In vitro dissociation and re-assembly of peroxisomal alcohol oxidases of *Hansenula polymorpha* and *Pichia pastoris*

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Abstract **We have studied the in vitro inactivation/dissociation and subsequent reactivation/re-assembly of peroxisomal alcohol oxidases (AO) from the yeasts *Hansenula polymorpha* and *Pichia pastoris*. Both proteins are homo-oligomers consisting of eight identical subunits, each containing one FAD as the prosthetic group. They were both rapidly inactivated upon incubation in 80% glycerol, due to their dissociation into the constituting subunits, which however still contained FAD. Dilution of dissociated AO in neutral buffer lead to reactivation of the protein due to AO re-assembly, as was demonstrated by non-denaturing PAGE. After use of mixtures of purified AO from *H. polymorpha* and *P. pastoris* active hybrid AO oligomers were formed. When prior to dissociation FAD was chemically removed from AO, reactivation or re-assembly did not occur independent of externally added FAD.**

Key words: Alcohol oxidase; Peroxisomal matrix protein; Protein assembly; Protein reactivation

1. Introduction

Alcohol oxidase (AO) is the key enzyme in methanol metabolism in methylotrophic yeasts and catalyses the conversion of methanol into formaldehyde and hydrogen peroxide. The active form of the enzyme is an octamer of approximately 600 kDa and is localized, often in a crystalline form, inside peroxisomes ([1,2]; for a review see [3]). Each subunit of this homo-oligomeric protein contains one flavin adenine dinucleotide (FAD) as the co-factor. AO monomers are synthesized on free cytosolic polysomes at their mature size and subsequently imported into peroxisomes, where assembly takes place [4,5]. Previously we showed that the import process, followed by AO octamerization, is dependent on the presence of FAD [6]. Despite considerable efforts during the last decade all attempts to re-assemble/reactivate AO in vitro following its chemical dissociation, sofar failed [3]. Most likely the chemical reagents used for AO dissociation/inactivation (e.g. urea or guanidine-HCl) induced alterations in the conformation of the protein subunits or loss of the co-factor FAD, which inhibited successful reactivation. In addition, in vivo studies using *Saccharomyces cerevisiae* for the heterologous expression of the *H. polymorpha* AO gene suggested that AO assembly/activation was not a spontaneous process but instead mediated by specific protein factors [7–9].

Here we show that AO from two sources (*H. polymorpha* and *P. pastoris*) was inactivated and dissociated into FAD-containing subunits after incubation in high concentrations of glycerol. Re-assembly into an enzymatically active conformation occurred spontaneously in vitro after dilution of these samples in phosphate buffer; furthermore, using mixtures of AO of both sources hybrid AO oligomers (consisting of both *H. polymorpha* and *P. pastoris* AO subunits) were formed, which were enzymatically active. Re-assembly was dependent on the intact binding of FAD and did not occur in experiments in which AO was used from which FAD was chemically removed.

2. Materials and methods

2.1. Proteins and inactivation/dissociation conditions

Alcohol oxidase (AO) enzyme proteins of *Pichia pastoris* and *Hansenula polymorpha* were purified as described previously [10]. AO was inactivated by dissociation in 10 mM potassium phosphate buffer (pH 7.2), containing 80% glycerol, at room temperature. Inactivation/dissociation occurred almost instantly under these conditions. Also FAD-lacking AO was used in these experiments. For this purpose FAD was removed from octameric AO by incubation of whole cells or crude extracts with 6 mM KCN for 2 h at 37°C [9].

2.2. AO reactivation and re-assembly

Re-activation of AO was achieved by dilution of the glycerol-treated samples with 10 mM potassium phosphate buffer (pH 7.2) (for details see 'Results section') at room temperature and subsequent incubation on ice for minimal 30 min. For analysis of re-assembly during native gel electrophoresis, samples were loaded directly onto the gels following dilution. Hybrid AO protein formation was achieved by mixing various ratios of glycerol-treated AO of both organisms, prior to re-association by either 8-fold dilution with buffer or gel electrophoresis.

KCN/glycerol-treated samples were 8-fold diluted in the absence or presence of 5 mM FAD.

