New insights into the reductive half-reaction mechanism of aromatic amine dehydrogenase revealed by reaction with carbinolamine substrates

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Aromatic amine dehydrogenase uses a tryptophan tryptophylquinone (TTQ) cofactor to oxidatively deaminate primary aromatic amines. In the reductive half-reaction, a proton is transferred from the substrate C1 to Asp128β O2, in a reaction that proceeds by H-tunneling. Using solution studies, kinetic crystallography and computational simulation we show that the mechanism of oxidation of aromatic carbinolamines is similar to amine oxidation, but that carbinolamine oxidation occurs at a substantially reduced rate. This has enabled us to determine for the first time the structure of the intermediate prior to the H-transfer/reduction step. The proton–Asp128β O2 distance is ~3.7 Å, in contrast to the distance of ~2.7 Å predicted for the intermediate formed with the corresponding primary amine substrate. This difference of ~1.0 Å is due to an unexpected conformation of the substrate moiety, which is supported by MD-simulations and reflected in the ~10^-fold slower TTQ reduction rate with phenylaminoethanol compared to that with primary amines. A water molecule is observed near TTQ C6 and is likely derived from the collapse of the preceding carbinolamine TTQ-adduct. We suggest this water molecule is involved in consecutive proton transfers following TTQ reduction, and is ultimately repositioned near the TTQ O7 concomitant with protein rearrangement. For all carbinolamines tested, highly stable amide-TTQ adducts are formed following proton abstraction and TTQ reduction. Slow hydrolysis of the amide occurs after, rather than prior to, TTQ oxidation and leads ultimately to a carboxylic acid product. The new structural data are consistent with the wide range of kinetic parameters observed with different amine substrates.

Aromatic amine dehydrogenase (AADH) and the related methylamine dehydrogenase (MADH) are inducible periplasmic quinoproteins produced by some Gram-negative bacteria. These enzymes allow growth on primary amines as a source of carbon and nitrogen [1,2]. In Alcaligenes faecalis, AADH is specific for phenylethylamines, but also reacts to a lesser extent with primary aliphatic amines [1,4]. On the other hand, MADH is highly specific for smaller amines such as methylamine and ethylamine [3]. Both enzymes exhibit an α2β2 heterotetrameric structure (α molecular mass 40 kD, β 12 kD), with each β-subunit possessing a covalently bound redox-active tryptophan tryptophylquinone (TTQ) cofactor [4]. The oxidative deamination reaction proceeds in two steps. In the reductive half-reaction, the TTQ is reduced by substrate leading to incorporation of the substrate-derived amino group into the TTQ cofactor, resulting in conversion of the TTQ from a quinone to N-quinol form (Fig 1) [4,5]. In the oxidative half-reaction, the TTQ cofactor is subsequently reoxidized by electron transfer to either the periplasmic type I blue copper protein azurin (AADH) or amicyanin (MADH) [6,7].

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Recently, we reported the crystal structures of key intermediates in both the AADH reductive and oxidative half-reactions [8,9]. We have shown that substrate oxidation is dominated by H-tunneling (intermediate IIIa to IVa in Fig. 1) and occurs via a multi-step mechanism involving several proton transfers (IVa to Va) followed by hydrolysis of the Schiff base-reduced TTQ adduct (Vla to VIIa). This results in formation of a S-carbinolamine-TTQ-adduct (VIIa) that is relatively stable. Product release is likely preceded by TTQ oxidation. A significant structural rearrangement is seen in the catalytic subunit upon formation of the N-quinol form [9]. This conformational rearrangement influences directly the structure of the AADH:azurin electron transfer complex, and also introduces a water molecule close to the TTQ O7 hydroxyl group where it can serve as the initial proton acceptor from the O7 hydroxyl group during the oxidative half-reaction similar to that we have proposed previously [9]. Crystals of the oxidized amide-AADH adduct (intermediate IIIb) were produced by soaking the form A crystals in a stabilizing solution containing 25% PEG MME 2K, 120mM ammonium sulphate, 100mM sodium cacodylate, pH 7.5 and 1mM phenylacetaldehyde for 30 min. Crystals of the AADH TTQ TTQ adduct crystals for 5 min in the stabilizing solution supplemented with 50 mM potassium ferrocyanide. Crystals containing tryptamine-derivatized adducts of AADH (intermediate Vc) were produced by soaking the form A crystals in a stabilizing solution containing 25% PEG MME 2K, 120mM ammonium sulphate, 100mM sodium cacodylate, pH 7.5 and 1mM phenylacetaldehyde for 30 min. Crystals of the AADH TTQ TTQ adduct crystals for 5 min in the stabilizing solution supplemented with 50 mM potassium ferrocyanide. Crystals containing tryptamine-derivatized adducts of AADH (intermediate Vc) were produced by soaking the form A crystals in a stabilizing solution containing 25% PEG MME 2K, 120mM ammonium sulphate, 100mM sodium cacodylate, pH 7.5 and 1mM phenylacetaldehyde for 30 min. Crystals of the AADH TTQ TTQ adduct crystals for 5 min in the stabilizing solution supplemented with 50 mM potassium ferrocyanide. Crystals containing tryptamine-derivatized adducts of AADH (intermediate Vc) were produced by soaking the form A crystals in a stabilizing solution containing 25% PEG MME 2K, 120mM ammonium sulphate, 100mM sodium cacodylate, pH 7.5 and 1mM phenylacetaldehyde for 30 min. Crystals of the AADH TTQ TTQ adduct crystals for 5 min in the stabilizing solution supplemented with 50 mM potassium ferrocyanide. Crystals containing tryptamine-derivatized amide intermediates were obtained by exposure of anaerobically-grown form A AADH:tryptamine co-crystals to atmospheric oxygen for several weeks.

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Materials. BisTris propane buffer, DCPIP (2,6-dichlorophenol indophenol; sodium salt), PES (phenazine ethosulfate; N-ethylbenzopyrazine ethyl sulfate salt), β-phenylethylamine, tryptamine, benzylamine, phenylacetaldehyde, formaldehyde and indole-3-acetaldehyde-sodium bisulfite addition compound were obtained from Sigma. Deuterated benzylamine HCl (C$_6$D$_5$CD$_2$CD$_2$NH$_2$ HCl, 99.6%) and deuterated phenylethylamine (C$_6$D$_5$CD$_2$CD$_2$NH$_2$, 98%) were from CDN Isotopes. The chemical purity of the deuterated reagents was determined to be > 98% by high performance liquid chromatography, NMR, and gas chromatography, by the suppliers.

