Atomic Level Insight into the Oxidative Half-reaction of Aromatic Amine Dehydrogenase

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The quinoprotein aromatic amine dehydrogenase (AADH) uses a covalently bound tryptophan tryptophylquinone (TTQ) cofactor to oxidatively deaminate primary aromatic amines. Recent crystal structures have provided insight into the reductive half-reaction. In contrast, no atomic details are available for the oxidative half-reaction. The TTQ O7 hydroxyl group is protonated during reduction, but it is unclear how this proton can be removed during the oxidative half-reaction. Furthermore, compared with the electron transfer from the N-quinol form, electron transfer from the non-physiological O-quinol form to azurin is significantly slower. Here we report crystal structures of the O-quinol, N-quinol, and N-semiquinone forms of AADH. A comparison of oxidized and substrate-reduced AADH species reveals changes in the TTQ-containing subunit, extending from residues in the immediate vicinity of the N-quinol to the putative azurin docking site, suggesting a mechanism whereby TTQ redox state influences interprotein electron transfer. In contrast, chemical reduction of the TTQ center has no significant effect on protein conformation. Furthermore, structural reorganization upon substrate reduction places a water molecule near TTQ O7 where it can act as proton acceptor. The structure of the N-semiquinone, however, is essentially similar to oxidized AADH. Surprisingly, in the presence of substrate a covalent N-semiquinone substrate adduct is observed. To our knowledge this is the first detailed insight into a complex, branching mechanism of quinone oxidation where significant structural reorganization upon reduction of the quinone center directly influences formation of the electron transfer complex and nature of the electron transfer process.

Aromatic amine dehydrogenase (AADH) is an inducible periplasmic quinoprotein produced by some Gram-negative bacteria to allow growth on primary aromatic amines as a source of carbon and nitrogen (1, 2). In Alcaligenes faealis, AADH is specific for phenylethylamines but also reacts with primary aliphatic amines, although to a lesser extent (2, 3). AADH exhibits an $\alpha_2\beta_2$ heterotetrameric structure with each $\beta$-subunit possessing a covalently bound redox-active tryptophan tryptophylquinone (TTQ) cofactor (4). In the reductive half-reaction, the substrate-derived amino group is incorporated into the AADH TTQ, resulting in conversion of the quinone form of the cofactor into N-quinol (Fig. 1, species A–E) (3, 4). The TTQ cofactor is subsequently reoxidized by two consecutive electron transfers to azurin, a periplasmic type I blue copper protein, with release of ammonia (5–7). AADH is structurally and functionally very similar to methylamine dehydrogenase (MADH), a different TTQ-containing periplasmic enzyme. MADH oxidizes methylamine to formaldehyde in many Gram-negative bacteria that grow on methylamine (8). The three-dimensional structures for MADHs from several species are known (9–11), and MADH is believed to share a common mechanism for the reductive half-reaction with AADH (3, 12) during which cleavage of the substrate C-H bond occurs by vibration-assisted tunneling (13–15). However, unlike AADH, most of the known MADHs are reoxidized by transferring electrons to amicyanin (8, 16). Biochemical data suggests that, despite the very close structural and functional similarity of AADH and MADH, their respective electron acceptor partners, azurin and amicyanin, are not functionally interchangeable (6).

Recently we reported atomic resolution structures of oxidized AADH and key intermediates in the reductive half-reaction with tryptamine (15). This revealed a complex, multistep mechanism involving several proton transfers (Fig. 1, species B, C, and F). Unexpectedly, the resulting TTQ N-quinol species at the end of the reductive half-reaction is protonated at the O7 function. Therefore, during the oxidative half-reaction, deprotonation of the O7 hydroxyl group is required concomitantly with electron transfer.

