain structures of the reduced domain, extensive crystallization set-ups were performed in the presence of NADH and/or sodium dithionite. To avoid oxidative damage and enzymatic turnover with oxygen, this was performed anaerobically using a Belle Technologies (Dorset, UK) glove box with oxygen levels below 20 ppm. Crystallization in the presence of NAD⁺ was performed aerobically, with the enzyme oxidized as purified.

The crystals were grown at 22 °C by the vapor diffusion method, by mixing 1 µl of protein solution (12–15 mg/ml, in 20 mM bis-tris propane pH 7.3, 150 mM NaCl, 0.002% phenylmethylsulfonyl fluoride, and 1% octyl-β-D-glucopyranoside) with 1 µl of reservoir solution (supplemental Table S1). The crystals were fully formed as large thin plates after ~5 days. They were cryo-protected by adding harvest solution containing reservoir solution with polyethylene glycol concentration increased by 2% and with the addition of 1% octyl-β-D-glucopyranoside and 25% ethylene glycol. The crystals were frozen by plunging them into liquid nitrogen. For reduced protein it was important to minimize the exposure of crystals to air during harvesting and freezing, with exposures of longer than 5 min leading to a gradual decline in the diffraction resolution and exposures of more than 20–30 min resulting in the gradual loss of electron density for the bound NADH. Crystals frozen with air exposures of less than 5 min did not suffer any loss in resolution and are referred to as being frozen anaerobically (“anaerobic” state). We have also shown by spectroscopic measurements that sodium dithionite at concentrations used for crystal harvesting (such as for RND and RD structures) remains nearly fully reduced for more than 5 min after exposure to air, thus preserving the reducing environment. Crystals frozen after exposure to air for ~30 min are referred to as being frozen aerobically (“aerobic” state).

**Data Processing and Structure Determination**—Data were collected at 100 K with ADSC Q315 CCD detectors at Beamlines ID29 and ID23-1 at the European Synchrotron Radiation Facility (Grenoble, France) or at I02 Beamline at the Diamond Light Source (Didcot, UK). The image data were processed with MOSFLM and SCALAM from the CCP4 suite (17). The crystals belonged to space group P2₁, with one exception (data set RD is in P1). For a range of data sets at intermediate resolutions (between 4.5 and 3.5 Å), electron density for NADH was usually present in molecular replacement solutions (performed with Phaser (18) using the known structure as a search model (Protein Data Bank code 2FUG (8)), whereas NAD⁺ was not seen despite being added to mother liquor at up to 30 mM concentration. This is consistent with low affinity of NAD⁺ to the oxidized enzyme (~1–2 mM (19)), which most likely results from overall shift in the nucleotide charge toward positive (compared with NADH), in the vicinity of the positively charged binding site surface (4). In contrast, NADH has high affinity for both the oxidized (~10 µM) and the reduced enzyme (~100 µM (4, 19).

The native data used for the previously published structure (protein oxidized as purified, 4 molecules/ASU (8)) had significant anisotropy and had subsequently been subjected to anisotropic scaling and ellipsoidal truncation using the diffraction anisotropy server (20), to the resolution limits of 3.15, 3.15, and 3.5 Å, where the F/σ ratio drops to ~2.6 along a*, b*, and c*, respectively (data set O4). Experimental electron density was then calculated with this data and previous iron peak/derivative data (8) using Sharp (21) and solvent flattening in DM from the CCP4 suite, as described previously (8). This lead to improved experimental and modeled electron density. Two of the new data sets, RND (supplemental Table S1), from the crystal of the reduced domain grown with NADH/dithionite, and data set O2, from the crystal of oxidized domain grown in the presence of NAD⁺ (but not containing any bound ordered nucleotide), were both in a novel packing arrangement containing 2 molecules/ASU, and both extended to 3.1 Å resolution. The O2 data set was collected from two different areas on the same crystal because of radiation damage, and so R factors for this structure are somewhat higher than for the others. This structure is especially useful for comparisons because the crystal packing is the same as in most structures of the reduced enzyme.