2.3. Analytical procedures

Non-denaturing gel electrophoresis was performed on 4–10% gradient gels [11] to separate oligomeric AO from the other forms. Gels were stained with Coomassie Brilliant Blue. Western blotting was performed as described by Kyhse-Andersen [12] using specific antibodies against AO. The presence of FAD was visualized by fluorescence of the gel during illumination with UV light [13]. AO activity was measured according to Verduyn et al. at 37°C for *H. polymorpha* AO and 30°C for *P. pastoris* and hybrid AO proteins [14].

3. Results

3.1. Dissociation of alcohol oxidase (AO) into inactive subunits

Addition of 80% glycerol to cell free extracts of methanol-grown *H. polymorpha* resulted in the almost complete dissociation of octameric AO into monomeric subunits (Fig. 1, lanes 1 and 2). Since larger volumes, containing high concentrations of glycerol, resulted in a distorted image of the protein bands on the gels, the experiments were repeated using small quantities of protein and detection of the AO protein by Western blotting using specific α-AO antibodies (Fig. 1, lanes 3 and 4).

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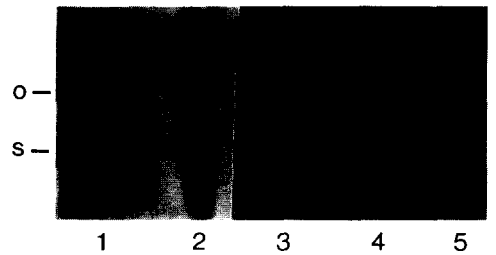

Fig. 1. Dissociation of AO in cell free extracts prepared from methanol grown *Hansenula polymorpha*. When 10% glycerol (lane 1 and 3) or 60% sucrose (lane 5) was used virtually all protein was present in the oligomeric form (O); upon addition of 80% glycerol the major part of AO protein was dissociated into subunits (S; lanes 2 and 4), as was evident after non-denaturing PAGE. Coomassie Brilliant Blue staining (lanes 1 and 2; 200 μg protein per lane); Western blotting using specific antibodies against AO (lanes 3–5; 30 μg protein per lane).

Addition of high concentrations of sucrose (up to 60%) did not result in dissociation of *H. polymorpha* AO (Fig. 1, lane 5).

Using purified AO from *H. polymorpha* a similar dissociation result was obtained; illumination of the protein bands in the native gel with UV light showed that both the octameric and the monomeric forms of AO still contained FAD (Fig. 2A,B). Although AO enzyme activity of the glycerol-treated samples, loaded on the gels, was reduced to less than 1%, a considerable amount of oligomeric AO was nevertheless detected after native gel electrophoresis. Below we show that this is apparently due to re-assembly of oligomers from their subunits during the process of gel-electrophoresis.

3.2. AO reactivation and re-assembly in vitro

Dilution of samples of inactive AO (in 80% glycerol) in phosphate buffer resulted in the reactivation of the AO protein (Fig. 3). The reactivation on ice was more efficient than at room temperature. Highest recoveries of AO enzyme activities were obtained by 8-fold dilution of the samples. Immediate dilution (1000-fold) into the enzyme assay mixture showed no significant reactivation.

Comparable concentration effects on the levels of re-assembly were observed when diluted samples of purified monomeric AO protein were subjected to native gel-electrophoresis (Fig. 4, lanes 1–6). In particular at low dilution rates (5–10 fold) a considerable re-assembly was observed as a result of the electrophoresis procedure (Fig. 4, lane 2). Reactivation and re-assembly of AO was prohibited when instead of native AO, AO

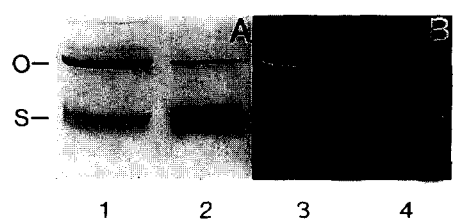

Fig. 2. Dissociation of purified AO from *H. polymorpha* and demonstration of the presence of the co-factor FAD in the subunits. Coomassie Brilliant Blue staining (Fig. 2A) and UV illumination (Fig. 2B) after non-denaturing PAGE of native (lanes 1 and 3) and glycerol treated AO (lanes 2 and 4), showing the position of AO oligomers (O) and subunits (S) (200 μg purified AO per lane).