However, the N-quinol O7 is buried in the interior of the protein with no obvious proton acceptor positioned nearby. Furthermore, previous biochemical studies indicated that the nature of the substituent at the C-6 position of the cofactor affects the reactivity of AADH with azurin. Kinetic analyses showed a 25-fold difference in electron transfer rate and 2.4-fold difference in apparent $K_d$ for the reactions of oxidized azurin with dithionite-reduced (O-quinol; Fig. 1, species D) and substrate-reduced (N-quinol; Fig. 1, species E) AADH (6). The enzyme must, therefore, have a mechanism for communicating the TTQ redox state to the enzyme surface to induce formation of a transient AADH-azurin complex or fine-tune the AADH-azurin interactions within a pre-formed complex for fast electron transfer. It is likely such a mechanism involves AADH...
structural changes concomitant with TTQ reduction, and this might allow a proton acceptor near the N-quinol O7 function.

To analyze how the structure of AADH responds to changes in the redox state of its cofactor and to identify structural factors that may determine specificity of AADH toward the redox partner azurin, we have determined high resolution crystal structures of the enzyme reduced either with sodium dithionite or with the substrate tryptamine in addition to the N-semiquinone form (Fig. 1, species H). We present a comparative analysis of the oxidized and reduced forms of AADH that reveals structural changes in the putative azurin-binding site of the substrate-reduced enzyme. We show that reduction to the N-quinol form leads to significant rearrangements in the protein structure in contrast to formation of the non-physiological O-quinol structure generated by reduction with dithionite. These structural changes are likely to improve binding of oxidized azurin to amine-reduced AADH. Surprisingly, despite the need for a second electron transfer to azurin in the oxidative half-reaction and, hence, the obvious benefits of retaining the "reduced" conformation in the N-semiquinone state, the structure of the N-semiquinone is virtually identical to that of oxidized AADH. Importantly, the structural rearrangement allows positioning of a water molecule close to the N-quinol O7 where it can serve as an initial proton acceptor during the first electron transfer of the oxidative half-reaction.

**MATERIALS AND METHODS**

**Protein Purification and Preparation of Crystals**—AADH from A. faecalis IFO 14479 was purified and crystallized in the oxidized form as previously described (4, 15). The crystals (hereafter designated as form A to be consistent with Masgrau et al. (15)) belong to space group P2₁ with unit-cell parameters \( a = 70.8, b = 89.1, c = 80.3 \ \text{Å}, \beta = 90.2^\circ \). These crystals contain a heterotetramer in the asymmetric unit. To produce free O-quinol AADH, these crystals were soaked for 1 h in a stabilizing solution that included 100 mM sodium dithionite, then coated in a 1:1 paraffin/Paratone-N mixture and flash-cooled in liquid nitrogen. The crystal complex of O-quinol AADH in complex with tryptamine was prepared by including 50 mM tryptamine in the stabilizing solution after dithionite reduction of the crystals. Crystals of substrate reduced N-quinol AADH were obtained either by soaking the form A crystals in a tryptamine solution as previously described (15) or by anaerobic co-crystallization with tryptamine. Crystallization trials with 12.5 mg/ml AADH and 5 mM (initial concentration) tryptamine with 19–22% (w/v) polyethylene glycol 2000 monomethyl ether, 100 mM ammonium sulfate, and 100 mM sodium cacodylate, \( pH \) 6.0, as a reservoir solution yielded crystals of N-quinol AADH that are isomorphous with the form A-oxidized AADH crystals. A second crystal form (form B) for tryptamine-reduced AADH was obtained using higher concentrations (25–27% (w/v)) of polyethylene glycol 2000 MME and the protein solution (12.5 mg/ml) that was incubated with 5 mM tryptamine for 15 min and dialyzed anaerobically overnight against 10 mM potassium phosphate, \( pH \) 7.5, to remove excess substrate before crystallization. These crystals belong to space group P2₁2₁2₁, with unit-cell parameters \( a = 89.8, b = 96.3, c = 118.9 \ \text{Å} \), contain a heterotetramer in the asymmetric unit, and can be cryoprotected by soaking for 10–15 s in a solution containing 10% polyethylene glycol 300, 24% (w/v), polyethylene glycol 2000 MME, 100 mM ammonium sulfate, and 100 mM sodium cacodylate, \( pH \) 6.0. Crystals of N-semiquinone AADH species were prepared by soaking the form A crystals of oxidized AADH in a mother liquor supplemented with excess benzylamine and, after complete reduction, exposing them to a bright UV light in the range of 320–395 nm for 1 min. The UV-visible absorption spectra of cryo-cooled crystals have been measured using the on-line microspectrophotometer on beamline ID 14.4 at European Synchrotron Radiation Facility (Grenoble, France).

