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Monomer Formation and Function of *p*-Hydroxybenzoate Hydroxylase in Reverse Micelles and in Dimethylsulfoxide/Water Mixtures

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It has previously been postulated that the dimeric form of the flavoprotein p*-hydroxybenzoate hydroxylase (PHBH) is important for catalysis. Here it is demonstrated that the monomeric form of PHBH is active. In a water/AOT/isooctane reverse micellar system, the function of the monomeric and dimeric forms of PHBH could be observed separately by varying the size of the micelles. A considerable decrease in the* K_M *value for* p*-hydroxybenzoate (POHB) was found for monomeric PHBH, accompanied by a 1.5-fold decrease in enzymatic activity. The same tendency was observed when monomers of PHBH were formed by adding DMSO to the buffer. The FAD in PHBH and PHBH labeled with the fluorescence dye Alexa488 was investigated by time-resolved fluorescence anisotropy to observe monomer formation in water/DMSO mixtures. Monomer formation of PHBH occurred gradually with increasing DMSO content in the mixture. Pure PHBH monomers were detected at DMSO concentrations of 30% (v/v) and higher.*

Introduction

para-Hydroxybenzoate hydroxylase (PHBH; EC 1.14.13.2) is a model flavoenzyme involved in the microbial metabolism of aromatic compounds.[1–3] PHBH catalyses the conversion of 4-hydroxybenzoate into 3,4-dihydroxybenzoate, using NADPH and molecular oxygen as cosubstrates. PHBH is a homodimer of about 88 kDa, with each monomer containing one FAD molecule. Although the mechanism of the action of PHBH has been extensively studied,[4–8] there are no direct indications of how enzyme catalysis is linked to the dimeric form. It has been suggested that the binding of *p*-hydroxybenzoate (POHB) to one of the subunits of the enzyme triggers conformational changes at the active site of the alternate subunit, thus generating a fully active enzyme.[2]

This work was aimed toward providing better insight into the role of subunit interactions in PHBH catalysis. Earlier experiments had indicated that the PHBH dimer is rather stable and that this stability is governed by the dissociation rate constant.[9] To modulate the oligomeric composition in PHBH we have applied a reverse micelle system, which can be regarded as a "nanoreactor" of molecular size.[10–13] The sizes of the inner cavities of the micelles where protein molecules are entrapped can be strictly controlled by varying the surfactant hydration degree (w_0), which is determined by the molar ratio of water to surfactant.[11,14] Reverse micelles of AOT in octane are a powerful tool with which to control the supramolecular structures of enzymes.[15–19] We have followed this approach to distinguish between the activities of the pure monomeric and dimeric forms of PHBH function at different degrees of hydration of the micelles. Water/DMSO mixtures were applied as a complementary system to induce PHBH monomer formation. DMSO is known to promote dissociation of the dimeric protein by weakening hydrophobic interactions between the subunits.[9,20]

Time-resolved fluorescence techniques were used to visualize the changes in the oligomeric composition of PHBH, since these methods provide detailed information on the microenvironment of the fluorescent molecule.[21] With the time-resolved fluorescence anisotropy technique the rotational motion of supramolecular protein complexes can be studied. Since the rotational correlation time is directly proportional to protein mass,[22] monomer formation can be observed through measurements of rotational correlation times that should be roughly two times shorter for a monomer than for a dimer. In addition, segmental motion can be distinguished from the overall rotation of protein complexes.[23–25] For PHBH we use two types of fluorophores: the FAD prosthetic group and the much brighter Alexa488 dye, which can be selectively attached to Cys116 through a maleimide derivative.[23]

In this study the monomeric form of PHBH was isolated by entrapment in reverse micelles and by addition of DMSO. The

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results obtained demonstrate for the first time that the PHBH monomer is active. The impact of this result for the molecular mechanism of the enzyme function is discussed.

Results

Regulation of the oligomeric composition of PHBH in reverse micelles

It has been found that an enzyme in a reverse micelle system normally shows a bell-shaped dependence of its catalytic activity on the degree of surfactant hydration—($w_0 = [H_2O]/[AOT]$)—with an optimum at conditions under which the enzyme size corresponds to the size of the micelle inner cavity.[11,12,14,16,19] From the position of the activity optimum (w_0) one can calculate the inner radius of the micelles (r_m), which can be used to deduce the enzyme maximal radius according to an empirical equation: $r_m(Å) = (1.5\,w_0 + 4)$.[31] Oligomeric enzymes show activity profiles with several optima, where each of these optima reflects a different oligomeric form of the enzyme.[15,16,18]

