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Yeast mitochondrial F_1F_0-ATP synthase exists as a dimer: identification of three dimer-specific subunits

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Using the technique of blue native gel electrophoresis, the oligomeric state of the yeast mitochondrial F_1F_0-ATP synthase was analysed. Solubilization of mitochondrial membranes with low detergent to protein ratios led to the identification of the dimeric state of the ATP synthase. Analysis of the subunit composition of the dimer, in comparison with the monomer, revealed the presence of three additional small proteins. These dimer-specific subunits of the ATP synthase were identified as the recently described subunit e/Tim11 (Su e/Tim11), the putative subunit g homolog (Su g) and a new component termed subunit k (Su k). Although, as shown here, these three proteins are not required for the formation of enzymatically active ATP synthase, Su e/Tim11 and Su g are essential for the formation of the dimeric state. Su e/Tim11 appears to play a central role in this dimerization process. The dimer-specific subunits are associated with the membrane bound F_0-sector. The F_0-sector may thereby be involved in the dimerization of two monomeric F_1F_0-ATP synthase complexes. We speculate that the F_1F_0-ATP synthase of yeast, like the other complexes of oxidative phosphorylation, form supracomplexes to optimize transduction of energy and to enhance the stability of the complex in the membrane.

Keywords: dimer/F_1F_0-ATP synthase/mitochondria/subunits e, g and k/yeast

Introduction

F_1F_0-ATP synthase complexes play a central role in the synthesis of ATP in all living organisms. This enzyme is present in the plasma membrane of bacteria, thylakoid membranes of chloroplasts and in the inner membrane of mitochondria. In each of these locations, the F_1F_0-ATP synthase functions to synthesize ATP in a manner which is coupled to the translocation of protons across the respective membranes. Two functionally distinct parts of this complex can be distinguished, the F_1 part, which performs the ATP synthesis and hydrolysis reactions, and the membrane bound F_0-sector, which mediates the proton transport.

The mitochondrial F_1F_0-ATP synthase, complex V of the respiratory chain, is composed of both nuclear and mitochondrial encoded subunits (Law *et al*., 1995; Walker *et al*., 1991, 1995). In the yeast *Saccharomyces cerevisiae*, three subunits, 6, 8 and 9 (Su 6, Su 8 and Su 9), are encoded by the mitochondrial DNA and represent essential subunits of the membrane-bound F_0 sector. The remaining subunits of the F_0-sector and all subunits of the F_1 sector are nuclear encoded. Recently, several small novel subunits of the F_1F_0-ATP synthase from yeast have been reported, the subunits h (Su h), subunit e/Tim11(Su e/Tim11) and subunit f (Su f; Arselin *et al*., 1996; Arnold *et al*., 1997; Spannagel *et al*., 1997). In addition, a putative homolog of the mammalian subunit g (Su g) has been reported; however, it has not yet been demonstrated as being a subunit of the yeast ATP-synthase complex (Prescott *et al*., 1997). Not all of these proteins appear to be essential for the activity of the F_1F_0-ATP synthase (Arnold *et al*., 1997; Prescott *et al*., 1997).

In this study we have re-analysed the subunit composition of the mitochondrial F_1F_0-ATP synthase from yeast. We used a mild detergent lysis procedure followed by blue native gel electrophoresis to isolate the ATP synthase. Using this procedure, the ATP synthase was found to exist as a dimer. Analysis of the subunit composition of the dimer by a high-resolution second dimension SDS–polyacrylamide gel electrophoresis (SDS–PAGE) lead to the identification of three novel proteins. These subunits present only in the dimeric form of the complex include Su e/Tim11, the putative Su g homolog (Prescott *et al*., 1997) and a new subunit, termed subunit k (Su k). Characterization of these subunits, in particular their role in the formation of the dimeric state of the F_1F_0-ATP synthase is presented here.

