Beyond the Protein Matrix: Probing Cofactor Variants in a Baeyer−Villiger Oxygenation Reaction

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ABSTRACT: A general question in biochemistry is the interplay between the chemical properties of cofactors and the surrounding protein matrix. Here, the functions of NADP+ and FAD are explored by investigation of a representative monooxygenase reconstituted with chemically modified cofactor analogues. Like pieces of a jigsaw puzzle, the enzyme active site juxtaposes the flavin and nicotinamide rings, harnessing their H-bonding and steric properties to finely construct an oxygen-reacting center that restrains the flavin peroxide intermediate in a catalytically competent orientation. Strikingly, the regio- and stereoselectivities of the reaction are essentially unaffected by cofactor modifications. These observations indicate a remarkable robustness of this complex multicofactor active site, which has implications for enzyme design based on cofactor engineering approaches.

KEYWORDS: cofactors, flavin, nicotinamide, monooxygenation, biocatalysis

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n the fields of biocatalysis and industrial biotechnology, there is a growing interest in the so-called “cofactor engineering”, the idea of tuning the enzyme/cofactor function to make it of interest for green chemistry and industrial processes. By combining suitable host proteins with artificial cofactors, biomimetic, new artificial biocatalysts have been generated.1−5 This approach obviously requires a thorough knowledge of the precise functioning of natural cofactors, and from this perspective, it should be noted that the literature on the functional consequences of chemical alterations of enzyme cofactors remains very limited compared with the wealth of studies based on protein engineering.

Along these lines, here, the effects on enzyme catalytic and substrate-specificity properties exerted by chemical modifications of cofactors are explored. As a model system, the FAD-containing and NADPH-dependent phenylacetone monooxygenase (PAMO), which catalyzes the Baeyer−Villiger oxidation of phenylacetone to benzylacetate, is used.6 PAMO is a prototype for a large class of flavoenzymes that perform the oxygenation of a variety of compounds, including oxygenation of soft nucleophiles by the drug-metabolizing flavin-dependent monoxygenases, hydroxylation of primary amines by the siderophore-synthesizing ornithine and lysine hydroxylases, and oxygenation of ketones and sulfides by Baeyer−Villiger monoxygenases.7,8 Their catalytic reaction starts with the reduction of FAD by NADPH. This is followed by the reaction of the reduced prosthetic flavin group with oxygen to form the crucial flavin (hydro)peroxide. The distal oxygen of this flavin intermediate is finally inserted into the substrate, this step taking place through a negatively charged tetrahedral intermediate in the case of Baeyer−Villiger monoxygenases such as PAMO (Scheme 1). The fact that NADP(H) and FAD synergistically act not only as redox cofactors (the textbook function of these molecules) but also as true catalytic elements in oxygen activation and substrate oxygenation makes these enzymes a most insightful test case for our approach.9−13 PAMO was reconstituted with four FAD derivatives, chosen for their varying properties. (see Supporting Information (SI))

Scheme 1. Flavin Peroxide (left) and the Postulated Criegee Tetrahedral Intermediate (right)

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Table 1. Steady-State Kinetic Parameters

<table>
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<tr>
<th></th>
<th>(V_0^{(i)}) (mV)</th>
<th>(k_{ad}(s^{-1}))</th>
<th>(K_M) (µM) NADPH</th>
<th>(k_{ad}(s^{-1}))</th>
<th>(K_M) (µM) phenylacetone</th>
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<tbody>
<tr>
<td>FAD</td>
<td>-208</td>
<td>0.060 ± 0.010</td>
<td>7.90 ± 0.01</td>
<td>2.98 ± 0.01</td>
<td>33.4 ± 2.4</td>
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<tr>
<td>FADrec</td>
<td>-208</td>
<td>0.055 ± 0.012</td>
<td>7.30 ± 0.01</td>
<td>2.48 ± 0.02</td>
<td>43.8 ± 4.4</td>
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<tr>
<td>7-Cl,8-nor-FAD</td>
<td>-128</td>
<td>0.257 ± 0.003</td>
<td>3.87 ± 0.27</td>
<td>0.62 ± 0.02</td>
<td>23.6 ± 3.8</td>
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<tr>
<td>8-Cl-FAD</td>
<td>-152</td>
<td>0.071 ± 0.004</td>
<td>39.27 ± 1.35</td>
<td>1.27 ± 0.06</td>
<td>49.6 ± 8.5</td>
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<tr>
<td>7,8-dCl-FAD</td>
<td>-126</td>
<td>0.025 ± 0.003</td>
<td>n.d.</td>
<td>0.48 ± 0.01</td>
<td>50.4 ± 5.0</td>
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<tr>
<td>1-deaza-FAD</td>
<td>-280</td>
<td>0.045 ± 0.003</td>
<td>18.87 ± 2.59</td>
<td>0.14 ± 0.01</td>
<td>11.9 ± 2.5</td>
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</table>

<table>
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<tr>
<th></th>
<th>(V_0^{(i)}) (mV)</th>
<th>(k_{ad}(s^{-1}))</th>
<th>(K_M) (µM) APADPH</th>
<th>(k_{ad}(s^{-1}))</th>
<th>(K_M) (µM) APADPH and phenylacetone</th>
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<tr>
<td>FAD</td>
<td>-208</td>
<td>0.080 ± 0.005</td>
<td>&lt;10</td>
<td>0.040 ± 0.005</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

“...nhibits some specific substrate orientation (e.g., H bonds) but, rather, represents a featureless surface that shapes the active site and establishes nonspecific van der Waals interactions with the substrate (Figures 1–2).”

