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# Formation and quantification of protein complexes between peroxisomal alcohol oxidase and GroEL

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We have studied the use of yeast peroxisomal alcohol oxidase (AO) as a model protein for in vitro binding by GroEL. Dilution of denatured AO in neutral buffer leads to aggregation of the protein, which is prevented by the addition of GroEL. Formation of complexes between GroEL and denatured AO was demonstrated by a gel-shift assay using non-denaturing polyacrylamide gel electrophoresis, and quantified by laser-densitometry of the gels. In the presence of MgAMP-PNP or MgADP the affinity of GroEL for AO was enhanced. Under these conditions up to 70% of the purified GroEL formed a complex with this protein. Release was stimulated at room temperature by MgATP, and was further enhanced by addition of GroES.

Alcohol oxidase; GroEL; HSP; Non-denaturing gel electrophoresis; Peroxisomal matrix protein; Protein complex formation

## 1. INTRODUCTION

In vivo the processes of folding and oligomerisation of nascent polypeptide chains into their native conformation must be tightly regulated and are often modulated by a heterologous group of components, collectively termed molecular chaperones [1]. These proteins have been implicated in a variety of cellular processes, such as maintaining the membrane-translocation-competent (unfolded) conformation of precursor proteins, bacterial DNA replication, and folding and assembly of proteins in various cellular compartments, including cytosol, mitochondria, chloroplasts and endoplasmic reticulum [2,3]. GroEL, mitochondrial hsp60 and the chloroplast ribulose bisphosphate carboxylase subunit binding protein (RBP) belong to a subclass of chaperones, the chaperonins, which have been demonstrated to mediate correct folding and assembly of proteins in ATP-dependent reactions in vivo and in vitro [4]. Of these chaperones the GroEL/GroES system of *Escherichia coli* has been extensively studied. Native GroEL is a tetradecamer of about 800 kDa and GroES a heptamer of 70 kDa, which can interact with GroEL during association with other proteins [5–10].

We have now studied alcohol oxidase (AO) from yeast for its use to bind to GroEL. The active form of AO is an octamer of 600 kDa and localized in peroxisomes [11,12]. AO monomers are synthesized on free cytosolic polysomes at their mature size and subsequently imported into peroxisomes, where assembly and activation takes place [13–15]. Both in vivo and in vitro experiments indicated that assembly of AO protein is not a spontaneous process [16–18]. It is likely that one (or more) chaperones are involved in the pathway from precursor synthesis to the mature octameric AO protein. If this assumption is correct monomeric unfolded AO should, in principle, be able to interact with these proteins.

In this paper we present a simple procedure to study GroEL–protein complex formation using AO as a model protein, taking advantage of the large size of the monomers (75 kDa) of this protein. Binding of AO caused a distinct change in the electrophoretic mobility of GroEL in non-denaturing gels, which allowed visualization and quantification of the formation of complexes. This procedure enables direct systematic studies on the nature and possible requirements of the GroEL complex formation and dissociation.

## 2. MATERIAL AND METHODS

### 2.1. *Proteins*

GroEL and GroES were purified as described previously [10,19]. Purified AO of *Hansenula polymorpha* was a gift from Unilever Research Laboratories. AO was denatured in a medium containing 6 M guanidine-HCl, 25 mM Tris-HCl (pH 7.4), 0.2 M KCl and 10 mM dithiothreitol for 2 h at room temperature.

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2.2. *Aggregation studies*

Aggregation of AO was determined by measuring light scattering at 320 nm [20]. Denatured AO was diluted 125-fold to a final concentration of 40 μg/ml (0.5 μM monomeric AO) in buffer A (25 mM Tris-HCl, pH 7.2, containing 50 mM KCl and 2 mM dithiothreitol) and incubated at 10°C in the absence or presence of a 2.5-fold molar excess of 14mer GroEL to monomeric AO.

