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Immunological identification of the alternative oxidase of *Acanthamoeba castellanii* mitochondria

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Abstract Mitochondria of the protozoa *Acanthamoeba castellanii* possess a cyanide-insensitive oxidase cross-reacting with monoclonal antibodies raised against the plant alternative oxidase. Immunoblotting revealed three monomeric forms (38, 35, and 32 kDa) and very low amounts of a single 65 kDa dimeric form. Cross-linking studies suggest that while in plants the alternative oxidase occurs as a dimer, in amoeba it functions as a monomer. Immunologically detectable protein levels change with the age of amoeba cell culture. Increased amounts of the 35 kDa protein are accompanied by an increase in the activity of cyanide-resistant respiration.

Key words: Alternative oxidase; Cyanide-resistant respiration; Mitochondria; GMP; *(Acanthamoeba castellanii)*

1. Introduction

Mitochondria of the free-living amoeba *Acanthamoeba castellanii* share many common features with mitochondria of higher plants [1,2], absent from their animal counterparts. These are: (i) the ability to oxidise cytosolic NADH via the rotenone-insensitive external NADH dehydrogenase, situated on the outer surface of the inner mitochondrial membrane [3]; (ii) the presence of two internal NADH dehydrogenases: the rotenone-sensitive complex I and the rotenone-insensitive dehydrogenase, involved in a non-electrogenic bypass for the electrons from the matrix pool of NADH to ubiquinone [1]; (iii) a complex oxidation of malate carried out by two matrix enzymes: malate dehydrogenase and NAD^+-linked malic enzyme (decarboxylating) [1], (iv) the presence of a cyanide (CN)- and antimycin-resistant alternative oxidase in addition to the conventional cytochrome *c* oxidase [4,5]. Mitochondria of many other microorganisms also possess a CN-resistant oxidase and the external NADH dehydrogenase, insensitive to rotenone [6], although it is unknown if they also possess the internal NADH dehydrogenase, insensitive to rotenone and if they oxidise malate in a similar way as higher plants and amoeba.

The striking bioenergetic similarities between amoeba and higher plant mitochondria are consistent with a hypothesis based on homology of ribosomal RNA, which suggests that amoeba might be considered as an evolutionary divergence point for higher plants and animals [7,8]. In this respect especially the characterisation of the CN-resistant oxidase in amoeba is of interest, since such an oxidase is present in plants, but not in animals.

The activity of this CN-resistant oxidase decreases during transition from the exponential to the stationary phase of culture in amoeba trophozoites [4,9,10] and is strongly controlled by the availability of iron in the culture medium [10]. Our previous results show that, as in plant mitochondria [2], CN-resistant respiration in amoeba mitochondria branches from the main respiratory chain at the level of the ubiquinone pool and the flow of electrons through this pathway is not coupled to oxidative phosphorylation [4]. Like in plant mitochondria, activity of the CN-resistant pathway varies with the substrate provided, being relatively low with external NADH as the substrate [11].

Not in all aspects the amoeba CN-resistant oxidase resembles the one in higher plants: while in higher plant mitochondria the alternative oxidase is stimulated by pyruvate [12,13], there is no effect of pyruvate on the amoeba CN-resistant oxidase, which, however, is strongly stimulated by the purine nucleoside 5′-monophosphates AMP [4,9,10,14], GMP (the lowest K_m of stimulation) and IMP [15]. The same effect of purine mononucleotides on the activity of the alternative pathway was observed in other micro-organisms such as *Euglena gracilis* [16], *Moniliella tomentosa* [17], *Paramecium tetraurelia* [18], *Neurospora crassa* [19] and *Pichia stipitis* [20].

