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method is applied to the LSU data, the archaebacterial tree is strongly favoured. This strong incongruency, the computer simulation, and the empirical test all indicate that the EP method is rather inefficient for phylogenetic reconstruction in this situation. By contrast, for the data under consideration, the NJ and MP methods appear to be highly efficient because they have a greater than 90% probability of recovering the model tree (Table 1). Because both methods support the archaebacterial tree regardless of whether the SSU or the LSU data are used, and because this tree has also been supported by other analyses of rRNA sequence data[4,11,13,17], we can conclude with confidence that it is the true tree. But this tree does not explain some molecular properties shared by eubacteria and halobacteria[19].

Currently there is much debate about the root of the tree. One view is that the archaebacteria represent the urkingdom from which the eubacteria and the eukaryotes have arisen independently, the former lineage from within the sulphur-dependent archaebacteria (eocytes) and the latter from the methanogen halophile group of archaebacteria[3]. (Lake's[6] view is similar to this, except he does not assume that the archaebacteria represent the urkingdom.) This view is obviously not compatible with the branching order shown in Fig. 1c. Another possibility[3,4] is that the root lies in the central branch of Fig. 1c. Both this view and the view[4] that the eukaryotic, eubacterial and archaebacterial lines were all derived from a progenote imply that the eukaryotic lineage is as old as the eubacterial lineage, but eukaryotes probably did not appear before 2,000 million years (Myr) ago, whereas eubacteria go back at least 3,500 Myr (ref. 20). A more plausible hypothesis is that the archaebacteria and the eukaryotes are sister groups because they share marked molecular and cellular resemblances[21]. For example, the archaebacterial genes coding for the RNA polymerase core subunits are much more similar to the eukaryotic than to the eubacterial counterparts[22]. Under this hypothesis the eocytes branched off shortly after the divergence between archaebacteria and eukaryotes, and *Methanococcus* branched off shortly after that. This supports the suggestion that the archaebacteria form a monophyletic but highly diversified group[2-4]. The greater lengths of the halobacterial and methanogen branches compared with that of the eocytic branch may explain why the resemblance between archaebacteria and eukaryotes is most pronounced in the case of the sulphur-dependent archaebacteria[3,6,19]. ☐

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A family of mitochondrial proteins involved in bioenergetics and biogenesis

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THE respiratory chain complexes of mitochondria consist of many different subunits, of which only a few partake directly in electron transport. The functions of the subunits that do not contain prosthetic groups are largely unknown[1]. The cytochrome reductase complex of *Neurospora crassa*, for example, consists of nine different subunits[2], of which the peripheral membrane proteins I and II (ref. 3) that are located on the matrix side of the mitochondrial inner membrane[4] are the largest subunits devoid of redox centres. Significantly, a cytochrome reductase fraction lacking these two subunits was inactive in electron transfer[5], and in yeast mutants with defective genes for either of the two subunits, assembly of the reductase is disrupted[6,7]. Most mitochondrial proteins are imported into the mitochondrion as precursor proteins, and two proteins are necessary for cleaving their presequences[8], namely the matrix processing peptidase (MPP) and the processing enhancing protein (PEP), the latter strongly stimulating the activity of the former[9]. Temperature-sensitive yeast mutants, which are affected in PEP or MPP, accumulate precursors at the non-permissive temperature[10-12]. We report here that subunit I of the cytochrome reductase complex of *N. crassa* is identical to PEP and that the protein is therefore bifunctional, participating in both electron transport and protein processing. The processing proteins and subunits I and II of cytochrome reductase can be grouped as members of the same protein family.