For data set RND, data from the same crystal were also collected at the iron peak wavelength. The initial positions of the iron-sulfur clusters were determined by molecular replacement with Phaser using the known structure as a search model (Protein Data Bank code 2FUG (8)). MAD phasing in Sharp, using iron peak and native data sets, was then performed essentially as previously described (8). Subsequent density modification using solvent flipping in CNS (22) produced significantly better maps than solvent flattening in DM, probably because only two molecules were available for NCS averaging, as compared with our original case of four molecules (when there was no significant difference between the two approaches). CNS maps were of very good quality (supplemental Fig. S1), showing the power of two-wavelength MAD phasing using iron-sulfur clusters. This allowed us to avoid model bias during the solution of the structure of the reduced domain. Improved resolution and the availability of experimental density in two different crystal forms allowed us to increase the completeness of the model and to improve its accuracy in some of surface-exposed, not very well ordered areas. In particular, residues 226–243 from subunit Nqo4 and residues 714–724 and 748–758 from Nqo3 were retraced. The register was adjusted in some residues neighboring these areas, as well as in residues 2–26 from Nqo1, residues 173–180 and 511–527 from Nqo3, residues 348–350 from Nqo4, and residues 2–13 and 107–140 from Nqo5. These changes do not involve any of the functionally important residues discussed previously (4, 8). Overall, these adjustments lead to improved geometry and lower R factors for the oxidized domain structure and for good quality models in new crystal forms (supplemental Table S1).
The improved resolution also allowed us to observe a partially disordered loop between two N-terminal β-strands in Nqo4, which led us to adjust register adjustment in these strands. Relatively poor electron density of this loop (residues 32–38) did not allow us to build it unambiguously, and so it is not included in the final models. This loop is facing the Q cavity in the vicinity of functionally important Tyr87. Although the loop is partly disordered, the remaining electron density appears to be stronger in the oxidized enzyme compared with the reduced state. This flexible loop contains three residues that are highly conserved and essential for activity (23), histidines 34 and 38, which may serve as intermediates in quinone protonation and could interact with Tyr87.

As noted previously, in all structures some of the residues with flexible side chains, exposed to the solvent at the surface of the molecule, have clear electron density for backbone atoms, but not for side chains. These have not been mutated to alanine but can be identified by high B factors (more than ~110 Å²). In the O2 and RND structures, unexplained electron density is present near the entrance to the putative iron-binding channel, in the vicinity of Glu94 and Asp98. It might represent several manganese or magnesium atoms (or possibly polyethylene glycol 400 in the O2 structure) and was not modeled because of the irregular shape of the density.

In the RND structure, Cys46 was not modeled as disconnected from cluster N2, despite the lack of electron density, because the nature of the replacement ligand is unknown. However, the length of the iron-sulfur bond was increased in refinement to place the cysteine backbone atoms closer to their electron density.

Model building was carried out in O (24), using the PHASER molecular replacement solutions as starting points. The higher resolution structures (O4, O2, and RND) were refined in CNS version 1.2 (22) using NCS restraints with 100 kcal/mol/Å² weight and restrained individual B factor refinement. Experimental phase information was used for refinement of the structures O4 and RND. The final round of refinement was carried out in Phenix (25), which resulted in slightly improved R factors and geometry. According to PROCHECK (26) and MOLPROBITY (27) analyses, these structures are of better than average quality of similar resolution structures. The models of the oxidized domain (O4 and O2) consist of the following residues: subunit Nqo1, 2–438; Nqo2, 3–180; Nqo3, 1–55, 73–143, and 150–777; Nqo4, 26–31 and 39–409; Nqo5, 1–196; Nqo6, 15–57 and 74–175; Nqo9, 26–179; and Nqo15, 3–129. Each model also contains seven [4Fe-4S] clusters, two [2Fe-2S] clusters, and one FMN molecule. The structure of the reduced domain (RND) contains an NADH molecule. It also contains residues Gly2 and Val25, whereas residue Arg27 is not included.

To verify whether the observed differences between structures of the oxidized and reduced domain are reproducible, we also determined structures from crystals grown with NADH only (without dithionite) at 3.7 Å resolution (data set RN) and from crystals grown with dithionite only also at 3.7 Å resolution (data set RD). Furthermore, two data sets at 4.2 Å resolution (RA1 and RA2 and supplemental Table S1) were collected from aerobically frozen crystals of NADH-reduced domain. Thus, we compared two structures of the oxidized enzyme, O4 and O2, with five structures of the reduced enzyme in somewhat different states. All low resolution structures (data sets RN, RD, RA1, and RA2) were refined by the same procedure in CNS, optimized by following Rfree. Molecular replacement solutions (obtained using the O2 structure of the oxidized domain) were subjected to a round of torsion angle dynamics-simulated annealing with starting temperature of 2500 K, followed by slow cooling and two macro cycles of 20 coordinate minimization steps and five group B factor refinement steps. NCS restraints with 100 kcal/mol/Å² weight were used throughout. To keep good model geometry it was important to use phi/psi restraints to the starting structure angles, as implemented in CNS. These restraints also lowered the Rfree and overall such a procedure resulted in a large drop of Rfree (between 3 and 8%) from the initial values. For structures with bound NADH, the refinement resulted in a movement of Nqo1 loop 202–207 and in shifts of neighboring α-helices similar to those seen in the higher resolution RND structure, validating such an approach. If the RND structure was used as a starting model instead of O2 (as a control), the final structures after refinement with any particular data set were very similar, again validating the procedure used. Overall, the refined structures fitted well to the calculated electron density. These low resolution structures were not rebuilt manually, because the main purpose was to check for gross shifts of α-helices, which were indeed observed. For comparisons, the structures were superposed using CCP4 program LSQKAB (17). The figures were prepared with PyMOL (DeLano Scientific).