Results

Dimeric structure of the yeast F_1F_0-ATPase

The subunit composition of the mitochondrial F_1F_0-ATPase from *S.cerevisiae* was analysed using blue native gel electrophoresis followed by resolution of protein constituents in a second dimension by SDS–PAGE. Mitochondrial membrane proteins were solubilized with a low concentration of Triton X-100 and the extract was applied to a blue native gel (Figure 1A). Two dominant complexes could be identified, one of molecular mass ~1000 kDa and a less abundant complex of 500 kDa. Second-dimension resolution of both of these complexes by SDS–PAGE resulted in a protein subunit profile characteristic of F_1- and F_0-sector subunits of the F_1F_0-ATPase complex (Figure 1B). The estimated molecular mass of the smaller complex corresponds well to that of the monomeric F_1F_0-ATPase complex. The larger and more predominant one apparently represents a dimer of the complex. The identity of both complexes was confirmed by protein

sequencing of individual subunits following SDS–PAGE (Table I) and by Western blotting using antisera specific for the subunits of the F_1-sector (results not shown; see Figure 5).

The protein subunit composition of the monomeric and dimeric forms of the F_1F_0-ATPase was very similar, with the exception of three small proteins, present exclusively in the dimeric form (Figure 1B; Table I). N-terminal sequencing of these proteins revealed them to be the recently described subunit e/Tim11 (Su e/Tim11) (Tokatlidis *et al*., 1996; Arnold *et al*., 1997), the putative Su g homolog (Prescott *et al*., 1997) and a novel protein which we term Su k. Although they are not entirely reliable indices of stoichiometry, the Coomassie staining intensities suggest a copy number of two per dimer for Su g and Su e/Tim11. Su k seems to be poorly stained by Coomassie, thus making it technically difficult to estimate its precise stoichiometry.

On the basis of these data we conclude that mild solubilization of yeast mitochondria yields a dimeric form of the F_1F_0-ATPase complex. This dimer contains three small proteins which are not found in the monomer.

