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Coenzyme Binding during Catalysis Is Beneficial for the Stability of 4-Hydroxyacetophenone Monooxygenase*

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The NADPH-dependent dimeric flavoenzyme 4-hydroxyacetophenone monooxygenase (HAPMO) catalyzes Baeyer-Villiger oxidations of a wide range of ketones, thereby generating esters or lactones. In the current work, we probed HAPMO-coenzyme complexes present during the enzyme catalytic cycle with the aim to gain mechanistic insight. Moreover, we investigated the structural role of the nicotinamide coenzyme. For these studies, we used (i) wild type HAPMO, (ii) the R339A variant, which is active but has a low affinity toward NADPH, and (iii) the R440A variant, which is inactive but has a high affinity toward NADPH. Electrospray ionization mass spectrometry was used as the primary tool to directly observe noncovalent protein-coenzyme complexes in real time. These analyses showed for the first time that the nicotinamide coenzyme remains bound to HAPMO during the entire catalytic cycle of the NADPH oxidase reaction. This may also have implications for other homologous Baeyer-Villiger monooxygenases. Together with the observations that NADP+ only weakly interacts with oxidized enzyme and that HAPMO is mainly in the reduced form during catalysis, we concluded that NADP+ interacts tightly with the reduced form of HAPMO. We also demonstrated that the association with the coenzyme is crucial for enzyme stability. The interaction with the coenzyme analog 3-aminopyridine adenine dinucleotide phosphate (AADP+) strongly enhanced the thermal stability of wild type HAPMO. This coenzyme-induced stabilization may also be important for related enzymes.

The interaction of proteins with small molecules, such as ligands and cofactors, often coincides with an increased stability of the protein due to the coupling of binding with the unfolding equilibrium (1–4). Thus, apart from their catalytic role, cofactors may also have a structural role. The importance of cofactor binding for protein stability is revealed for type I BVMOs form a newly identified superfamily of flavoprotein aromatic hydroxylating monooxygenases and eukaryotic flavin-containing monooxygenases, type I BVMOs contain FMN and use NADH (17). Type I BVMOs contain two Rossman folds, which are involved in the binding of the ADP moieties of FAD and NADPH (18). Together with bacterial N-hydroxylating monooxygenases and eukaryotic flavin-containing monooxygenases, type I BVMOs form a newly identified superfamily of flavoprotein monooxygenases (19), which is distinct from the family of flavoprotein aromatic hydroxylases. Recently, the crystal structure of phenylacetone monooxygenase from Thermobifida fusca was solved (20). This first structure of a type I BVMO revealed a two-domain architecture and highlighted the importance of an active site arginine, which seems critically involved in substrate oxygenation. Replacement of this conserved arginine in HAPMO (R440A) does not impair NADPH binding but results in complete enzyme inactivation (21). From site-directed mutagenesis, it was also established that Arg-339 and Lys-439 are involved in determining the coenzyme specificity of HAPMO. The R339A mutant showed a largely decreased affinity for NADPH, as judged from kinetic analysis and binding experiments. Saturation mutagenesis of Lys-439 suggested that this residue is involved in the binding of the 2’-phosphate of NADPH and allowed to change the coenzyme specificity of HAPMO in favor of NADH (21).

The abbreviations used are: HAPMO, 4-hydroxyacetophenone monooxygenase; AADP+, 3-aminopyridine adenine dinucleotide phosphate; BVMO, Baeyer-Villiger monooxygenase.
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**FIGURE 1. Proposed mechanism of action of HAPMO.** The mechanism is based on the kinetic analysis of both cyclohexanone monoxygenase (22) and HAPMO. S and P indicate substrate and product, respectively. In the experiments described in this report, no ketone substrate was present. Under these conditions, the enzyme peroxo-flavin intermediate slowly decays to form hydrogen peroxide while releasing NADP$^+$ acting as an NADPH oxidase (inner cycle).

