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Does aminotriazole inhibit import of catalase into peroxisomes by retarding unfolding?

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Fusion of complementary cell lines from patients with diseases of peroxisome biogenesis leads to peroxisome assembly in the heterokaryons and to uptake of cytosolic catalase by the newly assembled peroxisomes. Here we show that catalase import is inhibited by prior binding to catalase of the inhibitor 3-amino-1,2,4-triazole, which appears to retard unfolding of the protein.

Peroxisome; Zellweger syndrome; Catalase; Import of proteins; Unfolding

1. INTRODUCTION

Since their discovery as distinct organelles some three decades ago [1] peroxisomes have been shown to be involved in an ever increasing number of metabolic processes [2,3]. Their indispensability for normal cellular function has been stressed by the recognition in man of a number of hereditary disorders in which morphologically distinguishable peroxisomes are deficient, e.g. the cerebro-hepato-renal syndrome of Zellweger [4]. The primary defect in Zellweger syndrome and related disorders is thought to lie in the protein import machinery [5]. Consequently, the proteins that would normally be imported into peroxisomes remain in the cytosol and can either be rapidly degraded, as appears to be the case for the β-oxidation enzymes [6] and enzymes catalyzing the first steps in plasmalogen biosynthesis [7], or can remain in the cytosol in an active and stable form as is the case for catalase [8].

Complementation analysis after somatic cell fusion of cultured fibroblasts derived from patients with Zellweger syndrome and related diseases shows that the cell lines can be divided into different complementation groups, indicating that different genetic defects are responsible for the disorders [9,10]. Upon fusion of complementary cell lines, assembly of functional peroxisomes takes place in the heterokaryons, which includes transport of catalase from the cytosolic to the peroxisomal compartment. This phenomenon allows one to study in more detail the import of proteins into peroxisomes in vivo.

Our knowledge on protein import has advanced a great deal through the discovery of a specific signal sequence, consisting of the tripeptide serine-lysine-leucine (SKL) and generally located at the carboxy-terminus of the polypeptide, that directs certain proteins to peroxisomes [11]. Despite this, little is known about the machinery that is responsible for the actual import of proteins into peroxisomes. From the work of Eilers and Schatz [12,13] on protein import into mitochondria it is known that proteins most likely are not translocated across a membrane in a tightly folded conformation. Agents which can either stabilize the native conformation or mildly denature the protein can influence the rate of translocation across mitochondrial membranes [13]. In analogy with these studies on mitochondrial protein import we have investigated the effect of 3-amino-1,2,4-triazole (aminotriazole), a specific inhibitor of catalase that binds covalently to the enzyme [14], on the import of catalase into peroxisomes in heterokaryons formed after fusion of two complementing Zellweger cell lines. The results are described in this paper.

2. MATERIALS AND METHODS

2.1. *Cell lines*

Cultured skin fibroblasts were obtained from patients with the cerebro-hepato-renal syndrome (cell line W78/515: Tegelaers et al. [15], cell line GOM 85 AD: Wanders et al. [16]).

2.2. *Materials*

Ham's F10 medium and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Glasgow, UK);

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polyethyleneglycol (PEG) (M_r 1000) from Merck (Darmstadt, Germany); enzymes were from Boehringer (Mannheim, Germany); radiochemicals, streptavidin-fluorescein isothiocyanate and biotinylated donkey anti-rabbit Ig from Amersham International (Amersham, UK); protein A Sepharose CI4B beads from Pharmacia (Uppsala, Sweden) and 3-amino-1,2,4 triazole from Sigma (St. Louis, USA).

All other chemicals were of the purest grade available.

2.3. *Methods*

Cells were fused, fixed and processed for immunofluorescent staining for catalase exactly as described by Wiemer et al. [17]. In those samples that were treated with aminotriazole this compound was added at a concentration of 10 mM at least 24 h before the fusion. Aminotriazole was present in the same concentration during the incubations following the fusion.

For the coupling of aminotriazole to bovine liver catalase in vitro, approximately 400 μg catalase was incubated for at least 12 h at room temperature in the presence of 0.4 M aminotriazole and 5 mM ascorbate. In order to immunoprecipitate catalase, primary antibodies to this enzyme were coupled to protein A Sepharose beads that were pretreated by extensive washing in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl and 0.5% (by vol.) Triton X-100 (TENT), to which 50 mg/ml ovalbumin was added. After coupling of the antibodies (at least 2 h, 4°C), the beads were washed extensively and subsequently incubated with the antigen (at least 2 h, 4°C). The beads containing the bound antigen were spun down and the residual catalase activity was measured according to [8]. This procedure was followed using bovine liver catalase, as well as a mixture of the native enzyme plus a preparation inactivated previously by binding of aminotriazole followed by dialysis to remove excess inhibitor.

