The C-terminal Domain of 4-Hydroxyphenylacetate 3-Hydroxylase from Acinetobacter baumannii Is an Autoinhibitory Domain*§

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Background: The flavin reduction rate of C1 reductase is enhanced ~20-fold upon binding to HPA (effector).

Results: The truncated C1 variant lacking the C-terminal domain is reduced as fast as the wild-type enzyme in the presence of HPA.

Significance: These findings demonstrate a novel principle that may be used in a wide variety of oxygenases.

Cooperation of the transfer of reduced flavin or of electron transfer within or between flavin-dependent enzymes is important for minimizing both wasteful consumption of reducing agents such as NAD(P)H and production of reactive oxygen species such as hydrogen peroxide or superoxide. The control of interdomain electron transfer has been extensively studied for nitric-oxide synthase (NOS) (1) and cytochrome P450 reductase (2). Evidence suggests that these enzymes coordinate and control electron transfer processes via protein conformational changes (3, 4) and domain movements (5). NOS regulates reductase activities via an autoinhibitory domain that slows its redox reactions when calmodulin and Ca2+ are not present (6, 7). Among the two-component flavin-dependent monoxygenases in which reduced flavin transfer between reductase and oxygenase components is required as part of their catalyses there are three known mechanisms for transfer of reduced flavin. Bacterial luciferase and alkane-sulfonate monoxygenase (8–11) use protein-protein interactions to mediate reduced flavin transfer, whereas simple diffusion can account for the reduced transfer in p-hydroxyphenylacetate hydroxylase (HPAH)3 from Acinetobacter baumannii (12) and the oxygenase involved in actinorhodin biosynthesis (ActVA-B) (13). Styrene monoxygenase (14) and HPAH from Pseudomonas aeruginosa (15) use both modes of transferring reduced flavin.

However, little is known about how these transfers are regulated in these flavin-dependent enzymes.

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3 The abbreviations used are: HPAH, p-hydroxyphenylacetate hydroxylase; HPA, p-hydroxyphenylacetate; C1, reductase component of HPAH; C2, monoxygenase component of HPAH; PheA2, reductase component of phenol 2-hydroxylase from B. thermoglucosidasi A7; t178C1, C1 truncated at residue 178.
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putida (16, 17), Pseudomonas aeruginosa (15), Escherichia coli (18), Klebsiella pneumonia (19), Sulfolobus tokodaii (20), Thermus thermophilus (21, 22), and A. baumannii (12, 23–31), have been carried out. HPAH consists of a reductase component (C1) that catalyzes reduction of FMN to generate FMNH− for its oxygenase (C2), which catalyzes the oxygenation of HPA (12, 23). C2 has been shown to hydroxylate HPA via a C4a-hydroperoxyflavin typical of flavin-dependent hydroxylases (29–31). C1 can catalyze the reduction of FMN by NADH, and under aerobic conditions, the resulting FMNH− will react with oxygen to generate H2O2 (constituting NADH oxidase activity) (25). In the presence of HPA, the substrate for C2, the rate of reduction of FMN is greatly stimulated, implying that HPA acts as an effector of the C1 enzymatic activity. Such allosteric control of the reductase component by a substrate of the partner oxygenase has been reported only for C2 and for nitrolitrioc-tate and EDTA monooxygenases (32, 33) but not for HPAH from other organisms or reductase components of other two-component flavin-dependent enzymes (34). It has been speculated that the C-terminal half of C1, which does not contain any sequences for NADH or flavin-binding folds, is a regulatory domain that when bound to HPA permits rapid reduction of FMN (25).

The N-terminal domain(s) of C1 (residues 1–178; 18 kDa) is homologous with several smaller flavin reductases with known structures (HPAH systems from S. tokodaii (20) and from T. thermophilus (21) and the reductase component of phenol 2-hydroxylase from Bacillus thermoglucosidasius A7 (PheA2) (35)), and its structure is predicted to be similar. The remaining C-terminal residues (192–315) have ~20% identity to MarR proteins (36). MarR is a repressor protein that binds to its DNA target to control expression of MarA. MarA is a protein that regulates expression of the AcrAB-ToIC efflux pump that excretes xenobiotics from bacteria (37). Upon binding to an aromatic effector such as salicylate (and presumably antibiotics), MarR dissociates from DNA to derepress the expression of MarA and the AcrAB-ToIC efflux pump, thereby promoting excretion of antibiotics or aromatic xenobiotics (37, 38).