AADH crystallisation and crystal preparation. AADH was purified and crystallised as described previously [8]. Crystals of the AADH R-phenylaminoethanol TTQ adduct (intermediate IIIb) were produced by soaking the form A crystals in a stabilizing solution containing 25% PEG MME 2K, 120mM ammonium sulphate, 100mM sodium cacodylate, pH 7.5 and 1mM phenylacetaldehyde for 30 min. Crystals of the phenylacetic acid and formamide adducts of AADH (intermediate Vc) were produced by soaking the form A crystals in a stabilizing solution that included either 2 mM phenylacetaldehyde or 5 mM formaldehyde for 36 hours. Crystals of the oxidized amide-AADH adducts were obtained by soaking the amide-AADH adduct crystals for 5 min in the stabilizing solution supplemented with 50 mM potassium ferrocyanide. Crystals containing tryptamine-derivatized amide intermediates were obtained by exposure of anaerobically-grown form A AADH:tryptamine co-crystals to atmospheric oxygen for several weeks.

Data collection and structure determination. Diffraction data were collected at cryogenic...
temperatures on ESRF stations ID14-1 and 14-2 (Grenoble, France) and DESY-EMBL station X11 (Hamburg, Germany). All data were processed and scaled using the DENZO/SCALEPACK package [Table 1]. Model building and refinement were carried out using the programs TURBO-FRODO [12] and REFMAC [13]. High resolution Fourier difference maps unambiguously allowed identification of the species present in the active sites. In all models, hydrogen atoms were included at the last stages of refinement in their riding positions, as implemented in REFMAC.

Purification of enzymes. AADH was purified from Alcaligenes faecalis IFO 14479 as described previously [14]. MADH was purified from Methylophilus methylotrophus (sp. W 3A1) as described [15]. Prior to use in kinetic studies, enzymes were reoxidized with potassium ferricyanide and exchanged into 10 mM BisTris propane buffer, pH 7.5, by gel exclusion chromatography. AADH concentration was determined using an extinction coefficient of 27,600 M⁻¹ cm⁻¹ at 433 nm [5]. MADH concentration was determined using an extinction coefficient of 25,200 M⁻¹ cm⁻¹ at 440 nm [15].

Aldehyde-dependent reduction of AADH and MADH. Aldehydes were added to a 1 ml solution containing 5 µM oxidized AADH or MADH, 10 mM BisTris propane buffer, pH 7.5 and 50 mM ammonium sulfate (unless stated otherwise). Following addition of aldehyde, spectra were recorded for at least 24 hours using a Varian Cary 50 UV-visible spectrophotometer. Spectra were analyzed and intermediates of the reaction identified by global analysis and numerical integration methods using SPECFIT/32TM software.

Steady-state analysis. Steady-state kinetic measurements were performed with a 1 cm light path in 10 mM BisTris propane buffer, pH 7.5, at 25 °C in a total volume of 1 ml. Enzyme activity was measured using a PES/DCPIP coupled assay system [14]. Reduction of DCPIP was followed at 600 nm using a Perkin-Elmer Lambda 5 UV-visible spectrophotometer. Reaction mixtures with the enzyme pre-incubated in 1 mM phenylacetaldehyde and 50 mM ammonium sulphate typically contained 25 mM (for β-phenylethylamine and tryptamine dependent reactions) or 50 mM AADH (for benzylamine dependent reactions), 0.04 mM DCPIP, 5 mM PES and 500 µM substrate. MADH reactions with formaldehyde typically contained 300 mM MADH. Initial velocity was expressed as µmol product formed per µmol enzyme per second using a molar absorption coefficient at 600 nm of 22,000 M⁻¹ cm⁻¹ for DCPIP [16].

Stopped-flow kinetic analysis. Rapid kinetic studies were performed using an Applied Photophysics SX.18MV stopped-flow spectrophotometer. Experiments were performed by rapid mixing of oxidized AADH or MADH in 10 mM BisTris propane buffer, pH 7.5, containing 50 mM ammonium sulfate, with various concentrations of aldehydes, at 25 °C. Absorbance changes were followed at 456 or 481 nm for AADH and MADH, respectively. Data were analyzed by non-linear least squares regression analysis on an Acorn RISC PC using Spectrakinetics software (Applied Photophysics). For each reaction, at least three replicate measurements were collected and averaged, each containing 1000 data points. Absorbance changes were monophasic, and observed rates were obtained by fitting to the standard single exponential expression.

Computational simulations of the AADH/R-phenylaminoethanol adduct. Computational studies of the AADH/R-phenylaminoethanol derived iminium adduct (IIIb; Fig. 1) were performed based on the corresponding crystal structure, using methods described previously for the AADH/tryptamine system [8, 17]. The final QM/MM solvated system comprised a 25Å radius sphere centered on the substrate NT iminium atom (see Supplementary Scheme 1) – containing 7797 atoms, including 3723 waters. The simulations were performed using CHARMM [11], with the PM3 method used to describe the QM region (comprising 62 atoms) and the MM region described using the CHARMM22 all-hydrogen force field [18] for protein residues with TIP3 [19] type water molecules. The generalized hybrid orbital (GHO) [20] treatment was applied to link the QM and MM regions where covalent bonds exist – three boundary QM/MM atoms were used in the model, positioned at the Cα carbon atoms of Asp128β and of the two modified tryptophans forming the TTQ cofactor. The system was energy minimized, and then equilibrated using 30ps of PM3/CHARMM22 stochastic boundary molecular dynamics simulation [21] (β=300 K), followed by a production run of 60 ps, using a 1 fs time step and a non-bonded cutoff of 13 Å. Structures were saved every 0.5 ps for subsequent analysis.