It is known that the plant mitochondrial alternative oxidase is encoded by nuclear gene(s) and consists of one to three proteins between 32 and 39 kDa, depending on the tissue [21]. Monoclonal antibodies (AOA), developed against *Sauromatum guttatum* alternative oxidase were found to cross-react with alternative oxidase proteins in a wide range of thermogenic and non-thermogenic plant species, as well as in fungi and in trypanosomes [21,22], indicating that this protein is well conserved throughout the species.

In this paper it is shown for the first time that also the CN-resistant oxidase of the protozoa *A. castellanii* cross-reacts with the *S. guttatum* antibodies. The relationship between the activity of CN-resistant respiration and the nature and amount of alternative oxidase proteins detected throughout the culture period were investigated.

2. Materials and methods

2.1. Cell culture

Soil amoebae *Acanthamoeba castellanii*, strain Neff, were cultured in medium described by Neff et al. [23] with some minor modifications: 1.5% proteoso-peptone (Difco), 0.15% yeast extract (Difco), 30 mM $MgCl_2$, 30 mM $FeSO_4$, 27 mM $CaCl_2$, 1.5% glucose, 2.5 $mg \cdot l^{-1}$

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Abbreviations: AOA, monoclonal antibodies that react with the major polypeptides of the alternative oxidase and designated ‘alternative oxidase all’; DTT, dithiothreitol; h, hours of growth of amoeba culture following inoculation; EGS, ethylene glycol-bis-(succinimidylsuccinate); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SHAM, salicylhydroxamic acid; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate

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vitamin B_{12}, 1 mg·l^{-1} vitamin B_1, 0.2 mg·l^{-1} vitamin H. After about 60 h of exponential growth with a generation time of 7–8 h, amoeba cultures reach the stationary phase with a density of 10×10^6 cells·ml^{-1}. For the present studies trophozoites were harvested from cultures at different phases of growth, i.e. 12 h (1.5×10^6 cells·ml^{-1}, the early exponential phase), 24 h (4.0×10^6 cells·ml^{-1}, the middle exponential phase), 48 h (7.3×10^6 cells·ml^{-1}, the late exponential phase) and 78 h (10.5×10^6 cells·ml^{-1}, the stationary phase) following inoculation.

Potato tubers (*Solanum tuberosum*, cv. Bintje), used for comparisons with the plant alternative oxidase, were obtained from local stores and stored at 8°C until used. Ageing of potato tuber slices and callus induction was performed as described before [24].

2.2. Isolation of mitochondria and measurement of mitochondrial respiration

A. castellanii mitochondria were isolated by differential centrifugation [25] in an isolation medium containing: 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 0.5 mM EGTA and 0.2% (w/v) BSA. Mitochondria were purified on a 31% self-generating Percoll gradient and were washed without BSA and EGTA.

Mitochondria from aged potato tuber slices and potato tuber callus mitochondria were prepared as described before [24].

Oxygen consumption was measured with 10 mM succinate as the substrate in a Rank Bros. (Cambridge, UK) electrode at 25°C in 2 ml of reaction medium (20 mM Tris-HCl (pH 7.4), 120 mM KCl, 3 mM KH_2PO_4, 8 mM $MgCl_2$, 0.2% (w/v) BSA) with 1–2 mg of mitochondrial protein. State 3 respiration was determined in the presence of 0.17 mM ADP. Cytochrome pathway mediated respiration or CN-resistant respiration were measured in the presence of 1 mM SHAM or 1 mM KCN (±200 μM GMP) respectively.

Protein was estimated by the biuret method [26] with BSA fraction V as a standard.

