Polypeptide containing iron porphyrin and flavin showed aniline-hydroxylation activity in the presence of 1-benzyl-1,4-dihydronicotinamide and O₂. The increased activity at pH 5.5 compared with that at pH 7.0 suggests that the dissociation of His from the iron porphyrin favored the hydroxylation.

One of the key steps in the oxidation with cytochrome P-450 is the electron transfer to this enzyme from cytochrome P-450 reductase. Flavin derivatives reduce nearby hemes during the oxidation reaction catalyzed by enzymes or artificial α-helix bundle polypeptides. Unlike 4α-helix bundle, which seems to surround porphyrin too closely, we recently synthesized a βαβα-type polypeptide loosely packing porphyrin and reported its peroxide-dependent oxidation catalysis. In order to utilize O₂ as oxidant, we have attached flavin to this βαβα-type polypeptide containing iron porphyrin, which could successfully hydroxylate aniline under the aerobic conditions (Figure 1).

Figure 1. (a) Polypeptides 1–3 with the iron porphyrin and/or flavin. A, Ala; a, d-Ala; E, Glu; G, Gly; H, His; K, Lys; L, Leu; P, Pro, and V, Val. (b) Supposed βαβα-type structure of 1.

Polypeptides 1 consist of β-strand segments and α-helix segments linked by spacers (Figure 1a). The β-strand segment (≈1.2 nm) are of alternative hydrophobic (X₁, Val, and X₂)/hydrophilic (Glu/Lys) ion pair sequences and the α-helix segments (≈1.5 nm) are of hydrophobic (four Leu)/hydrophilic (two pairs of Glu/Lys) residues on the opposite side of the helix, both designed to cover the porphyrin (≈1.0 nm). When the hydrophobic faces of the helices come close to the porphyrin to coordinate with iron by His, the hydrophobic Val in the β-strands would also come close to the porphyrin and the whole molecule would arrange to give a structure with hydrophobic interior (porphyrin, Val, and Leu) and hydrophilic (Glu/Lys) exterior (Figure 1b). The flavin moiety exists at the molecular surface (see below), therefore, the redundant in the solution is expected to reduce flavin, and then the intramolecular electron transfer from the reduced flavin to iron porphyrin takes place efficiently in the globular structure.

Polypeptide 1 and the control compounds (2, 3) were synthesized via the condensations of the peptide fragments as reported. Flavin (7α-bromoacetyl-10-methylisoalloxazine) was attached to Cys side chain of the deprotected 49-mer polypeptide before iron insertion. After the final purification with HPLC using a Superdex Peptide HR 10/30 column, polypeptides 1–3 were characterized by MALDI-TOF-MS. Unfortunately, H NMR signals of the polypeptides were too broad to assign even before iron insertion.

UV/vis spectrum of 1 at pH 7.0 was characteristic for the low-spin Fe(III) complex with a narrow Soret band at 420 nm and broad Q bands at 550 and 575 nm (sh) (Figure 2a), which indicate the bis-His coordination as fifth and sixth ligands. The UV/vis spectra of 1 at pH 2.7 and 615 nm, which indicate the formation of high-spin Fe(III) species. The His side chains were protonated at pH 2.7 and did not coordinate to iron porphyrin. However, these spectral changes showed no clear isosbestic points and showed a bimodal profile in the Abs=420 vs pH plot (Figure 2b), which suggests some intermediate species at around pH 5.5. The CD spectra of 1 at pH 7.0 showed negative bands at 222 and 208 nm, characteristic of the polypeptide containing α-helices. The CD spectrum is indeed ambiguous in the polypeptides containing β-strand due to the overlapping of the small Cotton effect of β-strand. However, when the Θ=222 value of 1 (−12500 deg cm² dm⁻³) was employed to analyze the α-helicity, 1 was evaluated to be 34% α-helix, which coincided with the 37% (18/49) value assuming the α-helical structure for the nine residues of each α-segment. We have no direct evidences for the conformation of β-strands at present, therefore, we wish to denominate 1 as the ββαα-type polypeptide. However, CD spectra showed that the β-strand are not helical, consequently, it is at least sure that the porphyrin was loosely surrounded by the polypeptide at pH 7.0. The peptide 2 without flavin showed a CD spectrum (Θ=222 = −14000) similar to 1 (3.5 µM) (a) at pH 7.0, 4.0, and 2.7. (b) Absorbance at 420 nm of 1 vs pH.