A further point of investigation was the sensitivity to chemical modifications of NADPH, the other cofactor necessary for the reaction. We used an acetylpyridine analogue (APADPH) in which the amide group of the nicotinamide moiety is replaced by an acetyl group. The use of APADPH resulted in a relatively slow oxidase activity similar to that...
Table 2. Enantioselectivity of the Conversion of Bicyclo[3.2.0]hept-2-en-6-one and Thioanisole Using FAD and NADPH Analoguesa

<table>
<thead>
<tr>
<th>cofactors</th>
<th>conversion (%)</th>
<th>normal/abnormal</th>
<th>ee (%) normal</th>
<th>ee (%) abnormal</th>
<th>conversion (%)</th>
<th>ee (%)</th>
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</thead>
<tbody>
<tr>
<td>FAD + NADPH</td>
<td>44</td>
<td>71:29</td>
<td>94</td>
<td>74</td>
<td>33</td>
<td>24</td>
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<tr>
<td>7-Cl,FAD + NADPH</td>
<td>48</td>
<td>70:30</td>
<td>76</td>
<td>68</td>
<td>34</td>
<td>40</td>
</tr>
<tr>
<td>8-Cl,FAD + NADPH</td>
<td>46</td>
<td>70:30</td>
<td>92</td>
<td>72</td>
<td>38</td>
<td>36</td>
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<tr>
<td>1-deaza-FAD + NADPH</td>
<td>32</td>
<td>75:25</td>
<td>95</td>
<td>88</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>FAD + APADPH</td>
<td>8</td>
<td>72:28</td>
<td>45</td>
<td>77</td>
<td>40</td>
<td>15</td>
</tr>
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</table>

“First, PAMO converts the (1S,SR)-ketone and produces an excess of the "normal" lactone with ee in favor of the (1S,SR)-enantiomers for both lactones (SI Figure S4). As the (1R,SS)-substrate is depleted, the (1S,SR)-ketone is also converted, yielding mainly the abnormal lactone. Therefore, the ratio of the normal to the abnormal lactones and the enantiomeric excess of the abnormal product are conversion-dependent. However, in our experiments, reactions were stopped before 50% of conversion was reached, which allows fair comparison. The percent enantiomeric excesses (ee) are reported in favor of the (1S,SR) products. The 7,8-dicarboxymethylene FAD reconstituted enzyme exhibited a tendency to precipitate in the assay conditions (see SI), and therefore, no reliable observations could be made. Average of duplicate experiments. Estimated on the basis of the peak area of the product.

measured for NADPH (Table 1). However, the addition of phenylacetone did not result in a burst of APADPH consumption. Instead, phenylacetone triggered an even slower conversion of APADPH, indicating that phenylacetone conversion was, at best, very inefficient. Consistently, we found that the reaction is only 30% coupled, implying that flavin reoxidation occurs mostly through the abortive generation of hydrogen peroxide rather than through substrate oxygenation (either phenylacetone or thioanisole; Table S1). This is in sharp contrast with NADPH, which gives 100% coupling (i.e., for each molecule of oxidized NADPH, a substrate molecule is converted). Presteady state kinetics underpinned these observations, showing that with APADPH as an electron donor, there is a 20-fold decrease in the rate of formation of the flavin peroxide, which decays 100-fold more rapidly (SI Table S2). Furthermore, these rates are unaffected by the presence of phenylacetone. Thus, the high uncoupling results from the combination of decreased efficiencies in two critical catalytic steps, stable flavin-peroxide formation, and substrate oxygenation. Such a clear demonstration of the role of the bound pyridine dinucleotide in fine-tuning the reactivity with oxygen prompted us to investigate the crystal structures of both oxidized and reduced PAMO bound to APADPH+ (SI Figure S5, Table S3). In both cases, the binding of APADPH+ is virtually identical to that of NADPH (Figure 1 and SI S6); however, the presence of the less polar acetyl group induces a conformational change of Asp66, which is now oriented toward the active site with its carboxylate group positioned in direct contact with the flavin C4a atom. These features were confirmed by determining the structure of a PAMO mutant (SI Figure S7) and finely correlate with the kinetic properties of the APADPH-reduced enzyme: (i) the proximity of negative charges (e.g., Asp66) is known to generally decrease the reactivity of the reduced flavin with oxygen through destabilization of the proposed radical pair intermediate consisting of a complex of the superoxide anion and neutral flavin radical,21 and (ii) the relocated Asp66 can hamper stabilization of the oxygen-adduct on the C4a atom. This conformation of Asp66 and the associated alteration of the H-bonding environment around the oxygen-reacting and substrate-binding site can also possibly explain the observed slight changes in the enantioselectivity of the reaction with APADPH (Table 2).