Acyl-CoA:dihydroxyacetonephosphate acyltransferase (DHAP-AT) activity was measured as described by Schutgens et al. [7].

Urea gradient gels were prepared as outlined by Goldenberg and Creighton [18]. Acrylamide concentrations of 5–7.5% were used while urea gradients were established from 0 to 8 M. The gels contained a 1.2 M Tris-HCl buffer of pH 8.8, and the electrophoresis was carried out towards the anode in a buffer of 0.2 M glycine and 25 mM Tris, pH 8.5. All solutions were made without sodium dodecylsulphate or β-mercaptoethanol.

3. RESULTS AND DISCUSSION

Upon immunofluorescent staining of Zellweger cell lines using antibodies to catalase, a diffuse fluorescent pattern is observed (Fig. 1 and [17]), whereas in heterokaryons formed by fusion of two complementary cell lines (ZS1 and ZS2), a punctate fluorescent pattern is observed upon staining for catalase (Fig. 2, panels a–d; see also [17]). This change in staining of catalase represents the relocation of this enzyme from its cytosolic location in the Zellweger cells to the newly formed peroxisomes in the heterokaryons. However, if the fusion of the same cell lines is carried out in the presence of aminotriazole hardly any punctate fluorescence was noted even after 48 h following fusion (Fig. 2, panels e–h). This fluorescent pattern corresponds to a cytosolic localization of catalase in the heterokaryons. These findings suggest that binding of aminotriazole to catalase hinders the import of the latter enzyme into the peroxisome.

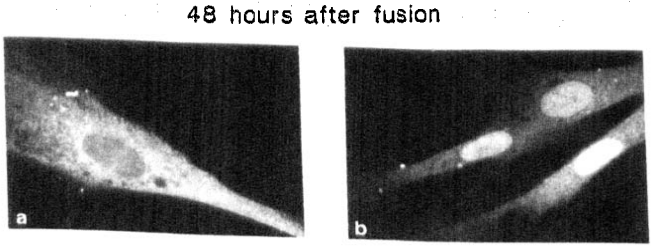

Fig. 1. Immunofluorescent staining of catalase in Zellweger fibroblasts. (a) Self-fusion of cell line ZS1 (GOM85AD). (b) Self-fusion of cell line ZS2 (W78/515). Cells were fixed and incubated successively with antibodies to catalase, a biotinylated conjugate of donkey anti-rabbit Ig and streptavidin-labelled FITC as described in [18].

In order to check if aminotriazole did not hinder the complementation process as such, we measured another parameter independent of the import of catalase, viz. the increase in activity of DHAP-AT that occurs 2 to 4 days after fusion of complementary cell lines [9]. Aminotriazole had no significant effect on this increase in activity (results not shown). Thus, the effect of aminotriazole seems to be limited to the import of catalase.

The effect of aminotriazole on the reactivity of catalase with its antibodies was investigated in the experiment of Fig. 3. In the left hand panel, different amounts of native catalase were preincubated with a fixed amount of immobilized antibodies. After precipitation of the catalase that was bound to the antibodies, the remaining catalase activity in the supernatant was measured. The curve relating catalase activity in the solution to the amount of protein added had the same slope with and without preincubation, indicating that a constant amount of enzyme was being precipitated. The right hand panel shows that this was also true for catalase which had been 50% inhibited by aminotriazole. The total amount of protein that could be immunoprecipitated was approximately the same whether native or partially inactivated enzyme was used. It can be concluded that aminotriazole has no significant effect on the binding of catalase to the antibodies.

The effect of aminotriazole on unfolding of catalase was investigated. The unfolding profile of bovine liver catalase was monitored in vitro on transverse urea gradient gels [18]. These gels possess a horizontal linear gradient of urea, perpendicular to the direction of migration. Native catalase and catalase pretreated with aminotriazole were applied as a straight band and during electrophoresis were exposed to a continuously varying concentration of urea (Fig. 4A, 4B, respectively). The unfolded molecules exhibit a decreased electrophoretic mobility due to an increase in hydrodynamic volume. An apparent difference between the unfolding patterns for catalase and for catalase bound to aminotriazole was observed; the latter experiences a transition from folded to (partly) unfolded conformation at a higher urea concentration than the former.