In this work the AOT/isooctane/water reverse micellar system was applied to monitor the functional properties of isolated monomeric and dimeric forms of PHBH. The dependence of PHBH activity on the degree of hydration (w_0; Figure 1) shows two optima, at hydration ratios of 13 and 18, which are tentatively assigned to the monomeric and dimeric forms of PHBH, respectively. Radii of micelles determined from these w_0 values are 23.5 and 31 Å. The radius (r_m) at $w_0 = 18$, corresponding to the optimal catalytic activity of PHBH, is in good agreement with the maximal radius (r_p) of the dimeric enzyme obtained from its 3D structure (Table 1). At the same time, for monomeric PHBH the optimal size of the micelles (r_m) is somewhat larger than the radius of the protein (r_p) calculated from the crystal structure data (Table 1). As discussed previously,[12] such deviations can be due to a nonspherical shape of the protein molecule (see also below).

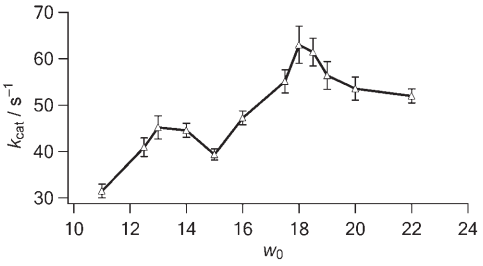

Figure 1. Dependence of the catalytic activity of PHBH on the degree of hydration (w_0; $w_0 = [H_2O]/[AOT]$) in the system of reversed micelles of AOT in isooctane. Experimental conditions: AOT (0.2 M), Tris/sulfate (100 mM), pH 8.0. POHB and NADPH concentrations were varied from 20 to 400 μM to determine the turnover numbers from the initial velocity of NADPH consumption. Kinetic data were analyzed by nonlinear least-squares fitting analysis.[29]

The kinetic parameters (k_{cat} and K_M for NADPH, as well as K_M for POHB) determined in AOT reverse micelles for the dimeric form of PHBH are close to those observed in aqueous solution (Table 2). Monomeric PHBH, however, shows a 1.5-fold decrease in k_{cat} and a considerable decrease in K_M for POHB, while K_M for NADPH is practically unchanged in relation to that of the dimer (Table 2).

Table 2. Kinetic parameters of PHBH determined with natural substrates at pH 8.0 in aqueous solution, in the reverse micellar system of AOT in isooctane at degrees of hydration of 13 and 18, corresponding to the function of the monomeric and dimeric forms of the enzyme, respectively, and in water/DMSO mixtures containing DMSO (20%, *v/v*). Turnover numbers are extrapolated to infinite concentrations of POHB and NADPH. Kinetic constants have maximal error values of 10%.

System	K_M NADPH [μM]	K_M POHB [μM]	k_{cat} [s^{-1}]	Oligomeric form
aqueous solution/ literature data[1]	37/23	18/11	61/57	dimer
AOT, $w_0 = 13$	52	0.6	45	monomer
AOT, $w_0 = 18$	25	25	63	dimer
DMSO, 20% (*v/v*)	60	10	30	monomer/dimer

The catalytic activity of PHBH in water/DMSO mixtures

We have previously shown that the stability of the PHBH dimer is governed by the dissociation rate constant and that the rate of dimer dissociation is increased in an aqueous solution containing DMSO.[9] When the catalytic performance of PHBH in water/DMSO mixtures is studied, the turnover rate shows a monotonous decrease as a function of DMSO concentration (Figure 2).

At a DMSO concentration of 20% (*v/v*) the activity of PHBH is halved, even though such a DMSO concentration is normally lower than the "threshold" concentration at which denaturation is observed for many enzymes.[32–34] It is remarkable that in 20% DMSO the K_M for POHB is lower than that in aqueous solution. A similar effect on the K_M for POHB was observed upon monomer formation of PHBH in reverse micelles. The decrease

Table 1. Comparison of the maximal radii (r_p) of dimeric and monomeric (as a subunit of the dimer) forms of PHBH obtained from its three-dimensional dimeric structure,[45] and the inner radius of the micelle (r_m) calculated from the position of the activity optimum ($w_{0,opt.}$) of the enzyme in water/AOT/isooctane reverse micelles.