**Data Collection and Structure Determination**—Diffraction data for all structures were collected at cryogenic temperatures on European Synchrotron Radiation Facility stations ID14-1, 14-2, and 14-4 (Grenoble, France). All the data were processed and scaled using the DENZO/SCALEPACK package (20). Data collection statistics are summarized in Table 1. The initial
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FIGURE 2. Comparison of the structures of A. faecalis AADH and Paracoccus denitrificans MADH (PDB code 2BBK). The TTQ cofactor atoms are depicted as magenta spheres, the α-subunits are depicted in blue, and the catalytic β-subunits are shown in green.

RESULTS

Overall Structure—The 1.2-Å-resolution crystal structure of oxidized AADH (RSCB PDB code 2AH1) has been reported elsewhere (15). In brief, the α2β2 AADH heterotetramer (α 39 kDa, β 13 kDa) comprises two αβ dimers that are related by a 2-fold axis. The large subunit has a 7-bladed β-propeller fold consisting of seven 4-stranded β-sheet motifs. The small subunit has a disulfide-rich mainly β2-layer sandwich structure and possesses a tryptophan TTQ prosthetic group. The quinol moiety of the TTQ (derived from Trp-109β) is covalently bound to the indole ring of Trp-160β and is located at the bottom of a very narrow active site pocket lined mostly by apolar and aromatic residues.

Superposition of the structures of AADH and MADH (PDB code 1MDA (25)) reveals that the structural similarity between the two enzymes extends over the entire length of the proteins (Fig. 2). Alignment of the αβ dimers of AADH and MADH reveals that 347 Cα atoms can be superimposed with an r.m.s.d. of 2.3 Å. Alignment of the small subunits alone results in a much better match whereupon 105 Cα atoms overlap with a r.m.s.d. of 0.9 Å and 43% sequence identity over equivalent positions.

Structures of Fully Reduced AADH Species—The structure of AADH reduced with tryptamine before crystallization under anaerobic conditions has been determined in two different crystal forms, to 1.3 Å (form A) and 1.55 Å (form B) (Table 1). Values for the lengths of the bonds C6-O6 and C7-O7 of the cofactor derived from the 1.3-Å resolution omit electron density maps are consistent with the single C-N (1.5 Å) and single C-O (1.4 Å) bond. In both forms the two αβ dimers comprising the heterotetramer have different crystallographic environments. In crystal form A, one of the TTQ-containing subunits forms three hydrogen bonds with a symmetry-related molecule through β-hairpin 110–125 (involving residues Pro-116β, His-117β, and Asp-118β). The other small subunit in the asymmetric unit of crystal form A as well as both small subunits in form B makes no direct crystal-packing contacts.

The reduced state of the cofactor in crystals soaked in sodium dithionite solution could be visually verified (reduced crystals are colorless in comparison to the dark-green oxidized crystals) and confirmed by unrestrained refinement of the C6-O6 and C7-O7 bond lengths against 1.8- and 1.5-Å-resolution data sets (Table 1). The average refined values (1.5 and 1.4 Å) for the C6-O6 and C7-O7 bonds, respectively, in both crystal forms are colorless in comparison to the dark-green oxidized crystals.

