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Octameric alcohol oxidase dissociates into stable, soluble monomers upon incubation with dimethylsulfoxide

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Abstract

Alcohol oxidase (AO) is a peroxisomal, homo-octameric flavoenzyme, which catalyzes methanol oxidation in methylotrophic yeast. Here, we report on the generation of soluble, FAD-lacking AO monomers. Using steady-state fluorescence, fluorescence correlation spectroscopy, circular dichroism and static light scattering approaches, we demonstrate that FAD-lacking AO monomers are formed upon incubation of purified, native octameric AO in a solution containing 50% dimethylsulfoxide (DMSO). Upon removal of DMSO the protein remained monomeric and soluble and did not contain FAD. Binding experiments revealed that the AO monomers bind to purified pyruvate carboxylase, a protein that plays a role in the formation of enzymatically active AO octamers in vivo.

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Keywords: Alcohol oxidase; Flavoenzyme; Dimethylsulfoxide; Spectroscopy

Methylotrophic yeast species, among which Hansenula polymorpha, are able to grow on methanol as sole source of carbon and energy. The enzyme alcohol oxidase (AO) catalyses the first step of methanol catabolism, namely the oxidation of methanol into formaldehyde with concomitant production of hydrogen peroxide. In vivo, AO protein is localized to peroxisomes together with catalase, which decomposes hydrogen peroxide, and dihydroxyacetone synthase, the first enzyme of the formaldehyde assimilatory pathway [for a recent review see 1].

Native, enzymatically active AO is a homo-octameric flavoenzyme of approximately 600 kDa, which consists of eight identical subunits, each containing one non-covalently bound flavin adenine dinucleotide (FAD) as a cofactor. The AO biosynthetic pathway—from the synthesis of inactive AO monomers in the cytosol to the formation of enzymatically active AO octamers in the peroxisomal matrix—has been the topic of extensive research, which revealed that FAD binding is not only important for the enzyme activity of AO, but also essential to allow import of the protein into peroxisomes [2–4]. The association of FAD to newly synthesized AO monomers requires the cytosolic protein pyruvate carboxylase (Hppyc1p) [5]. Mutational analysis revealed that not the enzyme activity of Hppyc1p, but another, novel function of this protein is required for this process [5]. For a recent review see [6].

Abbreviations used: AO, alcohol oxidase; FAD, flavin adenine dinucleotide; DMSO, dimethylsulfoxide; Pyc, pyruvate carboxylase; KP, potassium phosphate buffer; ABTS, 2,2'-Azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid; Trp, tryptophan; FCS, fluorescence correlation spectroscopy; SLS, static light scattering; CD, circular dichroism; Tm, melting points; AO + DMSO, AO treated with 50% DMSO; AO – DMSO, AO treated with 50% DMSO followed by removal of the DMSO by gel filtration.
In order to understand the molecular mechanism involved in HpPyc1p-mediated FAD binding to AO, we aim to reconstitute this process in vitro using purified components. However, despite numerous attempts nobody so far has succeeded in obtaining soluble, stable monomeric AO protein that lacks FAD. Upon denaturing using urea or guanidine, AO invariably formed insoluble protein aggregates upon removal of the denaturing compound. Incubation of native AO in 80% glycerol resulted in dissociation of the octamers into the constituting subunits. However, FAD remained bound to these subunits [7,8]. Moreover, the presence of a highly viscous solvent over-shadows the results of studies of the dynamic properties of proteins and is likely to exert unwanted side effects on HpPyc1p in reconstitution experiments.

Therefore, we now studied the use of dimethylsulfoxide (DMSO). This compound has been used to successfully dissociate dimeric p-hydroxybenzoate hydroxylase [9]. In this study, we used DMSO to dissociate native octameric \textit{H. polymorpha} AO into FAD-lacking monomers. Using a combination of spectroscopic techniques, we showed that upon removal of DMSO the AO protein remained monomeric, soluble and did not contain FAD.

Materials and methods

Sample preparation

AO was purified from \textit{H. polymorpha} as described previously [10]. Protein concentrations were determined from the absorbance at 280 nm using the corresponding molar extinction coefficient of \textit{H. polymorpha} AO calculated on the basis of the aromatic amino acid content of monomeric AO protein (ε$_{280}$ = 92,180 M$^{-1}$ cm$^{-1}$). Purified AO protein was incubated for 5 min at room temperature in 50 mM potassium phosphate buffer (KP), pH 7.2, supplemented with increasing concentrations of DMSO (ranging from 10% to 50% v/v). DMSO was removed by gel filtration using a PD-10 desalting column (Amersham Biosciences, the Netherlands) and 50 mM KP, buffer pH 7.2 as solvent. The protein concentration of the sample after gel filtration was calculated based on the absorbance at 280 nm. After gel filtration over 80% of the protein was recovered as soluble AO monomers.