2.3. SDS-PAGE and immunoblotting

Up to 80 μg of mitochondrial protein was solubilized in the sample buffer (1% (w/v) SDS, 60 mM Tris-HCl (pH 6.8), 10% glycerol, 0.004% (w/v) bromphenol blue, ±100 mM DTT) and boiled for 4 min. Electrophoresis (SDS-PAGE) was carried out in a manner similar to that of Laemmli [27] using a 5% polyacrylamide stacking gel and a 12.5% polyacrylamide resolving gel (non-urea gels) or a 10% polyacrylamide resolving gel (with 2.5 M urea), followed by Western blotting. The procedure of mitochondria treatment with DTT and diamide as well as cross-linking with SMCC and EGS was performed as described by Umbach and Siedow [28]. Bio-Rad prestained low molecular mass markers were used to estimate molecular mass and the efficiency of the electrotransfer. Antibodies against alternative oxidase proteins of *S. guttatum* (generously supplied by Dr. T.E. Elthon) were used at dilutions of 1:1000. Alternative oxidase bands were visualised using the Amersham ECL system.

3. Results and discussion

Alternative respiratory activity and alternative oxidase amounts were measured during batch culture in amoeba mitochondria. Immunoblotting of total mitochondrial proteins of amoeba with monoclonal antibodies (AOA) against alternative oxidase of *S. guttatum* shows a strong cross-reaction with the amoeba oxidase proteins. The quantity of immunologically detectable protein increased with amoeba cell age, reaching the highest level at 24 h after inoculation (Fig. 1). In older cells the expression of the alternative oxidase declined to very low levels in the 78 h culture.

Table 1
Uninhibited respiration (+0.17 mM ADP), respiration via the cytochrome pathway (+0.17 mM ADP +1 mM SHAM) and CN- (1 mM) resistant respiration (±200 μM GMP) in mitochondria isolated from amoeba cell cultures

Condition	Age of the culture (h)			
	12 (1.5×10^6)	24 (4.0×10^6)	48 (7.3×10^6)	78 (10.5×10^6
+ADP	59±4.6	105±7.2	120±6.6	77±4.3
+ADP +SHAM	60±4.0	98±4.2	116±3.6	78±4.0
+KCN	5±1.0	12±2.3	6±1.8	3±0.9
+KCN+GMP	41±2.7	95±5.1	48±4.5	16±1.9

Values in parentheses represent density of the culture (number of cells·ml^{-1}). Values of respiratory rates with 10 mM succinate as the substrate are in nmol O_2·min^{-1}·mg^{-1} protein.
Data are mean values±SD of three determinations.

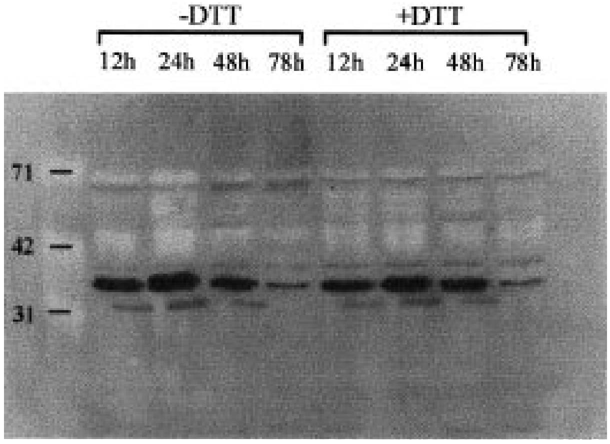

Fig. 1. Alternative oxidase amounts in mitochondria isolated from amoeba cultured for 12, 24, 48, and 78 h. From left to right the first four samples were prepared without DTT, the next four were prepared in the presence of 100 mM DTT. An immunoblot from 12.5% SDS-PAGE gel probed for the alternative oxidase protein using the AOA monoclonal antibodies is shown. The amount of protein loaded for all lanes was 36 μg. Molecular masses (in kDa) of the markers are indicated on the left.

Fig. 1 shows that immunoblots of amoeba mitochondria proteins with *S. guttatum* alternative oxidase antibodies revealed the expression of three lower molecular mass forms: 38, 35, and 32 kDa, and a single 65 kDa band. In *S. guttatum*, the alternative oxidase has been identified as a cluster of three polypeptides of molecular mass 37, 36, and 35 kDa [26]. Thus, the alternative oxidase appears to be remarkably conserved between *A. castellanii* and higher plants, considering the similar molecular masses and the ability to react with antibodies raised against the plant oxidase. This is of particular interest in the evolutionary context, since it has been postulated that the protozoa amoeba might be considered as an evolutionary divergence point for higher plants and animals [7,8], the latter missing the alternative oxidase.