A potential energy profile for the proton transfer reaction (IIIb to IVb) was calculated starting from the equilibrated structure. A reaction coordinate,
Results and Discussion

Atomic resolution structure of a novel intermediate following the reductive half-reaction with tryptamine. During the reductive half-reaction of AADH with tryptamine, the final S-carbinolamine-reduced TTQ adduct (VIIa; Fig. 1) is highly stable under anaerobic conditions [8]. We therefore sought to establish if oxidation of the cofactor leads to product dissociation. This involved exposing anaerobically produced crystals of intermediate VIIa to oxygen in the absence of free substrate to allow for slow oxidation of AADH by oxygen. It was expected that the AADH structure would return to the oxidized resting state following oxidation and product release. However, even after an exposure of several weeks, crystals remained colourless rather than return to the dark-green colour as observed for oxidized AADH crystals. Data to atomic resolution (1.05 Å) were collected from these oxygen exposed intermediate VIIa crystals, revealing a distinct and novel intermediate in both of the active sites present in the asymmetric unit (PDB code 2OIZ, Fig. 2a,b). Given the high resolution obtained, observed bond lengths and angles were used to unambiguously identify the intermediate present in the first β subunit (denoted chain H) as indol-3-acetamide covalently bound to the TTQ (Fig. 2a). The carbonyl oxygen–carbon bond is bent slightly out of plane with the TTQ system and points towards Asp128β O1. The amide carbonyl oxygen–Asp128β O1 distance (2.5 Å), as well as the electron density and refined bond lengths for Asp128β, indicate that Asp128β O1 is protonated. The overall conformation of the chain H β subunit is similar to the N-quinol AADH structure [9] with a partially occupied water molecule positioned near the TTQ O7 atom, indicating that the TTQ is predominantly in the reduced state [9]. In contrast, the second β subunit (denoted chain D) remains mainly in the oxidized AADH conformation, presumably as a consequence of crystal packing contacts as observed previously [9]. In this subunit, the active site electron density is again consistent with nearly full occupancy of the indol-3-acetamide adduct, but in two distinct conformations. The first conformation (accounting for ~30% of the active site electron density) is similar to that observed in the chain H β subunit, while the second conformation (~40% of the electron density) positions the amide carbonyl oxygen within hydrogen bonding distance of Asp128β O2 and Thr172β (Fig. 2b). The latter conformation is accommodated in the narrow active site tunnel by rotation of the TTQ along the Trp109β Cγ-Cβ bond, turning the C1 group away from Asp128β. In this case, both the C1–NT as well as the amide carbonyl oxygen–carbon bond are significantly out of plane in comparison to the TTQ, a conformation that is likely only accessible for the reduced amide adduct (see below). The remainder of the electron density corresponds to the uncomplexed quinone state (i.e. resting state) of the enzyme. Given the presence of multiple species in this active site, the protonation state of Asp128β cannot be assessed.

Reaction with carbinolamines leads to formation of amide-TTQ covalent adducts. Two alternative mechanisms can be considered that lead to formation of an amide-TTQ adduct during the reaction of AADH with aromatic amines. The first putative mechanism assumes that, following oxidation of the S-carbinolamine species VIIa (observed during the reductive half-reaction with amines [8]), the enzyme succeeds in oxidizing the bound S-carbinolamine via a second H-transfer rather than releasing the product. However, in contrast to the first H-transfer, Asp128β is incorrectly positioned to act as proton acceptor, given that the remaining proton is positioned on the opposite side of C1. In the second putative
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mechanism, the aldehyde product is released from the enzyme following oxidation of the S-carbinolamine-TTQ adduct VIIa (e.g. as proposed in [8,9]). In the presence of ammonia, the product aldehyde can form a (racemic) carbinolamine as part of the aldehyde/iminium equilibrium [22], and this carbinolamine can potentially act as substrate for the enzyme. While attack of the carbinolamine can potentially act as substrate for the aldehyde/iminium equilibrium [22], and this (racemic) carbinolamine as part of the presence of ammonia, the product aldehyde can form a TTQ adduct VIIa (e.g. as proposed in [8,9]). In the enzyme following oxidation of the mechanism, the aldehyde product is released from the acetamide-TTQ adduct structure in chain H in both sites, occupying a position equivalent to that of indol-3-amine oxidation (see [8] and Figs 1, 3). We have been able to reject the first mechanism by carefully measuring the number of electrons released per amine substrate molecule under standard assay conditions (two in all cases tested, data not shown) and by the fact that amide formation and hydrolysis are exceedingly slow (see below). Consequently, amide formation and hydrolysis cannot be an integral part of the steady-state mechanism for reaction with amines [14, 23]. We have demonstrated that soaking oxidized crystals of AADH with phenylacetaldehyde and ammonia for prolonged periods leads to AADH reduction. The corresponding crystal structure (PDB code 2I0T, to 1.4 Å, Fig. 2c) reveals a phenylacetamide-TTQ adduct in both active sites, occupying a position equivalent to that of indol-3-acetamide-TTQ adduct structure in chain H in both β subunits. This demonstrates that, under crystallisation conditions, aromatic amide-reduced TTQ adducts are formed following reaction with the R-carbinolamine.

Slow reduction of AADH during incubation with aldehydes in the presence of ammonia. To investigate whether the reaction of AADH with carbinolamines occurs in solution, AADH was incubated with phenylacetaldehyde in both the presence and absence of ammonia under aerobic conditions. No significant changes to the absorption spectrum were observed during prolonged incubation with 1 mM phenylacetaldehyde in the absence of ammonia (see supplementary Fig. 1S). However, in the presence of high concentrations of ammonium sulfate (50–100 mM, where the reaction rate is independent of ammonia concentrations see Fig. 1S) a rapid red shift in the spectrum was observed upon addition of 1 mM phenylacetaldehyde (Fig. 4a,b). This spectral change occurred at ~30 s and was essentially independent of substrate concentration used for stopped-flow analysis (Fig. 4c). Following this rapid shift, a slow decay over ~24–36 h yielded a species with absorption maxima at 417 and 587 nm. Absorbance changes representing enzyme reduction at 464 nm can be fitted to a single exponential expression resulting in a rate constant for reduction, k_{red}, of ~0.00013 s. Similar to reactions with phenylacetaldehyde, a very slow reduction of the TTQ center is observed when AADH is incubated with 10 mM formaldehyde in the presence of ammonium (Fig. 4d). However, in this case, no significant red shift is observed. Since the red shift in the absorption spectrum is only observed during reaction with the aromatic phenylacetaldehyde (the corresponding carbinolamines for which AADH presumably has high affinity) we posit this represents accumulation of a covalent TTQ adduct of the derived carbinolamine (intermediate IIIb, see Fig. 3) prior to TTQ reduction.