PHBH form	$a \times b \times c$ [Å][a]	r^*_p [Å][b]	$w_{0,opt.}$	r_m [Å] ($r_m = 1.5\,w_0 + 4$)	Difference between r_m and r_p [%]
monomer	21.1×22.6×37.4	18.7	13	23.5	25
dimer	21.1×58.7×31.4	29.3	18	31	5

[a] The geometrical size of the enzyme. [b] Half the length of the longest dimension of the PHBH dimer calculated from the 3D structure.[45]

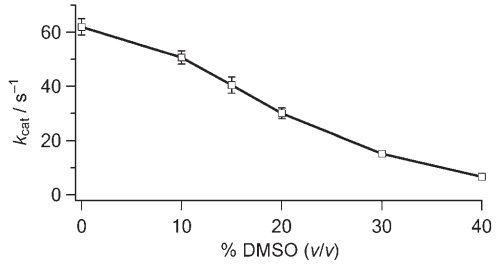

Figure 2. Dependence of the catalytic activity of PHBH on the DMSO concentration. Experimental conditions: Tris/sulfate (100 mM), pH 8.0. POHB and NADPH concentrations to determine the turnover numbers from initial velocity of NADPH consumption were 20–400 μM. Kinetic data were analyzed by nonlinear least-squares fitting analysis.[29]

in K_M POHB in both systems is even more noticeable when it is taken into account that both addition of organic solvents and entrapment in reverse micelles can result in higher apparent K_M values for aromatic substrates, due to a smaller preference for substrate extraction from solution to the enzyme active site.[11,12,32,34,35] Therefore, the presence of DMSO during catalysis most probably induces dissociation of PHBH. In 20% DMSO, the turnover rate is considerably less than that observed for the monomeric form of PHBH in reverse micelles (Table 2). The lower activity in DMSO also cannot be explained simply by the increased viscosity (Table 3). This suggests that there is some inactivation of the enzyme by DMSO, and that the monomeric enzyme is more sensitive to inactivation than the dimer.[9] A further increase in DMSO concentration up to 40% (*v*/*v*) results in a decrease in enzyme activity down to 15% of the initial level.

Time-resolved fluorescence and anisotropy of FAD-PHBH in water/DMSO mixtures

Another approach for demonstrating the existence of PHBH monomers in water/DMSO mixtures involves time-resolved fluorescence spectroscopy. In many flavoenzymes the fluorescence of the flavin prosthetic group shows low molecular brightness due to severe quenching.[36,37] Recently, we have systematically investigated the experimentally observed total flavin fluorescence decay of wild-type PHBH and its Y222A and Y222V muteins.[38] Although flavin fluorescence in PHBH is strongly quenched, the quenching is considerably less than in *E. coli* glutathione reductase (GR): the ratio of fluorescence quantum yields $Q_{PHBH}/Q_{GR} \approx 15$. For substrate-free PHBH in aqueous buffer the fluorescence lifetime pattern shows high heterogeneity, with at least four lifetime components covering a dynamic range between 15 ps and 3 ns.[38] This could be due to the various dynamic states previously reported for PHBH.[39] From the similarity in structure and fluorescence characteristics between PHBH and other tyrosine-containing flavoproteins such as glutathione reductase[36] and NADPH peroxidase,[40] the mechanism of quenching most probably involves photoinduced electron transfer from particular tyrosine residues to the light-excited flavin. Tyr222 is very likely to be the quenching residue.[38] In single-molecule studies the Y222A variant does not show the fluctuations in fluorescence due to open-to-in positional dynamics.[39]

Here, we used time-resolved fluorescence to characterize the excited-state properties of FAD-PHBH in different mixtures of water and DMSO. When DMSO was added, the average fluorescence lifetime $\langle\tau\rangle$ increased from 0.6 ns in aqueous buffer to 1.1 ns in 40% DMSO (Table 3). Fluorescence anisotropy decay of FAD-PHBH was used to monitor the rotational motion of the dimeric enzyme. Representative examples of fluorescence anisotropy decay of FAD-PHBH in aqueous solution and in 40% DMSO (*v*/*v*) and the analysis are presented in Figure 3. A single rotational correlation time (ϕ_{prot}) of 32 ns was obtained for FAD-PHBH in aqueous solution, and is in excellent agreement with that expected for a dimeric PHBH molecule.[22] Although the 1.6 ns and 3.1 ns fluorescence lifetime components only contribute to the total fluorescence decay by 7% and 2%, respectively,[38] they are the main carriers of the anisotropy signal in the nanosecond time region.