We isolated cytochrome reductase as a monodisperse protein-detergent complex[13] and by treating it with salt cleaved it into three distinct parts, one of which was a subcomplex of subunits I and II[4]. Following purification by sucrose gradient centrifugation[5], we dissociated the subcomplex by raising the pH and separated the single subunits by DEAE-chromatography and gel permeation chromatography. Pure subunit I was obtained with a yield of ~30%. We raised antibodies against the subunit[14] and used them in conjunction with hybrid-release translation to isolate positive clones from a *N. crassa* complementary DNA library[15]. Further screening by colony-filter hybridization was performed with the same library and in a cDNA-library in λgt11 (ref. 16). Surprisingly, we found that the cDNA sequence was identical to the sequence of the processing enhancing protein[9]. The amino-acid sequence deduced from the cDNA was verified by solid-phase sequencing of isolated subunit I and bromocyanogen peptides thereof (data not shown).

PEP and MPP have been isolated from the mitochondria of both *N. crassa*[9] and yeast[17]. MPP is a soluble matrix protein, accounting for ~0.03% of total mitochondrial protein, whereas PEP comprises 0.5% of total protein and is found in the matrix (~25%) as well as associated with the membranes (~75%) (ref. 9). We compared the abilities of subunits I and II to stimulate the processing activity with that of PEP itself. For the assay, a PEP-free MPP preparation was used to process the precursor of the β-subunit of ATP-synthase ($F_1\beta$). MPP alone showed low processing activity which was stimulated by either isolated PEP, isolated subunit I or whole cytochrome reductase. Subunit II, however, was inactive (Fig. 1). Immunoblotting revealed that antibodies to *N. crassa* subunit I, *N. crassa* PEP, and yeast PEP recognized *N. crassa* subunit I as well as *N. crassa* PEP (Fig. 2a).

To determine the number of homologous genes, we hybridized restricted genomic DNA of *N. crassa* with PEP/subunit I cDNA (Fig. 2*b*). The Southern blots showed only bands corresponding to the number of restriction sites expected from the cDNA sequence and from restriction patterns of genomic clones (unpublished data). Hybridization at lower stringencies showed increased background but no additional bands, indicating the absence of a cross-hybridizing DNA and the existence of a single gene for subunit I/PEP.

An alignment of the amino-acid sequences of MPP, PEP and subunits I and II all of yeast, and subunit I/PEP of *N. crassa* (Fig. 3*a*) reveals significant similarities among the sequences. Whenever one member of this family is compared with the other four members, *N. crassa* subunit I/PEP shows the highest degree of sequence identity, whereas yeast subunit II shows the lowest. The sequence of *N. crassa* MPP (unpublished) is also consistent with this alignment.

Although the evolutionary relationships of cytochrome reductase subunit I and II, PEP and MPP remain enigmatic, it is conceivable that the proteins are relics from a processing protease in the endosymbiotic prokaryotic ancestor of mitochondria. Alternatively, the processing activity may have evolved

from a protein that originally stabilized or facilitated the assembly of multi-protein complexes.

In yeast, subunit I, the *cor1* gene product[6] and PEP, the *mas1* gene product[11], are homologous but not identical. This prompted us to consider the possibility that the enhancement of processing associated with subunit I was the result of a residual ancestral function, and that *in vivo* processing could be mediated by a distinct, as yet unidentified component. There are good grounds for discounting this possibility and arguing that subunit I/PEP is indeed a bifunctional protein. First, isolated subunit I has a very high processing stimulation activity. As judged from the staining of bands on SDS gels, equimolar amounts of subunit I stimulate MPP to maximal activity. Second, subunit I/PEP of *N. crassa* shows considerable similarity to PEP of yeast (Table 1) and is more closely related to yeast PEP than to yeast subunit I. This close relationship is further indicated by the ability of an antibody against yeast PEP to recognize *N. crassa* subunit I/PEP. Third, the two functions of the *N. crassa* subunit I/PEP in respiration and precursor processing may be located on different domains of the protein. The possibility that a putative hydrophilic N-terminal domain points into the matrix and bears the PEP-function is supported by its remarkable similarity to

FIG. 1 Stimulation of the activity of the MPP by PEP, subunits I and II and whole cytochrome reductase.