Crystal structure of the R-carbinolamine TTQ adduct. As predicted, soaking of AADH crystals for a brief period (~3 mins) in phenylacetaldehyde in the presence of ammonia leads to formation of an iminium-oxidized TTQ adduct (PDB code 2I0T, Fig. 2d). The high resolution electron density obtained (1.35 Å) clearly reveals stoichiometric conversion to the R-carbinolamine-derived iminium-TTQ adduct (intermediate IIIb, see Fig 3). As with the S-carbinolamine-reduced TTQ adduct observed previously [8] (intermediate VIIa; Fig 1), the hydroxyl group of IIIb is within hydrogen bonding distance of Asp128O1 (2.5 Å) and the backbone amide nitrogen of Trp160β (3.0 Å). C1 to Asp128β distances of 3.6 Å and 3.8 Å for O1 and O2, respectively, are observed for IIIb. That AADH rapidly reacts with R-phenylaminoethanol to form IIIb is consistent with the red-shift observed in absorption spectroscopy during initial stages of the reaction of AADH with the racemic carbinolamine mixture. It also indicates that TTQ reduction by R-carbinolamine is limited by H-transfer to Asp128β rather than formation of the iminium-TTQ adduct. However, no significant red-shift is observed during the reaction of AADH with primary amines, where H-transfer is also rate-limiting [23]. This is consistent with the idea of no significant accumulation of a covalent TTQ intermediate prior to H-transfer in the reaction of AADH with primary amines – a possible explanation is that the equilibrium between the non-covalent enzyme-substrate complex and intermediate IIIa is poised towards the former. In contrast, the additional hydrogen bonds formed between the enzyme and the C1-OH hydroxyl group of IIIb, are likely responsible for shifting the equilibrium preceding
the H-transfer step towards the covalent enzyme-substrate complex.

In the structure of intermediate IIIb (Fig. 3), a partially occupied water molecule immediately adjacent to the TTQ, and within hydrogen bonding distance of both Asp128β O2 and the backbone carbonyl oxygen of Tyr126β, is observed in both active sites (water W1, Fig 5b). This water molecule is not observed in any of the previously determined AADH-complex structures obtained by reaction with primary amines [8,9]. It is in close contact with the TTQ C6 atom, suggesting that it is derived from dehydration of the transient carbinolamine species required to form IIIb. It is also ideally placed for an Asp128β O2 catalysed attack on the TTQ C6 of IIIb to form the transient carbinolamine species in the reverse reaction. No water molecule is observed at this position for either primary amine- or carbinolamine-derived intermediates that occur after the substrate C–H bond cleavage step. However, in the majority of these intermediates, a minor rearrangement in protein structure, as a consequence of TTQ reduction, has instead introduced a (partially occupied) water molecule near the TTQ O7 atom (see Fig. 5b and [9]).

The rate of reduction of the TTQ center by R-phenylaminoethanol is decreased by ~10-fold when compared with the reduction rate with primary amines [8,14,23]. This is despite the iminium-TTQ adduct (IIb) being the predominant species in solution on mixing R-phenylaminoethanol with AADH, implying that the geometry for C–H bond cleavage in IIIb is sub-optimal (e.g. geometry IIIb-A). With primary amines such as tryptamine, the H-Asp128β O2 separation decreases from ~2.7 Å to ~1.6 Å followed by proton tunneling over a distance of ~0.6 Å through the barrier in the bond cleavage step [8]. The experimentally deduced free energy of activation ~12.7 kcal mol⁻¹ and k ~3500 s⁻¹ agrees well with those calculated for this process (13.2 kcal mol⁻¹ and ~1500 s⁻¹, respectively [8]; a more recent study calculates these as 9.4 kcal mol⁻¹ and ~10⁶ s⁻¹, respectively [17]). A significant decrease in the reduction rate is predicted to occur in the absence of tunneling (i.e. for classical transfer [8, 17]). The crystal structure of intermediate IIIb indicates that the initial H-Asp128β O2 distance is ~3.7 Å (i.e. substantially larger than 2.7 Å with tryptamine). Thus, a significant rotation along the NT-C1 bond (Fig. 5a) is required to achieve a conformation (e.g. conformation IIIb-B) that is comparable to that proposed for intermediate IIIa formed in reactions with tryptamine [8]. This rotation also requires rotations along the substrate C1-C2 and C2-C3 bonds to avoid steric clash between the aromatic substrate moiety and the Phe169β sidechain. These additional conformational changes (e.g. IIIb-A to IIIb-B) – which could also involve reorganization of hydrogen bonding networks involving the hydroxyl group of the R-carbinolamine moiety – likely contribute to the substantially slower rate of C–H bond cleavage seen with carbinolamines compared with primary amines.

**QM/MM simulations of the AADH/R-phenylaminoethanol iminium complex IIIb.** The structure of IIIb-A (W1 present, averaged over the 60 ps production run, Fig. 6a) is in good agreement with the crystal structure (Cα RMSD = 0.37 Å). Importantly, the MD-averaged structure has the substrate moiety in the same conformation as that observed in the crystal structure, with the C2 carbon atom ~90° over the plane of the TTQ ring, and with similar (large) proton donor-acceptor distances: values for d(C1-H1), d(C1-O2) and d(O2-H1) are 1.13 ± 0.03 Å, 4.13 ± 0.28 Å and 3.88 ± 0.35 Å, respectively. For comparison, the modeled AADH/trypamine complex IIIa (a configuration more optimized for tunneling [8]) contains corresponding distances of 1.17 ± 0.04 Å, 3.52 ± 0.20 Å and 2.68 ± 0.22 Å, respectively (this model did not contain W1). When W1 is deleted from IIIb-A, the donor–acceptor distances are shortened only slightly. Therefore, the proton–acceptor distance is still too long for proton transfer, either classically or by tunneling, without the heavy atoms first repositioning. The contribution of selected active site residues to the QM/MM electrostatic interaction at different points along the reaction path with W1 present is given in Table 2. The profile of interactions for IIIb-A is very similar to that of IIIa for the AADH/trypamine system [17], with the main differences occurring in the contribution of Asp84β, Thr172β and W1. The presence of W1 in complex IIIb does not alter significantly the QM/MM interactions of the other residues (see Supplementary Table 1). To investigate the stability of W1 in its crystallographically observed position, the QM/MM dynamics simulations of IIIb-A were extended to a total of 500 ps – W1 remained close to its crystallographic position throughout the trajectory, consistent with its proposed role in catalysis.