Structural Differences between the O-Quinol and the N-Quinol AADH Species—Superposition of the crystal structures of oxidized and substrate-reduced AADH reveals that upon reduction, one of the two small subunits in both crystal forms undergoes minor but extensive structural change mainly involving the three-stranded antiparallel β-sheet formed by strands 109–115, 120–131, and 168–180 (Fig. 3, a and b). Repositioning of the side chains of the two residues in the immediate vicinity of the quinolated moeity of TTQ, Thr-172β and Tyr-126β, allows water molecule W1 to bind near the TTQ...
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O7 (2.6 Å), which is not accessible to the solvent in the oxidized form. W1 also forms hydrogen bonds to Thr-173 backbone carbonyl (3.0 Å) and the side chain of Thr-172 (2.5 Å). It likely that upon reduction, water molecule W1 is repositioned from W2 (observed in the structure of oxidized AADH but not in the reduced enzyme), an event linked to conformational rearrangement of the stretch of residues 172β-174β. To accommodate the Tyr-126β side chain in its new position, the stretch of residues 110β-112β is displaced with concomitant change in the side-chain conformation of residues Ile-110β, Thr-112β, and Leu-123β. Simultaneously, Tyr-126β exchanges its hydrogen-bonding partners from the backbone carbonyl of Arg-79β (2.7 Å) and the backbone nitrogen of Ala-82β (3.1 Å) in the oxidized structure to a newly introduced water molecule W3 (2.7 Å) that in turn forms a hydrogen bond to the Thr-112β backbone carbonyl (3.0 Å) in the substrate reduced structures. The combined effect of these changes is a restructuring of the molecular surface of AADH in the vicinity of the partially solvent-exposed Trp-160β moiety of the TTQ and the reorganization of the surface-bound water molecules.

The N terminus of the small subunit also undergoes significant structural change after reduction. In the structure of the oxidized enzyme, the 23 N-terminal residues are thermally disordered, with an interpretable electron density starting at residue 71β. In the structure of the tryptamine-reduced enzyme, an additional 12 residues at the N terminus of the small subunit that undergoes significant reorganization compared with the oxidized structure became ordered, forming multiple interactions with coiled region 73β-79β and β-hairpin 110β-125β.

Superposition of the structures of the conformationally altered αβ dimers in the form A and form B crystal structures of tryptamine-reduced AADH reveals that 478 Cα atoms can be overlapped with an r.m.s.d. of 0.3 Å (0.2 Å for 119 Cα atoms in the small subunit), indicating a very similar structural response to the change in the redox state in both crystal forms. Furthermore, superposition of the form A structure of AADH obtained by co-crystallizing with tryptamine and the crystal structure of the tryptamine complex produced by soaking of the form A crystals of oxidized AADH in a solution of tryptamine (PDB code 2AGW (15)) revealed that soaking and co-crystallization produces almost identical structural changes in the β-subunit that are not restrained by crystal packing (481 Cα atoms from the respective αβ dimers can be overlapped with an r.m.s.d. of 0.1 Å).

In contrast, the second small subunit in the form A crystal, restrained by crystal packing contacts involving β-hairpin 110β-125β, shows little or no change in the protein structure when compared with the oxidized protein. Similarly, one of the small subunits in the form B crystal of tryptamine-reduced AADH shows no structural change either. Analysis of the crystal packing shows that, despite the lack of direct crystal contacts made by this subunit, the lattice does not allow enough space for ordering of the N terminus as observed in the other subunit. Thus, analysis of the structure of tryptamine-reduced AADH in two different crystal forms shows that crystal packing forces restraining either β-hairpin 110β-125β or the N terminus in the small subunit hinder the structural re-organization of the latter. This suggests that restructuring of the three-stranded anti-parallel β-sheet formed by strands 109β-115β, 120β-131β, and 168β-180β and ordering of the N terminus upon reduction are co-operative.

