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ADP Competes with FAD Binding in Putrescine Oxidase*

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Putrescine oxidase (PuO, EC 1.4.3.10) is a flavin-containing amine oxidase involved in polyamine degradation. It catalyzes the oxidative deamination of putrescine (1,4-diaminobutane) into 4-aminobutanal with concomitant reduction of oxygen to hydrogen peroxide (Scheme 1). In addition, the oxidase also accepts other polyamines, like spermine and spermidine, which are involved in cell growth and differentiation and interact with nucleic acids (1, 2).

PuO was first reported in the 1960s when it was isolated from Micrococcus rubens (3, 4). This enzyme (PuO_{Mr}) is a soluble homodimeric protein and is unique among flavoprotein oxidases in that the isolated enzyme appears to contain only one noncovalently bound FAD molecule per dimeric protein (5). PuO_{Mr} was heterologously expressed in Escherichia coli (6) and is applied as a diagnostic enzyme for the detection of di- and polyamines (7, 8). Increased levels of polyamines in tissue and body fluids are related to cancer and thus can be used as tumor markers (9). PuO_{Mr} can also be used to monitor food freshness by detecting polyamines (10).

Recently we identified a novel putrescine oxidase from the actinomycete Rhodococcus erythropolis (PuO_{Rh}) (11). PuO_{Rh} shares 67% sequence identity with PuO_{Mr} and displays similar catalytic properties: it is highly specific for putrescine and is effectively inhibited by aliphatic monoamines and short di- and polyamines. Like PuO_{Mr}, PuO_{Rh} is a homodimer of about 100 kDa and, interestingly, also contains only one mol of noncovalently bound FAD per mol of dimeric protein. Such a low flavin cofactor occupancy is remarkable as inspection of the PuO sequences suggests that each monomer contains a Rossmann-fold domain capable of FAD binding.

To explain the reason of the unusually low FAD/protein ratio in PuO, more detailed structural information would be highly informative. Unfortunately, no crystal structure is available for a PuO. However, for the sequence-related human monoamine oxidase B (MAO-B), the crystal structure has been solved (12). MAO-B is a flavoprotein oxidase located at the outer mitochondrial membrane (13) and is involved in the oxidation of neurotransmitters (14). The human oxidase shares 32% sequence identity with PuO_{Rh} and catalyzes the oxidative deamination of aromatic monoamines like phenylethylamine and benzylamine. Based on the MAO-B structure we have constructed a structural model for PuO_{Rh} (11). By inspection of the model and subsequent site-directed mutagenesis studies, we have verified that one specific active site residue (Glu-324) in PuO_{Rh} is crucial for its preference for aliphatic diamine substrates. Like PuO_{Mr} and PuO_{Rh}, MAO-B is a homodimeric protein, but in contrast to both bacterial oxidases, each MAO-B subunit contains one covalently bound FAD cofactor. The FAD in MAO-B is covalently attached to a cysteine residue (Cys-397) via a C8α-linkage.

In this report, we describe our findings concerning a more detailed analysis of cofactor binding in PuO_{Rh}. The data reveal that ADP is competing with FAD binding. Furthermore, we found that by merely mutating an alanine residue into a cysteine at an analogous position with respect to Cys-397 in PuO_{Rh}, the enzyme reacts like PuO_{Mr} and displays proper ADP binding. Our results indicate that ADP can compete with FAD binding in PuO_{Rh}.
MAO-B, PuO<sub>Rh</sub> is able to form a covalent cysteine-FAD linkage. This is the first time that a noncovalent flavoprotein is converted to a mutant enzyme that forms a covalent flavin-protein bond similar to naturally occurring flavin-protein bonds. These findings suggest that covalent flavinylation may have evolved to prevent binding of ADP or related cellular compounds.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Oligonucleotides were from Sigma-Aldrich. Pfu DNA polymerase was from Stratagene. E. coli TOP10 electrocompetent cells were purchased from Invitrogen. E. coli ORIGAMI was obtained from NOVAGEN. Horseradish peroxidase was from Fluka. All other chemicals were of analytical grade. Constructs were sequenced at GATC Biotech (Kostanz, Germany).