A local minimum (corresponding to conformation IIIb-B; Fig. 6a) was also localized in the potential energy surface at Z0 = -0.65 Å (Fig. 6d). It has the H1 proton already oriented towards the acceptor O2, with d(C1-H1) = 1.15 Å, d(C1-O2) = 2.94 Å and d(O2-H1) = 1.85 Å. However, unrestrained MD of this structure led to it reverting to the IIIb-A conformation, illustrating the preference of the
system for IIIb-A. In going from IIIb-A to the “optimal conformation” IIIb-B, most of the reaction path motion corresponds to rearrangement of the substrate phenyl group and C1 and C2 atoms. From conformation IIIb-B to the maximum of the potential energy barrier \((Z_{O2} = 0.15 \text{ Å})\), which can be taken here to approximate to the classical transition state structure, the reaction path still suggests approach of donor and acceptor, but quickly becomes dominated by proton motion (Fig. 6b,d). At the maximum the potential energy barrier \((Z_{O2} = 0.15 \text{ Å})\), values for \(d(C1-H1)\), \(d(C1-O2)\) and \(d(O2-H1)\) are 1.44, 2.77 and 1.34 Å, respectively. Along the path, W1 stays close, and hydrogen bonded, to O2 until after the classical transition state, at \(d(C1-H1)\) = 1.43 Å, \(d(C1-O2)\) = 2.75 Å and \(d(O2-H1)\) = 1.33 Å. This juncture it is not possible to assess the net impact of all the effects on proton tunneling and on the observed KIEs (barrier shape [height/width] and energy of reaction).

As observed previously \([17]\), the hydroxy group of Thr172β has turned away from O2 and points towards O7 in the structure of the reaction product IVb (Fig. 6c). During this process, water W1 also breaks its hydrogen bond with O2. In fact, the length of the hydrogen bond formed with W1 is very similar to those formed with Thr172β \([d(O2-OH2-W1) = 3.58 \pm 0.35 \text{ Å and d(O2-OG1-Thr172β} = 3.55 \pm 0.38 \text{ Å}]\). Reflecting this, the contribution of Thr172β and W1 to the QM/MM energies is essentially the same (Table 2). The substrate C1 hydroxyl group, which remains hydrogen bonded to O1 along the reaction path, also adopts a new conformation during the molecular dynamics simulation of IVb (Fig 6c). We cannot rule out the possibility that the inclusion of thermal energy (i.e. running MD) along the whole reaction path would induce these conformational changes earlier in the reaction. With the hydrogen bonds to W1 and Thr172β broken, the Asp128β carboxylate has rotated in the unrestrained IVb structure (Fig. 6c) with respect to the IIIb conformation. This reorientation, which brings the transferred proton closer to O7, was also observed for the unrestrained product complex in the AADH/tryptamine system \([17]\). However, in that case – in which an equivalent to W1 was absent – we assumed that a local minima found at \(Z_{O2} \sim 0.7 \text{ Å}\) corresponded to the intermediate IVa which, by proton rearrangement involving NT, O7 and O2 (see Fig. 1), would lead to Va. In the light of the possibility of having a water molecule (W1) well positioned in the active site during the reaction, the step from IVb to Vb (and by analogy IVa to Va) could occur directly by proton rearrangement involving only O2 and O7 and mediated by W1 (see Fig. 3). In light of this, it is interesting to note that under steady-state conditions, the leaving group is likely to be ammonia rather than water \([24]\), which could perform a similar role to W1.

Rapid TTQ reduction occurs with indol-3-acetaldehyde. Substantial differences in TTQ reduction rates and corresponding KIE values have been observed for the reaction of AADH with tryptamine compared with β-phenylethylamine (the primary amine most similar to \(R\)-phenylaminoethanol). TTQ reduction is ~80 times more rapid with tryptamine and the KIE value (~55) is substantially inflated compared to the KIE (~12) measured with phenylethylamine \([8, 14, 23]\). The conformation of intermediate IIIa with tryptamine is distinct from – and more optimal for subsequent C-H bond breakage by – the IIIb-A conformation. Indeed, the IIIb-A conformation cannot be populated by the corresponding tryptamine complex as it would lead to severe steric clash of the indole aromatic moiety with Phe169β. To further investigate this issue, we have performed kinetic studies of TTQ reduction with indol-3-acetaldehyde and ammonia to form the corresponding indole-based carbinolamine. In this case, we find that the TTQ reduction rate is significantly increased compared with reactions using phenylethylaldehyde/ammonia \((k_{lim} 0.0148 \text{ s}^{-1} \text{ versus } -0.00013 \text{ s}^{-1} \text{ for indol-3-acetaldehyde and phenyacetaldehyde, respectively; Fig. 7})\). Also, unlike the reaction with phenyacetaldehyde+ammonia, but similar to the reaction with tryptamine, we did not observe a red shift in the spectrum prior to reduction of the TTQ centre. With indol-3-acetaldehyde and ammonia as substrates, this is consistent with the structure of the corresponding IIIb intermediate exclusively adopting conformation IIIb-B as proposed for reaction of AADH with tryptamine \([8]\). Soaking of
AADH crystals with ammonia and indol-3-acetaldehyde leads to relatively rapid formation of the amide adduct Vc (Fig. 3) in both subunits with no detectable accumulation of any other covalent species. The structure of amide adduct Vc (data not shown) has no significant differences from that obtained during the oxidation of crystals that have been soaked with tryptamine (PDB code 2OIZ).