The results of the fluorescence anisotropy decay analysis obtained for FAD-PHBH in aqueous solution and in water/DMSO mixtures are summarized in Table 3. The results indicate that FAD is rigidly bound to the protein and rotates together with the protein. The viscosities (η) of the water/DMSO mixtures increase at higher DMSO content. Therefore, to make valid comparisons between results from various water/DMSO mixtures, the experimental rotational correlation times must be divided by viscosity values. In water/DMSO mixtures the value of ϕ_{prot}/η for FAD-PHBH decreases with increasing DMSO concentration (Table 3). Although it looks as though the fluorescence anisotropy decay of FAD-PHBH in 40% DMSO (*v*/*v*) is slower than in aqueous buffer (see Figure 3B), after correction for viscosity, ϕ_{prot} decreases to a value of 21 ns. Time-resolved fluorescence anisotropy of FAD-PHBH can also be used as a diagnostic tool to investigate the release of FAD from the protein, be-

Table 3. Average fluorescence lifetimes ($\langle\tau\rangle$) and fluorescence anisotropy decay parameters (β_i, ϕ_{prot}) of FAD-PHBH in water/DMSO mixtures.[a]

Percentage of DMSO (*v*/*v*)	$\langle\tau\rangle$ [ns]	$r(0)$	ϕ_{prot} [ns]	Relative viscosity (η)	ϕ_{prot}/η [ns]
0	0.62 (0.58–0.67)	0.364	31.8 (30.1–33.6)	1.00	31.8 (30.1–33.6)
10	0.79 (0.74–0.85)	0.370	36.5 (34.4–38.7)	1.25	29.2 (27.5–31.0)
20	0.93 (0.90–0.96)	0.374	43.2 (40.9–45.7)	1.57	27.5 (26.0–29.1)
30	1.00 (0.91–1.13)	0.361	42.0 (39.9–44.2)	2.01	20.9 (19.9–22.0)
40	1.09 (1.01–1.23)	0.355	55.0 (37.0–65.0)	2.58	21.3 (16.3–25.2)

[a] The average fluorescence lifetime or amplitude-weighted fluorescence lifetime is defined as $\langle\tau\rangle = \sum_{i=1}^{N} \alpha_i\tau_i / \sum_{i=1}^{N} \alpha_i$; α_i is the amplitude of lifetime component τ_i. Values in parentheses were obtained after rigorous error analysis at the 0.67 confidence level in a global analysis of two separate experiments.

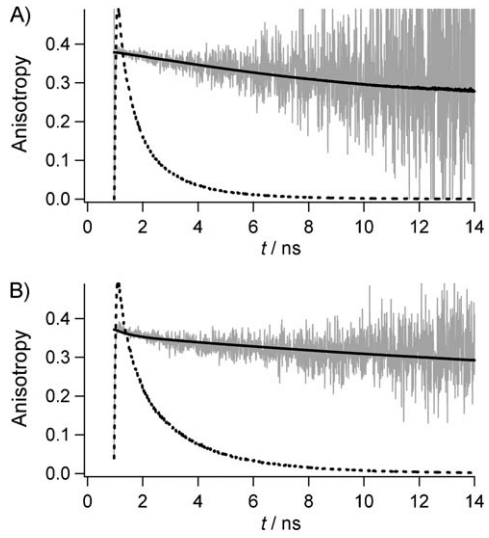

Figure 3. Fluorescence anisotropy decay of PHBH. A) Experimentally observed fluorescence anisotropy decay (gray) and corresponding theoretical curve (black) retrieved from the optimal monoexponential fit of PHBH (10 μM) in potassium phosphate (50 mM), pH 7.5, at 293 K. The recovered parameters are the initial anisotropy—$r(0) = 0.37$—and the rotational correlation time—$\phi = 31 \pm 2$ ns. The time-dependent total fluorescence intensity normalized to 0.5 (-----) has been included in the graph for clarity. A rotational correlation time of 31 ns agrees well with a protein of 88 kDa. B) Experimentally observed fluorescence anisotropy decay and corresponding theoretical curve (black) retrieved from a biexponential fit of wild-type PHBH (10 μM) in potassium phosphate (50 mM), pH 7.5 containing DMSO (40%, *v*/*v*) at 293 K. The recovered parameters are: $\beta_1 = 0.017$, $\phi_1 = 0.5$ ns, $\beta_2 = 0.355$, $\phi_2 = 58$ ns. The short correlation time of 0.5 ns arises from dissociated FAD, and the pre-exponential factor of 0.017 indicates the presence of about 5% dissociated FAD. The time-dependent total fluorescence intensity normalized to 0.5 (-----) has been included in the graph. The fluorescence intensity in the tail of the curve (> 10 ns) is approximately four times higher than that in (A). This explains the less noisy experimental anisotropy in relation to that in panel A.

cause free FAD will give rise to a rapid correlation time component in the time-dependent anisotropy. At 40% DMSO only a small fraction (5%) of FAD is released from the protein (see Figure 3B). The rotational correlation time of free FAD is based upon 0.5/2.58 = 0.19 ns [2.58 is the relative viscosity of 40% DMSO (*v*/*v*)], which is the experimental correlation time as reported previously.[41]