In this study, three truncated C1 variants as well as single site variants of residues Arg-21, Phe-216, Arg-217, Ile-246, and Arg-247 were constructed to investigate the role of the C-terminal domain in regulating HPA-stimulated C1 activity. The results show that residues 179–230 and particularly residue Phe-216 are important in the HPA-stimulated NADH oxidase activity. The data indicate that the C-terminal domain is an autoinhibitory domain that upon binding HPA undergoes conformational changes to allow more rapid flavin reduction and release of FMNH−.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals and reagents used were analytical grade and purchased from commercial companies. PCR primers were synthesized by Eurogentec (Singapore). FMN was synthesized from FAD using snake venom from Crotalus adamanteus and purified using C18 Sep-Pak cartridges (Waters) (39, 25). Concentrations of the following compounds were calculated on the basis of known extinction coefficients at pH 7.0: NADH, ε340 = 6.22 mm−1 cm−1; FAD, ε450 = 11.3 mm−1 cm−1; FMN, ε456 = 12.2 mm−1 cm−1; HPA, ε277 = 1.55 mm−1 cm−1; C1 and single site variants of C1, ε458 = 12.8 mm−1 cm−1; and t178C1, ε458 = 11.9 mm−1 cm−1.

Modeled Structure of C1—A three-dimensional structure of C1 was modeled using the Swiss-PdbViewer (or DeepView) program via SWISS-MODEL, a web site that analyzes and predicts three-dimensional structures of target amino acid sequences (40–42). The C1 structure obtained from this site was used with the PyMOL program (version 0.99) to calculate vacuum electrostatics and identify amino acids that could be important for interactions between the N- and C-terminal domains of wild-type C1 and would be suitable for application of site-directed mutagenesis.

Construction of Truncated C1 Variants—Genes of truncated variants, t178C1, t230C1, and t259C1, were amplified by PCR using a GeneAmp PCR system (Applied Biosystems, model 2004). The PCR contained 4 μg of C1-pET11a plasmid as a DNA template, 0.5 μM primers (supplemental Tables S1 and S2), a 200 μM concentration of each dNTP, and 2.0 units of Pfu DNA polymerase (Fermentas) in 1× Pfu buffer and sterile water in a total volume of 50 μl. The final PCR products were digested by Ndel and BamHI and ligated into the corresponding sites of pET11a using T4 ligase. The ligated plasmids were analyzed for their sequences by Macrogen (Korea) and kept at −80 °C until used.

Site-directed Mutagenesis—The genes encoding the variants R247A, I246W, R217A, F216A, and R21A were constructed according to the PCR protocol and primers described in supplemental Table S2. DpnI was added to the resulting PCR products to remove the template plasmids. Plasmids encoding for C1 variants were propagated in E. coli and purified according to the QIAprep Spin Miniprep kit protocols (Qiagen, Germany). The sequences of the purified plasmids were analyzed by Macrogen, 1st BASE Pte. Ltd. (Singapore), or the DNA Sequence Core (University of Michigan). The variant C1 plasmids were kept at −80 °C until used.

Protein Expression/Purification—Most of the C1 variants were expressed and purified according to the protocol reported for the wild-type C1 (24). For variants t230C1, t259C1, and R201A, only the crude extracts were prepared and tested for their NADH oxidation activities. After purification, the purities of the t178C1, R247A, I246W, R217A, F216A, and R21A variants were estimated by SDS-PAGE.

NADH Oxidase Activity—NADH oxidase activity of C1 was measured spectrophotometrically at 340 nm. A typical assay reaction contained enzyme (~0.02 μM; no extra FMN) and NADH (96 μM) in 50 mM sodium phosphate buffer, pH 7.0 at 25 °C. Reactions were carried out in the absence and presence of HPA to test whether variants exhibited HPA-stimulated NADH oxidation activities. After purification, the purities of the t178C1, R247A, I246W, R217A, F216A, and R21A variants were estimated by SDS-PAGE.