Slow turnover of aromatic carbinolamines by AADH can be observed indirectly. Samples taken at different time intervals from a solution of AADH that is incubating with 1mM phenylacetaldehyde and ammonia have decreasing specific activity with increasing incubation time when assayed with aromatic primary amines under standard conditions (Fig. 8a). This indicates that Vc (Fig 3) formed during the oxidation of carbinolamines by AADH is relatively stable. Unfortunately, aromatic aldehydes react with amides under standard conditions (Fig. 3). This indicates that the Vc (Fig 3) formed during the oxidation of carbinolamines is a dead-end reaction that is not possible. However, full AADH activity (with primary amines as substrate) is regained – for those samples taken from a solution of AADH incubating with phenylacetaldehyde and ammonia – following prolonged incubation in the presence of excess potassium ferricyanide but in the absence of any excess aldehyde (Fig. 8a). This demonstrates, albeit indirectly, that any intermediates formed during the reductive half-reaction with carbinolamines are eventually converted to the carboxylic acid product, with release of the active enzyme. In contrast to the aromatic aldehydes, formaldehyde is compatible with the PES/DCPIP used in steady-state assays of AADH and MADH. Slow turnover of formaldehyde is observed with MADH (Fig. 8b,c), although not with AADH owing to the very low binding affinity for this substrate. However, formaldehyde does reduce AADH in the presence of ammonia (Fig. 4d). The crystal structure (PDB code 2I0R, to 1.4 Å) of an AADH formamide TTQ-adduct was obtained by soaking crystals for 36 h in 5 mM formaldehyde (Fig. 9a). While the structure is essentially identical to the Vc adducts described above, the C1 amide is clearly much more solvent accessible. In following the oxidation of formaldehyde Vc crystals by soaking in potassium ferricyanide for ~5 mins, nearly all electron density corresponding to the formamide adduct is removed (PDB code 2OK6, Fig. 9b). In contrast, similar ferricyanide oxidation of phenylacetamide-AA DH crystals revealed that the protein returns to the oxidized conformation, and that there is no significant difference in the amide occupancy or conformation (PDB code 2OK4). A similar observation is made in the case of indol-3-acetamide TTQ-adduct crystal (PDB code 2OJY), although, in this case, the conformation of the amide in the chain D β subunit is altered from pointing predominantly towards Asp128β O2 to exclusively interacting with Asp128β O1 (a conformation compatible with the presence of a C6=NT double bond). These results suggest that amide hydrolysis preferentially occurs following oxidation of Vc to form Vlb, an intermediate with a significantly more electrophilic C1. Nevertheless, in case of non-formaldehyde derived amides, the hydrolysis rate is severely limited by access of water to a position close to the C1 amide atom.

Conclusions. The further oxidation of the aldehyde product in the presence of ammonium is a previously unforeseen reaction pathway for quinoprotein-catalysed amine oxidation. In contrast to the fast oxidation to the aldehyde rate limited by C-H bond breakage, further oxidation to the carboxylic acid is slow and limited by H-transfer and amide hydrolysis. Given the slow rates observed, it is highly unlikely that the latter pathway supports growth. However, the high similarity with the natural substrates (both in terms of structure and reaction mechanism as established in this work) and inherently slow rates allow us to study the AADH amine oxidation mechanism in greater detail, providing a more detailed model that provides answers to questions regarding the origin of the TTQ O7 proton acceptor and the reasons underpinning the wide range of kinetic parameters observed for distinct primary amines.

Based on our structural and kinetic studies, we postulate a mechanism for carbinolamine oxidation (Fig. 3) that is highly similar to the mechanism described previously for the AADH reductive half-reaction with tryptamine (Fig. 1, [8]). We propose that attack of the R-carbinolamine leads to the R-carbinolamine intermediate IIIb, in which proton abstraction from the substrate C1 atom by the O2 carboxylate oxygen of the catalytic base, Asp128β, leads to the conceptual enol intermediate (IVb). Structural determination of the catalytically competent R-phenylaminoethanol derived iminium-TTQ adduct (IIIb) reveals that this species preferentially occupies a conformation (IIIb-A) that is distinct from the catalytically more optimal conformation we proposed from our previous studies with tryptamine [8]. This is consistent with the substantial reduction in the rate constant for H-transfer rates with phenylaminoethanol as a substrate. In contrast, the reaction rates obtained with the
carbinolamine formed by indol-3-acetaldehyde and ammonia are closer to those obtained with tryptamine—we propose that this is the result of substrate adopting a more optimal conformation in the active site, similar to that postulated for intermediate IIIa with tryptamine substrate [8]. We suggest that these conformational differences observed with different substrates might account for the variation in KIE and reaction rate with distinct amine substrates [8,14,23]. Also, we suggest that intermediate IIIa of the smaller primary amines (e.g. non-indol compounds such as β-phenylethylamine) will need to undergo conformational reorganization (e.g. similar to IIIb-A to IIIb-B) to optimize the geometry for H-transfer. Additionally, our data suggest that in the case of the reaction of AADH with primary amines, covalent intermediates prior to the reduction step do not accumulate to a significant extent (i.e. no red-shift is observed) and are thus not amenable to crystallographic analysis.

Our previous computational analysis of the reaction with tryptamine [8,17] did not take into account the water molecule (W1) that resides close the TTQ C6 and Asp128β in intermediate IIIb (and by analogy possibly in intermediate IIIa). While the net result of all the effects on proton tunneling and on the observed KIE remains to be determined, we speculate this water molecule could function to facilitate consecutive hydrogen transfer steps involved in formation of Vb (and by analogy Va). Furthermore, it is possible that concomitant with protein rearrangement upon TTQ reduction [9], water W1 relocates to the position near O7 (e.g. from W1red to W1ox in Fig. 5b). Given that under steady-state conditions the leaving group during formation of IIIa is proposed to be ammonia (rather than water [24]), it is a distinct possibility that ammonia will function as the proton acceptor required during oxidation.

Our model for amine oxidation by AADH as presented in [8] is thus extended (for both primary amines and carbinolamines, Fig. 3) to include the possibility of conformational heterogeneity for intermediate III as well as the fact that the product water/ammonia of formation of intermediate III fulfills key roles during protein transfers in the reductive half reaction and as TTQ O7 proton acceptor during the oxidative half-reaction.
Figure legends.