For fluorescence correlation spectroscopy (FCS) measurements, purified AO was labelled with Alexa Fluor 488 (succinimidyl ester) using the protein labelling kit of Molecular Probes (Leiden, The Netherlands). Labelled protein was dialyzed against 50 mM KPi buffer, pH 7.2, until it contained less than 5% free Alexa Fluor 488 dye. FCS measurements were performed using labelled AO in 50 mM KP buffer, pH 7.2, in the same buffer supplemented with 50% (v/v) DMSO (AO + DMSO) or using AO treated with 50% DMSO followed by removal of DMSO by gel filtration (AO – DMSO).

\textit{In vitro} binding assays

For \textit{in vitro} protein binding experiments, immobilized \textit{H. polymorpha} pyruvate carboxylase (HpPyc1p) was used, which was obtained by incubating crude cell extracts of \textit{Escherichia coli} cells producing C-terminal His$_6$ tagged HpPyc1p [5] with Ni-NTA resin for 1 h. The immobilized HpPyc1p-His$_6$ protein was incubated for 1 h at 4 °C with native AO protein in buffer 50 mM KP, pH 7.2 or with AO protein incubated with 50% DMSO followed by removal of DMSO by gel filtration (see above at “Sample preparation”) in the same buffer. After incubation for 1 h at 4 °C the resin was extensively washed with 50 mM KP, containing 100 mM NaCl pH 7.2, supplemented with increasing concentrations of imidazole (from 10 mM up to 40 mM), followed by elution with the same buffer containing 500 mM imidazole to elute the HpPyc1p-His$_6$ protein together with the bound proteins. The fractions from the wash and elution steps were further analyzed by Western blotting using anti-AO antibodies.

Activity measurements

\textit{AO} activity measurements were performed on a standard spectrophotometer at 37 °C according to protocol described in [11], which is based on the oxidation of 2,2’-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) that is monitored by measuring the absorbance at 420 nm. \textit{AO} activities were measured in \textit{AO} preparations containing increasing concentrations of DMSO and in samples containing 80% glycerol as a control. All samples were kept at 37 °C prior to the measurements. The concentration of \textit{AO} in all assays was 12.6 nM.

Steady-state fluorescence measurements

\textit{Steady-state} fluorescence experiments were performed on a Fluorolog 3.2.2 (Horiba Jobin Yvon Ltd., The Netherlands) spectrophotometer equipped with a thermostatically controlled cuvette holder using a 10 mm light-path fused silica cuvettes (Hellma GmbH, Germany). Measurements were performed at room temperature. The total absorbance of the samples at the excitation wavelength (295 or 450 nm) was kept below 0.1 to minimize inner filter effects. Excitation and emission slits were kept at 2 nm. The spectrum of a blank solution, containing all components except protein, was subtracted from each sample spectrum. All collected spectra were corrected for wavelength-dependent instrumental response characteristics.

Fluorescence correlation spectroscopy (FCS) measurements

The ConfoCor2 (Carl Zeiss, Jena, Germany), a dual channel system based on a Zeiss Axiosvert 100 M inverted microscope, was used to acquire fluorescence fluctuation data. The system is equipped with an argon ion laser line of 488 nm and a 40 × 1.2 water immersion apochromatic objective and is controlled by AIM3.1 software (EMBL Heidelberg, Germany). The system was calibrated by optimizing the position of the pinhole and the correction ring of the objective lens using a solution of 5 nM Alexa Fluor 488.

Samples were measured in 96-well plates with borosilicate bottom. Autocorrelation traces were acquired during 10 s at room temperature and repeated 20 times. For data analysis the FCS data processor 1.3 software package (Scientific Software Technologies Center, Belarusian State University, Minsk, Belarus (http://www.sstcenter.com) was used [12]. Autocorrelation curves were globally analyzed to multi-component three-dimensional Brownian motion models, which included triplet kinetics:

\[ G(t) = 1 + \frac{1}{(N)} \times \frac{1 - T - T e^{-\Delta t/T}}{(1 - T)} \sum_j x_j \frac{1}{(1 + \frac{t_{\text{dif}}}{\tau_{\text{irr}}})^{3/2}} \]  

(1)

where \( N \) is the average number of molecules, \( T \) is the fraction of molecules in the triplet state, \( 1/T \) the corresponding triplet decay rate, \( \tau_{\text{dif}} \) is the diffusion time of molecular species \( j \), \( \omega_{\text{xy}} \) and \( \omega_{\text{z}} \) are the equatorial and axial radii of the confocal volume set up by the laser beam, and \( \omega_{\text{xy}}/\omega_{\text{z}} \) is the structural parameter. Eq. (1) is written in a multi-component form with \( x_j \) the fraction of molecular species \( j \).