Reduction of C1 Variants by NADH and FMN—NADH oxidation of reduced t178C1 by oxygen, and the transfers of reduced flavin from t178C1 to C2 or to cytochrome c were investigated using a stopped-flow spectrophotometer (TgK Scientific). All experiments were carried out in 50 mM sodium phosphate, pH 7.0 at 4 °C unless noted otherwise. Experiments and preparations for anaerobic solutions were similar to the protocols in previous reports (12, 25, 26). For the
reductive half-reaction, anaerobic solutions of oxidized enzyme (20 μM) were mixed with equal volumes of anaerobic solutions of NADH (100 μM to 10.4 mM) in the stopped-flow spectrophotometer. The oxidative half-reactions were studied by mixing anaerobic solutions of reduced t178C1 (20 μM) with buffers equilibrated with gas mixtures of oxygen and nitrogen ([O2] = 0.13, 0.33, 0.65 and 1.03 mM, concentrations after mixing). Rates of reduced flavin transfer from t178C1 to C2 or to cytochrome c were measured by mixing a solution of reduced t178C1 with a solution containing C2 and equilibrated with various concentrations of oxygen or mixed with a solution containing cytochrome c and equilibrated with oxygen-free N2 or argon.

Data Analysis—Observed rate constants (kobs) from kinetic traces were calculated by fitting data to exponential functions in Program A. Rate constants were determined from plots of kobs and NADH concentrations using Marquardt-Levenberg nonlinear fitting algorithms included in KaleidaGraph (Synergy Software) according to Equation 1.

\[
\frac{k_{\text{obs}}}{K_d} = \frac{k_3[NADH]}{K_d + [NADH]} \quad (\text{Eq. 1})
\]

RESULTS

Modeled Structure of Wild-type C1—The SWISS-MODEL web site (40–42) was used to predict the three-dimensional structure of C1 from its sequence. The N-terminal domain of C1 (residues 14–168) is 38% identical to the reductase component of PheA2 (Protein Data Bank code 1RZ0), and the C-terminal domain was modeled according to the structure of the MarR protein (Protein Data Bank code 3BPV) using SWISS-MODEL (37–39). A is a ribbon diagram of C1, with side chains of residues subjected to mutagenesis displayed in blue and green. B presents the N-terminal (lower) and C-terminal (upper) domains with electrostatic fields (+, blue; −, red). The dashed line indicates residues (169–191) that link the end of the N-terminal domain and the beginning of the C-terminal domain.

PyMOL was used to analyze the modeled C1 structure for surface electrostatics (Fig. 1 B) and to identify amino acids that might be important for controlling the C1 reaction. This analysis identified Arg-21 and Arg-217 as important for the interactions between the N- and C-terminal domains that likely participate in the regulatory function by HPA. MarR structures (Protein Data Bank codes 3BPX and 1JGS) (36, 38) show that Arg-16 and Arg-86, which are homologous to Arg-21 and Arg-217 in C1, bind to the carboxylate of its salicylate regulatory ligand perhaps analogously to C1 binding HPA. Therefore, several variants around Arg-21 and Arg-217 positions (R247A, I246W, R217A, F216A, R201A, and R21A) were constructed to test their functional roles.

Enzyme Preparations of Truncated C1 Variants—Three variants of C1 truncated at the C-terminal domain (t178C1, t230C1, and t259C1; Fig. 2) were constructed and overexpressed in E. coli (“Experimental Procedures” and supplemental data). The effect of HPA in stimulating NADH oxidase activity was ascertained by assaying activities of the truncated variants in the absence and presence of HPA (“Experimental Procedures”) (23, 25). The t230C1 and t259C1 variants exhibited markedly increased rates of NADH oxidation in the presence of HPA similar to wild-type C1 (data not shown), implying that these truncated variants can bind HPA normally. Therefore, we con-
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clude that residues 230–315 are not critical for regulating C1 activities. In contrast, the t178C1 variant catalyzed NADH oxidation at the same rate both in the absence and presence of HPA, implying that the C-terminal residues 179–229 are important for HPA binding and are stimulating C1 activity. Single site mutations of positively charged residues in this region were constructed to more precisely probe their roles in regulating C1 activity (results shown below).