**Figure 1.** Schematic overview of the mechanism of amine oxidation by AADH. For clarity, only part of the TTQ cofactor is represented, while the side chain of the different primary amines is indicated by an R. We refer the reader to supplementary scheme 1 for a full description of the substrate-TTQ-enzyme adduct. Intermediates during the reductive half-reaction are numbered by roman numerals in accordance with ref. [8]. Intermediates during the oxidative half-reaction are labelled by letters, with A representing the N-quinol state, B the N-semiquinone state and C the N-quinone state (see also [9]).

**Figure 2.** Stereo view of the distinct intermediates observed during reaction of AADH with aromatic aldehydes in the presence of ammonia. Aldehyde-derived carbon atoms are depicted in yellow while enzyme carbon atoms are depicted in green. Key residues in the active site and the TTQ cofactor are shown, with the sigmaA weighted 2F_o-F_c map contoured at 1.5 σ. For clarity, only panel A has been labelled as all panels are in a similar orientation. **A.** Indol-3-acetamide intermediate Vc observed in the chain H β subunit of air exposed intermediate VIIa crystals. **B.** Amide TTQ adduct Vc showing both conformations observed in the chain D β subunit of air exposed intermediate VIIa crystals. **C.** Phenylacetamide intermediate Vc obtained by reduction with phenylacetaldehyde in the presence of ammonia. **D.** R-carbinolamine adduct IIIb obtained by short soak in the presence of phenylacetaldehyde and ammonia.

**Figure 3.** Schematic overview of the proposed mechanism for AADH R-carbinolamine oxidation. For ease of comparison with the previously proposed amine oxidation mechanism, similar notation is used to Fig. 1. For clarity, only part of the TTQ cofactor is represented, while the side chain of the different R-carbinolamines is indicated by an R. We refer the reader to supplementary scheme 1 for a full description of the substrate-TTQ-enzyme adduct. The active site water molecule (or ammonia in case of a steady state mechanism, see[24]) is denoted W1. Whether a conformational equilibrium between IIIb-A and IIIb-B occurs depends on the nature of the R side chain.

**Figure 4.** Spectral species observed upon incubation of AADH with aldehydes in the presence of ammonium. **A.** Spectral changes observed upon incubation of AADH with phenylacetaldehyde. The initial spectrum (blue) corresponds to enzyme in the presence of 50 mM ammonium sulfate and the absence of phenylacetaldehyde. Following addition of phenylacetaldehyde, a similar – but red-shifted – spectrum is observed (red). This species decays slowly over 24 hours to yield the final spectrum (magenta). Inset, absorbance change representing enzyme reduction at 464 nm [~0.00013 s⁻¹ determined from fitting to the standard single exponential expression]. **B.** Spectral intermediates identified during incubation of AADH with phenylacetaldehyde. Spectral changes accompanying enzyme reduction are as in panel A. Intermediates were identified by global analysis and were best described by a three step kinetic model A (blue, oxidized enzyme) → B (red, assigned to IIIb see Fig. 3) → C (green, assigned to Vc) → D (magenta, assigned to VIb). **C.** Stopped-flow kinetic data for the formation of the AADH red shifted species during reaction with phenylacetaldehyde in presence of ammonium. A monophasic decrease in absorbance was observed (corresponding to the shift in maximum absorbance from 456 to 464 nm) on addition of phenylacetaldehyde. Observed rates were obtained by fitting to the standard single exponential expression. Rate constants (~30 ± 0.8 s⁻¹) were independent of phenylacetaldehyde concentration (investigated range 0.2 – 10 mM). **D.** Spectral changes observed upon incubation of AADH with formaldehyde. The initial spectrum (blue) corresponds to enzyme in the presence of 50 mM ammonium sulfate and the absence of formaldehyde. Unlike with phenylacetaldehyde, no significant red shift is observed on addition of formaldehyde (red). The enzyme slowly reduces as in panel A. Inset, absorbance change representing enzyme reduction at 456 nm.

**Figure 5.** **A.** Comparison of IIIb-A conformation as observed in the crystals (phenylacetaldehyde derived carbon atoms in cyan) with a more optimal configuration (IIIb-B) based on the modelled intermediate IIIa (Fig.1) during tryptamine reduction ([8]; phenylacetaldehyde derived carbon atoms in yellow). Putative hydrogen bonding interactions made between IIIb-B conformation and active site residues are shown by dotted lines. Key active site residues and TTQ cofactor are displayed with green carbons. **B.** Overlay of crystal structures of IIIb-A (with green carbons) and Vc (with cyan carbons) for phenylacetaldehyde and...
ammonia as substrates. The active site water molecule situated close to C6 and O7 is shown as a red sphere (labelled W1ox and W1red, respectively).

**Figure 6.** Representation of the AADH/R-phenylamine-ethanol complex active site for the computationally modelled structures of (A) intermediate IIIb-A ($Z_{O2} = -2.71$ Å) and the more optimal structure IIIb-B at $Z_{O2} = -0.65$ Å (in yellow), (B) structure IIIb-B (yellow) and potential energy maximum ($Z_{O2} = 0.15$ Å, in cyan), and (C) $Z_{O2} = 0.15$ Å (in cyan) and product complex IVb ($Z_{O2} = 2.2$ Å, in orange). The structures for the reactant and the product are averaged over their corresponding molecular dynamics trajectories. The other two are taken from the calculated potential energy profile. D. Potential energy profile (left-hand axis) for proton transfer to O2 for IIIb-A (*i.e.* for formation of IVb) in the presence (black line with crosses) and absence (grey line with squares) of the active site water molecule W1. Distances along the reaction path in the presence of W1 are also shown (right-hand axis): $d(C1-H1)$ solid black line, $d(C1-O2)$ dashed grey line and $d(O2-H1)$ solid grey line. All energies are in kcal mol$^{-1}$ and distances in Å.

**Figure 7.** Kinetic data for the reaction of AADH with indole-3-acetaldehyde in presence of ammonium. A. Stopped-flow kinetic data for the reaction of AADH with indole-3-acetaldehyde. Absorbance changes were followed at 456 nm and observed rates were obtained by fitting to the standard single exponential expression. The fit shown is to the standard hyperbolic expression ($k_{lim}: 0.0148 \pm 0.0002$ s$^{-1}$; $K_d: 0.10 \pm 0.01$ mM). B. Spectral intermediates identified during incubation of AADH with indole-3-acetaldehyde. Spectral changes accompanying enzyme reduction are as in Figure 3D for formaldehyde. Intermediates were identified by global analysis and were best described by a two step kinetic model A (blue, oxidized enzyme) $\rightarrow$ B (green, assigned to Vc) $\rightarrow$ C (magenta, assigned to VIb).