METHODS. Increasing amounts of PEP, subunits I or II or cytochrome reductase (amount of added protein based on subunit I content) were added to the processing assay (15 μl), which contained 30 mM Tris-HCl, pH 8.2, 2 mM $MnCl_2$, 1 mM PMSF and precursor to ATPase $F_1\beta$, synthesized from the cDNA in pGEM plasmid as substrate[8]. The reaction was started with 50 ng MPP and carried out for 30 min at 25°C. The processing product was analysed by SDS-PAGE, fluorography and quantification by laser densitometry. For isolation of subunit I, 30 mg of a subcomplex of the subunits I and II (ref. 5) was dialysed for 6 h against 50 mM Tris-HCl, pH 8.0, and 50 mM NaCl, and loaded onto a 1.5 × 35-cm DEAE Sepharose CL-6B column (Pharmacia) in the same buffer. Subunit II passed through; subunit I and undissociated subcomplex were eluted at 200 mM NaCl using a 500 ml gradient from 50–500 mM. Peak fractions were pooled, concentrated 10-fold by ultrafiltration and gel-filtered on a 1.0 × 50-cm Ultrogel AcA 34 column (LKB) in 50 mM Tris-HCl, pH 7.0. Subunit I (3 mg ml^{-1}) eluted as a protein of relative molecular mass 50,000. PEP was isolated from a mitochondrial extract, prepared by sonication and centrifugation (40,000 r.p.m. for 60 min). The supernatant (50 mg protein) was loaded onto a 2.5 × 5.0-cm hydroxyapatite column (Biorad) in 10 mM Mops, pH 7.2, 50 mM NaCl and 0.1 mM EDTA. In the presence of a 300 ml gradient from 0–200 mM Na-phosphate, PEP eluted at 110–140 mM. The protein was loaded onto a MonoQ column (Pharmacia) and chromatographed with 10 ml of a gradient from 50–400 mM NaCl. PEP (0.05 mg) eluted at 210–250 mM NaCl and was gel-filtered on a Superose 12 column (Pharmacia) in 50 mM Tris-HCl, pH 7.5, and 100 mM NaCl. MPP was enriched from a cell extract of *N. crassa* hyphae by DEAE cellulose and Zn-Chelat chromatography[9].
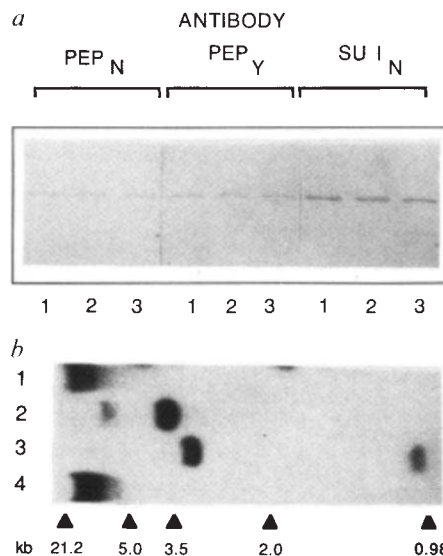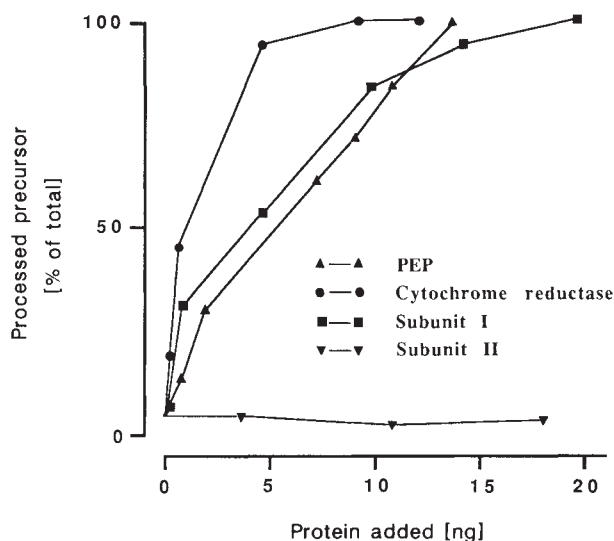

FIG. 2 *a*, Immunoblotting of PEP (1), subunit I (2) and cytochrome reductase (3) from *N. crassa* with antisera against *N. crassa* PEP (PEP$_N$), yeast PEP (PEP$_Y$, *mas1* protein) and *N. crassa* subunit I (SUI$_N$). *b*, Southern hybridization of restricted *N. crassa* genomic DNA with PEP cDNA.