2.3. *Binding studies*

In order to determine optimal binding conditions varying amounts of denatured AO were diluted 50-fold in buffer A containing 0.1 μM GroEL 14mer to obtain molar ratios of AO monomeric protein to GroEL 14mer ranging from 0.2 to 11. Upon dilution the samples were mixed on a Vortex stirrer and subsequently incubated for 10 min at room temperature. The effect of temperature on binding was determined by diluting AO 50-fold (final concentration 20 μg/ml; 0.25 μM monomeric AO) in buffer A containing 0.1 μM GroEL 14mer and incubating for 10 min at room temperature, 15 and 10°C (2.5-fold molar excess monomeric AO to 14mer GroEL). The reproducibility of the binding was tested in a series of experiments at 10°C with a 2.5-fold molar excess of AO. The effect of nucleotides on binding was investigated by addition of MgAMP-PNP or MgADP (10 mM final concentration) to buffer A containing GroEL, prior to dilution of a 2.5-fold molar excess of denatured AO at both room temperature and 10°C.

2.4. *Kinetics of release*

Binding was performed in a volume of 1.25 ml containing 0.1 μM GroEL with a 2.5-fold molar excess of monomeric AO to GroEL. The material was then divided in separate reaction vessels and the release was initiated by addition of magnesium acetate and ATP (10 mM final concentrations), GroES in a 2.5-fold molar excess and casein in a 4-fold molar excess (with respect to GroEL 14mer) at 10°C and room temperature. Samples were taken at different time intervals; reactions were stopped by adding 1,2-cyclohexane-diamine-tetra-acetic acid (CDTA; 50 mM final concentration) and cooling the samples on ice.

2.5. *Analytical procedures*

After binding of denatured AO to GroEL non-denaturing gel electrophoresis was performed on 4–10% gradient gels [21] to separate the GroEL/AO complex (upper band) from the unbound GroEL (lower band). Gels were stained with Coomassie brilliant blue and scanned with a laser densitometer using the GelScan XL program. Binding is defined as the ratio of the signal of the upper band to the sum of the signals of the upper and lower band expressed in percentages. SDS-PAGE was performed according to Laemmli [22].

## 3. RESULTS

3.1. *Aggregation and formation of complexes*

Denatured alcohol oxidase (AO) aggregated rapidly upon dilution in buffer A, however, this aggregation was almost completely prevented in the presence of a 2.5-fold molar excess of GroEL 14mer (Fig. 1). Direct physical interaction between AO and GroEL was demonstrated by non-denaturing PAGE. Stable complexes were readily separated from unbound GroEL (Fig. 2A; lane 3). Both bands observed in the native gel were excised and analyzed by SDS-PAGE. The upper band contained both the 75 kDa AO and the 60 kDa GroEL, whereas in the lower band only GroEL was detected (Fig. 2B). The location of AO was confirmed by Western blotting of a native gel using specific antibodies raised against AO (data not shown). The upper band solely contained a complex of AO bound to GroEL, and

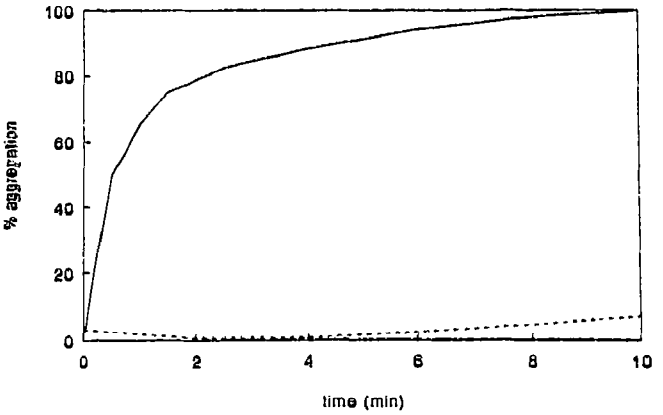


Fig. 1. Aggregation and suppression of aggregation at 10°C upon dilution of denatured alcohol oxidase (AO) in 25 mM Tris-HCl, pH 7.2, supplemented with 50 mM KCl and 2 mM DTT (buffer A) in the absence (---) or presence (—) of a 2.5-fold molar excess of GroEL. The aggregation in the absence of GroEL after 10 min is defined as 100%.

lacked native or reconstituted AO octamers because these migrated to a different position in the gel (Fig. 2A, lane 1). Guanidine-denatured AO, when applied to a native gel, aggregated in the wells.