The t178C1 truncated variant was purified according to the protocol used for the wild-type enzyme (24). The subunit molecular mass of the purified t178C1 was estimated to be ~20 kDa from SDS-PAGE analysis (supplemental Fig. S2A), and the native molecular mass determined via gel filtration analysis is ~45 kDa (supplemental Fig. S2B). These results indicate that like wild-type C1, and PheA2 (35) the truncated enzyme is also a homodimer (23). Table 1 compares biochemical and biophysical characteristics of t178C1 and wild-type C1. In general, the properties of t178C1 are similar to those of wild-type C1 (12, 25, 26). Most of the properties of C1 regarding catalytic and redox activities are retained in the N-terminal domain of the t178C1 truncated variant, but there is no regulation by HPA. These data are therefore consistent with the C-terminal domain being a regulatory domain that controls HPA-stimulated activity.

Reductive Half-reaction of t178C1 — The kinetics of reduction of the t178C1-bound FMN by NADH were investigated using stopped-flow spectrophotometry. Fig. 3 shows the kinetic traces recorded at 458 and 690 nm of the reduction by NADH of t178C1-bound FMN in the absence and presence of HPA. HPA had no measurable effect on the kinetics. Moreover, these traces are nearly indistinguishable from those for the reduction of wild-type C1 in the presence of 0.2 mM HPA (after mixing). For reference, the kinetic trace of wild-type C1 in the absence of HPA is overlaid (Fig. 3).

Traces monitored by absorbance changes at 458 and 690 nm were biphasic, and the data were analyzed according to the model used for analysis of wild-type C1 in the presence of HPA (see Scheme 2 in Ref. 25). The first phase of the reaction (dead time, ~0.003 s) resulted in formation of a charge transfer complex (C1-FMN-HPA-NADH) as noted by the absorbance increase at 690 nm. The second phase (0.003–0.01 s), demarked by a large decrease in absorbance at 458 nm and a small increase in absorbance at 690 nm, is due to reduction of FMN to form a second charge transfer species (C1-FMNH2-HPA-NADH). Apparent rate constants describing this phase were dependent on NADH concentration, and the value of k3 (flavin reduction) was analyzed according to Equation 1. The third phase (0.01–0.05 s) resulted in the decay of the second charge transfer species, and this phase was independent of HPA concentration.

The kinetic parameters of t178C1 are summarized in Table 2. The rate constants for reduction of FMN by t178C1 (276 ± 6 s⁻¹) were not affected by the presence of HPA. This result implies that in wild-type enzyme the C-terminal part of C1 (residues 179–315) inhibits reduction of FMN in the N-terminal domain, and HPA binding to the C-terminal domain alleviates this inhibition. Without the C-terminal domain, t178C1 is constitutively active with or without HPA present.

Oxidative Half-reaction of t178C1 — The oxidation of reduced t178C1 by oxygen was investigated using stopped-flow experiments and compared with the results for wild-type C1. Studies with wild-type C1 showed that superoxide dismutase (SOD), which catalyzes the oxidation of superoxide radicals, inhibits C1 activity without HPA present.

### Table 1

| Biochemical and biophysical properties | t178C1 | Value ratio (HPA/no HPA) | Wild-type C1 | Value ratio (HPA/no HPA) |
|--------------------------------------|--------|-------------------------|--------------|-------------------------|
| Extinction coefficient at 458 nm | 11.9 _±_ 0.3 x 10^3 | _—_ | 12.8 x 10^3 | _—_ |
| Kc (apoenzyme and free FMN) | 0.02 x 10^-3 | 0.034 ± 0.004 | 1.7 | 0.006 ± 0.001 |
| Subunit molecular mass (kDa) | 45 ± 1 | _—_ | 68 ± 1 | _—_ |
| Redox potential (mV) | 252 ± 1 | 252 ± 1 | 1 | 236b |
| Specific activity (units/mg⁻¹) | 7.1 ± 0.4 | 8.0 ± 0.7 | 1.1 | 15.2 ± 0.1 |

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*Data for wild-type C1 (25).  
*One unit is the amount of enzyme that catalyzes the oxidation of 1 μM of NADH min⁻¹. For the specific activity assay, enzyme (2 μM) was added to NADH (96 μM) in sodium phosphate buffer, pH 7.0 containing 100 μM HAP at 25 °C.
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motesthe wild-type enzyme to shift toward the faster reacting species that releases FMNH\(^{-}\) at a greater rate.