RESULTS AND DISCUSSION

Structures—To determine the structure of the reduced domain, the crystals were grown anaerobically in the presence of NADH and/or dithionite. The structure with bound substrate NADH has been determined to 3.1 Å resolution in a novel packing arrangement of 2 mol/ASU in space group P21, (NADH and dithionite, data set/structure RND; supplemental Table S1). Additional structures were determined to 3.7 Å resolution: RN (NADH, 2 mol/ASU in P21) and RD (dithionite, 4 mol/ASU in P1) (supplemental Table S1). These crystals were frozen with minimal exposure to oxygen (<5 min) during cryo protection to ensure that a fully reduced (anaerobic) state is represented. Some crystals were also grown anaerobically in the absence of NADH but were harvested with longer exposures to oxygen (~30 min). The resulting structures (NADH aerobic, 4.2 Å resolution, 4 mol/ASU in P21, data sets RA1 and RA2; supplemental Table S1) were included in this analysis because they appear to represent a redox state similar to the RD structure. One of the crystals was grown aerobically in the presence of NAD+ and the resulting structure of the oxidized domain represents, usefully, a similar packing arrangement to the reduced domain structures (2 mol/ASU in P21, 3.1 Å resolution, data set O2; supplemental Table S1). It does not contain any ordered NAD+ because of its low affinity to the oxidized enzyme. The resolution of the original structure of the oxidized domain (4 mol/
ASU in P2₁; Protein Data Bank code 2FUG) was improved to 3.15 Å (from 3.3 Å) by anisotropic scaling (supplemental text) (data set O4; supplemental Table S1). The availability of experimental electron density in two different crystal forms and the increased resolution allowed us to improve the completeness (from 2333 to 2369 residues) and the accuracy of the original model in some of surface-exposed areas (see “Experimental Procedures”). Altogether seven structures have been used in the analysis, and any features discussed here have been reproduced at least twice.

Mode of NADH Binding—Electron density for a bound nucleotide, adjacent to the FMN, was identified in the RND structure (supplemental Table S1 and Fig. S1) and has been modeled as NADH (rather than NAD⁺) in accordance with crystal growth conditions (supplemental text). The FMN is also likely to be reduced in these conditions (supplemental text). Hydrophobic stacking interactions are formed between the nicotinamide ring of NADH and the exposed face of the isoalloxazine ring of the bound FMN, similarly to many other nucleotide-binding flavoenzymes (29, 30). The adenine ring of NADH is stabilized by stacking interactions with the aromatic rings of three conserved phenylalanines (Fig. 1A and supplemental Figs. S2 and S3). NADH binding induces movement of the loop formed by Nqo1 residues 202–207, which puts Phe205 ~1.7 Å closer to the adenine ring, promoting a stacking interaction (Fig. 1B). The glycine-rich loop formed by Nqo1 residues 65–71 (as well as the preceding N-terminal α-helical domain) moves ~0.7 Å away from NADH, allowing it to occupy the binding pocket. In contrast to most enzymes interacting with nucleotides (29), this exposed glycine-rich loop (which is not part of classic Rossmann fold (8)) is not involved in hydrogen bonding with the pyrophosphate moiety, despite being in close proximity to it. Instead, upon NADH binding, conserved Lys202 loses an H-bond to Glu184 and makes an H-bond to O²P of the pyrophosphate. The carbonyls of Gly66 and Gly67 from the glycine-rich loop interact with N⁷N of the carboxamide group and O³P of the nicotinamide ribose. Additionally, conserved Lys75 loses H-bonds to FMN O⁵ and O³P and instead makes an H-bond to O¹B of the adenosine ribose. The carboxyl group of conserved Glu485 also interacts with the adenosine ribose, in a similar manner to the aspartate conserved in most dehydrogenases (29). Altogether, ~10 putative hydrogen bonds are formed between NADH and the protein (Fig. 1A and supplemental Fig. S2). These hydrogen bonds and the stacking interactions extend over the whole length of nucleotide, which fits tightly in its extended form into the binding pocket, explaining the high affinity of complex I for its substrate. The O¹B atom of adenosine ribose points into the protein interior, but there is some limited unoccupied space in this area, which may accommodate the extra phosphate of NADPH. This explains the observable (but low) activity of complex I with NADPH as a substrate (31, 32). The N⁶A atom of the adenine moiety points into solvent, without any interactions with the protein, which explains why complex I can effectively use deamino-NADH (in which this atom is replaced by oxygen), unlike many other dehydrogenases.