Fig. 1 shows the proposed mechanism of action of BVMOs (22). After flavin reduction by NADPH and reaction with oxygen, the generated flavin C4a-peroxide attacks the carbonyl carbon of the substrate. After product formation, water is released from the resulting hydroxyflavin, regenerating the oxidized enzyme. For cyclohexanone monoxygenase, there is some indirect evidence derived from kinetic studies that the initially formed NADP$^+$ remains bound during the substrate oxygenation process. In fact, release of NADP$^+$ from the reoxidized enzyme is the last step to complete the catalytic cycle and is the rate-determining step in catalysis (22). Cyclohexanone monoxygenase does, however, not act on aromatic compounds and lacks the N-terminal domain of HAPMO. A similar kinetic mechanism has been proposed for pig liver monooxygenase that when no suitable substrate is present, the flavin peroxo-flavin intermediate slowly decays to form hydrogen peroxide while releasing NADP$^+$ acting as an NADPH oxidase (inner cycle).

The data in this report have provided the first direct evidence that the enzyme binding to HAPMO and its effect on protein stability. The major size-exclusion chromatography as the principal tools to monitor coenzyme binding of 4-hydroxyacetophenone monoxygenase.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Protein Expression, and Purification**—NADPH, NADP$^+$, and 3-aminoptyridine adenine dinucleotide phosphate (AADP$^+$) were obtained from Sigma. The *hupE* gene from *P. fluorescens* ACB encoding HAPMO was expressed in *Escherichia coli* strain TOP10 containing the vector pBADNK/*hupE*. The two genes encoding the mutant proteins R339A and R440A were generated as recently described (21). For efficient expression, *E. coli* cells were grown at 20°C with 0.002% (w/v) arabinose and 50 μg/ml ampicillin, essentially as described before (21). Purification of wild type HAPMO and the R339A and R440A variants was performed as reported earlier (13).

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**RESULTS**

**Quaternary Structure of HAPMO Variants**—Wild type HAPMO is a homo-dimeric flavoprotein with each monomer containing a noncovalently tightly bound FAD cofactor (13). To determine accurate masses of wild type, R339A, and R440A HAPMO and to investigate whether these proteins remain in their native form during ionization, the...
HAPMO variants (1 μM) were analyzed by nanoflow electrospray ionization mass spectrometry in 50 mM ammonium acetate, pH 6.8 (Fig. 2). As can be seen from Fig. 2A, ionization of wild type HAPMO revealed two major ion series around 4,500 and 6,000 mass-to-charge (m/z) corresponding to molecular masses of 77,610 ± 10 and 145,232 ± 21 Da, respectively (TABLE ONE). These species represent the monomeric and dimeric form of HAPMO, with the dimeric species being the dominant form. The molecular masses of the monomeric and dimeric form are in close agreement with the calculated masses including the FAD cofactors. Besides the native species containing two FAD molecules, small amounts of the dimeric apo-protein and dimeric protein with one FAD molecule were also observed. Thus, the mass spectrometry data showed the dimeric nature of HAPMO with tight binding of FAD.

Mass determination of R339A and R440A variants revealed molecular masses of 145,103 ± 43 and 145,092 ± 44 Da, respectively. Again, these molecular masses are in close agreement with the calculated masses of the dimers, including two FAD molecules (TABLE ONE). The HAPMO mutants were essentially present as holo-dimers, and only minor amounts of holo- and apo-monomer and apo-dimer were observed (Fig. 2, B and C). The mass spectrum of R440A revealed some more monomeric protein when compared with wild type and R339A HAPMO, which may indicate that in this particular HAPMO variant, the interaction between the monomers is somewhat weaker. Interestingly, in the electrospray process, the R339A mutant obtained fewer charges than wild type and R440A HAPMO. Whereas R339A yielded 21+ to 25+ ions for the dimeric form, both wild type and R440A yielded series of 23+ to 27+ ions. The observation that R339A obtains one charge less per monomer may indicate that the side chain of Arg-339 is exposed to solvent and is protonated in wild type enzyme, or alternatively, it may reflect that the mutation causes a small conformational difference.