Kinetic traces for the oxidation of the flavin of t178C\(_{1}\) monitored at 458 nm in the absence and presence of HPA were single exponential (Fig. 4). Therefore, the reduced t178C\(_{1}\) reacted with O\(_{2}\) in a simple bimolecular reaction with the rate constant of 247 ± 2 m\(^{-1}\) s\(^{-1}\) (Table 2 and Scheme 1), which is 50% of that of wild-type enzyme (25). The presence of HPA had no effect on the oxidation of the t178C\(_{1}\) variant (Fig. 4). The experiment in Fig. 4 did not contain superoxide dismutase because superoxide dismutase introduced complicated multiphasic kinetics on t178C\(_{1}\) oxidation (supplemental Fig. S3). Therefore, oxidations of reduced wild-type C\(_{1}\) and of t178C\(_{1}\) occur via different mechanisms in which superoxide might be accumulated in the oxidation of the wild type but not significantly in the t178C\(_{1}\) variant. Nevertheless, these results for t178C\(_{1}\), like those for flavin reduction (Fig. 3), show no effects of HPA.

\[
\text{(HPA)}\text{t178C}_{1}^{\text{red}} + O_2 \rightarrow \text{(HPA)}\text{t178C}_{1}^{\text{ox}} + H_2O_2 \\
245 \text{ M}^{-1} \text{s}^{-1}
\]

**SCHEME 1**

**Flavin Transfer from t178C\(_{1}\) to C\(_{2}\)—**Using the stopped-flow spectrophotometer, a solution of reduced t178C\(_{1}\) was mixed with solutions of C\(_{2}\) containing various concentrations of O\(_{2}\).

![Kinetic traces for the reduction of t178C\(_{1}\), by NADH in comparison with C\(_{1}\) wild-type. t178C\(_{1}\) (20 μM final concentration) was reduced by NADH (6.4 mM final concentrations) in the absence and presence of HPA. The reactions were monitored at 458 (for oxidized FMN) and 690 nm (for charge transfer complexes) with a stopped-flow spectrophotometer. Solid lines are kinetic traces of the truncated variant in the absence and presence of HPA (traces from both conditions are overlaid). The dashed lines are kinetic traces of wild-type C\(_{1}\) in the absence (upper line) and presence (lower line) of 200 μM HPA (concentration after mixing) monitored at 458 nm. The reduction rate constants of truncated C\(_{1}\) in the absence and presence of HPA are nearly the same (276 ± 6 and 285 ± 4 s\(^{-1}\)) and also similar to that of wild-type C\(_{1}\) in the presence of HPA.**

![Kinetic traces for the reduction of t178C\(_{1}\), by NADH in comparison with C\(_{1}\) wild-type. t178C\(_{1}\) (20 μM final concentration) was reduced by NADH (6.4 mM final concentrations) in the absence and presence of HPA. The reactions were monitored at 458 (for oxidized FMN) and 690 nm (for charge transfer complexes) with a stopped-flow spectrophotometer. Solid lines are kinetic traces of the truncated variant in the absence and presence of HPA (traces from both conditions are overlaid). The dashed lines are kinetic traces of wild-type C\(_{1}\) in the absence (upper line) and presence (lower line) of 200 μM HPA (concentration after mixing) monitored at 458 nm. The reduction rate constants of truncated C\(_{1}\) in the absence and presence of HPA are nearly the same (276 ± 6 and 285 ± 4 s\(^{-1}\)) and also similar to that of wild-type C\(_{1}\) in the presence of HPA.**

**FIGURE 3. Kinetic traces for the reduction of t178C\(_{1}\), by NADH in comparison with C\(_{1}\) wild-type. t178C\(_{1}\) (20 μM final concentration) was reduced by NADH (6.4 mM final concentrations) in the absence and presence of HPA. The reactions were monitored at 458 (for oxidized FMN) and 690 nm (for charge transfer complexes) with a stopped-flow spectrophotometer. Solid lines are kinetic traces of the truncated variant in the absence and presence of HPA (traces from both conditions are overlaid). The dashed lines are kinetic traces of wild-type C\(_{1}\) in the absence (upper line) and presence (lower line) of 200 μM HPA (concentration after mixing) monitored at 458 nm. The reduction rate constants of truncated C\(_{1}\) in the absence and presence of HPA are nearly the same (276 ± 6 and 285 ± 4 s\(^{-1}\)) and also similar to that of wild-type C\(_{1}\) in the presence of HPA.**