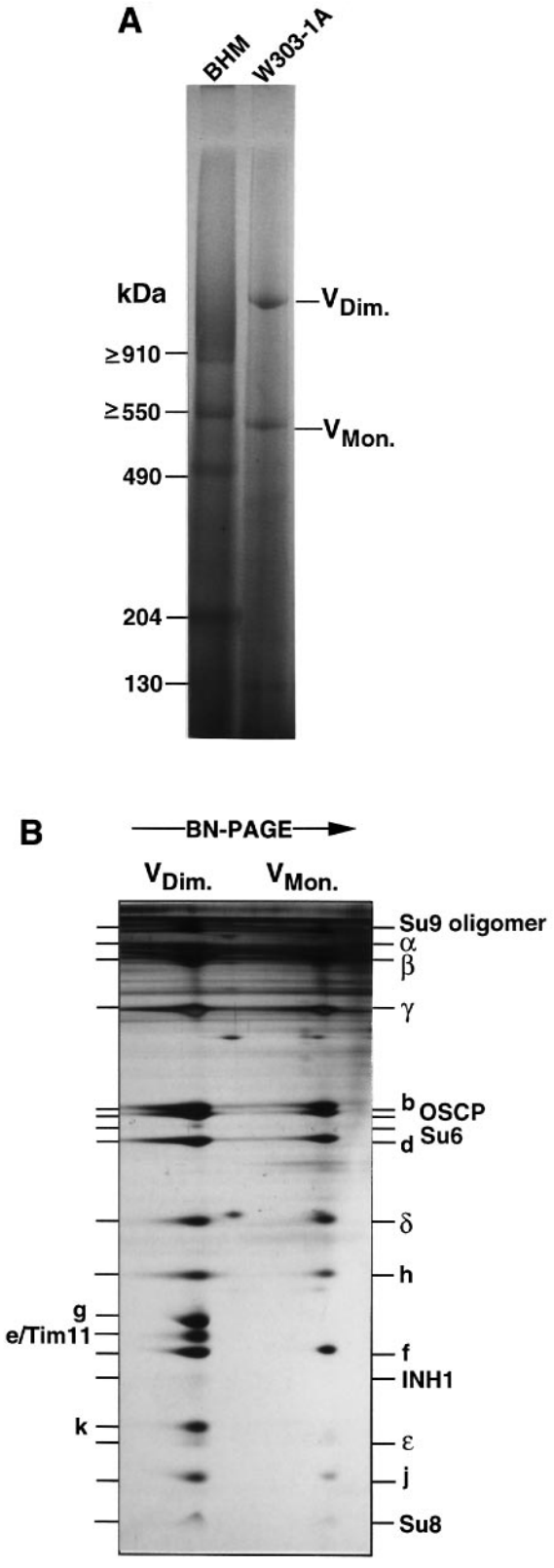

Fig. 1. One- and two-dimensional resolution of dimeric and monomeric ATP synthase from *S.cerevisiae* mitochondria. (**A**) ATP synthase from yeast mitochondria was solubilized at a low Triton X-100:protein ratio (0.6 g/g) and separated by BN–PAGE. A dimeric and a monomeric form of ATP synthase were identified using complexes I–V from bovine heart mitochondria (BHM) as molecular mass standards. Complex I, ⩾910 kDa; complex V, ⩾550 kDa; complex III, 490 kDa; complex IV, 204 kDa; complex II, 130 kDa. (**B**) Polypeptide composition of monomeric and dimeric ATP synthase. The dimeric and monomeric forms of the complex resolved by BN–PAGE were analysed in a second dimension by Tricine-SDS–PAGE. The resulting gel was silver stained. The protein subunits from monomeric ATP synthase and three dimer-specific Su g, e/Tim11, and Su k were identified by N-terminal protein sequencing and Western blotting. Abbreviations: $V_{Dim.}$ and $V_{Mon.}$, the dimeric and monomeric forms, respectively, of complex V of the respiratory chain, the F_1F_0-ATP synthase.

Topology of dimer-specific components and function in the catalytic activity of the F_1F_0-ATPase

Su e/Tim11 is known to span the inner membrane once with an N_{in}–C_{out} orientation and requires the presence of the mitochondrially encoded F_0-subunits for stable expression (Tokatlidis *et al*., 1996; Arnold *et al*., 1997). We cloned the corresponding genes for the other two dimer-specific subunits and raised specific polyclonal antisera against them (Figure 2). The hydropathy plots for Su g and Su k suggests that they are membrane proteins with a single transmembrane domain. Su g- and Su k-specific antibodies were raised against peptides corresponding to the C-terminal regions of both subunits. With these antibodies it could be shown that both Su g and Su k, like Su e/Tim11 (Arnold *et al*., 1997), were inaccessible to exogenously added protease in intact mitochondria (Figure 2). Opening of the outer membrane by hypotonic swelling rendered the subunits accessible to the added protease. Furthermore, Su g appears to be an integral inner-membrane protein, as it was largely retained with the mitochondrial membranes upon alkaline extraction. In contrast, Su k was solubilized by this procedure, indicating that it was probably peripherally associated with the inner membrane. The submitochondrial localization and association of these subunits with the inner membrane would suggest they are probably part of the F_0-sector.

These three subunits are not essential for the catalytic activity of the F_1F_0-ATPase complex, as yeast strains harbouring deletions of the individual genes remained respiratory competent (Figure 3A). A weak petite phenotype, however, was observed, in particular for the Δ*tim11* and Δ*su g* strains. Growth of these strains on non-