Complex Formation between Oxidized HAPMO Variants and NADP+ or AADP+. In the following, we aimed to monitor noncovalent HAPMO variant-nicotinamide coenzyme complexes present during the catalytic cycle. To study these protein-ligand interactions by mass spectrometry, we used NADP+ and the coenzyme analog AADP+.

For these studies, we could not use NADPH as the reduced coenzyme because of the difficulties of directly observing these complexes. Electrospray ionization mass spectrometry is an ideal direct method to char-

**TABLE ONE**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Species</th>
<th>Cofactor</th>
<th>Average M&lt;sub&gt;e&lt;/sub&gt;</th>
<th>Calculated mass *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Dimer</td>
<td>FAD</td>
<td>145,232 ± 21</td>
<td>145,222</td>
</tr>
<tr>
<td>R339A</td>
<td>Dimer</td>
<td>FAD</td>
<td>145,103 ± 43</td>
<td>145,052</td>
</tr>
<tr>
<td>R440A</td>
<td>Dimer</td>
<td>FAD</td>
<td>145,092 ± 44</td>
<td>145,052</td>
</tr>
</tbody>
</table>

* Mass calculated on the basis of the primary sequence including the FAD cofactor.
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**FIGURE 3.** Nanoflow electrospray mass spectra of HAPMO variants in the presence of AADP. Enzyme (1 μM) in 50 mM ammonium acetate, pH 6.8, was mixed with AADP and infused in the electrospray source. Spectra were collected in positive ion mode. A, wild type enzyme mixed with a stoichiometric amount of AADP; B, R440A HAPMO mixed with a stoichiometric amount of AADP; C, R339A HAPMO mixed with a stoichiometric amount of AADP; D, R339A HAPMO mixed with a 10-fold molar excess of AADP, one molecule of FAD; E, one molecule of AADP.

characterize such interactions; however, it is difficult to preserve anaerobic conditions within the source of the electrospray ionization probe. Therefore, we monitored the binding of the nicotinamide coenzyme to the enzyme under aerobic conditions in the absence of ketone substrate.

By measuring the redox state of the flavin cofactor during enzyme turnover, information can be obtained about the rate-limiting step in catalysis. These experiments have shown that under aerobic conditions and in the presence of sufficient NADPH, but in the absence of a ketone substrate, the flavin is mainly in a reduced form in both wild type and R440A HAPMO (100 and 97%, respectively), whereas in the R339A, most of the FAD is in the oxidized form. These results indicated that, at least in the wild type enzyme and R440A variant, flavin reduction is relatively fast (21). As a consequence, the predominant enzyme form present during the reaction of substrate-free HAPMO with NADPH under aerobic conditions may be a complex of a reduced or oxygenated enzyme species and NADP (Fig. 1, right part of the cycle). This biochemical property allowed us to directly monitor the interaction between NADP and substrate-free reduced wild type and R440A HAPMO during turnover by mass spectrometry.

In a typical experiment, we mixed 1 μM HAPMO enzyme with excess NADPH and measured noncovalent protein complex formation and the amounts of NADP and NADP in real time up to 50 min at room temperature. First, we monitored the mass spectra of wild type HAPMO and R440A variant during incubation with 10 μM NADPH (Fig. 4, B and C). Upon mixing with NADPH, the mass spectrum revealed two new ion series, which corresponds with the binding of one and two molecules of the pyridine nucleotide to the dimeric species of wild type and R440A variants. Full saturation of the wild type enzyme and R440A variant with NADPH(H) was not reached due to the relatively low concentration of NADPH used. Increasing the molar excess may, however, induce nonspecific complex formation. As the enzyme-bound flavin is mainly in the reduced or oxygenated state during steady-state conditions, the species we observed in the mass spectrum most probably reflected the reduced enzyme in complex with NADP. Unfortunately, the high molecular mass of the HAPMO dimer did not allow us to differentiate between reduced, oxygenated, or oxidized HAPMO. For R339A, we did not observe any interaction between the protein and the nicotinamide coenzyme.