Mechanism of Hydride Transfer—The B-face of the nicotinamide ring is stacked (slightly at an angle) against the re-face of FIGURE 1. The NADH-binding site. A, the site viewed from the solvent-exposed side. FMN and residues involved in NADH binding are shown as sticks with carbon in yellow and NADH with carbon in salmon. Hydrogen bonds are shown as dotted lines in green, stacking interactions are in gray, and the hydride transfer path is in red. The van der Waal’s contact between Glu184 and C⁴N of NADH is shown in gray. Prefixes before residue names indicate the subunit number. B, conformational changes in the nucleotide-binding site upon reduction by NADH. Superposed structures of the oxidized (O2, green) and NADH-reduced (RND, yellow) domain are shown. C, surface representation without NADH; FMN is shown as sticks with carbon in cyan. D, surface representation with bound NADH, colored salmon.
the isoalloxazine ring, in a position that is typical for direct hydride transfer in flavoproteins. This arrangement is consistent with the known stereospecificity of the complex I reaction for the 4B hydrogen of NADH (31, 33). Upon NADH binding, the side chain of conserved Glu97 moves away from the flavin, allowing the nicotinamide ring to occupy its position. The carboxyl group of Glu97 is involved in several hydrogen bonds to the protein backbone and the side chain of the conserved Tyr180, which may stabilize the observed conformation. The Cβ atom of this glutamate is in van der Waal’s contact (3.2 Å) with the CεN atom of the nicotinamide, likely pushing it closer to the Nε atom of FMN and thus promoting hydride transfer from CεN to Nβ. The distance between these two atoms (3.2 Å) is slightly shorter than average for flavoenzymes (30), consistent with fast rates of hydride transfer in complex I (34). The particular location of CεN, as a site of oxidative attack, relative to the isoalloxazine ring (its projected position falls between the two lines defined by the CεA-CεA and Nε-Nε atoms of FMN), is very similar to that seen in other flavoenzymes, consistent with a notion of highly conserved, stereochemically optimized geometry of the active site (30). Upon NADH binding, the isoalloxazine ring shifts ~0.5 Å away from neighboring iron-sulfur cluster N3. This is consistent with the observed line width narrowing of the EPR signal of flavosemiquinone radical in the presence of NADH, suggested to occur because of diminished dipole-dipole interaction with cluster N3 (35). This movement of the flavin is likely due to an overall shift of the structure around the glycine-rich loop away from the NADH.

Thus, this structure shows how an unusual Rossmann fold domain of complex I, which evolved to bind both flavin and NADH by the addition of an extra glycine-rich loop (8), incorporates familiar elements of nucleotide binding in a different structural environment. It explains the kinetic properties of NADH oxidation by complex I, such as high affinity, stereospecificity, substrate specificity, and fast hydride transfer rates. All of the residues involved in interactions with the cofactors and most residues lining the binding cavity are very well conserved (supplemental Fig. S3), suggesting that the mechanism of dehydrogenation by complex I is conserved throughout the species from bacteria to humans. During turnover in vivo ($k_{cat} = 200 \text{s}^{-1}$), most of the iron-sulfur clusters are reduced (36), and the likely rate-limiting steps are NAD+ release (34) and/or quinone/quinol binding/release.

Implications for Generation of ROS by Complex I—Flavin is now generally regarded as a main source of ROS in complex I (4, 37–39), although cluster N2 (40) and (semi)quinone (41) are also being discussed. In the absence of NADH, the isoalloxazine ring is exposed to the solvent (Fig. 1C), and so if the flavin is reduced, ROS formation is inevitable. However, when a nucleotide is bound, the flavin is shielded from the solvent (Fig. 1D). Therefore, during normal turnover, ROS formation would be minimal (~1% of electrons escape to oxygen (37, 39)), because electron transfer from flavin down the redox chain is faster than NAD+ release (34). However, under conditions when reoxidation of flavin is slow (the presence of complex I inhibitors, a highly reduced quinone pool, or reverse electron transport), the rate of ROS production increases (39), consistent with our structural interpretation. Therefore, an attractive candidate for a drug directed at minimizing ROS production by complex I would be a compound with high affinity for the reduced flavin site but low affinity toward oxidized enzyme, to avoid interference with the main physiological activity.

Conformational Changes upon Reduction—From a mechanistic point of view it is of particular interest to know whether any long range conformational changes occur upon reduction of the enzyme. Comparison of the structure of NADH-reduced hydrophilic domain (RND) to the oxidized form (O2 or O4) does not reveal major rearrangements (root mean square deviation = ~0.6 Å). However, some reproducible differences are observed, mostly at the extremities of the structure (Fig. 2, A and B), near the nucleotide-binding site (tops of the panels) and near the interface with the membrane domain (bottoms of the panels). Changes near the nucleotide-binding site appear to be directly related to NADH binding, as discussed above.

Near to the interface with the membrane domain, reduction by NADH leads to a shift of ~1 Å in the positions of helices H1 and H2 (residues 18–35 and 143–160 of Nqo6) toward the tip of the membrane domain. There is also a concerted shift (of ~1 Å) of a four-helix bundle in Nqo4 (residues 110–297) toward the membrane interior (Fig. 2C). Even though these shifts are small, they are determined unambiguously at our resolution and are reproducible (supplemental text). The presence or absence of dithionite did not lead to any significant differences between the two structures of the NADH-reduced domain (RND and RN).

Cross-linking studies have suggested that, upon reduction, contacts between the subunits of complex I in the bovine, Escherichia coli and Thermotoga enzymes are diminished (32, 42, 43). Consistently, the number of intersubunit contacts (calculated using the CCP4 program CONTACT with 3.5 Å cutoff) is reduced by ~7–8% in our structures of the reduced enzyme (RND and RN) as compared with the oxidized form (O4 and O2). This likely reflects small adjustments occurring upon the reduction of iron-sulfur clusters, some of which build up to the shifts of helices described above. It cannot be excluded that the extent of these movements might be different in the intact complex. However, our cross-linking studies have shown a similar degree of change in cross-linking patterns upon reduction both for the intact complex and for the isolated hydrophilic domain (32).