Densitometric scanning of the native gels resulted in quantifiable graphs. Under the experimental conditions employed highest binding was obtained at 10°C; the optimal molar ratio for maximum binding of GroEL, as determined by a saturation curve, amounted to a 2.8-fold molar excess of AO (Fig. 3). The reproducibility of the binding was within a 5% range (data not shown).

3.2. *Effect of nucleotides on binding*

Both at 10°C and room temperature binding is increased by a non-hydrolysable ATP analogue or ADP in the presence of magnesium acetate (Table 1).

3.3. *Kinetics of release and stability of the complex*

At 10°C the complex is stable and hardly influenced

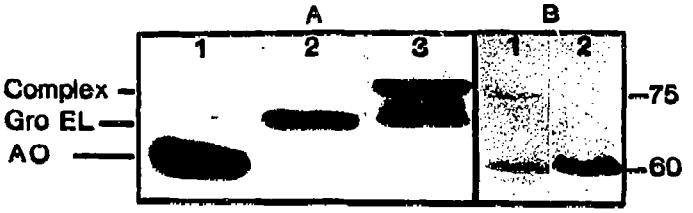


Fig. 2. (A) Coomassie brilliant blue staining after non-denaturing PAGE of a 4–10% gradient gel, showing the position of the GroEL/AO complex (lane 3) compared to native AO (lane 1) and GroEL (lane 2) alone. (B) Coomassie brilliant blue staining after SDS-PAGE of both bands excised from a non-denaturing gel, as shown in Fig. 2A, lane 3, demonstrating the presence of both AO and GroEL in the upper band (Fig. 2B, lane 1) and presence of solely GroEL in the lower band (Fig. 2B, lane 2).

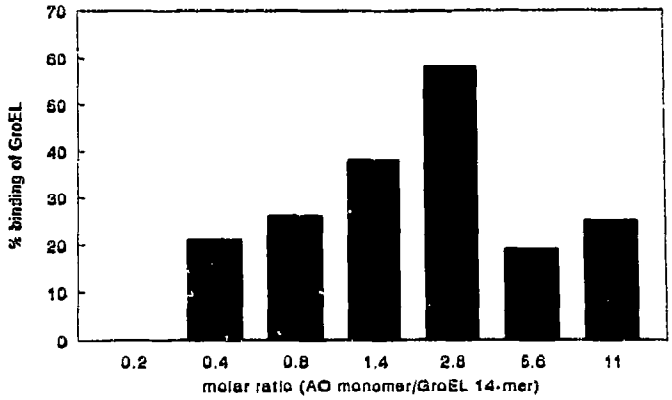


Fig. 3. Binding percentages of GroEL upon dilution of different amounts of denatured AO in buffer A, containing 0.1 $\mu$M GroEL 14mer to obtain molar ratios as indicated. Data are determined by scanning of non-denaturing gels (as shown in Fig. 2A, lane 3), and given as the ratio of the signal of the upper band to the sum of the signals of the upper and the lower band, expressed as percentages.