The alternative oxidase of plant mitochondria appears to exist in the membrane as a dimer that can be either non-covalently linked (more active reduced state) or covalently linked (less active oxidised state) [28]. In plant mitochondria, the oxidised dimer (higher molecular mass form) of the oxidase can be visualised only by omitting the reductant from the SDS-PAGE sample buffer [28]. In contrast to the situation in plant mitochondria, in amoeba mitochondria some of the protein was still in the high molecular mass form (65 kDa) in the presence of DTT (Figs. 1 and 2). Under both conditions (with or without the reductant) there was no significant difference in the quantity of the low molecular mass forms of the oxidase (38, 35, 32 kDa). Fig. 2 shows immunoblots with AOA *S. guttatum* antibodies of total mitochondrial proteins of amoeba and aged potato tuber slices (as a control) in the presence and absence of the reductant DTT. In aged potato mitochondria, in which the alternative oxidase appears to be a single protein of about 36 kDa [29], the oxidised, high molecular mass species were detected by antibodies only when reductant was omitted from the gel sample buffer, as reported for other plant mitochondria [28,30]. Moreover, the reduced form of the alternative oxidase was detected only in the presence of DTT. In amoeba, however, the omission of DTT from the sample buffer did not result in conversion of the low molecular mass species to the higher molecular mass species, suggesting that the formation of a disulfide bond does not occur in the alternative oxidase protein of amoeba mitochondria under those conditions. This was further confirmed by experiments in which we incubated samples with diamide, a reagent that oxidises thiols to disulfides [28]. In diamide-treated plant mitochondria the alternative oxidase is oxidised and this effect can be reversed by subsequent incubation of the mitochondria with DTT, while diamide-treatment given after incubation with DTT converts the protein into the oxidised form again [28]. In amoeba mitochondria, however, independent of the absence or presence of DTT and/or diamide and the order of the treatments, all of the alternative oxidase protein remained in the lower mass form (Fig. 3). This raises the intriguing question whether the alternative oxidase exists in the amoeba mitochondrial membrane as a reduced dimer, in which the monomers cannot be linked by a disulfide bridge, or whether the amoeba alternative oxidase, in contrast to its counterpart in plant mitochondria occurs as a monomer. It therefore became of interest to know what the effect is of chemical cross-linkers. We used SMCC, a hydrophobic, heterobifunctional reagent with two reactive groups, of which one reacts with Lys and the other with Cys, provided that those two amino acids are in proximity [28,32] and EGS, a hydrophobic homobifunctional, Lys-specific cross linker [28,33]. Again, in contrast to the situation in plant mitochondria, in amoeba mitochondria only the lower mass species were observed (Fig. 4), strongly suggesting that in the amoeba mitochondrial membrane, the alternative oxidase occurs as a monomer. A small band at 65 kDa appeared to be present, however, suggesting the presence of some dimeric species, but the amounts were always very low and did not change with any reducing, oxidising or cross-linking treatment, so at present it seems most likely that this band represents an artefact, created during heating of the sample [34]. A comparison between Figs. 3 and 4 shows a difference with respect to the relative amounts of especially the 35 and 32 kDa bands. In Fig. 3 the 35 kDa band is dominant, while in Fig. 4 the 32 kDa band is almost as pronounced as the 35 kDa band (although scanning shows that the 35 kDa band is still the largest) and both bands appear broader. For both gels the same mitochondrial preparation was used, albeit that after running the gel presented in Fig. 3 the mitochondria were stored at −70°C for 2 days, before they were used for the gel presented in Fig. 4. Such a treatment, however, was often applied before and hardly affected the relative distributions of the bands. The difference therefore, is more likely to be related to the nature of the gel used: for the cross-link studies (Fig. 4) urea was included in the gels, conform the procedure of Umbach and Siedow [28], in order to have the protein as denatured as possible to rule out any artifactual dimerisation. It is not clear at present what causes this different behaviour. Nevertheless, independent of the gel type used, it appears that in amoeba mitochondria, different from the situation in plant mitochondria, the alternative oxidase exists in lower mass forms of which the 35 kDa monomer is predominant. It is