Surprisingly, movements near the interface with the membrane domain are not accompanied by any significant changes in the central part of the structure, and so they do not appear to be communicated directly by protein interactions. Therefore, it is likely that these shifts of the helices are driven by a change in the redox state of nearby iron-sulfur clusters (N2, N6a, and N6b (8)). The structure of the dithionite-reduced domain (RD) shows similar movements of helices H1 and H2, but interestingly, no shifts in the four-helix bundle of Nqo4 (supplemental Fig. S4B). Dithionite reduces complex I clusters slowly, on a time scale of minutes (44), and so it is likely that in our RD case, after a short exposure of crystal to air during harvesting, cluster N2 remains reduced because of its high potential, whereas other clusters are oxidized. On the other hand, NADH reduces most of complex I clusters, including N6b, on a millisecond time scale (34), and so they will remain reduced in RND and RN.
structures, despite a similar (short) exposure of crystals to air. After a prolonged exposure, as in the RA1 and RA2 structures, the density for bound nucleotide becomes weaker than in the anaerobic state (RND and RN). It is likely that in the aerobic state, as NADH is consumed to produce ROS (37) and NADH/NADH ratio in the solution drops, most of the redox chain in the complex is oxidized, whereas cluster N2 remains reduced. Indeed, EPR experiments on the Neurospora crassa enzyme have shown that after reduction of the complex by low amounts of NADH, cluster N2 remained reduced after exposure to air, whereas other clusters were reoxidized (45). Similar N2 coordination patterns (see below) suggest that the redox state of N2 and neighboring clusters in the NADH aerobic state (RA1 and RA2) is similar to that of the dithionite state (RD). Consistently, in the NADH aerobic state helices H1/H2 undergo similar shifts as in the RD (and RND) structures, whereas the four-helix bundle of Nqo4 shows limited or no movement (as in the RD structure). This suggests that the H1/H2 movement is mainly driven by the reduction of cluster N2, to which these helices are connected either directly (H2) or through a short β-strand (H1).

EPR studies of the intact complex suggest that although in the NADH-reduced enzyme cluster N6b is likely to be reduced in addition to N2, N6a may not be (34, 46, 47). Therefore it is likely that the shift of the four-helix bundle, which occurs in this case (RND structure), is driven by the reduction of cluster N6b or N6b/N2 as a pair. Contribution from N6a (or even more distant clusters) cannot be excluded. However, the large distance from cluster N6a to the preceding N5 (8), combined with the possibility of a low potential of N6a (47), may allow only very transient reduction of N6a under steady state conditions, so that the N6b/N2 cluster pair works with electrons delivered effectively in pairs from NADH (and donated in pairs to quinone). Cluster N6b is hydrogen-bonded to semi-conserved His41, whereas N6a does not have such a partner and so may have a lower potential. The conclusion that the reduction of iron-sulfur clusters drives conformational changes, rather than nucleotide binding per se, is consistent with our cross-linking data (32). In the intact complex, helix H1 is likely to extend toward the antiporter-like subunits Nqo12–14 (4). The long and rigid four-helix bundle, with its apparent concerted movement into the membrane domain, and the amphipathic helix H1 moving like a “lever” along the surface of the membrane domain (Fig. 2C), together may be able to drive rearrangements of transmembrane helices, leading to proton translocation.

Environment of Cluster N2—The terminal cluster N2 is the electron donor to quinone (2, 8, 46). It is coordinated, uniquely, by two consecutive cysteines of Nqo6 subunit (“tandem cysteine motif”). This leads to an unfavorable, strained geometry of the side chains of the two cysteines. Recently, a similar cluster

FIGURE 2. Conformational changes upon reduction by NADH. A and B, the structure of the NADH-reduced domain (RND) was superposed with that of oxidized domain (O2) and colored according to Cα deviations between the structures - blue to green to yellow as deviation increases. Higher deviations near the nucleotide-binding site (top) and at the interface with the membrane domain (bottom) are evident. NADH is shown as spheres in magenta. C, close-up of the interface with the membrane domain (structures RND and O4 were superposed). The approximate (expected) border of the membrane domain is shaded in blue. Helices H1 and H2 from Nqo6 subunit and the four-helix bundle from Nqo4 are indicated. Clusters N2 and N6b are shown as spheres. The arrows indicate movements of helix H1 and the four-helix bundle upon reduction.
Coordination was discovered in adenosine 5'-phosphosulfate reductase (48). The motif is fully conserved in complex I and so is likely to be important for the function. The strained geometry is likely to lead to flexibility in this area, which appears to be important for the conformational changes that we observe, particularly around helices H1/H2. Another possibility is that such coordination may allow the cluster to be protonated upon reduction, because its redox potential is pH-dependent (redox-Bohr effect) (46).