**TABLE 2**

Summary of kinetic parameters of t178C\(_{1}\)

| Kinetic constants          | t178C\(_{1}\) Value ratio | Wild-type C\(_{1}\) Value ratio |
|----------------------------|----------------------------|---------------------------------|
| Flavin reduction (s\(^{-1}\)) | 276 ± 6 285 ± 4 1          | 116* and 3.1* 300*              |
| Flavin oxidation (m\(^{-1}\)s\(^{-1}\)) | 247 ± 2 246 ± 1 1         | 820 (50%)* and 320 (50%)*       |
| Reduced flavin transfer to C\(_{2}\) (s\(^{-1}\)) | 42 ± 2 42 ± 4 1          | 0.35* 74                        |
| Reduced flavin transfer to cyt c (s\(^{-1}\)) | 75 ± 1 76 ± 1 1          | 0.35* 80*                      |

* Data from wild-type C\(_{1}\) (25).

* Value could not be calculated.

* Data from wild-type C\(_{1}\) (12).

*Cytochrome.
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in the absence of HPA and 80 s\(^{-1}\) in the presence of HPA (\~\textasciitilde230-fold difference; Table 2). These results again confirmed that the autoinhibition and the effect of HPA on stimulation of FMNH\(^-\) release in \textit{C}_1 were absent in the t178C\(_2\) variant.

\textit{Enzyme Preparation of Single Site Variants of \textit{C}_1}—All of the above results suggest that the C-terminal domain of \textit{C}\(_1\) is a regulatory domain for controlling the production of FMNH\(^-\). The results in Figs. 3 and 4 suggest that for the wild-type \textit{C}_1, the C-terminal domain acts to inhibit both the reduction of the flavin and the release of FMNH\(^-\) to the solution. In the presence of HPA, we suggest that domain movement occurs to relieve the inhibition, resulting in \~\textasciitilde20-fold faster flavin reduction and \~\textasciitilde230-fold faster release of FMNH\(^-\). Because the crystal structures of \textit{C}_1 with and without HPA bound are currently not available, putative binding sites of HPA on the C-terminal domain of \textit{C}_1 were postulated from its homology with the x-ray structure of the MarR-salicylate complex (Protein Data Bank codes 3BPX and 1JGS). The Arg-86 and Arg-16 residues of MarR undergo a conformational change upon binding salicylate to optimize its ability to bind its target DNA (36, 38). On the basis of its homology to MarR and the results obtained with the truncated \textit{C}_1 variants (Figs. 2 and 3), we propose that the HPA binding site is located near residues 193–201 and 220–264 of \textit{C}_1. In addition, we deduced that a negatively charged moiety of HPA would interact with positively charged residues in that region. Therefore, residues Arg-21 and Arg-217, which are located between the N- and C-terminal domains, were identified as putative residues that are important for controlling the NADH oxidation activity of \textit{C}_1. Single site variants of these residues and of other residues located in the same region (Arg-21, Arg-201, Phe-216, Arg-217, Ile-246, and Arg-247) were constructed, changing these residues to Ala or Trp (for Ile-246) (Figs. 1 and 2 and supplemental Table S1). Most of these variants (R247A, I246W, R21A, R217A, and F216A) were expressed and purified using the same protocol as used for the wild-type enzyme, and their flavin reduction kinetics were measured in the absence and presence of HPA (see below). With R201A, only the crude extract was tested for the NADH oxidation activity in the absence and presence of HPA. R201A is as active as the wild-type \textit{C}_1, and its NADH oxidase activity can be enhanced by the presence of HPA just as with the wild-type enzyme (supplemental Fig. S4). Therefore, the involvement of this residue with the HPA binding was ruled out.