Time-resolved fluorescence and anisotropy of Alexa488-PHBH in water/DMSO mixtures

PHBH from *Pseudomonas fluorescens* contains a surface-accessible cysteine (Cys116), which can be specifically covalently labeled with maleimides without influencing the enzyme activity.[9,42] As described previously, we modified PHBH with Alexa488 maleimide to yield one strongly fluorescent label per enzyme subunit.[23] The total fluorescence decay of Alexa488-PHBH was best fitted with a biexponential function with a major component with a lifetime of 3.7 ns and a minor component with a lifetime of around 1 ns, leading to an average fluorescence lifetime of 3.2 ns.[23] The average fluorescence lifetime of Alexa488-PHBH is unchanged with addition of DMSO up to 20% (*v*/*v*), while at DMSO concentrations of 30% (*v*/*v*) the average fluorescence lifetime becomes slightly longer (3.4 ns; Table 4).

The important advantages of using Alexa488-PHBH instead of native PHBH in time-resolved fluorescence anisotropy measurements are a much greater brightness of the fluorescence and a longer fluorescence lifetime. Analysis of the fluorescence anisotropy decays of Alexa488-PHBH in aqueous solution yielded a short correlation time that reflects rapid segmental motion and a longer rotational correlation time that reflects protein rotation.[23] Important to note is that the overall rotational correlation time seems somewhat shorter than that observed for FAD-PHBH. As discussed previously, the longer measured correlation time is shorter than the correlation time for overall protein rotation because of contributions from the segmental motion.[23] Similar results are obtained for Alexa488-PHBH in water/DMSO mixtures. In Table 4 we present the values of the long rotational correlation time of Alexa488-PHBH at increasing DMSO concentrations, together with those that are normalized by the relative viscosity of the system. In water/DMSO mixtures the ϕ_{prot}/η value clearly decreases with increasing DMSO concentration, indicating gradual monomerization of Alexa488-PHBH. The twofold decrease in ϕ_{prot}/η suggests complete monomer formation at DMSO concentrations of 30% (*v*/*v*).

Dose-response analysis

The most direct way to obtain fractions of oligomeric species is by analysis of the fluorescence anisotropy decays. Suppose

Table 4. Average fluorescence lifetimes ($\langle\tau\rangle$) and long correlation times (ϕ_{prot}) of Alexa488-PHBH in water/DMSO mixtures.[a]

Percentage of DMSO (*v*/*v*)	$\langle\tau\rangle$ [ns]	β_2	ϕ_{prot} [ns]	Relative viscosity (η)	ϕ_{prot}/η [ns]
0	3.08 (3.00–3.18)	0.20	19.3 (16.2–22.3)	1.00	19.3 (16.2–22.3)
10	3.14 (3.02–3.18)	0.20	17.5 (14.2–23.0)	1.25	14.0 (11.4–18.4)
20	3.19 (3.06–3.28)	0.20	19.2 (15.4–26.3)	1.57	12.3 (9.8–16.7)
30	3.41 (3.32–3.49)	0.20	20.8 (15.7–26.8)	2.01	10.4 (7.8–13.3)
40	3.42 (3.36–3.50)	0.20	25.5 (19.9–33.2)	2.58	9.8 (7.7–12.9)

[a] The average fluorescence lifetime or amplitude-weighted fluorescence lifetime is defined as $\langle\tau\rangle = \sum_{i=1}^{N} \alpha_i\tau_i / \sum_{i=1}^{N} \alpha_i$; α_i is the amplitude of lifetime component τ_i. Values in parentheses were obtained after rigorous error analysis at the 0.67 confidence level in a global analysis of two separate experiments. The initial anisotropy $r(0)$ is on average 0.33 ± 0.01.

that we have two rotating protein species in equilibrium: dimers with rotational correlation time ϕ_1 and monomers with rotational correlation time ϕ_2. The apparent observed rotational correlation time can then be regarded as a harmonic mean correlation time:[42]

$$\phi_{obs} = \sum_{i=1}^{2} \beta_i / \sum_{i=1}^{2} (\beta_i/\phi_i)$$

For quantitative analysis the amplitudes β_i must be weighted by the fluorescence efficiencies of both species. However, if it is assumed that the fluorescence efficiency of a dimer is twice that of a monomer, then the amplitude β_i immediately represents the fractional contribution of the oligomeric protein species *i*. If we further take ϕ_1 as the first point (no DMSO present) and ϕ_2 as the last point (40% DMSO) in the titration with DMSO and, noting that $\beta_1+\beta_2=r(0)$ (see Table 3), then β_1 can be expressed in all three correlation times and

$$r(0):\ \beta_1 = r(0) \times \phi_1 \times \left(\frac{1}{\phi_{obs}} - \frac{1}{\phi_2}\right) / \left(1 - \frac{\phi_1}{\phi_2}\right)$$

