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

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The NADPH-dependent dimeric flavoenzyme 4-hydroxyacetophenone monoxygenase (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 monoxygenases. 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 the octameric protein vanillyl-alcohol oxidase, it was demonstrated that cofactor binding influences the quaternary architecture of the enzyme (7). The H61T mutant is purified as apo-enzyme and mainly exists as a dimeric species. The binding of FAD to the enzyme restores the octameric conformation (7, 8). Similarly, for lipoamide dehydrogenase, it was shown that FAD binding increases the protein melting temperature from 35 to 80 °C (9). Coenzymes such as NAD(P)H and NAD(P)H can have, besides from their function in electron transfer, also a structural role. Several liver and pectoral muscle enzymes are substantially protected by NAD(P)H against heat and proteolysis by trypsin (10). Phosphate dehydrogenase is stabilized by NAD+ (11), and DNA ligases become more compact upon anchoring coenzyme NAD+ (12).

In the present work, we have studied the noncovalent interactions between the oxidized or reduced nicotinamide coenzyme (NADP+ or NADPH) and the flavoenzyme 4-hydroxyacetophenone monoxygenase (HAPMO).6 HAPMO from Pseudomonas fluorescens ACB is a Baeyer-Villiger monoxygenase (BVMO), which converts a wide range of ketones into the corresponding esters or lactones (13). The enzyme is also able to perform enantioselective sulfoxidations (14). In recent years, BVMOs have received increasing attention, representing a promising alternative for chemical oxidations reactions allowing highly selective oxygen insertion reactions (15, 16).

HAPMO is a so-called type I BVMO. These enzymes contain FAD as a cofactor and use NADPH as a source for electrons, whereas Type II 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 monoxygenases and eukaryotic flavin-containing monoxygenases, type I BVMOs form a newly identified superfamily of flavoprotein monoxygenases (19), which is distinct from the family of flavoprotein aromatic hydroxylases. Recently, the crystal structure of phenylacetone monoxygenase 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).

6 The abbreviations used are: HAPMO, 4-hydroxyacetophenone monoxygenase; AADP+, 3-aminopyridine adenine dinucleotide phosphate; BVMO, Baeyer-Villiger monoxygenase.

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4 The abbreviations used are: HAPMO, 4-hydroxyacetophenone monoxygenase; AADP+, 3-aminopyridine adenine dinucleotide phosphate; BVMO, Baeyer-Villiger monoxygenase.
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 peroxyflavin 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-peroxidase 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 flavin-containing monoxygenase (23), suggesting that continued association of coenzyme is a recurrent theme for this class of monoxygenases, regulating enzyme activity. It has also been found for these monoxygenases that when no suitable substrate is present, the flavin C4a-peroxidase will slowly decay to form hydrogen peroxide while releasing NADP$^+$ (Fig. 1), thereby acting as an NADPH oxidase. The observed oxidase activity of HAPMO in the absence of an aromatic substrate is 0.1 s$^{-1}$ (13), which may suggest that also with HAPMO, NADP$^+$ remains bound throughout the oxidative half-reaction. Kamerbeek et al. (21) do not address the issue of protein-coenzyme complexes present during the catalytic cycle. The suggested interaction may also be beneficial for the enzyme from a structural point of view.

In this study, we aimed to gain mechanistic insight in HAPMO. For this, we selected wild type HAPMO and two HAPMO mutants (R339A and R440A). We used electrospray ionization mass spectrometry and size-exclusion chromatography as the principal tools to monitor coenzyme binding to HAPMO and its effect on protein stability. The major advantage of mass spectrometry is that it allows the direct observation of the noncovalent interactions between protein and ligand in real time even when a stoichiometric heterogeneous mixture is formed (24–26).

EXPERIMENTAL PROCEDURES

**Chemicals, Protein Expression, and Purification**—NADPH, NADP$^+$, and 3-aminopyridine adenine dinucleotide phosphate (AADP$^+$) were obtained from Sigma. The *hapE* gene from *P. fluorescens* ACB encoding HAPMO was expressed in *Escherichia coli* strain TOP10 containing the vector pBADN/K/hapE. 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).

**Nanoflow Electrospray Ionization Mass Spectrometry**—Enzyme samples for nanoflow electrospray ionization mass spectrometry experiments were prepared in aqueous 50 mM ammonium acetate, pH 6.8. Wild type, R339A, and R440A HAPMO were introduced into a nanoflow electrospray ionization orthogonal time-of-flight mass spectrometer (Micromass LCT, Waters, Manchester, UK) modified for high mass operation and operating in positive ion mode. For wild type HAPMO and variants, mass determinations were performed under conditions of increased pressure in the source and intermediate pressure regions in the mass spectrometer (Pirani gauge 7 milibars and Penning gauge 1.2 × 10$^{-6}$ milibars) (27, 28). Nanoflow electrospray voltages were optimized for transmission of larger multimer protein assemblies (capillary voltage 1450–1650 V and cone voltage 100–125 V). For the mass determinations of NADPH, NADP$^+$ and AADP$^+$ electrospray voltages were optimized for transmission of smaller organic molecules (capillary voltage 1050–1150 V and cone voltage 30 V). Borosilicate glass capillaries (Kwik-Fil, World Precision Instruments Inc., Sarasota, FL) were used on a P-97 puller (Sutter Instrument Co., Novato, CA) to prepare the nanoflow electrospray capillaries with an orifice of about 5 μm. The capillaries were subsequently coated with a thin gold layer (−500 Å) by using an Edwards ScanCoat Six Pirani 501 sputter coater (Edwards High Vacuum International, Crawley, UK). For the kinetic experiments with NADPH, an automated chip-based electrospray source (Nanomate, Advion) was used. The automated source was programmed to aspirate 4 μl of sample into the conductive pipette tip and then to deliver the sample to the inlet of the electrospray chip. This chip consists of 10 × 10 nozzles in a silicon wafer and a channel extending from the nozzle to an inlet side at the opposite face of the chip. Electrospray was initiated by applying an electric voltage of 1850 V, a head pressure of 0.2 p.s.i. to the sample in the pipette tip, and a cone voltage of 30 V.