\textit{Kinetics of Flavin Reduction of R247A, I246W, R21A, R217A, and F216A in the Absence and Presence of HPA}—Solutions of oxidized R247A, I246W, R21A, R217A, and F216A variants were mixed anaerobically with various NADH concentrations in the absence and presence of HPA using the stopped-flow spectrophotometer, and the reactions were monitored by the absorbance changes at 458 and 690 nm. Figs. 7 and 8 show time courses for the reduction of these variants by 6.4 mM NADH in the absence and presence of HPA. The data were analyzed as described for Fig. 3. For most variants, the presence of HPA increased the rate constant for the reduction of enzyme-bound
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flavin about 17–24-fold, which is similar to the effect of HPA observed with the wild-type C1 (~20-fold; Table 3). Therefore, these residues are not directly involved in the HPA binding. The only exception was observed for the F216A variant in which there was no stimulation of flavin reduction by HPA. This result implies that Phe-216 is likely involved with the HPA binding and/or the stimulation of flavin reduction by HPA.

Further attempts were carried out to evaluate the properties of the F216A variant. Gel filtration chromatography data indicated that F216A is a dimeric enzyme similar to the WT and the truncated variant t178C1. CD spectra of wild type and the F216A variant were similar and were unaffected by addition of HPA. These results imply that the presence of HPA does not cause any significant change in secondary structures of the proteins. The rate constants obtained from these traces are given in Table 3. The inset shows kinetic traces of F216A mutant at 458 and 690 nm.


dashed lines for wild-type C1 in the absence (upper) and presence (lower) of 200 μM HPA (after mixing). The rate constants obtained from these traces are given in Table 3. The inset shows kinetic traces of F216A mutant at 458 and 690 nm.

**DISCUSSION**

The results reported herein imply that the C-terminal domain of C1 is an autoinhibitory domain that suppresses the redox activity of C1 in the absence of its effector, HPA. Upon binding HPA, the repression of C1 activity is alleviated presumably by a protein conformational change(s) that moves the C-terminal domain away from the active site. The regulatory mechanism illustrated for C1 is unusual among the two-component flavoprotein monoxygenases. Most of the flavin reduction rate constants of each variant are shown in Table 3. The rate constants of each variant are shown in Table 3. The inset shows kinetic traces of F216A mutant at 458 and 690 nm.

**TABLE 3**

Summary of flavin reduction rate constants of C1 variants and wild-type

| Enzyme       | Reduction rate constant a (s⁻¹) | Value ratio (HPA/no HPA) |
|--------------|-------------------------------|--------------------------|
| Wild-type C1 | 14.7 a                        | 20                       |
| t178C1       | 276 ± 6                       | 285 ± 4                  |
| F216A        | 287 ± 2                       | 286 ± 4                  |
| R217A        | 17 ± 1                        | 285 ± 4                  |
| J246W        | 13 ± 1                        | 263 ± 4                  |
| R247A        | 11 ± 1                        | 267 ± 2                  |

a Calculated from the reductive half-reaction traces that contained 6.4 mM NADH in anaerobic 50 mM phosphate buffer, pH 7.0 at 4 °C.

b Data for wild-type C1 (25).

c The C-terminal domain of C1 is an autoinhibitory domain that suppresses the redox activity of C1 in the absence of its effector, HPA. Upon binding HPA, the repression of C1 activity is alleviated presumably by a protein conformational change(s) that moves the C-terminal domain away from the active site. The regulatory mechanism illustrated for C1 is unusual among the two-component flavoprotein monoxygenases. Most of the flavin reduction rate constants of each variant are shown in Table 3. The rate constants of each variant are shown in Table 3. The inset shows kinetic traces of F216A mutant at 458 and 690 nm.

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**DISCUSSION**

The results reported herein imply that the C-terminal domain of C1 is an autoinhibitory domain that suppresses the redox activity of C1 in the absence of its effector, HPA. Upon binding HPA, the repression of C1 activity is alleviated presumably by a protein conformational change(s) that moves the C-terminal domain away from the active site. The regulatory mechanism illustrated for C1 is unusual among the two-component flavoprotein monoxygenases. Most of the flavin reduction rate constants of each variant are shown in Table 3. The rate constants of each variant are shown in Table 3. The inset shows kinetic traces of F216A mutant at 458 and 690 nm.
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The C-terminal domain of C1 has homology to MarR proteins whose structures are characterized by a helix-winged protein fold for binding to DNA targets (38). The binding of salicylate and other aromatic compounds to MarR pushes apart the two DNA binding lobes, thereby abolishing the ability of MarR to bind to DNA (36). Because binding of HPA can alleviate the autoinhibitory effect of the somewhat homologous C-terminal domain of C1, we suggest that conformational changes similar to those of MarR may also occur in C1 upon binding HPA. We suspect that the binding of HPA would disrupt interactions between the C- and N-terminal domains of C1 so that the enzyme-bound flavin becomes more accessible to NADH and to solvent (Figs. 1 and 9). The observation that other aromatic compounds in addition to HPA can stimulate C1 activity (23) to solvent (Figs. 1 and 9). The observation that other aromatic compounds in addition to HPA can stimulate C1 activity (23) suggests that this regulatory mechanism of C1 is a general means for HPAH to increase the rate of reduced flavin production when a potential substrate for the oxygenase component is present.