In both of our structures of the oxidized domain, the electron density connecting both tandem cysteines to the cluster is very clear (Fig. 3, A and B, and Ref. 8). Remarkably, in the NADH-reduced domain (structures RND and RN), the density connecting Cys to the cluster disappears completely (Fig. 3, C and D). It cannot be excluded that Cys remains a coordinating ligand but with much increased flexibility. However, on the basis of the electron density (the backbone density for the cysteine is too far from the potential S/H position; Fig. 3, C and D), it is more likely that the iron atom of the reduced cluster is now coordinated by another ligand, such as an hydroxide or a water molecule, as seen in aconitase (49). In principle, solvent-coordinated iron atom might be protonated directly. However, importantly, such a disconnection is likely to lead to protonation of the free Cys.

Strikingly, in the structure of dithionite-reduced domain (RD), Cys becomes disconnected instead, whereas Cys retains good connecting density (Fig. 3E). In both structures of aerobically harvested NADH-reduced enzyme (RA1 and RA2), the density connecting Cys to the cluster also comes back fully (i.e. is similar to that in oxidized enzyme), whereas density connecting Cys to the cluster also disappears (Fig. 3F). Therefore, RA1, RA2, and RD structures are likely to represent a similar redox state, consistent with crystal harvesting conditions, as discussed above. This suggests that when cluster N2 is reduced, one of the tandem cysteines disconnects, the choice being dependent on the redox state of neighboring clusters. When only N2 is reduced (RD, RA1, and RA2 structures), Cys is disconnected, but when N6b is also reduced (RN structures), Cys is disconnected instead. The redox state of N6b may be communicated to N2 via conformational changes that we observe (shift of four-helix bundle in RND structure, as compared with RD). Furthermore, the interaction may also be mediated via charged residues. For example, Arg, which is conserved and essential for activity (50), is hydrogen-bonded to cluster N2 but is also only 6 Å away from cluster N6b ligand Cys and may be hydrogen-bonded to the main chain carbonyl of His, located between the two Cys ligands of N6b.

Similarly to Cys in the NADH anaerobic state, cluster coordination by Cys in the dithionite anaerobic (or NADH...
Mechanism of Respiratory Complex I

aerobic) state is likely to be replaced by a solvent ligand. We have found previously that locally bound nonchelatable metal may be available for the Fenton reaction near cluster N2 (32), consistent with the possibility of cluster ligation by solvent. Under no conditions did we observe a structure with both tandem cysteines disconnected. The similarity of dithionite anaerobic and NADH aerobic states suggests that the latter is not the result of oxidative damage. The disconnection of tandem cysteines is also not likely to result from radiation damage during data collection (supplemental text).

Thus, it is likely that during the catalytic cycle $\varepsilon$Cys$^{45}$ and $\varepsilon$Cys$^{46}$ would become disconnected from the cluster (and so protonated) in sequence when cluster N2 is reduced. $\epsilon$Cys$^{45}$ is next to invariant and essential for activity $\epsilon$Tyr$^{87}$ (50), which points into the quinone-binding cavity, and so one can envisage a $\epsilon$Cys$^{45}$ → $\epsilon$Tyr$^{87}$ → (Q-site) proton delivery pathway. Although $\epsilon$Tyr$^{87}$ is one of likely secondary proton acceptors, there are other possibilities, such as nearby conserved $\epsilon$Arg$^{83}$, which is very well positioned for this role, making apparent hydrogen bonds to the sulfurs of the cluster and $\epsilon$Cys$^{45}$. It will be of interest to study the role of this arginine by mutagenesis.

Protons used during quinone reduction in complex I are delivered from the cytosol side in bacteria (or matrix in mitochondria). However, the quinone-binding site resembles a funnel pointing into the membrane (8) and may not be directly accessible from the solvent in the intact complex. Interestingly, a densely packed path of conserved ionizable residues connects the cytosol-exposed part of the Nqo4 subunit to the $\epsilon$Tyr$^{87}$-$\epsilon$Cys$^{45}$ area (supplemental Fig. S5), possibly representing a proton delivery pathway. A similar pathway, with at least four residues conserved between complex I and hydrogenases ($\epsilon$Arg$^{350}$, $\epsilon$Asp$^{401}$, $\epsilon$His$^{299}$, and $\epsilon$Arg$^{279}$), was suggested for proton transfer in the homologous [Ni-Fe]-hydrogenases (51). Other proton delivery pathways to either one of the tandem cysteines cannot be excluded without further mutagenesis data. In the large subunit of [Ni-Fe]-hydrogenases, invariant Glu$^{18}$ is essential for proton transfer (51) and superposes by structural alignment to $\epsilon$Tyr$^{87}$ in complex I, consistent with the possible role of this tyrosine in proton delivery. Thus, proton transfer toward the Q-site may be controlled, through tandem cysteines, by the redox state of clusters N2/N6b. Such a control is not necessary for simple “chemical” protonation of quinone but would be needed as a part of coupling mechanism (see below).