Aniline-Hydroxylation Activity of a Flavin-linked βαβα-Type Polypeptide Packing an Iron Porphyrin

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Next, we examined aniline hydroxylation in the presence of \( \text{O}_2 \) (air) catalyzed by 1-3 with 30 equivalents of the reductant (1-benzyl-1,4-dihydronicotinamide, BNAH) at pH 7.0 (Eq 1). The amount of the product \( p \)-aminophenol formed after the 15 min reaction, the only one product detected by HPLC, was determined by the literature method. The formation of \( p \)-aminophenol with 1 was six times greater than that with 2 at pH 7.0 (Table 1), which showed efficient electron relay via the attached flavin in 1. To verify the electron relay through flavin we monitored the UV/vis spectra of the solution of 1 and 2 after addition of BNAH under Ar atmosphere. For polypeptide 1, the absorptions at 432, 536, and 568 nm increased after the addition of BNAH, which correspond to the peaks of Fe(II) porphyrin. However, the Fe(II) species formation was significantly slow for 2 in the presence of BNAH. This clearly illustrates the importance of the flavin moiety in the polypeptide for the efficient electron transfer. Aniline hydroxylation hardly occurred with 3, indicating that the hydroxylation was actually catalyzed by the iron porphyrin. Although hydroxylation of aniline occurs with Fe(III) porphyrin/\( \text{H}_2\text{O}_2 \), the reactivity of 1 was not changed by the addition of an \( \text{H}_2\text{O}_2 \) scavenger, catalase (350 unit \( \text{cm}^{-3}\)), or an \( \text{O}_2^- \) scavenger, superoxide dismutase (100 unit \( \text{cm}^{-3}\)). These results showed that 1 utilizes \( \text{O}_2 \) and electrons derived from flavin and not using \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) generated from the reducing system. Although excess amounts of BNAH was added, \( \approx \)20 equivalents of BNAH was consumed after 15 min in the reactions including 1 and 3. However, BNAH was less consumed in the reaction with 2 (\( \approx \)5 equivalents after 15 min). Although BNAH reduces flavin at the surface of 1, aerobic reoxidation of the reduced flavin may occur to consume excess BNAH (Eq 2). This may be the reason of the low conversion of aniline and the low yield of \( p \)-aminophenol.

The formation of \( p \)-aminophenol with 1, indicating that the flavin attachment induce only slight changes to the conformation of the polypeptide. This fact supports the hypothesis that the flavin moiety is at the surface of the molecule (Figure 1b) due to the hydrophilic nature of the \( \text{isoalloxazine} \) ring. The bis-His coordination to Fe(III) in 1 and 2 stabilize their folded structures. However, polypeptide 3 without flavin almost did not show the activity of hydroxylation hardly occurred with 3, whereas aerobic reoxidation of the reduced flavin may occur to consume excess BNAH (Eq 2). This may be the reason of the low conversion of aniline and the low yield of \( p \)-aminophenol.

Saturation kinetics for aniline hydroxylation was observed for 1 at the \( \approx \)40 mM aniline concentration (Figure 3a). The Michaelis-Menten parameters of 1 are, \( k_{cat} = 1.2 \times 10^{-2} \text{ min}^{-1} \) and \( K_M = 2.6 \times 10^{-3} \text{M} \). Such small \( k_{cat} \) and large \( K_M \) values of 1 are attributed to its-His coordination in 1, which prevents the

![Figure 3](image-url)

**Table 1.** Aniline hydroxylation catalyzed by 1-3 at 37°C

<table>
<thead>
<tr>
<th>Peptide</th>
<th>pH</th>
<th>([p\text{-Aminophenol]}) after 15 min/(\mu\text{M})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>0.63</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>0.10</td>
</tr>
<tr>
<td>3</td>
<td>7.0</td>
<td>no reaction</td>
</tr>
</tbody>
</table>

\([\text{Peptide}] = 7.0 \mu\text{M}, [\text{aniline}] = 10 \text{mM}, [\text{BNAH}] = 210 \mu\text{M}\).