Table I. Proteins associated with dimeric ATP synthase of *S.cerevisiae*

Band in SDS gel	Assignment	Gene	Mature protein			Staining ratio[d]	SWISS-PROT accession No.
			N-terminal sequence[a]	AA[b]	Mass (Da)[c]		
1	Su 9 oligomer	ATP9	[f] MQLVLA	76	7759	0.31	P00841
2	Su α	ATP1	ASTKAQPTEV	510	54 952	2.37	P07251
3	Su β	ATP2	ASAAQSTPIT	478	51 254	2.14	P00830
4	Su γ	ATP3	ATLKEVEMRL	278	30 614	1.00	P38077
5	Su 4 or Su b	ATP4	MSSTPEKQTD	209	23 249	1.03	P05626
6	Su 5 or OSCP	ATP5	ASKAAAPPPV	195	20 870	0.93	P09457
7	Su 6 or Su a	ATP6	no sequence	249	27 956	0.10	P00854
8	Su d	ATP7	sLAKsAANKL	173	19 677	1.06	P30902
9	Su δ	ATP16	AEAAAASSGL	138	14 553	0.59	Q12165
10	Su h	ATP14	DVIQDLYLRE	92	10 408	0.51	Q12349
11	Su g	ATP20	MLSRIQNYTSGLV	115	12 921	0.96	Q12233
12	Su e or Tim11p	ATP21/TIM11	[ac] STVNVLRYSALGL	95	10 744	0.71	P81449
13	Su f	ATP17	VSTLIPPKVV	95	10 565	1.22	Q06405
14	Inhibitor protein	INH1	sEGsTGtPRG	63	7383	0.33	P01097
15	Su k	ATP19	MGAAYHFMGKAIPP	68	7533	0.39	P81451
16	Su ε	ATP15	sAwRKAGI	61	6611	1.01	P21306
17	Su j	ATP18	MLKRFPTPILKVY	59	6687	0.83	P81450
18	Su 8	ATP8	[f] MPQLVPFYF	48	5822	0.40	P00856

[a]Proteins were identified by direct Edman degradation, or after deformylation ([f]), or deacylation ([ac]), except Su 6 which was identified by Western blotting. Lower case letters indicate amino acids which were not identified.
[b]AA, number of amino acids.
[c]The masses of the mature proteins do not include N-terminal modifications.
[d]The staining ratios were obtained by densitometric quantification of Coomassie-stained SDS gels. The stain intensities (arbitrary units) were divided by the molecular masses of the individual subunits, and normalized to Su γ.

fermentable carbon sources was inhibited in the presence of oligomycin, demonstrating that none of these subunits played a role in conferring oligomycin sensitivity on the F_1F_0-ATPase complex (results not shown). This observation was supported by the enzymatic measurement of the oligomycin-sensitive ATP hydrolysis activity of the F_1F_0-ATPase complex in mitochondria from the various deletion strains (Figure 3B). The protein amounts of the ATP synthase monomer (Δ*tim11* and Δ*su g* strains) and the sum of the dimer and monomer (wild-type and Δ*su k* strains) which were densitometrically quantified in two-dimensional gels, were reduced in the Δ*tim11*, Δ*su g* and Δ*su k* mitochondria, but were proportional to the reduction in the measured ATP hydrolysis activities (Figure 3B). Thus the specific activities of the monomer and dimer forms of the F_1F_0-ATP synthase appear to be similar.

In conclusion, Su e, Su g and Su k are non-catalytic subunits of the F_1F_0-ATPase complex and their presence is not required for the assembly of an enzymatically active complex.

Expression of dimer-specific subunits

We next addressed whether these subunits were interdependent on each other for stable expression and if their levels were influenced by the presence of the mitochondrially encoded subunits of the F_0-sector. Mitochondria were isolated from the three deletion yeast strains and a rho^0 strain, which lacks a mitochondrial genome, and were analysed by Western blotting together with mitochondria from the corresponding wild-type strain. The levels of Su e/Tim11, Su g and Su k were analysed and compared with other mitochondrial proteins, including other known subunits of the F_1F_0-ATPase complex (Figure 4). The presence of Su e/Tim11 influences the stable expression of both Su g and Su k, as they were not detected in mitochondria isolated from the Δ*tim11* strain. On the other hand, Su e/Tim11 was present in the Δ*su g* mitochondria, albeit at reduced levels, whereas stable expression of Su k clearly was affected in the absence of Su g. Deletion of Su k, however, had no appreciable effect on the steady state levels of either Su e/Tim11 or Su g. Furthermore, the levels of another bona fide F_0-sector subunit, Su f, was reduced significantly in the absence of both Su e/Tim11 and Su g, but appeared to be unaffected by the absence of Su k. The levels of the α-subunit of the F_1-sector and an unrelated control protein, cytochrome b_2 were unaffected in the various deletion mutants.