Bound Divalent Cations—Each of the three higher resolution structures described here has been solved in the presence of different divalent cations, Ca$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$, mostly in an effort to improve the resolution. This has also allowed us to identify cations bound to the protein. In the O4 structure we could resolve at least four ions bound to each molecule (Fig. 4A). The presence of 0.1 M CaCl$_2$ in the mother liquor, B factor refinement, the geometry and nature of coordination ligands (carboxyls of Glu and Asp, main chain carbonyl and N$^\#$ of His) allowed us to assign the bound metal as Ca$^{2+}$. Interestingly, three of four cations are bound in the previously identified acidic groove (Fig. 4A), a negatively charged protein surface between the C-terminal domain of Nqo3 and the rest of the complex (4). We have suggested it as a possible area of interaction with cations, which are known to modulate complex I activity (4). The fourth Ca$^{2+}$ atom is involved in crystal contacts (as well as some of Mg$^{2+}$ and Mn$^{2+}$ atoms in the other two structures), explaining why high concentrations of divalent cations are necessary for crystallization.

In the RND structure (crystals grown in 0.1 M MgCl$_2$), we could resolve 5–7 bound cations for each molecule, three of which were also found in the acidic groove (Fig. 4A), confirming that this area is involved in interactions with divalent cations (4). Most cations refined well as Mg$^{2+}$ atoms, except for one tightly bound (almost buried under the surface) at the interface.
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FIGURE 5. A model of complex I mechanism, based on structures described here. Iron-sulfur clusters N2 and N6b are depicted as oxidized or reduced by empty or filled circles, respectively. C46 and C45 indicate the tandem cysteines from Nqo6 subunit, whereas Q/QH2 indicate quinone/quinol. One of possible proton acceptors from C45 is Tyr87 (Y-O) and from C46-Glu49 (E-O). H-path indicates proton delivery pathway from the cytosol to tandem cysteines, such as that shown in supplemental Fig. 5, which may be the same for both cysteines (although other pathways are also possible). H1 and 4HB indicate helix H1 from Nqo6 and the four-helix bundle from Nqo4, respectively, and are shown on dark backgrounds when helices are shifted relative to oxidized state. The proposed catalytic cycle is described in detail in the text.

of subunits Nqo3 and Nqo15 (Fig. 4B). B factor refinement suggested that it is a heavier atom, with a mass similar to Ca2+. This atom is present in all three structures. It appears to stabilize the Nqo3-Nqo15 interaction, and it is known that calcium stabilizes bacterial complex I (32). On the basis of these observations, we tentatively assigned this atom as Ca2+ in all structures, on the assumption that it remains tightly bound to the complex throughout purification and crystallization. This remains to be confirmed by further investigation. The only other cation found in the same position in all three structures is coordinated by the carboxyl group of Asp302 and the main chain carbonyl of Leu274, also in the acidic groove. It cannot be excluded that this is a tightly bound Ca2+ as well, although B factor refinement was less conclusive in this case.

In the O2 structure (crystals grown in 0.1 M MnCl2), six to eight cations bound per molecule were resolved. Again, three cations were found in the acidic groove, whereas one Mn2+ ion (only in the first copy of the molecule in ASU) was found in the previously identified putative iron-binding channel (8) at the interface of subunit Nqo15 with the rest of the complex (Fig. 4C). A bound Mg2+ atom was identified in the RND structure of Nqo15 but interacts with His350, Glu123, and Ser68. This puts the metal within ~10 Å of cluster N1a. This cluster might have unique role as an antioxidant (4, 8), and if iron binds in this location, it could be used for regeneration of the cluster in case of iron loss. Overall, the structure is consistent with iron being delivered by frataxin multimers, because monomers do not permanently bind to its partner (4, 16). Frataxin deficiency in humans leads to Friedreich’s ataxia, a severe neurodegenerative disorder (52). However, exactly how frataxin delivers iron (Fe2+) to its protein partners is not known, and T. thermophilus complex I may provide such a model. Iron and manganese are neighboring transition metals with similar properties. Often, they can be exchanged in biological reactions, and so Mn2+ can be used to mimic easily oxidized Fe2+. The fact that Mn2+ was identified as a bound metal in the putative iron-binding channel suggests that iron is also likely to bind in the same location.

The metal ion shown in Fig. 4C is not directly coordinated by residues of Nqo15 but interacts with His350, Glu123, and Ser68. This puts the metal within ~10 Å of cluster N1a. This cluster might have unique role as an antioxidant (4, 8), and if iron binds in this location, it could be used for regeneration of the cluster in case of iron loss. Overall, the structure is consistent with iron being delivered by frataxin multimers, because monomers do not permanently bind to its partner (4, 16). Frataxin deficiency in humans leads to Friedreich’s ataxia, a severe neurodegenerative disorder (52). However, exactly how frataxin delivers iron (Fe2+) to its protein partners is not known, and T. thermophilus complex I may provide such a model. Iron and manganese are neighboring transition metals with similar properties. Often, they can be exchanged in biological reactions, and so Mn2+ can be used to mimic easily oxidized Fe2+. The fact that Mn2+ was identified as a bound metal in the putative iron-binding channel suggests that iron is also likely to bind in the same location.