The results for the FAD-PHBH anisotropy data have been collected in Figure 4. The points have been fitted to a sigmoid function (for details see legend of Figure 4) to obtain the concentration at which 50% of PHBH dimers are present ($[DMSO]_{1/2}$). We obtained $[DMSO]_{1/2}=23.5\pm6.8\%$ (*v*/*v*). We have also subjected the Alexa488-PHBH data to the same analysis, which leads to a value with larger error: $[DMSO]_{1/2}=17.5\pm12.2\%$ (*v*/*v*; for graph see Figure S1 in the Supporting Information). The larger error is due to the fact that the dynamic range of the anisotropy is smaller than that for FAD-PHBH by almost a factor of two ($\beta_2=0.2$; see Table 4).

The dependence of the fluorescence lifetimes on DMSO concentration has also been analyzed in terms of a sigmoid model function. $[DMSO]_{1/2}$ then signifies the concentration at which a 50% change in lifetime range is observed. The changes in the average fluorescence lifetime of FAD as function of DMSO concentration were too gradual to give a reliable midpoint concentration, since the standard deviation is much larger than the actual value: $[DMSO]_{1/2}=4.9\pm35.3\%$ (*v*/*v*; Figure S2). A plausible explanation is that the average fluorescence lifetime is dominated by the very short fluorescence lifetime component arising from picosecond quenching.[38] This lifetime component then undergoes a gradual, noncooperative change with a midpoint at 20%. On the other hand, the long, unquenched fluorescence lifetime component is more sensitive to changes in the direct isoalloxazine environment. Indeed, when the long fluorescence lifetime component of FAD-PHBH is analyzed, a reliable midpoint value is obtained: $[DMSO]_{1/2}=18.1\pm6.3\%$ (*v*/*v*; Figure S3). This also applied for the average lifetime of Alexa488-PHBH, leading to $[DMSO]_{1/2}=22.7\pm6.4\%$ (*v*/*v*; Figure S4). The combination of these results leads to $[DMSO]_{1/2}=20.6\pm2.7\%$ (*v*/*v*).

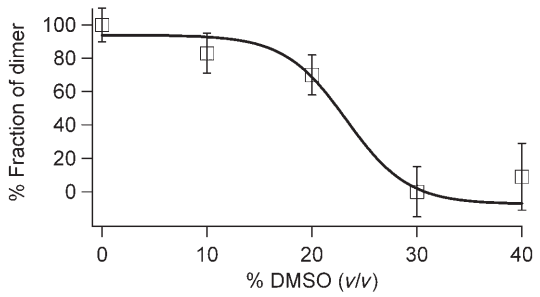

Figure 4. Fraction of dimeric PHBH as recovered from analysis of the observed rotational correlation times obtained from fluorescence anisotropy decay of FAD-PHBH as a function of [DMSO]. The standard deviations in the data points are estimated from the confidence intervals of the observed rotational correlation time (see Table 3) with the assumption of precise values of *r*(0). The experimentally determined data points, weighted by their standard deviations, are fitted to a sigmoid equation to determine the concentration of DMSO at which 50% of dimeric species is present—$[DMSO]_{1/2}$. The sigmoid equation has the following form: $y=\text{base}+\text{max}/\{1+\exp[(x_{half}-x)/\text{rate}]\}$. The fit parameters were base $=94\pm8$, max $=-101\pm54$, rate $=3.0\pm4.5$, and $x_{half}=23.5\pm6.8$ ($x_{half}=[DMSO]_{1/2}$). The fitting procedure was performed with the aid of Igor Pro (Wavemetrics Inc., Lake Oswego, OR, USA).