It can be envisaged that the C-terminal autoregulatory domain of C1 is an important adaptation to minimize production of reactive oxygen species for two-component flavin-dependent monoxygenases. Another means for minimizing reactive oxygen species is to use protein-protein interactions as found in the bacterial luciferase system (8). Several different potential allosteric substrate-like effectors presumably can activate the autoregulatory domain of C1. This would permit a single reductase to be useful to and be regulated by several different molecules that would undergo oxygenation by their respective oxygenases.

Site-directed mutagenesis and kinetic investigations have identified residue Phe-216 as being important for HPA to exert its regulatory function. Results from truncated mutants have shown that residues 230–315 are not directly involved in the autoinhibitory mechanism in C1 because mutants truncated in this region behaved similarly to the wild-type enzyme (data not shown). Of the mutations of residues Arg-21, Phe-216, Arg-217, Ile-246, and Arg-247, only the F216A variant lacks the stimulatory effect by HPA. These C1 variants were chosen from analogies to the MarR Protein Data Bank structures 1JGS (38) and 3BPX (36), noting how MarR binds to the carboxylate of salicylate via Arg-16 and Arg-86. The mutations listed in Table 3 were focused on the location in which the C- and N-terminal domains are postulated to interact (Fig. 1). The results in Table 3 indicate that changes in residues Arg-21, Arg-217, Ile-246, and Arg-247 did not affect the kinetic properties of the reductase, thus eliminating consideration of their involvement in controlling the autoinhibition of C1. However, the F216A variant, which according to the modeled structure is located in a flexible loop region near a hydrophobic zone, may be involved in promoting tighter interactions between the N- and C-terminal domains; for example, the F216A variant may not allow the N- and C-terminal domains to interact as tightly as in wild-type C1. This variant thereby permits rapid flavin reduction in the absence of HPA. When the x-ray structure of C1 is available, the data reported here will become more useful for interpreting the functional roles of these residues. Recently, the crystalization and preliminary x-ray analysis of C1 have been reported (44).

A similar controlling mechanism for flavin reduction and the subsequent electron transfer via an autoinhibitory domain were found for the reaction of endothelial and neuronal nitric-oxide synthases, which contain P450 heme (oxygenase) and diflavin (reductase) domains. Binding of calmodulin/Ca2+ increases the rates of interdomain electron transfer from reduced FAD to FMN and from reduced FMN to heme iron in the oxygenase domain (1, 6). Deletion of the autoinhibitory domain in NOS resulted in an enzyme that is reduced faster and has greater NO synthesis activity (7, 45–47). In addition, single site charge reversal variants of neuronal NOS, such as K842E (6) and R1229E (48), display faster flavin and heme reduction kinetics in the calmodulin-free enzyme. These substituted residues might not promote autoinhibitory interactions between domains and thus could result in enzyme variants with more accessible redox-active sites. In this respect, the control of C1 activity by the C-terminal domain and the fact that a single site variant (F216A) can abolish the autoinhibitory feature resemble the results reported for NOS reactions.

In conclusion, this study has shown that in the absence of HPA the two-component flavin-dependent HPAH from A. baumannii utilizes the C-terminal domain of C1 as an autoinhibitory domain to suppress both the rate of reduction of FMN and the rate of release of FMNH2 from C1. This avoids wasteful consumption of NADH and formation of reactive oxygen species. Binding to HPA alleviates the autoinhibition by inducing conformational changes that allow C1 to generate reduced flavin for the oxygenase domain. This catalytic feature is unusual among known two-component flavin-dependent monoxygenases but may be common in flavin reductases harboring the extra C-terminal domain.

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