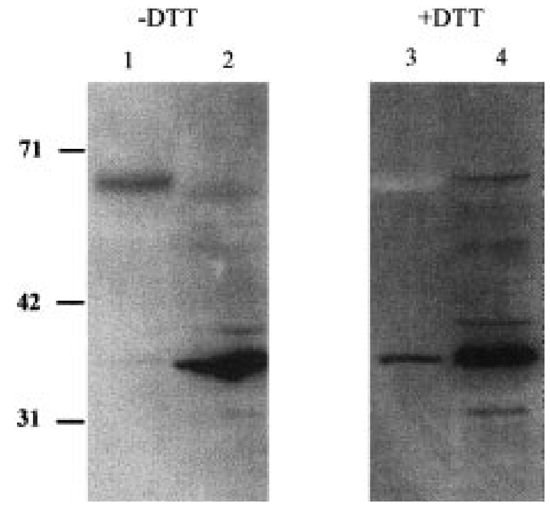

Fig. 2. Effects of a DTT treatment on mitochondrial alternative oxidase proteins in mitochondria isolated from aged potato tuber slices (lanes 1 and 3) and of amoeba cultured for 24 h (lanes 2 and 4). An immunoblot from a 12.5% SDS-PAGE gel probed with the AOA monoclonal antibodies is shown. Gel samples for lanes 1 and 2 were prepared without (−) DTT in the sample buffer; samples for lanes 3 and 4 were prepared in the presence (+) of 100 mM DTT. In all cases, 20 μg of protein was loaded per lane. Molecular masses of the markers (in kDa) appear on the left.

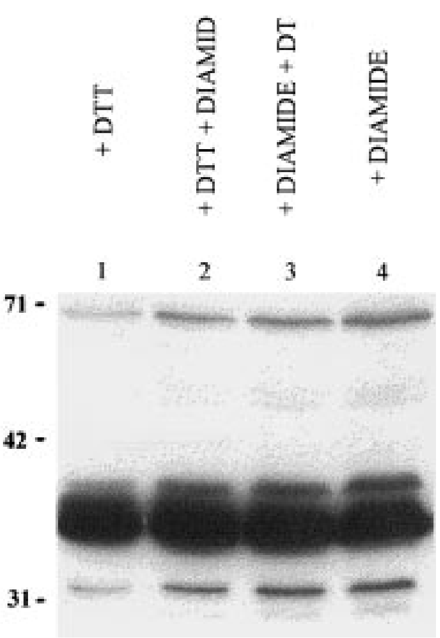

Fig. 3. Effects of DTT and diamide treatment. Mitochondria isolated from amoeba cells cultured for 24 h were treated on ice with 20 mM DTT, 3 mM diamide, or both reagents sequentially. An immunoblot from 12.5% SDS-PAGE gel probed for the alternative oxidase protein using the AOA monoclonal antibodies is shown. All samples (80 μg of protein in each lane) were prepared without DTT in the sample buffer. Mitochondrial incubations were with: lane 1, 20 mM DTT; lane 2, 20 mM DTT followed by 3 mM diamide; lane 3, 3 mM diamide followed by 20 mM DTT; lane 4, 3 mM diamide. Washing steps, in order to remove excess DTT or diamide prior to addition of the second compound to the samples for lane 2 and 3, were performed as described by Umbach and Siedow [28]. Molecular masses (in kDa) appear on the left.