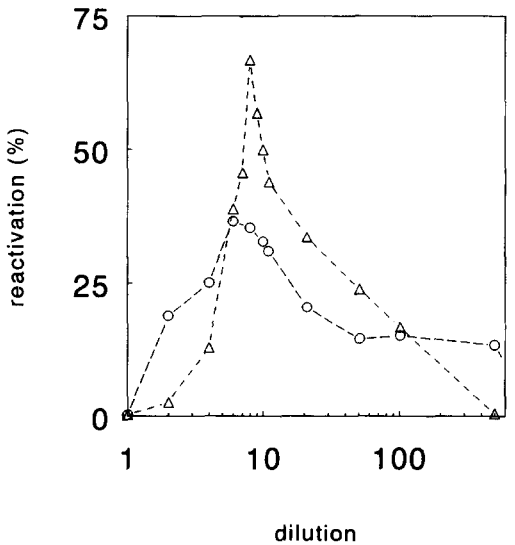

Fig. 3. Reactivation of dissociated AO from *H. polymorpha* (△) and *P. pastoris* (○) following dilution of glycerol-treated protein samples. These samples (4 mg/ml) were diluted with buffer to the indicated dilution values and incubated on ice, before measurement of AO enzyme activity. Data are expressed as the percentage of activity of the reactivated proteins, compared to untreated AO.

was used from which FAD was chemically removed by KCN. As expected, also the FAD-containing protein band which is so evident upon dissociation of native AO (Fig. 4, lane 7) is absent and instead a vague band lower in the gel appears, representing AO monomers, which lack FAD, which remains unaltered when FAD is added to the diluted samples (Fig. 4, lane 8).

From this we conclude that re-assembly/reactivation depends on binding of FAD to the monomers and furthermore, that this FAD-binding process does not occur spontaneously under the prevailing experimental conditions.

3.3. Formation of hybrid oligomers; re-assembly during gel-electrophoresis

Hybrid protein oligomers of AO were formed when glycerol-treated AO from *H. polymorpha* and *P. pastoris* were mixed prior to re-assembly by dilution or during electrophoresis. The existence of these hybrids was visualized on non-denaturing acrylamide gels as bands of protein oligomers with an electrophoretic mobility intermediate between *H. polymorpha* and *P. pastoris* native pure AO (Fig. 5, lanes 1, 2 and 4). When the proteins were allowed to re-assemble by dilution, prior to mixing, these intermediates were barely detectably (Fig. 5, lane 3). When mixed and immediately loaded on the gels, hybrid proteins were also formed during gel-electrophoresis (Fig. 5, lane 2).

Seven intermediate bands of hybrid proteins could be detected using non-denaturing gel electrophoresis, constituting approximately 70% of the AO protein. The total number of 9 protein bands most probably represent the 2 homo-oligomeric and 7 hybrid proteins. The protein bands of both homo-oligomeric forms of AO were a little more intense than the hetero-oligomeric intermediate bands, showing that there was only a slight species specific preference in oligomerization. Reactivation of mixtures containing hybrid proteins was up to

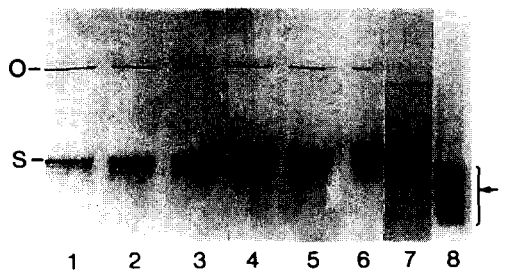

Fig. 4. Diluted samples of monomeric AO protein, obtained by glycerol treatment of purified octameric AO (4 μg), were assayed for re-assembly due to native gel-electrophoresis. Samples were diluted and immediately loaded on the gels. Dilutions: lane 1, undiluted; lane 2, 5-fold; lane 3, 10-fold; lane 4, 50-fold; lane 5, 100-fold; lane 6, 200-fold. Re-assembly is most efficient at low dilution rates (5–10 fold, lanes 2 and 3) as is evident from the intensity of the octameric bands (indicated by O). Lanes 1–6; Coomassie Brilliant Blue staining. Re-assembly of AO in 8-fold diluted glycerol-treated cell free extracts (lane 7, 40 μg) is prevented when prior to dissociation, FAD was removed from octameric AO, independent of the addition of 5 mM FAD to the sample (lane 8, 40 μg). FAD-containing protein bands are also absent under these conditions; instead a vague band slightly lower in the gel representing AO monomers, which lack FAD, appears (lane 8, arrow). O, octameric AO; S, monomeric AO.