Discussion

This is the first report on a systematic investigation of monomer formation in dimeric PHBH. Reverse micelles were successfully applied to measure the catalytic activity of isolated PHBH monomers in a nondenatured state. A 30-fold decrease in the Michaelis constant for POHB and a 1.5-fold decrease in turnover rate were found for PHBH monomers (20-fold increase in catalytic efficiency). This result can be explained by consideration of the catalytic mechanism of PHBH.[1,2] PHBH catalyzes its reaction in two ways: firstly by reduction of the enzyme cofactor—FAD—by NADPH in response to binding POHB to the enzyme, and secondly by oxidation of reduced FAD by dioxygen to form a flavin hydroperoxide, which oxygenates POHB to form 3,4-dihydroxybenzoate. These reactions are achieved through conformational rearrangements of the isoalloxazine ring and protein residues within the protein structure. It is believed that three conformations of the enzyme provide a foundation for efficient catalysis: *in*, *out*, and *open*.[2] During PHBH catalysis the isoalloxazine ring of the FAD moves *in* and *out* of the hydroxylation pocket.[4,5] In the oxidized state, the flavin swings *out* for the reaction with NADPH. The *in* form is adopted upon reduction of FAD in response to POHB binding. This closed conformation is required for substrate hydroxylation, which must occur in the absence of solvent. The *open* conformation is adopted (dynamically) without POHB bound and is responsible for providing solvent access required for substrate and product exchange.[2,39] It has previously been demonstrated that the equilibrium between PHBH conformers is important for the enzyme function. For instance, it has been observed that a PHBH mutant form with a more stable *in* conformation than the wild-type enzyme is characterized by a higher affinity for POHB and slower substrate and product exchange.[2] One can then suppose that the equilibrium between *in* and *open* conformations of PHBH is affected upon monomer formation. Therefore, the low K_M of POHB and the decrease in enzyme activity observed for monomeric PHBH might be due

to stabilization of the *in* conformation. As a result, the monomer ($k_{cat}/K_M = 75\ \mu M^{-1} s^{-1}$) has a much higher catalytic efficiency than the dimer ($k_{cat}/K_M = 3\ \mu M^{-1} s^{-1}$). It should be taken into account that these kinetic parameters were obtained in a reverse micellar system, where each protein molecule is accommodated in its individual compartment. On the one hand, this allows the properties of the isolated oligomeric forms of the enzyme to be monitored. On the other hand, under such conditions the optimal conformation for enzyme catalysis might be stabilized by the micellar matrix.[44] According to the crystal structure,[45] the near-globular PHBH dimer is composed of two ellipsoid monomers with dimension of $21 \times 23 \times 37$ Å per monomer. Our results suggest that in reverse micelles the isolated monomeric form of PHBH adopts a slightly more expanded conformation than the dimer.

Time-resolved anisotropy measurements of FAD-PHBH and Alexa488-PHBH in water/DMSO mixtures revealed that PHBH dissociates into the monomeric form at higher DMSO concentrations. In the case of FAD-PHBH, FAD is rigidly bound to the protein and remains so up to 40% DMSO. The rotational correlation time therefore reflects the rotational motion of the whole protein. After correction for viscosity, the rotational correlation time of the monomeric protein is 21 ns. The relatively longer correlation time of the monomer seems to be in agreement with the ellipsoid conformation reported above. The Alexa488 residue is flexibly bound to PHBH, resulting in shorter and less accurate rotational correlation times of the protein. Addition of DMSO makes the FAD fluorescence lifetimes of PHBH longer, indicating that fluorescence quenching by electron transfer is slightly perturbed. The real nature of this process is difficult to explore. Both structural fluctuations of quenching groups and changes in local dielectric constants might play a role.

Monomer formation by PHBH occurred gradually with increasing DMSO content in the mixture. Analysis of fluorescence parameters (rotational correlation times and fluorescence lifetimes) of PHBH as a function of DMSO concentration yields a midpoint value of 20.6% DMSO (*v*/*v*) for the dimer–monomer equilibrium of PHBH in the resting state. This is the same value as found for the catalytic activity of PHBH in water/DMSO mixtures (Figure 2). At this DMSO concentration both monomeric and dimeric forms exhibit catalytic activity, which results in an average K_M value for POHB of 10 μM. Pure PHBH monomers were detected at DMSO concentrations of 30% (*v*/*v*) and higher. However, under these conditions the activity of the PHBH monomer dropped considerably, most likely due to inactivation by DMSO.

Conclusions

In summary, in this study we have been able to isolate the monomeric form of PHBH, which is not achieved under conventional conditions. The PHBH monomer is catalytically more efficient than the PHBH dimer. Further studies are required in order to shed more light on the molecular mechanism of the function and structural organization of the PHBH monomer.

Experimental Section

Materials and reagents: Alexa488 maleimide was purchased from Molecular Probes Europe BV (Leiden, The Netherlands). NADPH was obtained from Boehringer (Mannheim, Germany). All other chemicals used were of the highest purity available. Buffers were made from nanopure-grade water (Millipore) and were filtered through a 0.22 μm filter (Millipore) before use.