**Construction of PuO<sub>Rh</sub>A394C**—To introduce covalently linked FAD, the mutant PuO<sub>Rh</sub>A394C was constructed by QuikChange PCR (Invitrogen). This mutation was inserted by replacing the GCC codon by a TGT codon using the following primers: puA394C<sub>fw</sub>: 5′-GGACCGCGGTGTACCGCGCGAGC and puA394C<sub>rv</sub>: 5′-GCTCGCCGGTAAACACCGC GGGTTC (mutated codon underlined). This resulted in pBAD-PuO<sub>Rh</sub>A394C.

**Expression and Purification of Wild-type PuO<sub>Rh</sub> and PuO<sub>Rh</sub>A394C**—Expression of PuO<sub>Rh</sub> and PuO<sub>Rh</sub>A394C in E. coli TOP10 was performed as described before (11). For expression of PuO<sub>Rh</sub>, PuO<sub>Rh</sub>A394C in E. coli ORIGAMI, these cells were transformed with pBAD-PuO<sub>Rh</sub>A394C and cultivated in TB medium supplemented with 50 μg/ml ampicillin, 15 μg/ml kanamycin, 12.5 μg/ml tetracycline, and 0.4% (w/v)L-(+)-arabinose at 30 °C for 24 h. Purification of wild-type PuO<sub>Rh</sub> and PuO<sub>Rh</sub>A394C was performed as described before (11).

**ESI-MS Experiments**—For nanoflow ESI-MS analysis of wild-type PuO<sub>Rh</sub> and PuO<sub>Rh</sub>A394C, enzyme samples in 50 mM ammonium acetate, pH 6.8, were used (final enzyme concentration 1 μM). For analysis under denaturing conditions, enzyme samples were diluted in 50% acetonitrile and 0.2% formic acid (final enzyme concentration ~2 μM). Analysis was performed on a LC-T nanoflow ESI orthogonal TOF mass spectrometer (Micro-mass, Manchester, UK). All measurements were performed by operating in the positive ion mode and by using gold-coated needles, made from borosilicate glass capillaries (Kwik-Fil; World Precision Instruments, Sarasota) on a P-97 puller (from Sutter Instruments, Novato). The needles were coated with a gold layer, which was performed by an Edwards Scancoat six Pirani 501 sputter coater (Edwards Laboratories, Milpitas). Mass spectra were calibrated using cesium iodide in water (25 mg/ml).

**Preparation of apoPuO<sub>Rh</sub> and FAD Reconstitution**—ApoPuO<sub>Rh</sub> was prepared at room temperature based on the hydrophobic interaction chromatography method (15).

**Analytical Methods**—All absorbance spectra were recorded in 50 mM Tris-HCl pH 8.0, at 25 °C on a PerkinElmer Lambda Bio40 spectrophotometer. Measurement of PuO<sub>Rh</sub>A394C activity was done by using a coupled horseradish peroxidase assay as described before (11).

**RESULTS**

**Structural Properties of Wild-type PuO<sub>Rh</sub>**—To determine the exact molecular mass of wild-type PuO<sub>Rh</sub>, we analyzed the purified protein (2.0 μM) by nanoflow ESI-MS. Under denaturing conditions (50% acetonitrile, 0.2% formic acid), the enzyme is highly charged, which enables accurate mass determination. The mass spectrum of the denatured wild-type enzyme (Fig. 1A) shows one major peak corresponding to a mass of 49,254 ± 14 Da. This value agrees well with the theoretically calculated protein mass based on the primary PuO<sub>Rh</sub> sequence minus the N-terminal methionine residue (49,244 Da). The determined mass spectrum of unfolded PuO<sub>Rh</sub> confirms that PuO<sub>Rh</sub> contains noncovalently bound FAD: besides for a species that matches the primary sequence of PuO<sub>Rh</sub>, a species is also observed with a mass of 785 Da, which corresponds to one FAD molecule.

By gel filtration experiments, it was already established that PuO<sub>Rh</sub> is mainly present as a homodimer of about 100 kDa (11). However, it was found that only 1 mol of FAD was bound per mol of dimer. To further investigate the oligomeric state and cofactor content of PuO<sub>Rh</sub>, we analyzed the enzyme in its native form by native ESI-MS (16). The resulting spectrum reveals only species corresponding to dimeric forms of PuO<sub>Rh</sub> while no monomers or other oligomeric states were observed (Fig. 1B). Three major dimeric species can be recognized: (∗, 99,358 Da; ○, 99,714 Da, and △, 100,074 Da). Minor amounts of some other PuO species (3 kDa larger) are also present because of translation of PuO by E. coli using an alternative stop codon (11). The three major and distinct dimeric forms are likely the result of variations in incorporation of the FAD cofactor in the monomers.