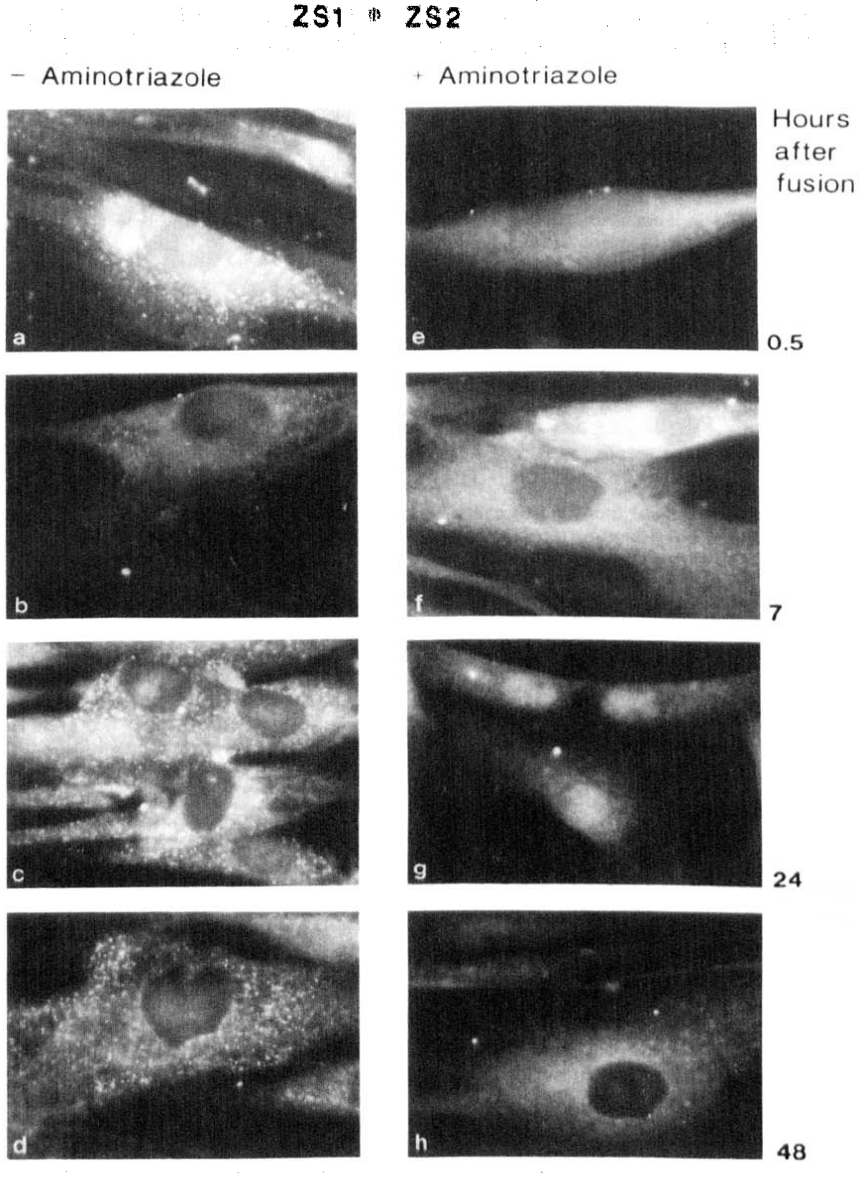

Fig. 2. Presence and subcellular localization of catalase as shown by immunofluorescence after fusion of two complementing Zellweger cell lines (ZS1 ×ZS2). (Panels a–d) Catalase staining after fusion without aminotriazole. (Panels e–h) Catalase staining after fusion in the presence of aminotriazole. Cells were fixed at the indicated times after fusion.

From studies on protein import into isolated mitochondria [12,13,19], it is now clear that signal sequences play an important role in determining the fate of a protein (reviewed by Verner and Schatz [20] and Hartl et al. [21]). In addition to this, unfolding of the protein to be imported seems to be an essential phenomenon for the actual membrane translocation [12,13]. Moreover, the unfolding of proteins in order to cross a membrane does not seem to be limited to mitochondrial membranes, but seems to play a role also e.g. for translocation across the membrane of the endoplasmatic reticulum [22].