Second, we analyzed the amounts of NADP and NADPH present during the HAPMO-mediated NADPH oxidase reaction. It should be noted here that by electrospray ionization mass spectrometry, NADP is detected as the M ion (744 atomic mass units) as it has a fixed positive charge in the nicotinamide ring, whereas NADPH is detected as the [M + H] ion (746 atomic mass units) (33, 34). It was found that under the experimental conditions used, a freshly prepared solution of NADPH contained about 8% of NADP. However, no significant spontaneous further oxidation occurred within the time frame of the experiment. The enzyme-mediated oxidation of NADPH to NADP was evidenced by the fact that we observed an increase in the amount of NADPH (744 atomic mass units) in time and a decrease in the amount NADPH (746 atomic mass units) (Fig. 5). Under the experimental conditions applied, wild type HAPMO oxidized about 70% of the added amount of NADPH within 50 min. The fact that the reaction did not reach completion can be attributed to product inhibition by NADP. The R440A mutant did not oxidize NADPH.

To validate the mass spectrometry data and to obtain some information about the binding site of NADP during turnover, we measured the...
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inhibition behavior of NADP\(^+\) with respect to NADPH. For wild type enzyme, it was found that NADP\(^+\) behaves as a weak competitive inhibitor with a \(K_i\) of 390 \(\mu\)M. The relatively large difference in \(K_m\) for NADPH (12 \(\mu\)M) (21) and \(K_i\) for NADP\(^+\) indicates that the oxidized enzyme is efficient in discriminating between the two states of the coenzyme. Furthermore, the fact that NADP\(^+\) competes with NADPH for binding is in line with the kinetic mechanisms that have been elucidated for sequence related monoxygenases (22). If a similar mechanism would be operative for HAPMO, NADP\(^+\) would dissociate from reoxidized enzyme while being tightly bound to the reduced enzyme.

In summary, the results clearly showed that NADP\(^+\) forms a stable intermediate complex with reduced and/or oxygenated enzyme but not with oxidized enzyme. Moreover, we demonstrated that the coenzyme, either in the reduced or in the oxidized form, is continuously associated with HAPMO.

Coenzyme Binding Increases the Stability of HAPMO—The observed interaction of the nicotinamide coenzyme with HAPMO during the entire reaction cycle might also be of importance for the enzyme from a structural point of view. Therefore, we probed the thermal stability of HAPMO variants in the absence or presence of pyridine nucleotide derivatives. When the free wild type enzyme (10 \(\mu\)M) was incubated at 36 °C, we observed a rapid loss of enzyme activity, which coincided with loss of dimeric structure. After a 120-min incubation at 36 °C, we observed a loss of enzyme activity, which coincided with loss of dimeric structure. After a 120-min incubation at 36 °C, we observed a rapid loss of enzyme activity, which coincided with loss of dimeric structure. After a 120-min incubation at 36 °C, we observed a rapid loss of enzyme activity, which coincided with loss of dimeric structure. After a 120-min incubation at 36 °C, we observed a rapid loss of enzyme activity, which coincided with loss of dimeric structure.

For these studies, we focused on three HAPMO variants with different biochemical properties. Wild type HAPMO and R440A interacted strongly with NADPH, but only wild type enzyme was fully active, and R339A interacted weakly with NADPH but could still perform catalysis by size-exclusion chromatography. The binding of AADP\(^+\) to HAPMO appeared to have a dramatic effect on the denaturation process. The addition of the coenzyme analog clearly stabilized the dimeric holoenzyme as evidenced by size-exclusion chromatography analysis (Fig. 6A). In the presence of an excess of free FAD, the aggregation process of wild type HAPMO was slowed down to some extent, suggesting that release of the cofactor plays a role in the inactivation process (Fig. 6A). However, the effect was far less dramatic when compared with AADP\(^+\).