A closer inspection of the dimeric species indicates that 36% is present as a holo dimer, containing two noncovalently bound FAD molecules (∗, as its mass corresponds well to the combined mass of 2 apomonomers and 2 FAD molecules (785 Da): 100,074 Da versus expected 100,078 Da. The other two dimeric species, ○ (21%) and ○ (43%), do not correspond to any apodimeric form (one or no FAD bound) as the differences in mass between the three dimers are not consistent with FAD (360 Da instead of 785 Da). These observations suggest binding of only the adenosine 5′-diphosphate (ADP) (427 Da) part of the FAD cofactor in these PuO<sub>Rh</sub> dimers. In fact, the species ○ (99,358 Da) corresponds nicely with the calculated mass of dimeric PuO<sub>Rh</sub>, while species ○ (99,714 Da) matches dimeric PuO<sub>Rh</sub>, containing one FAD and one ADP (99,721 Da).

**FAD Reconstitution of PuO<sub>Rh</sub>**—Because we observed that the ratio between the three different dimeric species was not constant for several batches of PuO<sub>Rh</sub>, we tried to saturate PuO<sub>Rh</sub> with FAD by incubating it with an excess of FAD. For this, 1.0...
FIGURE 1. Mass spectra of wild-type PuORh and PuORh A394C. Wild-type PuORh under denaturing conditions (A) and in its native state (B). PuORh A394C under denaturing conditions (C) and in its native state (D). Observed enzyme species are indicated with symbols and corresponding masses (open and closed symbols for PuORh and PuORh A394C, respectively).
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μM of PuORh was incubated with a 20-fold excess of FAD at room temperature for 6 h and subsequently incubated at 4 °C for another 12 h. ESI-MS analysis of this sample showed no change in the ratio of FAD/ADP binding. Incubation of PuORh with an excess of ADP also did not change the ratio in favor of the ADP-containing species. The addition of 1.0 mM ADP or FAD did also not influence the activity of PuO.

Another method for the preparation of apoflavoproteins is the use of hydrophobic interaction chromatography (15). To obtain apoPuORh we used a phenyl-Sepharose column. In the presence of 1 M KBr did not result in any FAD release. Surprisingly, it was also impossible to unfold the enzyme and release the cofactor by using up to 6.0 M urea. This is in line with the observation that upon SDS-PAGE PuORh still contains FAD as evidenced by in-gel flavin fluorescence (11). Only by using 6.0 M guanidinium chloride was it possible to unfold the enzyme. The unfolded enzyme was subsequently separated from its released cofactors by using a desalting column. Unfortunately, incubation of the unfolded protein for 12 h in a 100 μM FAD containing buffer did not yield any flavin-containing enzyme. Another method for the preparation of apoflavoproteins is the use of hydrophobic interaction chromatography (15). To obtain apoPuORh we used a phenyl-Sepharose column. In the presence of 1.5 M ammonium sulfate, PuORh binds to the column. By lowering the pH, it was possible to elute the flavin cofactor. The unfolded protein, bound to the phenyl-Sepharose, was then incubated at neutral pH with FAD to initiate refolding and FAD binding. When PuORh was eluted from the column, the protein fraction did not contain any FAD, which indicates that, again, proper refolding failed.

Introduction of Covalent FAD in PuORh A394C—In a previous study we constructed a model of PuORh that is based on the structure of the sequence-related MAO-B (11). In MAO-B, the FAD cofactor is covalently attached at the C8α position to the protein via a thio-ether linkage, involving Cys-397. Inspection of the PuORh model revealed that an alanine residue (Ala-394) is located at the analogous position, which precludes covalent binding of FAD at the C8α position of the isoalloxazine ring (Fig. 2). To probe if we could create a covalent cysteinyl-FAD linkage in PuORh, we mutated Ala-394 into a cysteine residue. This mutant (PuORh A394C) was well expressed in E. coli TOP10. Whereas purified wild-type PuORh displays a yellow color, it was found that purified PuORh A394C has an orange color. This visual inspection already confirms that the mutation induced a significant change in the microenvironment near the isoalloxazine ring of the flavin cofactor. The UV/VIS spectra of both PuORh variants show a clear change in the absorbance features (Fig. 3). Activity measurement with PuORh A394C revealed that the enzyme is still active, albeit with a slightly increased $k_{cat}$ value for putrescine and a significant decrease in $k_{cat}/K_m$ value (Table 1). The loss in catalytic efficiency is likely due to a change of the position of the isoalloxazine ring compared with the wild-type, resulting in a less favorable positioning of the substrate amine group to be oxidized.