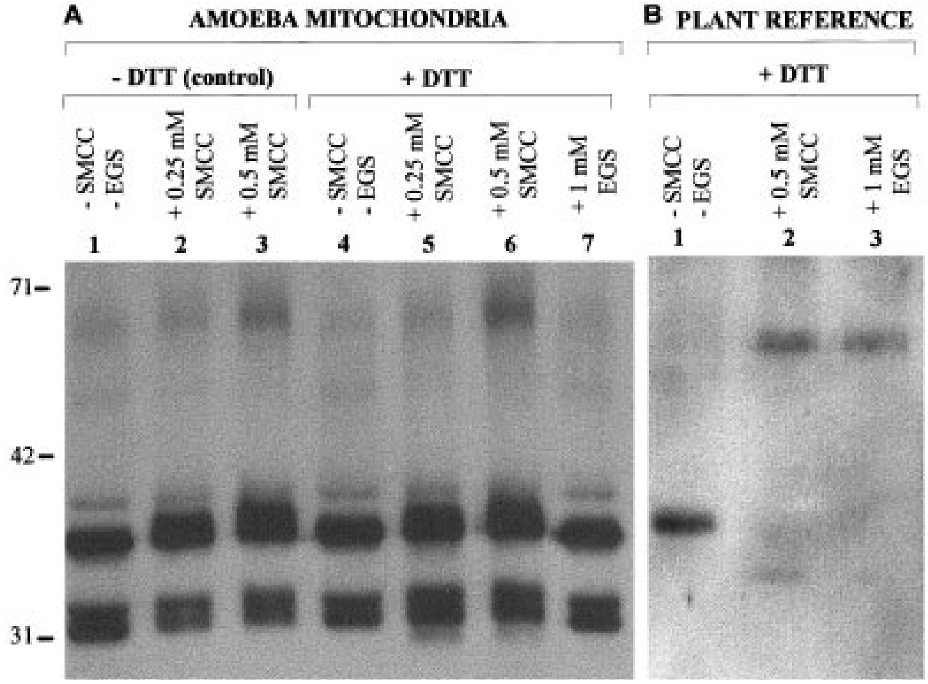

Fig. 4. Chemical cross-linking of alternative oxidase protein species with SMCC and EGS in mitochondria from amoeba cells cultured for 24 h (A, lanes 1–7) and potato tuber callus mitochondria (B, lanes 1–3). Mitochondria after treatment without (control) or with (+DTT) 20 mM DTT for 1 h on ice were cross-linked with indicated SMCC concentrations or with 1.0 mM EGS. Immunoblots from 10% SDS-PAGE urea gels probed with the AOA monoclonal antibodies are shown. For all samples, 100 mM DTT was present in the gel sample buffer. Lanes 1–3 (A), control amoeba mitochondria; lanes 4–7 (A) and lanes 1–3 (B), 20 mM DTT-treated mitochondria; lanes, A1, A4 and B1, mitochondria incubated without cross-linkers; lanes A2, A3, A5, A6 and B2 incubated at the indicated SMCC concentrations; lanes A7 and B3 incubated with 1.0 mM EGS. Lanes in part A were loaded with 50 μg of protein, and in part B were loaded with 30 μg of protein. Molecular masses (in kDa) are indicated on the left.

reasonable to propose that this monomeric form corresponds with the highest activity of the enzyme (Fig. 1 and Table 1). Changes in the amount of the 35 kDa oxidase protein were accompanied by changes in the activity of the CN-resistant respiration (Table 1). The highest activity of the alternative oxidase (in the presence of KCN) was observed in the 24 h culture, both in the absence and presence of GMP, while the uninhibited state 3 respiration and the cytochrome pathway mediated respiration reached maximal rates at 48 h of culture.