To probe whether the stabilizing effect of AADP\(^+\) can be related to ligand binding, the same experiments were performed with the HAPMO mutants. Again, a similar stabilizing effect of AADP\(^+\) was found for the R440A variant (data not shown). In line with earlier observations in this report that R339A interacts only weakly with AADP\(^+\), the addition of AADP\(^+\) had no effect on the stability of R339A. Thus, these results confirmed that the HAPMO structure is stabilized by AADP\(^+\) binding and that the continued association of the nicotinamide coenzyme enhances enzyme stability.

DISCUSSION

In this study, we have reported the first direct analysis of the noncovalent interactions of a flavin-containing Baeyer-Villiger monoxygenase with its nicotinamide coenzyme during the enzyme catalytic cycle. This was accomplished by the application of nanoflow electrospray ionization mass spectrometry. In addition, we studied the effect of coenzyme binding on the thermal stability of the enzyme by size-exclusion chromatography.

Complex Formation between HAPMO and Pyridine Nucleotides—For these studies, we focused on three HAPMO variants with different biochemical properties. Wild type HAPMO and R440A interacted strongly with NADPH, but only wild type enzyme was fully active, and R339A interacted weakly with NADPH but could still perform catalysis...
at high coenzyme concentrations. Electrospray ionization mass spectrometry data under native conditions showed that the three HAPMO variants have a dimeric quaternary structure and bind up to two molecules of FAD/dimer. Our data provided strong evidence that the quaternary structures of these three enzyme variants are similar. The R339A variant had a slightly different mass spectrum with one charge less per monomer than the two other HAPMO variants. These differences in charge either reflected small conformational differences between the enzyme variants or were directly related to the replacement of Arg-339 into Ala, thereby removing one potential protonation site during the electrospray process (4, 35).

Very recently, the crystal structure of phenylacetone monooxygenase, a BVMO from the thermophilic bacterium *T. fusca*, has become available (20). This enzyme has 30% sequence identity with HAPMO and is expected to be homologous from a structural point of view. Phenylacetone monooxygenase exhibits a two-domain architecture resembling that of disulfide oxidoreductases (36). Both the FAD-binding domain and the NADPH-binding domain exhibit the typical dinucleotide-binding fold (37). Unfortunately, no crystallographic model for the enzyme in complex with a pyridine nucleotide is available. On the basis of the crystallographic model of phenylacetone monooxygenase, a structural model of HAPMO was constructed. In this model, residue Arg-339 in HAPMO aligned with residue Arg-217 in phenylacetone monooxygenase, which has been assigned as a residue for interacting with the adenine part of NADPH. This nicely agreed with the observed effects on coenzyme recognition when this residue is replaced in HAPMO. It also indicated that Arg-339 in HAPMO is not involved in monomer-monomer subunit interaction. The published crystallographic model of the related BVMO (20) also suggests that Arg-339 in wild type HAPMO is positioned at the surface of the protein and may well be protonated in the electrospray process. The Arg-440 residue of HAPMO can also be pinpointed in the phenylacetone monooxygenase structure and corresponded with Arg-337 in the monooxygenase. It is located in the active site and is of crucial importance for catalysis as evidenced by the inability of the HAPMO R440A variant to perform Baeyer-Villiger reactions. This agreed with the fact that the R440A variant is still able to bind the coenzyme, whereas it is unable to complete the catalytic cycle. The suggested location of Arg-440 in HAPMO would also agree with the observation that the replacement of this residue does not result in a decrease of the number of charges during the electrospray process.