We conclude that Su e/Tim11 plays a central role in the assembly and/or stability of both Su g and Su k. The accumulation of Su e/Tim11 does not depend on the presence of Su g and Su k. On the other hand, the presence of the Su g appears to enhance the stability of Su e/Tim11. Su k, although not required for the accumulation of Su e/Tim11 and Su g, appears to need these two subunits for its own assembly and/or stability.

Dimerization of the F_1F_0-ATPase

The involvement of the novel subunits in the dimerization of the F_1F_0-ATPase complex was addressed. Wild-type mitochondria were solubilized with the detergent digitonin and the oligomeric state of the F_1F_0-ATPase was analysed by blue native gel electrophoresis followed by Western blotting (Figure 5A). Similar to the solubilization procedure in Triton X-100, both dimeric and monomeric forms of the F_1F_0-ATPase complex were resolved. Both forms of the complex could be shown to contain the α-subunit of the F_1-sector, whereas the Su e/Tim11 and Su g were located exclusively in the dimeric form of the complex.

The role of the novel subunits in the formation of

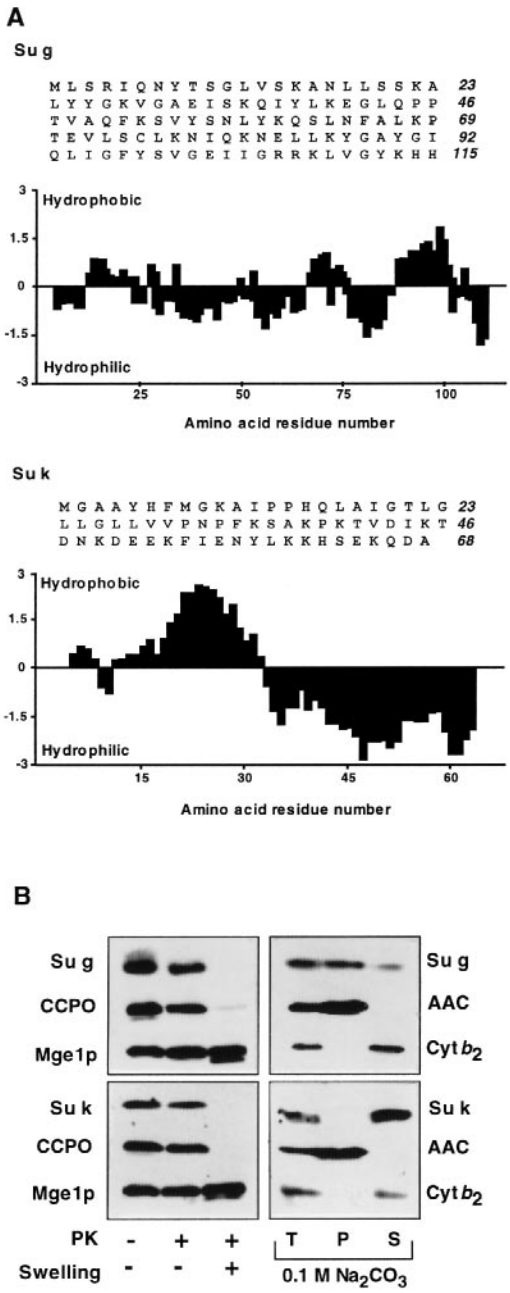

Fig. 2. Amino acid sequence of Su g and Su k of the mitochondrial F_1F_0-ATPase from *S.cerevisiae*; hydropathy profiles and submitochondrial localization of both proteins. (**A**) The amino acid sequences of Su g and Su k from *S.cerevisiae* together with their hydropathy profiles, are shown. (**B**) Submitochondrial localization of Su g and Su k. Mitochondria and mitoplasts generated from hypotonic swelling were incubated for 30 min on ice in the presence or absence of proteinase K (PK, 200 µg/ml), as indicated. Mitochondria were subjected to alkaline extraction (0.1 M Na_2CO_3, pH 11.5) for 30 min on ice. The sample was divided, one half was directly TCA-precipitated (total, T) and the other was separated by centrifugation (60 min at 226 000 *g*) into pellet (P) and supernatant (S) fractions, and then all samples were subjected to TCA precipitation. Samples were analysed by SDS–PAGE and Western blot analysis, using specific antisera for cytochrome *c* peroxidase (CCPO) and cytochrome b_2 (Cytb_2), both soluble proteins of the intermembrane space; Mge1p, a matrix-localized soluble protein; the ADP/ATP carrier protein (AAC), an integral inner membrane protein; and Su g and Su k.