**Figure 8.** A. Steady-state turnover of AADH incubated in phenylacetaldehyde and ammonium. Activity was regained following incubation (for ~48 hours) with excess potassium ferricyanide. Filled triangles, protiated tryptamine dependent activity; filled circles, protiated β-phenylethylamine dependent activity; open circles, deuterated β-phenylethylamine dependent activity; filled squares, protiated benzylamine dependent activity; open squares, deuterated benzylamine dependent activity. Inset, plot of KIE versus time. Filled circles and squares, KIE for β-phenylethylamine and benzylamine, respectively. B. Steady-state turnover of formaldehyde by MADH. Filled circles, formaldehyde dependent activity; open circles, background reaction (in the absence of formaldehyde); filled triangles, background reaction (in the absence of MADH). C. Stopped-flow kinetic data for the reaction of MADH with formaldehyde. Absorbance changes were followed at 481 nm rather than 440 nm (owing to a shift in the oxidized MADH spectrum in the presence of ammonium). Observed rates were obtained by fitting to the standard single exponential expression.

**Figure 9.** Stereo view of the distinct intermediates observed during reaction of AADH with formaldehyde in presence of ammonia. The formaldehyde derived carbon atom is depicted in yellow while enzyme carbon atoms are depicted in green. Key residues in the active site as well as the TTQ cofactor are shown with the sigmaA weighted 2Fo-Fc map contoured at 1.5 $\sigma$. For clarity, only panel A has been labelled. A. Formamide intermediate Vc obtained by reduction with formaldehyde in the presence of ammonia. B. Electron density obtained following a short soak of the formaldehyde Vc crystals in ferrocyanide.
References

Table 1.

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1 Rmerge = Σhkl | I_í-I_m | / ΣhklI_m, where I_m is the mean intensity of the reflection. Numbers in parentheses indicate values for the highest resolution shell.
2 Rcryst = Σhkl (|F_í|-|F_calc|) / Σhkl|F_í|
3 R_free was calculated on 5% of the data omitted at random.
Table 2. Contribution to the QM/MM electrostatic interaction energy\(^a\) for selected residues in the active site from analysis of the PM3-CHARMM22 calculations with water W1 present

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\(^a\)Negative energies indicate a stabilizing contribution and positive energies a destabilizing contribution. All energies are in kcal mol\(^{-1}\), numbers in parentheses indicate the average residue contribution across the ensemble of structures in the trajectories generated for reactant and product, numbers in square parentheses and italics are the average contributions for the complex IIIa in the AADH/tryptamine system [18], and numbers in bold are the respective energy relative to that in the reactant complex.

\(^b\)Residue Asp84\(\beta\) interacts with both the substrate and the TTQ co-factor. To provide a more detailed description of the interactions of this residue along the reaction coordinate, this residue is divided into three separate electrostatic groups: the carboxylate sidechain (CO\(_2\)\(^-\)), the NH group of the backbone (NH\(^-\)) and the carbonyl group of the backbone (C=O).
Carbinolamine reaction with AADH

Figure 1
Carbinolamine reaction with AADH

Figure 2 a,b,c,d
Carbinolamine reaction with AADH

Figure 3
Carbinolamine reaction with AADH

Figure 4 a,b,c,d
Carbinolamine reaction with AADH

Figure 5 a,b
Carbinolamine reaction with AADH

Figure 6 a, b, c, d
Carbinolamine reaction with AADH

Figure 7 a,b

A

![Graph showing the relationship between [Indole-3-acetaldehyde] (mM) and $k_{obs}$ (s$^{-1}$).]

B

![Graph showing absorbance against wavelength (nm).]
Carbinolamine reaction with AADH

Figure 8 a,b,c

A

B

C

Figure 8 a,b,c
Figure 9 a,b

[Image of molecular structures with labels W109β, W160β, D128β, O2, T172β]
Supplementary Scheme 1. Schematic of the active site of iminoquinone (IIIb), illustrating the QM/MM partition used in the calculations. The QM region is in black and the surrounding MM region in grey.
**Supplementary Table 1.** Contributions to the QM/MM electrostatic (electr) and van der Waals (vdw) interaction energies for selective residues in the active site from analysis of the PM3-CHARMM MD calculations with water W1 deleted.

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</table>

Negative energies indicate a stabilizing contribution and positive energies a destabilizing contribution. All energies are in kcal mol$^{-1}$, numbers in parentheses indicate van der Waals energies from the reaction path with W1 present.
Fig. 1S Ammonia dependence of the red-shifted species in AADH and MADH. Panel A, Stopped-flow kinetic data for the formation of the AADH red-shifted species during reaction with phenylacetaldehyde in presence of ammonia. Reactions were performed using 5 \( \mu \)M AADH (reaction cell concentration) in 10 mM BisTris propane buffer with 1 mM phenylacetaldehyde, pH 7.5, at 25 °C. A monophasic decrease in absorbance was observed (corresponding to the shift in maximum absorbance from 456 to 464 nm) on addition of phenylacetaldehyde. Observed rates were obtained by fitting to the standard single exponential expression. Observed rate constants (\( \sim 29 \pm 0.9 \text{ s}^{-1} \)) were independent of ammonia concentration (investigated range 10 – 100 mM), and similar to kinetic data shown in Fig. 4c. It was not possible to measure a shift in maximum absorbance with less than 10 mM ammonium sulphate (as indicated). Panel B, Steady-state turnover of formaldehyde by MADH as a function of ammonia concentration. Assays were performed as described in Materials and Methods with 300 nM MADH in 10 mM BisTris propane buffer, pH 7.5, and 10 mM formaldehyde, at 25 °C. Turnover numbers are consistent with those reported in Fig. 8b.
New insights into the reductive half-reaction mechanism of aromatic amine dehydrogenase revealed by reaction with carbinolamine substrates
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