The quality of the fits and performance of the optimization methods was improved by fixing the parameters that are known \textit{a priori} from independent experiments. The relation between diffusion time and diffusion constant (\( D \)) is shown in Eq. (2):

\[ \tau_{\text{dif}} = \frac{\omega_{\text{xy}}^2}{4D} \]  

(2)

where \( \omega_{\text{xy}} \) is obtained from the calibration measurements (\( \omega_{\text{xy}} \) in our experiment was 0.186 µm). \( \tau_{\text{dif}} \) for Alexa 488 in our set up varied between 33 and 35 µs.
The diffusion coefficient of a spherical particle scales inversely to the hydrodynamic radius \( r_h \) of the particle according to the Stokes–Einstein relation as shown in Eq. (3):

\[
r_h = \frac{kT}{6\pi \eta D}
\]

where \( \eta \) is the viscosity, \( T \) the absolute temperature, and \( k \) the Boltzmann constant. Assuming that \( r_h \) of the particle is proportional to the cubic root of its molecular mass \( M \), \( D \) can be rewritten as shown in Eq. (4):

\[
D^{-1} \approx \frac{6\eta\sqrt{M}}{kT}
\]

Assuming a more or less globular shape of the molecules, Eq. (4) allows us to derive the molecular mass of a protein and compare the relative mass differences of monomers and octamers. The diffusion constant was corrected for the viscosity increase of DMSO-containing buffer solutions.

**Static light scattering (SLS) measurements**

AO protein was used at a concentration of \( \sim 1 \) mg/ml, based on the extinction coefficient of 92,180 M \(^{-1}\) cm \(^{-1}\) at 280 nm. Samples of AO were then either diluted 1:1 with 10 mM KP, buffer pH 7.2 or with DMSO. After incubation for 5 min, equal aliquots of each sample (2 ml) were applied to a Superdex 200 16/60 gel filtration column (GE Healthcare, Sweden) pre-equilibrated in 10 mM KP, buffer, pH 7.2. A MiniDAWN static light scatterer (Wyatt Technologies, USA) was used in-line to analyze the protein molecular weight as it eluted from the gel filtration column. Analysis of the static light scattering data was performed using the ASTRA software provided by the manufacturer.

**Circular dichroism (CD)**

AO samples in 10 mM KP, buffer pH 7.2, recovered from static light scattering experiments were used in CD experiments. Protein concentrations were adjusted to 0.1 mg/ml. Measurements were conducted on a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control system (Jasco Inc., USA). In all experiments a 0.1 cm path length quartz cuvette was used. Far-UV scans were conducted at 20°C, with spectral bandwidths of 1 nm, accumulation times of 2 s and scan speeds of 100 nm/min. Three scans were averaged for each sample and standard deviations were derived from three separate samples. Buffer blanks were subtracted and secondary structure composition estimated using the program CONTIN [13]. Temperature scans were conducted at a wavelength of 222 nm, with scan rates of 2°C/min and accumulation times of 4 s. Melting points (\( T_m \)) were estimated as the temperature at which 50% AO is unfolded. Standard deviations were determined from three separate scans.

**Results and discussion**

**Dissociation of octameric AO into inactive monomers lacking FAD**

Incubation of purified native AO in buffer containing increasing concentrations of DMSO resulted in decreasing AO enzyme activities (Fig. 1). At a concentration of 50% DMSO the AO enzyme activity was not detectable anymore. The AO enzyme activity remained below the limit of detection upon removal of DMSO by gel filtration, indicating that the inactivation was irreversible at the experimental conditions used.

Fluorescence spectroscopy pointed to DMSO-induced changes in the protein conformation. AO monomers contain 9 Trp and 31 Tyr residues, but the fluorescence emission spectrum of native AO is dominated by Trp fluorescence upon excitation at 295 nm (Fig. 2) [8]. DMSO treatment resulted in a shift of the emission maximum to the longer wavelength amounting 5 nm upon incubation in 50% DMSO. This shift indicates that the average Trp environment became more polar. After removal of DMSO the fluorescence emission spectra still showed the same spectral shift of Trp fluorescence, which suggests that the Trp environment remains exposed (data not shown).