METHODS. *a*, PEP, subunit I, (each 0.1 μg) and cytochrome reductase (0.5 μg) were subjected to SDS-PAGE, transferred to nitrocellulose paper[18] and immunodecorated with the different antibodies. Bound antibodies were visualized using alkaline phosphatase coupled to antibodies against rabbit IgG[19]. *b*, 10 μg of total DNA extracted from *N. crassa* wild-type 74A were restricted with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3), *Pst*I (lane 4) and electrophoresed on a 0.8% agarose gel. Fragments were blotted onto nylon filters and hybridized with a nick-translated probe of the PEP cDNA[9] which represented the complete coding and 3'-untranslated region[20]. Hybridization was in 10 ×Denhardt's solution, 2 ×SSC, 0.1% SDS at 65°C for 20 h. Washing was performed twice with 0.1% SSC at 65°C for 10 min.
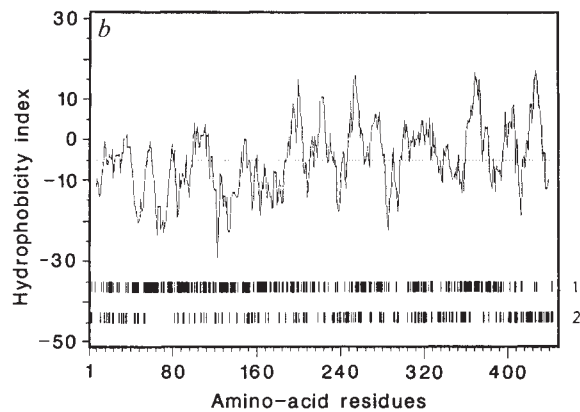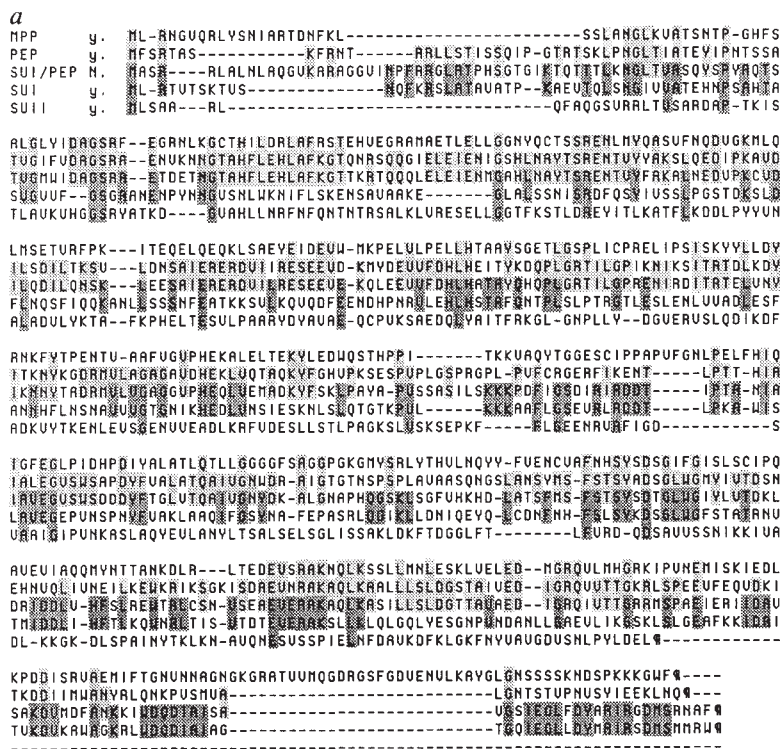
a