Structure of O-Quinol AADH Species—We have not been able to crystallize dithionite-reduced AADH. Thus, crystals of the O-quinol form of AADH were obtained by soaking the form A crystals of oxidized AADH in a solution of sodium dithionite. In contrast to the N-quinol form, the structure of O-quinol AADH shows no significant difference from the structure of oxidized AADH (for the reduced and oxidized structures, 939 Cα atoms can be superimposed with an r.m.s.d. of 0.2 Å). We have verified that the changes in the structure of AADH co-crystallized with tryptamine are linked to incorporation of the substrate amino group into the TTQ center rather than to the presence of the substrate in the active site. This was achieved by determining the structure of AADH in the form A crystal after reduction of the crystal with dithionite and soaking with tryptamine solution (Fig. 3c; supplemental Fig. 1). Superposition of this structure with the crystal structure of the tryptamine complex (produced by soaking of the form A crystals in excess tryptamine (15)) reveals a similar mode of tryptamine binding in the active site of N-quinol and O-quinol AADH. However,
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the protein structure of the O-quinol AADH complex with tryptamine did not exhibit any of the redox-linked changes observed in N-quinol AADH. Comparison of the structures of N-quinol and O-quinol AADH reveals the distinctly different hydrogen-bonding patterns around their respective TTQ C6 substituents (Fig. 3a). In the O-quinol form we postulate that a single hydrogen atom of the hydroxyl group at position 6 is donated to the carboxyl oxygen of Asp-128β (3.0 Å), whereas a lone electron pair of O6 is oriented toward the hydroxyl at position 7, a geometry optimal for the formation of an “intramolecular” O7(H)···O6 hydrogen bond. In contrast, the NH2/NH3+ group of the N-quinol has the potential to interact with three H-bond acceptors: the backbone carbonyl of Asp-84β (2.9 Å), water molecule W4 (2.7 Å), and carboxyl oxygen of Asp-128β (2.7 Å). The O7 atom of the TTQ accepts a hydrogen bond from the amide group of Asp-84β (2.9 Å) and donates a hydrogen bond to water molecule W1 (2.6 Å). This water molecule also acts as a donor of one hydrogen bond to Thr-173β backbone carbonyl (3.0 Å) and a second hydrogen bond to the side chain hydroxyl group of Thr-172β (2.5 Å), which in turn donates a hydrogen bond to the Asp0128β carboxylate (2.7 Å). This hydrogen-bonding network links a total of four different secondary structure elements in the small subunit of the substrate-reduced enzyme and is, therefore, likely to play a crucial role in stabilization of the new conformation. Thus, different hydrogen-bonding properties of NH2/NH3+ and OH substituents at position 6 of the quinol ring appear to be the key molecular factor behind the observed structural differences between the N-quinol and O-quinol forms of AADH.

Structure of N-Semiquinone AADH—Zhu and Davidson (19) previously demonstrated that on exposure to long range UV, MADH is oxidized via a relatively stable semiquinone intermediate. We have established that irradiation of the substrate
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The oxidative half-reaction of AADH can occur either directly from the 5-carboxylamidine intermediate (species B) as proposed in Masgrau et al. (15) or after product release from the 5-quinol (species F). We postulate a water molecule (W1) present in both cases to act as an initial proton acceptor to the TTQ 07. The second electron transfer from the 5-semiquinone (species C) can occur before (leading to species G) or after reaction with substrate (leading to species D) and occurs in the absence of any large-scale structural rearrangements. Protonation of the N6 group (conversion from F to E) can occur in the absence of substrate or monovalent cations that bind directly above the N6. Species A is the oxidized O-quinone, B is the 5-carboxylamidine-reduced TTQ adduct, C is the 5-semiquinone, D is the covalent substrate 5-semiquinone adduct, E is the protonated 5-quinol, F is the neutral 5-quinol, G is the protonated 5-quinone, and H is the first Schiff-base oxidized TTQ adduct.

Reduced AADH crystals with UV light in the range of 320–395 nm similarly allow for nearly stoichiometric preparation of the 5-semiquinone form as judged by spectral changes. The 1.45-Å structure of the 5-semiquinone form is essentially identical to the oxidized AADH structure, revealing that concomitant with the first electron transfer to oxygen (and by similarity to azurin), the protein structure returns to the conformation observed for the oxidized AADH with expulsion of the water molecule in the vicinity of O7. UV irradiation was carried out in the presence of excess benzylamine to avoid the possibility of significant amounts of quinone AADH being generated (benzylamine rapidly reduces crystalline quinone AADH without accumulation of covalent intermediates as is observed for tryptamine). Surprisingly, electron density in the active site clearly corresponds to partial occupancy of the benzylamine substrate as well as a covalent benzylamine TTQ-adduct (Fig. 3c). The electron density of the benzylamine adduct and analysis of the bond lengths and geometry strongly suggests this to be a semiquinone-substrate adduct species, a finding that is further corroborated by spectral analysis of the crystals, which confirms full stoichiometric conversion to the 5-semiquinone form before x-ray data collection. This establishes the 5-semiquinone has similar reactivity to the quinone species.