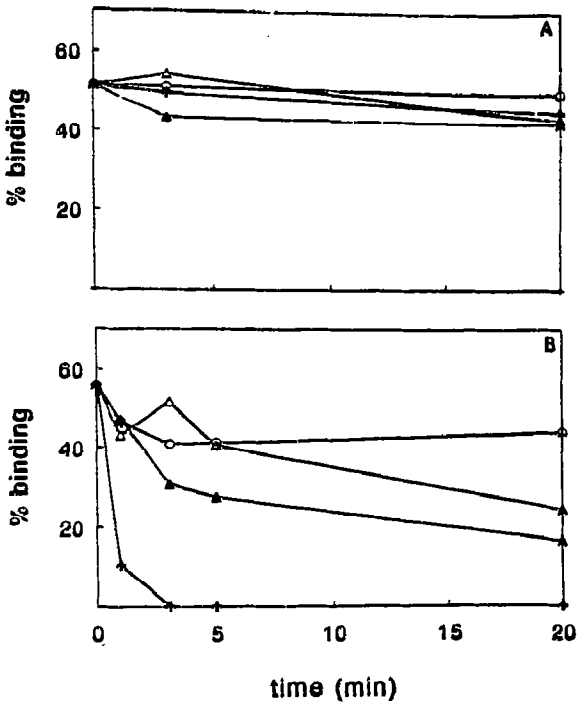


Fig. 4. Influence of MgATP and MgATP in the presence of GroES and casein on dissociation of GroEL/AO complexes at 10°C (A) and room temperature (B). ○, control; △, MgATP; ▲, MgATP = GroES; +, MgATP + GroES + casein. Data are expressed as indicated in Fig. 3.

by addition of MgATP, GroES and casein (Fig. 4A). At room temperature approximately 20% of the complex seems to be unstable and dissociates within 5 min. Partial release is furthermore observed in the presence of MgATP within 20 min; this effect was enhanced in the presence of GroES. Total release was accomplished within 3 min when casein was added as a competitive substrate for denatured AO (Fig. 4B).

## 4. DISCUSSION

In this study we presented evidence for the in vitro formation of complexes between denatured alcohol oxidase (AO) and GroEL. The assay used has several advantages compared to previously described methods. Firstly, only small amounts of GroEL and AO are required. Secondly, complex formation and stability can be directly monitored and accurately quantified. Thirdly, full prevention of aggregation of the substrate (AO) is in principle not essential since these aggregates, which remained in the wells during non-denaturing gel electrophoresis, do not interfere with the quantification method. This allows the addition of excess AO to maximize the percentage of GroEL bound, however, at high concentrations of AO aggregates may form too rapidly for maximal binding, and consequently only a small part of the denatured AO is bound by GroEL under these conditions. Therefore, the observed optimal molar ratio of the proteins for maximal binding of GroEL, namely a 2.8-fold molar excess of AO, does not reflect a binding stoichiometry of 3 molecules of AO bound by one GroEL 14mer; instead, based on the fact that part of the denatured AO has indeed aggregated, a stoichiometry of 2:1 or 1:1 is more likely. At 10°C binding of denatured AO was very efficient and the complex formed was quite stable at this temperature. Almost no release was observed after addition of MgATP. This implies that the process of binding is spontaneous, whereas release is dependent on ATP hydrolysis by GroEL. At room temperature only part of the complex formed at 10°C appeared to be stable. Addition of MgATP and GroES results in an enhanced, but not complete, release. Probably partial rebinding occurs under these conditions. These cycles of release and rebinding may be interrupted by addition of casein, a protein which is known to bind to GroEL, as a competitor; under these conditions all of the AO is released within minutes. Preliminary data indicate that the re-

Table 1

Influence of ADP and the non-hydrolysable ATP analogue, AMP-PNP, on the percentage of binding of GroEL to denatured alcohol oxidase at 10°C and room temperature, respectively, as determined after scanning of non-denaturing gels (compare Fig. 2A, lane 3)

| | Room temperature | 10°C |
|---|---|---|
| Control | 38 | 49 |
| MgADP | 55 | 70 |
| MgAMP-PNP | 58 | 58 |

Data are given as the ratio of the signal of the upper band to the sum of the signals of the upper and lower band and expressed in percentages.

leased AO is not active but aggregates. Probably additional factors are required for refolding/reactivation.

The high binding rates of a purified assembly factor (GroEL) to denatured AO (70%), and the simple assay based on non-denaturing PAGE, makes AO an eligible choice for further investigations of interactions with other molecular chaperones, and may develop into a general system for identifying and purifying these proteins from various sources, including peroxisomes.

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