Remarkably, precipitation of PuORh A394C in the presence of 5% trichloroacetic acid resulted in a yellow pellet and a yellow supernatant. When treating wild-type PuORh in the same way, the protein pellet was white. This suggests that part of the FAD is covalently tethered in the mutant enzyme while part of the FAD is still dissociable and released upon protein precipitation.

To confirm the presence of covalently bound FAD in PuORh A394C, we analyzed this mutant by ESI-MS under denaturing and native conditions. The mass spectrum under denaturing conditions (Fig. 1C) reveals the presence of two species. The major species ■ (85%) corresponds to a mass of 49,286 ± 13 Da, which agrees well with the theoretical mass of the PuORh A394C mutant enzyme minus the N-terminal methionine (49,276 Da). The minor species ● (15%) has a mass of 50,071 ± 16 Da, which corresponds to a 785-Da increase in mass when compared with species ■. This increase matches the mass of one FAD molecule and confirms that a significant portion of the FAD in the PuORh A394C mutant is covalently bound.

Mass spectra under native conditions for the PuORh A394C mutant (Fig. 1D) show a similar picture as for the wild-type enzyme (Fig. 1B); PuORh A394C is mainly present as a dimer, although a small amount of monomer can also be seen. Again, three different dimeric species (●, ○, and ▲) are observed that

FIGURE 2. Active site of MAO-B and modeled PuORh. In MAO-B, the flavin is covalently attached to Cys-397 via a C8 α-thioether linkage. The model of PuORh contains an alanine residue at this position (Ala-394) which precludes covalent FAD binding. The figure was prepared using PyMol software.
experiments we observed that PuORh is present in three differ-
ent dimeric species (32% homodimer containing 2 FAD. No apoenzyme is observed. The ratio of 1:2 for this flavoprotein oxidase is the result of compet-
tition binding, both being present in the cytoplasm. Both cofactors are tightly bound by PuORh. The binding of ADP in an FAD binding domain is not unique for PuORh. In NikD, a flavoprotein involved in nikkomycin biosynthesis, significant amounts of ADP derivatives were found to be present in the isolated protein, instead of FAD (20). Binding of ADP leads to an inactive enzyme, as it replaces the FAD redox cofactor. Therefore, ADP acts as a regulator for PuO activity. This could be of physiological relevance. However, as ADP is always present in the cytosol, PuO would always be partially inhibited. The level of inhibition may depend on the metabolic activity as this will be related to the concentration of ADP.

PuORh exhibits 32% sequence identity with human MAO-B and 30% sequence identity with human monoamine oxidase A (MAO-A). In contrast to MAO-B and MAO-A, PuORh does not contain 8α-S-cysteinyl-FAD, because it contains an alanine (Ala-394) instead of a cysteine residue at the respective position (Fig. 2). Also in PuORh and in other putative putrescine oxi-
dases, an alanine is present instead of a cysteine (11). The soluble monoamine oxidase from Aspergillus niger (MAO-N) also contains an alanine residue instead of a cysteine residue and is known to contain noncovalently bound FAD (21). In general, several roles can exist for the covalent linkage(s) between a protein and its flavin cofactor (22–24). For MAO-A and MAO-B, the role of the covalent flavin linkage has been extensively studied (25–29), and the effect of replacing the linking cysteine residue (Cys-406 in MAO-A and Cys-397 in MAO-B) has been investigated (30, 31). These mutagenesis studies revealed that, while replacement of the cysteine prevents coval-
tent FAD incorporation, the covalent flavin linkage is not essen-
tial for catalytic activity, neither for targeting and insertion of the protein in the mitochondrial membrane. However, it does play a role in the protein stability. Our observation that ADP competes with FAD binding in putrescine oxidase hints to another role for the presence of a covalent flavin linkage in MAO and sequence-related enzymes. By covalent tethering of FAD, the enzyme is assured of full incorporation of the flavin cofactor, outcompeting the (reversible) binding of ADP. Thereby, the enzyme can attain its fully active form. Such a pressure for discriminating between FAD and ADP binding can correspond to, respectively, the homodimer containing 2 ADP, the heterodimer containing both 1 ADP and 1 FAD, and the homodimer containing 2 FAD. No apoenzyme is observed. The distribution of these three different species (32% ◻, 40% ●, and 28% ▲) is similar to that of wild-type enzyme.