**Analytical Procedures**—The enzyme activity of HAPMO was spectrophotometrically determined by monitoring the consumption of NADPH at 340 nm (13) in the absence or presence of 4-hydroxyacetophenone. The consumption of NADPH and formation of NADP$^+$ in the absence of 4-hydroxyacetophenone was also monitored by mass spectrometry. The inhibition constant of NADP$^+$ with respect to NADPH was determined spectrophotometrically with varying concentrations of NADP$^+$. The thermal stability of wild type HAPMO and R339A and R440A variants was analyzed by enzyme inactivation studies and size-exclusion chromatography. HAPMO (10 μM) in 50 mM ammonium acetate, pH 6.8, was incubated at 36 °C in the absence or presence of FAD (200 μM) or AADP$^+$ (50 μM). At various time points, aliquots were taken and analyzed for enzyme activity and hydrodynamical behavior. Analytical size-exclusion chromatography was performed by using a Superdex 200 HR 10/30 column (Amersham Biosciences) by using 50 mM ammonium acetate, pH 6.8. Aliquots of 100 μl were loaded on the column and eluted at a flow rate of 0.8 ml/min. Apparent molecular masses were determined by using a calibration curve made with standards from a molecular mass marker kit (Bio-Rad) containing thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B$_{12}$ (1.35 kDa).

**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 \( \mu \text{M} \)) 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 for the dimeric form a series of 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 would slowly react with oxidized HAPMO. AADP⁺ is a nonreducing coenzyme analog in which the amide function of the nicotinamide moiety is replaced by a primary amine function. This compound has often been used to probe coenzyme binding (29–31).

Mixing the wild type enzyme (1 \( \mu \text{M} \)) with NADP⁺ (10 \( \mu \text{M} \)) and subsequent analysis by nanoflow electrospray ionization mass spectrometry did not reveal the presence of a complex between protein and NADP⁺. Only at a 5-fold higher concentration of coenzyme was some NADP⁺ binding observed (< 5% saturation/monomer). Weak binding of NADP⁺ to the oxidized enzyme was supported by UV-visible absorbance difference spectroscopy. The addition of 1.5 mM NADP⁺ to 10 \( \mu \text{M} \) HAPMO did not influence the absorption properties of the flavin cofactor, indicating that NADP⁺ does not bind, or at least not in the vicinity of the isoalloxazine ring.

AADP⁺ is known to bind tightly to wild type HAPMO and R440A with dissociation constants of 0.4 and 0.1 \( \mu \text{M} \), respectively (21, 32). When we mixed wild type HAPMO (1 \( \mu \text{M} \)) with AADP⁺ (1 \( \mu \text{M} \)), we observed two new ion series in the mass spectrum (Fig. 3A). Deconvolution of these two ion series revealed molecular masses of 145,773 and 146,229 Da, strongly indicating the presence of dimeric protein in complex with one and two molecules of AADP⁺. Similarly, the mass spectrum of a mixture between R440A and AADP⁺ revealed complexes between protein and one and two molecules of AADP⁺ (Fig. 3B). The spectra clearly show that in an equimolar ratio, R440A was nearly saturated with AADP⁺, whereas wild type enzyme was saturated for about 30% with this coenzyme analog. Wild type HAPMO became fully saturated with AADP⁺ when the enzyme (1 \( \mu \text{M} \)) was mixed with a 4-fold excess of AADP⁺ (4 \( \mu \text{M} \)) (data not shown). The mass spectra also indicated that AADP⁺ stabilizes the quaternary structure of wild type and R440A HAPMO as no apo-protein was observed when the coenzyme analog was bound. The R339A mutant, on the other hand, did not display significant binding of AADP⁺ (Fig. 3C). Even at a 10-fold excess of the compound, hardly any protein-AADP⁺ complex was formed (Fig. 3D). These results agreed well with earlier studies, which indicate that R339A only weakly interacts with AADP⁺. In summary, none of the oxidized HAPMO variants were able to bind specifically to NADP⁺, whereas wild type and R440A HAPMO, but not R339A, interacted strongly with the coenzyme analog AADP⁺.

**Complex Formation during NADPH Oxidation** —The interaction between NADP⁺ and reduced HAPMO has not yet been analyzed because of the difficulties of directly observing these complexes. Electrospray ionization mass spectrometry is an ideal direct method to char-

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**TABLE ONE**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Species</th>
<th>Cofactor</th>
<th>Average ( M_r )</th>
<th>Calculated mass ( Da )</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.
and R440A variant during incubation with 10 μM NADPH and measured noncovalent protein complex formation and the amounts of NADP⁺ and NADPH 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 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 NADP⁺ (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 monooxygenases (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, wild type HAPMO was completely inactivated. By size-exclusion chromatography of the same incubations, we observed a loss of quaternary structure (Fig. 6A), which accompanied enzyme inactivation. The size-exclusion chromatography data also showed that during the inactivation process, protein aggregates are formed and that the FAD cofactor is released (Fig. 6B). We have to mention here that it is very likely that most of the aggregates formed were too large and/or heterogeneous to be detected 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 monooxygenase 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 this 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 ($k_{cat} = 0.10 s^{-1}$) 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**