AO-bound FAD has a much lower quantum yield than free FAD. FAD bound to AO mainly acts as an acceptor of tryptophan fluorescence because of the significant overlap between its second electronic absorption band and the tryptophan fluorescence spectrum [8]. Increasing DMSO concentrations resulted in enhanced Trp and FAD fluorescence (Fig. 3a and b). The largest increase of Trp and FAD fluorescence is observed at

![Fig. 1](image1.png)

Fig. 1. Relative AO enzyme activities upon incubation of native, octameric AO in the presence of different compounds. (1–5) Incubation of AO with increasing concentrations of DMSO in potassium phosphate buffer pH 7.2. (1) 0% DMSO, (2) 10% DMSO, (3) 30% DMSO, (4) 40% DMSO and (5) 50% DMSO. (6) Incubation of AO in 50% glycerol, which is known to result in dissociation of AO octamers (15). (7) Labelling of AO with Alexa Fluor 488. AO enzyme activities were determined using methanol as substrate at a final concentration of 0.1 M.

![Fig. 2](image2.png)

Fig. 2. Normalized fluorescence spectra of AO in buffer (solid line) or in 50% DMSO (dashed line) upon excitation at 295 nm.
50% DMSO solution. Upon removal of DMSO, FAD fluorescence was not detectable anymore in the AO protein sample, confirming that FAD had dissociated from AO (data not shown). Therefore the progressive increase of Trp fluorescence at increasing DMSO concentration (Fig. 3 a) is indicative for decreasing energy transfer from tryptophan to FAD because of release of FAD into the solution. Similarly, the increase in FAD fluorescence upon increasing DMSO concentration (Fig. 3 b) can be ascribed to gradual dissociation of FAD into the buffer having a much higher fluorescence intensity.

Fluorescence correlation spectroscopy (FCS) measurements using Alexa Fluor 488 labelled AO indicated that DMSO treatment resulted in the formation of AO monomers. In Fig. 4, an example of normalized fitted autocorrelation data is shown for each of the three protein samples (native AO, AO + DMSO and AO after removal of DMSO). Note that AO in the presence of 50% DMSO shows the slowest decay of the autocorrelation function (or longest diffusion time). This is entirely due to the much higher viscosity of 50% DMSO (η = 3.24) as compared to that of water (η = 1) [14]. The best fits were obtained using a two-component diffusion model, where the shortest diffusion time was attributed to free Alexa dye (fixed to 34 ns in the analysis, in case of buffer solution). As shown in Table 1, the diffusion time of native AO was 339 µs, which, after using Eqs. (2) and (4), corresponds to a molecular mass of 637 kDa and is in line with the mass of octameric AO. In the presence of DMSO, AO shows slow decay, but dividing the by a factor of 3.24 as mentioned above, it brings the corrected diffusion time to around 156 ns, a value that is in agreement with a 62 kDa protein (Table 1), and hence, monomeric AO. The mass calculated for the sample from which DMSO was removed (84 kDa) is also indicative for AO monomers.

**Static light scattering (SLS)**

SLS measurements were performed to accurately determine the molecular mass of the different AO protein preparations. As gel filtration separates different oligomeric states on the basis of their hydrodynamic radius, SLS can be used to examine the mean molar mass of each species. SLS allows a direct determination of molecular mass from the scattering of the species, provided the protein concentration is known. In these experiments the extinction coefficients of the proteins are well known and the 280 nm absorption spectrum provides a direct measurement of protein concentrations.

**Table 1**

<table>
<thead>
<tr>
<th>Form of AO</th>
<th>τ_D (µs)</th>
<th>D (µm² s⁻¹)</th>
<th>Calculated MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO</td>
<td>339 (322–369)</td>
<td>25.2 (23.2–26.6)</td>
<td>637 (546–821)</td>
</tr>
<tr>
<td>AO + DMSO</td>
<td>156 (153–167)</td>
<td>54.8 (51.2–55.9)</td>
<td>62 (58–76)</td>
</tr>
<tr>
<td>AO - DMSO</td>
<td>173 (162–191)</td>
<td>49.5 (44.8–52.8)</td>
<td>84 (69–113)</td>
</tr>
</tbody>
</table>

Concentrations of all sample was 5 nM. For calculations Eqs. (2) and (4) were used.