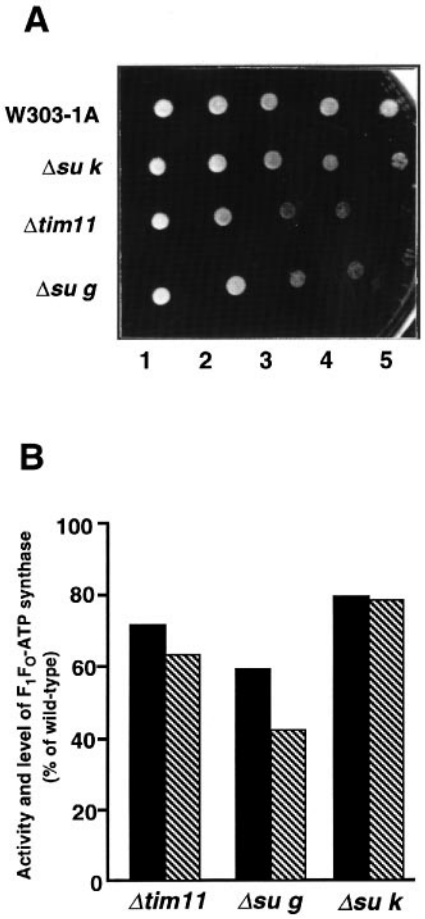

Fig. 3. Su g, Su k and Su e/Tim11 of the F_1F_0-ATPase are not essential for growth on non-fermentable carbon sources. (**A**) Yeast cells of strains Δ*tim11*, Δ*su g* and Δ*su k* and corresponding isogenic wild-type W303-1A, grown on YPD (glucose) medium were resuspended in sterile water at a concentration of 10 OD_{578}/ml. A dilution series was generated by serially diluting this suspension 10-fold each time. Two microlitres of each of the resulting dilutions were spotted onto a YPG (glycerol) plate (spots 1–5) and were incubated at 30°C for 2 days. (**B**) Oligomycin-sensitive ATP hydrolysis measured with isolated mitochondria (full bars) and correlated to the amount of ATP synthase (determined following BN–PAGE and second-dimension electrophoresis) (shaded bars) from deletion and wild-type strains were compared (determinations with three different mitochondrial preparations each; $n = 3$). Wild-type levels were set to 100%.

the dimeric state of F_1F_0-ATPase complex was then investigated. Mitochondria isolated from the three individual deletion yeast strains were isolated and membrane proteins were solubilized with digitonin and analysed by blue native gel electrophoresis (Figure 5B). In contrast to the isogenic wild-type, the dimeric form of the complex was absent in the Δ*tim11* and Δ*su g* mitochondria. On the other hand, the ratio of dimeric to monomeric complex in the Δ*su k* mitochondria was unaltered when compared with the wild-type. A similar result was obtained if the mitochondria were solubilized with Triton X-100 prior to analysis by blue native electrophoresis (Figure 5C) and followed by a second-dimension resolution by SDS–PAGE (Figure 5D).

We conclude that Su e/Tim11 and Su g play an essential role in the formation of the dimer of the F_1F_0-ATPase complex. In contrast, the presence of Su k appears not to be necessary.