In plant mitochondria the activity of the alternative oxidase has been reported to correlate not only with the total amount of the protein present, but also with the relative portion of the protein present in the active, reduced form. For instance in soybean mitochondria, the alternative pathway activity declined with age, which was accompanied by a relative increase in oxidised form of the protein, while the total amount of the protein remained unchanged [30]. During ageing of potato tuber slices, however, the rise in alternative respiratory activity is correlated with the amounts of alternative oxidase [29]. However, contrary to potato tuber mitochondria, where all of the alternative oxidase protein is synthesised de novo during ageing, polypeptides detected by immunoblotting with AOA antibodies seem to be present constitutively (although there are variations in expression levels) in the mitochondria of amoeba. This is consistent with our previous results showing that trophozoites of *A. castellanii* display activity of the CN-resistant alternative pathway at all phases of growth [10].

The function of the two other monomeric proteins (38, 32 kDa), of which the amounts (much less than the amount of 35 kDa protein, at least when gels without urea were used) do not change with the growth of amoeba culture (Fig. 1), is still unclear. Recently, however, evidence is beginning to accumulate suggesting that in plant mitochondria the multiple bands are isozymes, which are products from different genes [22,31].

The results of the present study can be summarised as follows:

Monoclonal antibodies to the alternative oxidase of the higher plant *S. guttatum* cross-react with the oxidase proteins of *A. castellanii*, revealing three lower molecular mass forms, i.e. 38, 35, and 32 kDa, and a single 65 kDa band. Especially the amount of the monomeric 35 kDa form decreases dramatically with ageing of the culture (78 h) after a maximum at 24 h; likewise, the (GMP-stimulated) CN-resistant respiration shows a maximum at 24 h and drops after 3 days.

Although accurate quantitative estimates of amounts of protein are difficult to make using highly exposed ECL immunoblots as presented in Fig. 1, these results nevertheless suggest a parallel between alternative oxidase activity and the amount of the 35 kDa isomer, indicating a clear regulation of the activity by protein expression that decreases with the age of the amoeba cell culture. The lower mass forms of amoeba oxidase are predominant independent of the presence or absence of reducing or oxidising agents and chemical cross-linkers, suggesting that, in contrast to the situation in plants, the amoeba alternative oxidase exists in the membrane as a monomer. During the preparation of this manuscript, it came to our knowledge that also in the fungus *N. crassa* and in the yeast *P. stipitis* the alternative oxidase appears to function as a monomer (A.L. Umbach and J.N. Siedow, personal communication). Another intriguing property, that distinguishes amoeba alternative oxidase from the plant alternative oxidase, is that it is activated by purine nucleoside 5′-monophosphates, but not by keto acids [4,9,10,14,15]. And again, also this phenomenon has been observed in the fungus *N. crassa* [19] and in the yeast *P. stipitis* [20]. In this respect, the comparison of the amino acid sequences of plant and non-plant sources becomes of interest. All of the known plant alternative oxidase protein sequences possess two highly conserved cysteines in the amino-terminal hydrophilic region. It has recently been suggested [35] that the cysteine residue just upstream of the first membrane spanning region is likely to be the site of pyruvate action (via the formation of a thiohemiacetal) whereas the sulfydryl responsible for redox regulation may be the more N-terminal of the two. Interestingly, in *N. crassa* both cysteines are not conserved, while in *P. stipitis* the cysteines are a few residues displaced, and located in an otherwise non-conserved region [36,37]. It is therefore tempting to speculate that also in the amoeba alternative oxidase these cysteines will not be strictly conserved. Another question still to be answered concerns the mechanism of the purine nucleoside 5′-monophosphate stimulation, compared to pyruvate stimulation in plant cells. The next steps in this research will therefore be (i) isolation and characterisation of cDNA encoding for the alternative oxidase from amoeba and (ii) studying the mechanism of the activation of the alternative oxidase in amoeba by purine nucleoside 5′-monophosphates.

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