At present, the retarded import of catalase bound to aminotriazole into peroxisomes can be explained by two possible mechanisms: firstly, aminotriazole could provoke a conformational change in the tetrameric catalase molecule, which could result in the masking of the peroxisomal targeting signal. Secondly, aminotriazole might hinder the unfolding of catalase, a process that could be of major importance for import of proteins into peroxisomes, as it is for import into mitochondria. The latter point seems even more relevant considering the phenomenon that, at least in Zellweger cells, but possibly also in control cells, catalase is assembled into the active tetramer of approximately 240 kDa in the cytosol [9]. The transport of such a bulky molecule over the peroxisomal membrane might therefore well require unfolding.

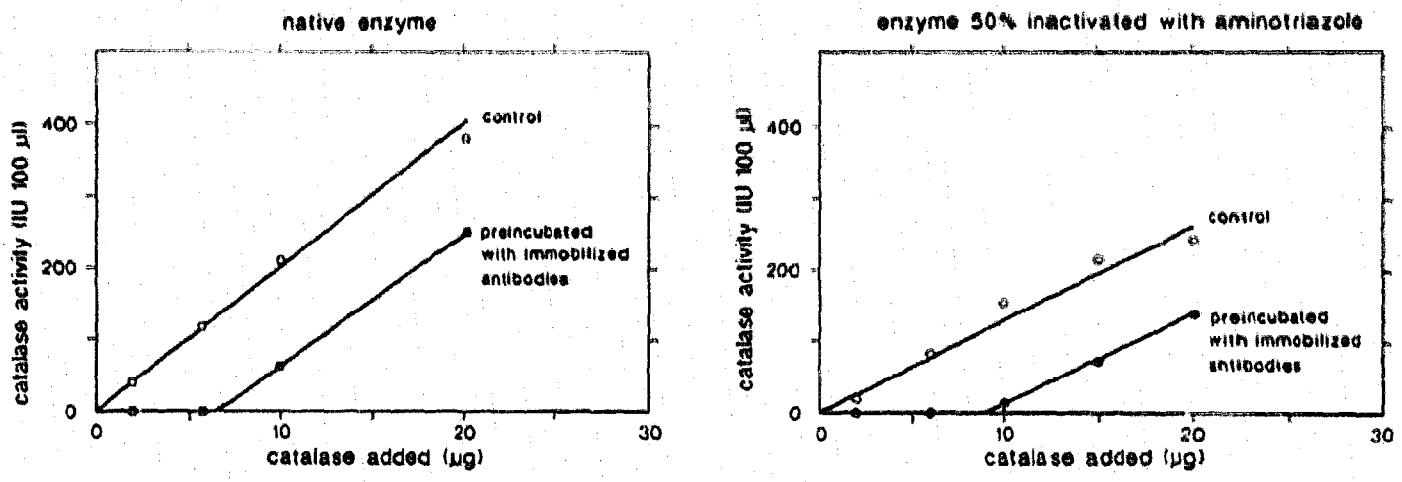

Fig. 3. Immunoprecipitation of native bovine liver catalase and of enzyme partially inactivated by aminotriazole using antibodies coupled to protein A Sepharose beads. After incubation of different amounts of catalase with a fixed amount of antibodies, the beads were spun down and the residual catalase activity was measured in the supernatant. For further experimental details see Materials and Methods.

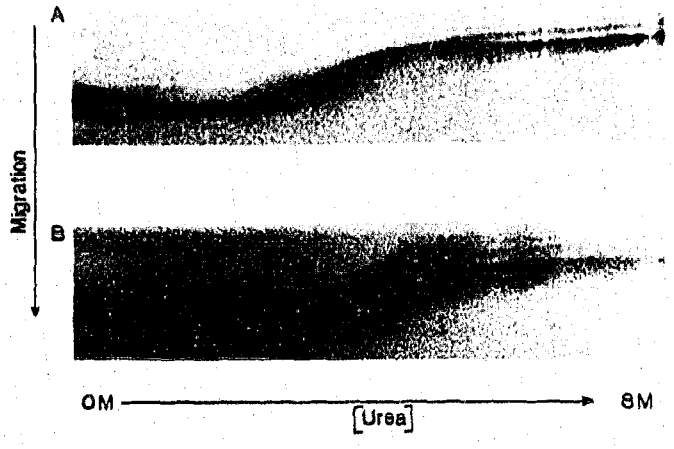

Fig. 4. Urea gradient gel electrophoresis of native catalase (A) and catalase coupled to aminotriazole (B). For a detailed description of the procedure see the Materials and Methods section.

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