The Michaelis-Menten constant of NADPH for wild type HAPMO is relatively low ($K_m = 12 \mu M$), suggesting that NADPH interacts strongly with the oxidized enzyme. Our mass spectrometry experiments with wild type enzyme and R339A and R440A variants showed that NADP$^+$ does not tightly interact with the oxidized enzyme, indicating that HAPMO can differentiate between oxidized and reduced pyridine nucleotides. These data were validated by biochemical inhibition studies, which revealed an inhibition constant of 390 $\mu M$ for NADP$^+$ for wild type HAPMO. Our studies also revealed tight binding of the coenzyme analog AADP$^+$ to wild type and R440A HAPMO but not to R339A. Apparently, the enzyme has evolved a very specific recognition site for binding reduced coenzyme to favor catalysis.

HAPMO catalyzes the conversion of ketones into their corresponding esters or lactones by using NADPH as external electron donor (Fig. 1). In the absence of a suitable ketone, substrate consumption of NADPH by HAPMO results in forming and stabilizing a peroxy-enzyme intermediate containing a bound NADP$^+$ molecule (13). The consumption of NADPH in the absence of aromatic substrate is slow ($k_{cat} = 0.10 \text{ s}^{-1}$) and relates to the slow decay of the peroxy-enzyme intermediate yielding hydrogen peroxide as product. These results were confirmed by enzyme-monitored turnover experiments, revealing that during catalysis, the flavin cofactor in wild type enzyme is mainly in the reduced and/or oxygenated state (21), awaiting the binding of a suitable substrate. It has been postulated for sequence-related monooxygenases that NADP$^+$ remains bound to the enzyme until the peroxy-enzyme intermediate has been converted (22, 38).

To characterize the noncovalent interactions of the nicotinamide coenzyme to HAPMO during turnover, we followed the reaction of HAPMO variants with NADPH under aerobic conditions in real time by mass spectrometry. The mass spectra clearly evidenced that HAPMO, when functioning as an NADPH oxidase (Fig. 1, inner cycle), is in complex with its coenzyme throughout the catalytic cycle. This strongly suggested that we observed the binding of NADP$^+$ to a reduced or oxygenated enzyme. This was the first direct evidence that during catalysis, a BVMO is virtually continuously occupied by the reduced or oxidized coenzyme, and only during the exchange of NADP$^+$ with NADPH is the enzyme in the free form.

**Stability of HAPMO**—The coenzyme analog AADP$^+$ was used to probe the influence of pyridine nucleotides on the stability of HAPMO. Data from size-exclusion chromatography showed that this analog prevents to a great extent aggregation and inactivation of HAPMO variants (wild type and R440A) that are able to bind this coenzyme analog. This indicated that the effect is not related to the intrinsic properties of the AADP$^+$ molecule as the R339A mutant showed no difference in stability upon the addition of the coenzyme analog. The stabilizing effect suggested that coenzyme binding induces a more rigid tertiary and/or quaternary structure due to specific noncovalent interactions between coenzyme and its highly specific binding site. The mass spectrometric observation that the binding affinity of FAD is enhanced upon the binding of AADP$^+$ to wild type HAPMO and R440A may also indicate that the enzymes adopt a more rigid structure.

In conclusion, this study has established the noncovalent interactions between pyridine nucleotides and HAPMO during catalysis. This coenzyme binding resulted in a dramatic increase in the stability of the enzyme. Based on the results of this study, it can be expected that most of the time, HAPMO will be occupied by coenzyme when it is in its natural intracellular environment. This indicated that the enzyme is *in vivo* more stable when compared with the isolated enzyme, which might explain the relatively poor stability of many reported isolated coenzyme-dependent enzymes. For example, a well studied homolog of HAPMO, cyclohexanone monooxygenase, has been shown to inactivate quite rapidly at room temperature (39). The concept of enzyme stabilization by AADP$^+$ binding might be exploited for enzyme purification or storage. The obtained results also showed that the electrospray ionization technique is well suited to detect noncovalent interactions between HAPMO subunits and between HAPMO and ligands and to monitor these interactions in real time (25, 26).

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**REFERENCES**


*Coenzyme Binding of 4-Hydroxyacetophenone Monooxygenase*