[Figure 3a: protein sequence alignment — dense amino-acid sequence blocks for MPP y., PEP y., SUI/PEP N., SUI y., SUII y.]

b

[Figure 3b: Hydrophobicity index plotted against Amino-acid residues (axis ticks at 1, 80, 160, 240, 320, 400; y-axis 30, 10, 0, −10, −30, −50)]

FIG. 3 *a*, Sequence alignment of the protein family comprising MPP from yeast (MPP$_y$)[12,21], PEP from yeast (PEP$_y$)[11], *N. crassa* subunit I/PEP (SUI/PEP$_N$) and the subunits I and II of yeast cytochrome reductase (SUI$_y$, SUII$_y$)[6,7]. Alignment was performed using the program ALNED by David George, PIR-NBRF. The light, hatched areas show the identity between *N. crassa* subunit I/PEP and yeast MPP and PEP, whereas the dark, hatched areas show identity between *N. crassa* subunit I/PEP and yeast cytochrome reductase subunit I and subunit II. *b*, Hydrophobicity profile of *N. crassa* subunit I/PEP and identity pattern between *N. crassa* subunit I/PEP and yeast PEP (1), and yeast subunit I (2). Hydrophobicity is plotted according to ref. 22, using a window of 15 amino acids. Identical residues are represented by vertical bars.

the N-terminal half of yeast PEP (65%), and its more distant relationship to the N-terminal half of yeast subunit I (23%) (Fig. 3*b*). A more hydrophobic C-terminal domain, which probably contacts other subunits of cytochrome reductase, could exert respiratory function. Compared with the N-terminal domain, this domain is less similar to the C-terminal half of yeast PEP (43%) but more similar to the C-terminal half of yeast subunit I (40%). Fourth, if a distinct *N. crassa* PEP that differs from subunit I exists, it should have a high degree of similarity to yeast PEP and, therefore, also to *N. crassa* subunit I. The PEP gene should be detected on hybridization of restricted *N. crassa* DNA with subunit I/PEP cDNA. Finally, cDNA of *N. crassa* subunit I/PEP, when transformed into a yeast *mif-1* mutant lacking PEP activity, equivalent to *mas1*, complements processing deficiency (A. Horwich, personal communiction), whereas when it is transformed into a *cor1* mutant of yeast[6] it does not complement respiratory deficiency (unpublished observations).

These observations support the opinion that respiratory and PEP function in *N. crassa* reside in a single protein. The situation in yeast where two distinct proteins have evolved to function as subunit I or PEP may be related to the exceptional ability of this organism to repress respiration under anaerobic conditions. The promitochondria of fermenting cells lack respiratory chain complexes, but still perform protein import and processing.

Thus, the requirement for PEP in repressed yeast might have led to the divergence of PEP and subunit I.

Our results contribute to understanding the roles of protein components in mitochondrial respiratory chain complexes that do not contain redox centres and are absent from the related bacterial enzymes. Some of these subunits may have functions that are not directly related to respiration. Proteins that stabilize and assist the assembly of oligomeric complexes may belong to families that have such functions in unrelated reaction pathways. In the case of PEP, one function could reside in an interaction with the targeting sequences, thereby facilitating import and processing. □

TABLE 1 Sequence similarities among the members of the PEP/MPP/cytochrome reductase subunit I/II family

	MPP$_N$	PEP$_y$	SUI/PEP$_N$	SUI$_y$	SUII$_y$
MPP$_y$	41.6	20.9	22.6	16.6	15.5
MPP$_N$		21.0	26.1	18.0	11.5
PEP$_y$			51.4	24.1	12.1
SUI/PEP$_N$				32.2	12.4
SUI$_y$					9.1

The percentages of identical residues of the members of the protein family in *N. crassa* and yeast based on the alignment of Fig. 3*a*.

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