Labeled protein preparation: Recombinant PHBH was expressed and purified as described previously.[26] The enzyme was pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and was stored at −70 °C in potassium phosphate (pH 7.0, 50 mM), containing EDTA (0.5 mM) and dithiothreitol (DTT; 0.5 mM). Immediately before labeling, DTT was removed by gel filtration on a Superdex 200 HR 10/30 column (Amersham Biosciences, The Netherlands), equilibrated in potassium phosphate (pH 7.0, 50 mM), containing EDTA (0.5 mM). Alexa488 maleimide labeling of PHBH was carried out as described previously.[23] All spectroscopic measurements were carried out in potassium phosphate (10 mM), EDTA (0.5 mM), pH 7.0 at 20 °C. The concentrations of protein-bound dye were determined by using a molar absorption coefficient $\varepsilon_{493} = 72\ mM^{-1} cm^{-1}$ for Alexa488 and a molar absorption coefficient ε_{450} of $10.2\ mM^{-1} cm^{-1}$ [26] for the unlabeled protein. The resulting degree of labeling was almost 100%, corresponding to one label per monomer. The catalytic activity of the enzyme was unchanged after labeling.

Enzyme activity: PHBH activities in water/DMSO mixtures and in reverse micelles [0.2 M solution of sodium bis-2-ethylhexyl sulfosuccinate (AOT) in isooctane] were measured by use of air-saturated Tris/sulfate (pH 8.0, 100 mM), containing EDTA (0.5 mM). For the experiments in reverse micelles the hydration ratio ($w_0 = [H_2O]/[AOT]$) of the micelles was varied by addition of different amounts of buffer to the air-saturated AOT solution in isooctane.[15] The concentration of POHB or NADPH was varied between 200 and 400 μM (depending on the DMSO concentration) of the fixed substrate.[23,27] The rates of NADPH oxidation in both systems (water/DMSO mixtures and reverse micelles) were followed by recording the absorption decrease at 340 nm. The experiments in water/DMSO mixtures were performed in duplicate; those in reverse micelles were performed in triplicate. Turnover numbers (k_{cat}) and (K_M) values were determined from the initial velocities of NADPH consumption by extrapolation of the reaction rates to infinite concentrations of POHB and NADPH. Kinetic data were analyzed by nonlinear least-squares fitting routines as described previously.[29] The concentration of active enzyme was determined spectrophotometrically by use of a molar absorption coefficient (ε_{450}) of $10.2\ mM^{-1} cm^{-1}$ for protein-bound FAD in aqueous solution.[21]

It should be mentioned that the reaction cycle of PHBH can in some cases be complicated by nonproductive catalysis with formation of stoichiometric amounts of hydrogen peroxide.[28] To take possible nonproductive catalysis upon monomer formation into account, the hydroxylation efficiency of PHBH in the water/DMSO system was determined from oxygen consumption measured with an oxygen electrode. The experiments were performed either in the absence or in the presence of catalase (150 units, to monitor the release of hydrogen peroxide),[28,29] under the same conditions as used for the spectroscopic method. The oxygen consumption curves measured either in the absence or in the presence of catalase were identical over the entire range of DMSO concentrations studied. Furthermore, with limiting NADPH concentrations, stoichiometric amounts of NADPH and oxygen were consumed. Thus,

one can conclude that no uncoupling of hydroxylation occurred upon monomer formation.

Polarized time-resolved fluorescence: Time-resolved fluorescence measurements were carried out with mode-locked lasers for excitation and time-correlated photon counting as detection technique as previously described.[30] The excitation wavelengths were 488 and 450 nm for Alexa488-PHBH and FAD-PHBH samples, respectively. The samples were contained in 1 mL and 10 mm light path fused silica cuvettes (Hellma GmbH, Müllheim, Germany, model 110-QS), placed in a temperature-controlled (20 °C) sample holder. The emission light was passed through Schott OG 515 (FAD-PHBH) or Schott GG 530 (Alexa488-PHBH) cut-off filters combined with a Schott model IL 526.0 nm, width 12.6 nm. The concentrations of the enzyme were 0.2 μM for Alexa488-PHBH and 1.5–25 μM for FAD-PHBH samples. To obtain a dynamic instrumental response of the experimental setup, the fast single exponential fluorescence decay of erythrosine B in nanopure water (with a known single lifetime of 90 ps) was recorded. Measurement and data analysis protocols have been described in detail previously.[23, 30]

Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase; POHB, *p*-hydroxybenzoate; TRFA, time-resolved fluorescence anisotropy; AOT, sodium bis-2-ethylhexyl sulfosuccinate.

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Keywords: enzyme catalysis · micelles · polarized fluorescence spectroscopy · subunit dissociation · water/DMSO systems

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