Complex I Mechanism—The two main models of coupling between electron transfer and proton translocation in complex I, conformational and direct coupling, are not mutually exclusive. To account for the high proton/electron stoichiometry, it was proposed that a combination of the two mechanisms is possible (8, 11, 53). As shown by our structures, upon reduction some rather small but significant and concerted shifts of several α-helices occur at the interface with the membrane domain. These movements may be sufficient to drive proton translocation by inducing conformation changes in transmembrane helices. Indeed, our cross-linking studies indicated that the interface between two hydrophobic subunits is changed upon reduction of E. coli complex I (32). Because there are three sim-
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ilar antiporter-like subunits in the membrane domain, it is possible that such conformational coupling would lead to the translocation of three protons (one/each subunit) in one catalytic cycle. The remaining fourth proton (accounting for the 4H+/2e− stoichiometry) could be translocated near the Q-site with the participation of cluster N2/tandem cysteines.

A putative model of such a mechanism, based on our structures, is shown in Fig. 5. As discussed above, the main determinant appears to be the redox state of clusters N2 and N6b (although contribution from more distant clusters cannot be excluded). Starting from the oxidized state 1 (O4 and O2 structures), the arrival of the first electron from NADH via the redox chain to cluster N2 will result in disconnection and protonation of εCys45. In this state, state 2 (dithionite aerobic/NADH aerobic structures, RD, RA1, and RA2), helices H1/H2 of Nqo6 are shifted, and the proton is delivered to εCys45. Although we illustrate the figure with two proton delivery pathways for two tandem cysteines, there may be only one pathway, such as that suggested in supplemental Fig. S5, bifurcating near the cysteines. The reduction of cluster N6b by the second electron will result in state 3, where εCys45 is reconnected to the cluster N2 (and so deprotonated), whereas εCys46 is disconnected and so protonated (NADH anaerobic structures, RND and RN). The four-helix bundle of Nqo4 is shifted; the proton leaving εCys45 is transferred to one of the residues nearby, such as εTyr87 or εArg88 (Y-OH). Then, after cluster N2 donates the first electron to quinone, creating a semiquinone radical, it is quickly reduced again by cluster N6b (because of the high potential of N2), and state 4 is created. The redox state of the clusters here is similar to state 2, and so εCys46 is reconnected to the cluster, whereas εCys45 is disconnected and so protonated again. The proton leaving εCys46 is transferred to one of ionizable groups nearby, such as εGlu49 (E-OH). This glutamate is essential for activity of E. coli complex I, and as discussed previously (54), the rotation of the side chain can bring it to within hydrogen-bonding distance of εCys46. In the next step, N2 donates an electron to the semiquinone, which is protonated by the Y-OH and E-OH groups, so that quinol is produced. Helices H1/H2 return to their starting positions (because N2 is oxidized), and this step is linked to the translocation of three protons through antiporter-like subunits Nqo12–14 via conformational changes. Conformational coupling probably involves the four-helix bundle as well and so is initiated at earlier steps. Its depiction at this step reflects its final stage and the fact that semiquinone radical is strongly stabilized by the proton-motive force (55). Because cluster N2 is likely to be oxidized first, before Y-OH donates its proton to the quinone, the proton released upon εCys45 reconnection to the cluster can take a different pathway from that shown in step 3, and so it can be ejected into the periplasm. The exit pathway for this “pumped” proton is likely to be located in the adjacent hydrophobic subunits. Finally, quinol is released from the binding site, quinone binds instead, and the cycle restarts.

The sum of the reactions depicted in Fig. 5 will be the transfer of two electrons from NADH and two protons from the cytoplasm to the quinone, coupled to the translocation of four protons across the membrane, consistent with known stoichiometry. In our “combined” complex I mechanism, the unique coordination of cluster N2 by tandem cysteines is central both for conformational coupling (allowing movements of helices H1/H2) and for direct coupling via cysteine protonation. One of the consequences of our mechanism would be pH dependence of the midpoint redox potential of cluster N2, because its reduction is accompanied by the protonation of εCys45 (which is thus predicted to act as a redox-Bohr group). Such pH dependence of N2 potential is indeed observed (46), consistent with our proposal. An alternative candidate for the redox-Bohr group has been discussed, but the evidence is ambiguous (supplemental text). Previously, the redox-Bohr effect has been repeatedly proposed to play some role in the proton translocation mechanism of complex I, and our mechanism shows the likely details of the process. Mutagenesis of the residues involved (tandem cysteines (28), εTyr87 (50), and εGlu49 (54)) leads to the loss of oxido reductase and proton-translocation activity, consistent with our model.

Elements of the mechanism suggested here may be applicable to other enzymes harboring a similar cluster coordinating motif (48). It appears that in complex I, unfavorable and unstable coordination of the cluster was evolutionarily selected because of the associated high efficiency of proton pumping. The full details of the coupling mechanism can be established by solving the structure of the intact enzyme and by further mutagenesis studies.

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