AO, native AO in KP buffer; AO + DMSO, AO in buffer supplemented with 50% (v/v) DMSO; AO – DMSO, AO incubated in buffer containing 50% DMSO followed by removal of DMSO by gel filtration. τ_D, diffusion time; D, diffusion constant; MW, molecular weight.

b Values between parentheses are the results of a detailed error analysis at the 67% confidence level.

c Value was corrected for viscosity of the solution of 50% DMSO.
Assuming an extinction coefficient of 92,180 M$^{-1}$ cm$^{-1}$ at 280 nm, untreated native AO protein eluted from the gel filtration column with a measured molecular mass of 588.7 kDa (±1%) (Fig. 5). This is in good agreement with the calculated molecular weight of octameric AO (593 kDa). However, the majority (~88%) of the AO protein that was treated with 50% DMSO eluted as a monomer, with a measured molecular mass of 75.4 kDa (±7%), consistent with the calculated mass of AO monomers (74.1 kDa). The remainder of the sample (about 12% of total AO) eluted with a measured molecular mass of 587.3 kDa (±8%), indicating that a small portion of DMSO-treated AO remains octameric. The ratio of these two measured molecular mass values for DMSO-treated AO is 7.8, consistent with an octamer-to-monomer transition of AO upon treatment with 50% DMSO.

CD measurements

Peak fractions from the SLS experiment, containing either octameric native AO or monomeric AO (Fig. 5), were further subjected to analysis by CD. CD spectra of AO (both treated and untreated with DMSO) have been recorded over the 185–260 nm spectral range (Fig. 6). These data indicate that the treatment by DMSO causes some disruption of AO secondary structure. While octameric AO is estimated [15] to contain about 27% α-helical and 44% β-sheet structure (representing a total of about 71% regular secondary structure), monomeric AO is estimated to contain only about 18% α-helical and 30% β-sheet structure (in total 48% regular secondary structure). Thus, the estimated secondary structure content of octameric AO—but not of monomeric AO—is very similar to that of the previously described homology model [8], which contains 30.1% helical structure and 41.7% β-structure, as determined by WHATIF [16]. Finally, we determined the melting temperatures ($T_m$) of native, octameric AO and DMSO-treated monomeric AO (data not shown). These experiments revealed that the $T_m$ of octameric AO is 72 ± 1 °C and that of AO monomers 60 ± 2 °C. Although monomeric AO that lacks FAD can be regarded, therefore, as a rather thermostable protein, it is of considerably lower stability than octameric AO. Taken together with the far-UV CD data, monomeric FAD-free AO can thus be described as containing a decreased content of regular secondary structure.

In vitro binding experiments

Finally, we analyzed binding of different AO preparations to HpPyc1p in vitro. To this purpose we immobilized His$_6$-tagged HpPyc1p to Ni-NTA resin and incubated the
of the two solvents the molecular, energetic and dynamic dissociation into subunits. Because of the different nature of the two solvents the molecular, energetic and dynamic aspects of the dissociation process remain poorly understood. It can be concluded from this work that the solvents act differently leading to permanent perturbation of the FAD binding site in case of DMSO. The lack of detailed structural features of AO hampers in obtaining more molecular insight.

Our current model [6] of the biosynthesis of AO of yeast H. polymorpha predicts that newly synthesized AO monomers bind in the cytosol to HpPyc1p, which mediates FAD-binding to AO monomers. The data presented here show that HpPyc1p binds monomeric, FAD lacking AO, but not AO octamers are fully in line with this model.

**Concluding remarks**

We show that incubation of native, octameric AO in a neutral phosphate buffer containing 50% DMSO results in the formation of inactive, FAD-lacking AO monomers. A wealth of data is available on the effect of glycerol on protein stability (for a review see [17]). The cosolvent glycerol is preferentially excluded from the protein–solvent interface and can therefore induce a decrease in volume and compressibility of the protein interior [13]. It has been suggested that a balance between repulsion of glycerol from hydrophobic surfaces of the protein and interaction with polar regions, is in fact the origin of the well known stabilizing effect of glycerol [18,19]. Besides its function as a viscogen glycerol can also induce structural changes in a protein, but this may arise from either an indirect effect of glycerol on the hydration layer or the same repulsion mechanism [20]. Much less is known of the effects of DMSO on protein stability and structure. DMSO can be considered as a kind of amphiphilic agent, since it contains both a non-polar dimethyl group and a polar sulfoxide. It is well known that both electrostatic and van der Waals’ interactions are responsible for the stability of oligomeric proteins. The driving force for the association between subunits is the hydrophobic interaction. This seems to be confirmed by the effects of glycerol and DMSO that provoke dissociation into subunits. Because of the different nature of the two solvents the molecular, energetic and dynamic interactions are responsible for the stability of oligomeric proteins.

Our current model [6] of the biosynthesis of AO of yeast H. polymorpha predicts that newly synthesized AO monomers bind in the cytosol to HpPyc1p, which mediates FAD-binding to AO monomers. The data presented here show that HpPyc1p binds monomeric, FAD lacking AO, but not AO octamers are fully in line with this model.

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**References**