It was found that the extent to which PuORh A394C contains covalent FAD varies depending on the conditions used. Depending on the medium used (LB or TB medium), the level of covalent flavinylation varied between 10 and 40%. However, while the percentage of covalently bound FAD varied, no sig-
nificant change in the ADP:FAD ratio was observed. The molecular basis for the different levels of covalent flavinylation may be related to e.g. a change in redox state of the cytosol, level of intercellular metabolites that affect protein folding, or the concentrations of cofactors, and remains to be established.

**DISCUSSION**

In this article we describe our findings concerning cofactor binding in putrescine oxidase from *R. erythropolis*. By ESI-MS experiments we observed that PuORh is present in three different dimeric species which contain: 1) two FAD molecules, 2) one FAD and one ADP molecule, or 3) two ADP molecules per dimer. These observations reveal that the unusual FAD/protein ratio of 1:2 for this flavoprotein oxidase is the result of competitive binding of ADP. It also shows that the enzyme is able to bind two FAD molecules per dimer while it was previously suggested that the dimeric enzyme could only bind one FAD. Binding of an FAD or ADP cofactor in each subunit is in line with structural model that has been build for PuO (11). The model reveals that each subunit contains a single Rossman-fold domain, which is able to accommodate an ADP or FAD mole-
cule. PuORh has been overexpressed in *E. coli* TOP10, which represents a different situation than expression in *R. erythropo-
lis*. However, also for the orthologous enzyme PuO_Mes, a 1:2 FAD/protein ratio was observed when the enzyme was purified from its original host, *M. rubens* (18). Expression of PuO_Mes in *E. coli* also yielded a similar cofactor/protein ratio (6). This indicates that the partial incorporation of ADP, instead of FAD, in PuORh, is not an artifact of heterologous expression in *E. coli*.

The binding of ADP instead of FAD as cofactor results in a catalytically non-active species of the protein and thus prevents maximal catalytic efficiency. Our study shows that the ADP is tightly bound in PuORh as all attempts to increase the FAD content failed. This suggests that PuORh has a high affinity for both FAD and ADP. This represents a different situation when compared with another FAD-containing oxidase from a *Rhodo-
coccus* species: *i.e.* eugenol oxidase. For this enzyme, it was observed that incomplete FAD incorporation leads to the pres-
ence of apoenzyme rather than ADP-containing protein (19).

**Cofactor Incorporation in Putrescine Oxidase**

![FIGURE 3. Absorbance spectrum of wild-type PuORh (solid line) and PuORh A394C (dashed line).](image-url)

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Steady-state kinetic parameters of wild-type (WT) PuORh and PuORh A394C for putrescine at pH 8.0</th>
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<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
</tr>
<tr>
<td>PuORh WT</td>
<td>$8.2 \pm 0.5$</td>
</tr>
<tr>
<td>PuORh A394C</td>
<td>$24.1 \pm 2.9$</td>
</tr>
</tbody>
</table>

Depending on the medium used (LB or TB medium), the level of covalent flavinylation varied between 10 and 40%. However, while the percentage of covalently bound FAD varied, no sig-
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certainly be envisaged when considering the location of the human MAO isozymes: both enzymes are located in mitochondria and, as a result, are surrounded by relatively high amounts of ADP and ATP.

To investigate if a covalent flavin link, as present in human MAOs, could be introduced in PuORh the mutant PuORh A394C was constructed. This mutant indeed contains for a significant part covalently bound FAD. However, most of the protein contains noncovalently bound FAD. This may be explained by the observation that in MAO-B the FAD-linking cysteine is involved in a peptide backbone flip (Fig. 2). Such an energetically unfavorably isomerization of the polypeptide backbone requires an appropriate protein microenvironment. The inability to facilitate formation of such a peptide backbone flip in PuORh may lower the efficiency of covalent FAD incorporation. Some more extensive enzyme redesign could alleviate this problem and may result in a fully covalently flavinylated PuO. Nevertheless, the observed formation of some covalently bound FAD in PuORh A394C shows that a covalent protein-flavin linkage can be formed autocatalytically. To our knowledge, this is the first example of the introduction of a covalent protein-flavin linkage in vivo in a flavoprotein that originally does not contain such a covalent bond.

REFERENCES