**DISCUSSION**

The rate of the oxidative half-reaction of AADH with the physiological electron acceptor azurin is dependent on the exact nature of the reduced AADH species. Electron transfer from 5-quinol AADH to azurin is fast and linked to proton transfer, but electron transfer from O-quinol AADH is slow and not linked to proton transfer (3, 6, 28). The coupled proton transfer and electron transfer from the N-quinol is a feature of both AADH and MADDH enzymes (18, 27) and was suggested to represent the deprotonation of the N-quinol to yield a highly reactive species. Electron transfer from this reactive species was suggested to be faster than from either unactivated 5-quinol or O-quinol. This assumes that the O7 group remains unprotonated throughout the catalytic cycle. However, the immediate environment of the TTQ O7 oxygen does not stabilize the formation of an oxyanion species upon reduction by substrate, and recent work in our group proposes the O7 group is protonated in the aminoquinol form (15).

Consequently, the conversion of TTQ to the semiquinone state in the oxidative half-reaction must be accompanied by proton abstraction from O7. That said, examination of the oxidized AADH structure reveals there is no suitable base in the vicinity of O7 to perform this role. Our structural analysis suggests that the structural rearrangement after reduction by the substrate allows for a shift of a water molecule W2 to a position within hydrogen-bonding distance of O7 (W1). The W1 water molecule can serve as an initial proton acceptor during the first electron transfer to azurin and can be transferred to the bulk solution through position W2 by the reverse rearrangement of the protein structure. This rearrangement occurs after the first electron transfer as revealed by the structure of the 5-semiquinol AADH leaving the second electron transfer to occur with the AADH overall structure already resembling the oxidized state.

In previous work we showed that the tryptamine carboxylamidine intermediate is long-lived both in crystals and in solution (15), with very slow rates of aldehyde product and N-quinol AADH formation in crystals. We have postulated that under physiological conditions oxidation of the carboxylamidine-TTQ precedes aldehyde product formation (Fig. 4). However, none of the available structures for any of the covalent tryptamine-TTQ intermediates reveals a significant change in the overall protein conformation when compared with the oxidized state. Yet, similar to N-quinol oxidation to 5-semiquinone, oxidation of a carboxylamidine-reduced TTQ intermediate must be accompanied by proton abstraction from O7. We propose that the
individual conformations observed in isolation for the oxidized and substrate-reduced AADH are in fact in rapid equilibrium regardless of TTQ redox state, allowing for transient positioning of a water molecule close to O7, thus allowing for proton transfer. In contrast, the relative ratio in occupancy of both conformations can depend on TTQ redox state. Thus, our data suggest that deprotonation of O7 is coupled to electron transfer from N-quinol AADH to azurin.

After formation of an AADH-azurin complex, the electrons are believed to be transferred from the quinolated moiety of the TTQ cofactor, buried within the protein, to the copper ion of azurin via a second, partially solvent-exposed indole ring of the TTQ (Trp-160β). Analysis of the structure of N-quinol AADH reveals that the surface residues that undergo conformational changes after reduction (Ile1-10β, Gly-111β, Thr-112β, and Leu-123β) form part of an extensive hydrophobic patch that surrounds Trp-160β. As shown in Fig. 5a, the location of this hydrophobic patch on the AADH heterotetramer is very close
to the location of the predominantly hydrophobic amicyanin-binding site on the surface of MADH. In fact, several of the MADH residues that interact with amicyanin overlap with those AADH residues that undergo significant repositioning upon substrate reduction (Fig. 5b). Structural and functional similarity between MADH and AADH implies that this hydrophobic patch on AADH serves as a recognition site for the electron acceptor partner, azurin. This is in line with the previous analysis that showed that the core of the interface in various electron transfer complexes generally consists of a patch of hydrophobic side chains surrounding exposed ligands of the redox site (26). This proposal is further supported by the previous kinetic studies that indicated that the apparent $K_m$ for azurin as an electron acceptor for AADH decreased with increasing ionic strength, suggesting that AADH-azurin association is mainly stabilized by hydrophobic interactions (6).