42.8% of the activity of the native mixtures, a value intermediate between the results obtained for the individual proteins indicating that the hybrid proteins were enzymatically active. By varying the ratios of the amounts of proteins used (e.g. 2:1 or 10:1) the spectrum of oligomeric intermediates shifted towards the protein of highest concentration and homo-oligomers of the second AO protein could no longer be detected, indicating that the rate of dissociation was virtually 100% (Fig. 5, lane 5–8).

4. Discussion

In this study we presented evidence for the in vitro dissociation of native oligomers of peroxisomal alcohol oxidase (AO) into inactive FAD-containing subunits during incubation in 80% glycerol. Dilution in buffer resulted in the re-assembly of active oligomers with electrophoretic properties, identical to the native proteins. No increase in reactivation was found when instead of a one-step dilution a careful multi-step dilution was performed. These observations indicated that high concentrations of glycerol disrupted the binding between the AO subunits, thereby causing dissociation of the protein without chemical modification of the substructure or loss of FAD binding. This procedure could be applied because no disulphide bonds exist between the subunits.

The efficiency of re-assembly of the proteins into the active conformation following dilution and incubation on ice decreased at relatively high glycerol concentrations (>10%). At enhanced dilutions of the dissociated proteins, the lowered protein concentration seemed to be the limiting factor for efficient reactivation and re-assembly (Fig. 3).

Dissociation of FAD from the octameric protein by KCN treatment inhibited the spontaneous reactivation/re-assembly of the subunits. In vitro the spontaneous rebinding of FAD was also not observed. This, together with the previous finding that apparently, FAD cannot rebind to apo-octamers in vivo inside peroxisomes [9], suggests that FAD binding in vivo occurs to monomers and most probably is an active process dependent on (a) peroxisomal factor(s).

The facts that (i) under certain conditions inactive FAD-containing AO subunits can spontaneously oligomerize and (ii) AO octamers can spontaneously form crystals in vitro [15], indicates that also in vivo, inside peroxisomes, where high concentrations of AO are present these processes may occur independent from the activity of specific chaperones or assembly factors.

On the other hand, the availability of FAD is essential for translocation of AO monomers into the peroxisomal matrix [6], where the actual binding with the FAD co-factor takes place.

Assuming that inactive FAD-containing, oligomerization competent AO monomers are the real intermediates in the in vivo AO folding pathway, would mean that the requirement for specific factors involved in the AO assembly pathway is limited to factors essential to mediate formation of these FAD-containing AO monomers. This is currently studied in our laboratory.

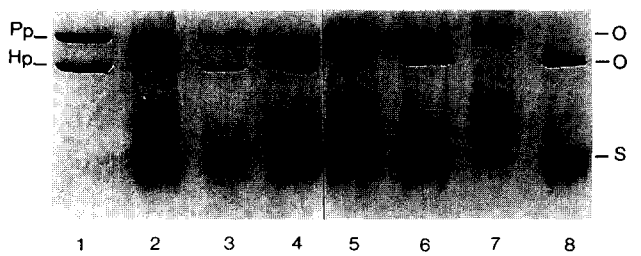

Fig. 5. In vitro formation of seven AO protein hybrids with intermediate electrophoretic mobility between purified AO of *H. polymorpha* (Hp) and AO of *P. pastoris* (Pp) (native proteins are mixed in lane 1). Hybrids were formed when re-assembly was allowed of mixtures containing identical amounts of the two glycerol-treated samples (4 mg/ml) on the gels (lane 2) or by dilution (lane 4). When re-assembly of the proteins was initiated by a 8-fold dilution with buffer prior to mixing of the two protein samples no hybrid formation occurred (lane 3). When the ratios of the amounts of *H. polymorpha* and *P. pastoris* proteins used were altered (respectively, 1:2, lane 5 and 2:1, lane 6) the spectrum shifted towards the most abundant protein. When the ratios were enhanced (1:10, lane 7 and 10:1, lane 8) homo-oligomers of the protein of lowest abundance were absent. Coomassie Brilliant Blue staining after non-denaturing PAGE. O, oligomers; S, subunits.

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