These findings demonstrate that even subtle alterations in the configuration of the oxygen-reacting center can drastically affect the outcome of the oxygen reaction. The comparative analysis of the three-dimensional structures of members of the class of Baeyer–Villiger and similar enzymes22–27 shows that the shape of the active site is such that the oxygen molecule is steered toward the flavin to promote an approach that is suited for the formation of the oxygen-cova lent adduct (Figure 2).28 In more detail, inspection of the active site geometries of these enzymes consistently indicates that oxygen is seemingly forced to approach the cofactor C4a–N5 locus from the flavin ring face (i.e., side-on) rather than the edge (edge-on). This is the geometry expected to support the formation of the flavin peroxide, whose oxygen atoms protrude out the cofactor plane. Once the flavin peroxide is formed, the distal oxygen of the intermediate is restrained to be oriented toward the ribityl chain, away from the flavin N5 atom. This location (at the very rear of the active site) protects the intermediate from decay, which is primarily triggered by protonation of the peroxide proximal oxygen by either water or protein groups, as shown by computational studies.29 The hydrogen-bonding and steric properties of the NADP+ amide and ribose are the pillars that hold up this active site configuration through their interactions with the flavin N5 atom and nearby side chains. In this context, it is instructive to compare this mechanistic strategy with that of aromatic hydroxylases, a class that is distinct from that of PAMO.30 In these flavoenzymes, the flavin peroxide forms only after (rather than before, as in PAMO) binding of the aromatic substrate, which affords a highly coupled hydroxylatation, despite the fact that the intermediate has limited stability (a half-life of milliseconds rather than minutes). This difference reflects the opposite geometry of the oxygen attack compared with PAMO and related monoxygenases: the protein environment of aromatic hydroxylases forces the peroxide distal oxygen to be oriented toward the flavin N5 (compare Figure 2 and SI Figure S8).31 This orientation makes the intermediate oxygen accessible to protonation, specifically including the possibility of a water-mediated proton transfer from N5, which would inevitably cause $\text{H}_2\text{O}_2$ elimination.32

Thus, PAMO (and generally, the related flavoenzyme class) features the distinctive ability to optimally harness the steric and hydrogen-bonding properties of NADP+ to both enhance the reaction of the reduced flavin with oxygen and, even more crucial, most effectively prevent the decay of the labile oxygen-activating intermediate that underlies substrate oxygenation. The uncovered delicate roles of the nicotinamide and flavin

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Figure 2. Comparison of Baeyer–Villiger enzyme three-dimensional structures22–27 shows that they all feature very similar, if not identical, oxygen-reacting centers formed by the juxtaposition of the nicotinamide–ribose of NADP+ and the flavin (with reference to the so-called “closed” conformation,23 which is thought to represent the protein state reacting with O2). Such a conservation is particularly significant, given the limited (<30%) sequence identities and the high diversity of the substrates used by these enzymes. This is outlined by modeling the flavin peroxide intermediate in three exemplifying NADP-bound enzyme structures analyzed in their reduced state (i.e., the state that reacts with O2): PAMO (top; 2YLS), bacterial FMO (middle; 2VQB), and ornithine hydroxylase SidA (bottom; 4B65). The geometry of the flavin peroxide was modeled by using as reference the three-dimensional structures of a flavin hydroxide bound to pyranose oxidase33 and of a covalent C4a-inhibitor adduct in monoamine oxidase B.34 The van der Waals surfaces of the flavin ring, NADP+, and side chains interacting with the C4a-OOH atoms (Asp66 and Arg337 in PAMO, Asn78 in FMO, Gln102 in SidA) are depicted. The drawing highlights the conformation (FMO) or narrow range of conformations (PAMO, SidA) permitted for the intermediate distal oxygen within the niche formed by the nicotinamide–ribose moiety and adjacent side chains. The substrate-binding site is located on the right-hand side of the intermediate oxygen above the pyrimidine ring of the flavin, as outlined by ornithine (carbons in orange) bound to SidA. A clip movie representing the geometry and environment of the flavin peroxide bound to SidA is available in the HTML version of this article on the ACS Web site.

cofactors in enzyme catalysis will be of help in future enzyme redesign efforts that include cofactor engineering.

**ASSOCIATED CONTENT**

3 Supporting Information

Experimental procedures for chemical syntheses, enzyme purification and reconstitution, kinetic studies, and X-ray analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

**Web-Enhanced Feature**

A movie clip representing the geometry of the flavin peroxide intermediate in PAMO is available in the HTML version of this article.

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Notes

The authors declare no competing financial interest.

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**REFERENCES**

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