Modeling of AADH-azurin complex on the basis of MADH-amicyanin complex is difficult because the folds of amicyanin and azurin are related but different. Compared with amicyanin, the azurin has more extensive loops above the copper site and a deeper burial of the copper ion as well as a different location of a hydrophobic patch around the copper-binding site with respect to the fold (25, 29).

Our analysis of the structure of the enzyme in two different redox states reveals that the putative azurin-binding site undergoes significant structural reorganization upon reduction of the quinone center by substrate. Restructuring of the molecular surface of AADH in the vicinity of the partially solvent-exposed Trp-160β and the reorganization of the surface-bound molecules occurs as a consequence of minor but extensive changes around the aminoquinol moiety of the TTQ (Trp-109β) that mainly involve the three-stranded antiparallel $\beta$-sheet carrying Trp-109β. In contrast to formation of physiologically relevant N-quinol form, the structure of the O-quinol form of AADH produced by chemical reduction with sodium dithionite shows no features different from the structure of the oxidized enzyme.

Together with previous observations that the electron transfer rate to azurin and the affinity of the AADH for azurin are significantly less for dithionite-reduced compared with amine-reduced AADH, our observations suggest that the structural changes are likely to favor productive complex formation by improving binding of oxidized azurin to the $N$-quinol MADH species. A recent crystallographic study of Pseudomonas putida [2Fe-2S] ferredoxin (Pdx) (17) presents an alternative example of redox-linked structural reorganization at the putative docking site for a redox partner. In this case the protein partner is cytochrome P450cam. A change in the redox state of one of the iron atoms in the active site is transmitted to the protein surface via structural changes on a scale similar to those seen in AADH. In time it seems likely that further examples of redox-linked partner recognition will emerge. Detailed structural and solution analysis of reduced and oxidized redox partner proteins are now required to establish if redox-linked structural change at the interface of physiological electron transfer protein complexes is a general mechanism for facilitating complex formation and dissociation and rapid electron transfer in biological redox chemistry.

The picture that emerges from our structural studies of the AADH oxidative half-reaction is as follows (Fig. 4). After formation of the reduced carbinolamine species during the reductive half-reaction, the reaction can proceed via either (relatively slow) hydrolysis of the carbinolamine (Fig. 4, species B) to form the $N$-quinol (Fig. 4, species F) or transfer of the first electron with concomitant product release. To form the $N$-semiquinone (Fig. 4, species C) the O7 hydroxyl proton needs to removed, an event that can likely occur when the protein adopts the reduced conformation and a water molecule is positioned in the O7 vicinity to serve as initial proton acceptor. Conversion of the $N$-quinol that results from carbinolamine hydrolysis can similarly proceed via the reduced conformation. The $N$-semiquinone (Fig. 4, species C) (which preferentially adopts the oxidized conformation) can either react with a substrate amine to form the $N$-semiquinone-substrate adduct (Fig. 5, species D) or be further oxidized via a second electron transfer to azurin to form the $N$-quinone (Fig. 4, species G). The $N$-quinone can be hydrolyzed to the O-quinone (Fig. 4, species A) or react directly with a new substrate molecule to form the Schiff base-oxidized TTQ adduct (Fig. 4, species H). Formation of the $N$-semiquinone-substrate adduct occurs using a similar mechanism but results in a molecular species where the substrate C1 atom is distant from the TTQ plane and the Asp-128β proton acceptor. Oxidation of this species leads to formation of the Schiff base-oxidized TTQ adduct with repositioning of the C1 atom in the TTQ plane. An atomic description of the events during the reductive half-reaction after formation of the Schiff base-oxidized TTQ adduct can be found elsewhere (15). Given the complexity of this branching mechanism and in the absence of determined microscopic rate constants for each individual step, it is not possible to predict the flux through the individual pathways under steady-state conditions.

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