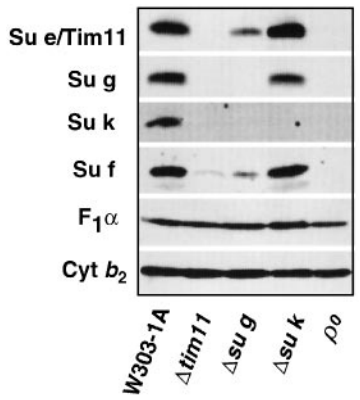

Fig. 4. Expression of F_1F_0-ATPase subunits in the absence of Su e/Tim11, Su g and Su k. Mitochondria (50 μg protein) isolated from the Δ*tim11*, Δ*su g* and Δ*su k* yeast strains and corresponding isogenic wild-type W303-1A strain were subjected to SDS–PAGE and analysed by Western blotting for the presence of ATP synthase subunits and cytochrome b_2 (Cytb_2), as indicated.

The dimeric F_1F_0-ATPase complex can be dissociated into intact monomers. Isolated wild-type mitochondria were solubilized with increasing Triton X-100 concentrations and the F_1F_0-ATPase complex was analysed by blue native gel electrophoresis. Increasing the detergent concentration resulted in a decrease of the dimer and a corresponding increase in the monomeric form (Figure 6A). Western blotting and decoration with $F_1\alpha$-specific antisera confirmed the presence of dimeric F_1F_0-ATPase at low Triton X-100 and its conversion to monomer with increasing amounts of the detergent (Figure 6B, upper panel). Dissociation of the dimer into the monomeric form resulted in the loss of Su e/Tim11 and Su g from the dimer (Figure 6B, lower panel). Loss of Su k from the dimer was also observed as confirmed in silver-stained two-dimensional gels and by microsequencing (results not shown; the titer of the antibody was not sufficiently high for use in blots of blue native gels). The three subunits were not recovered with the resulting monomer F_1F_0-ATPase complexes. Whether Su e/Tim11, Su g and Su k are released together as a subcomplex from the dimer was subsequently analysed. Gel-filtration analysis of Triton X-100 solubilized mitochondria was performed, using conditions where only the monomeric form of the ATP synthase was present. The three dimer-specific subunits did not co-elute from the column (results not shown). We therefore conclude these three subunits are not released in a subcomplex together upon dissociation of the dimer.

Discussion

In the present study we report the observation that the F_1F_0-ATP synthase of yeast mitochondria exists as a dimer. The dimeric form is observed under conditions when mitochondrial membrane proteins are solubilized by low detergent to protein ratios. Analysis of the protein composition of the dimer of the F_1F_0-ATP synthase by high-resolution second dimension electrophoresis revealed the presence of three additional small proteins, which are not observed in the monomeric form. Sequencing of these proteins indicated them to be the recently identified Su e/Tim11, the putative homolog of mammalian Su g proteins and a third novel protein, Su k.

Disruption of the respective genes for these proteins demonstrated them to be non-essential subunits for an enzymatically active F_1F_0-ATP synthase. Although the mitochondrial content of the ATP synthase complex was reduced in the absence of these proteins, in particular in the Su e and Su g deletion strains, the specific oligomycin-sensitive ATP hydrolysis activity remained unaltered. The functional relevance of these proteins was revealed following the electrophoretic analysis of the composition of the F_1F_0-ATP synthase in their absence. Mitochondria isolated from the Δ*tim11* and Δ*su g* yeast strains contained the monomeric form of the F_1F_0-ATP synthase, whereas the dimer was absent. We conclude therefore that Su e/Tim11 and Su g are essential for the formation of the dimeric form of the complex.

What mediates this dimerization? The dimer-specific subunits were found to be associated with the membrane and exposed to the intermembrane space. They appear to be associated with the F_0-sector of the ATPase complex. As Su e/Tim11 and Su g are required for the formation of the dimer, we predict that the dimerization is mediated by the F_0-sector and probably involves two monomeric F_1F_0-ATP synthase complexes (Figure 7). Su e/Tim11 apparently plays a central role in this dimerization process for the following reasons: (i) the sequence of Su e/Tim11, like its mammalian counterparts, predicts the ability to form a coiled-coil structure, often the basis for homo-dimerization events; and (ii) the presence of Su e/Tim11 was essential for the stable expression of the other two dimer-specific subunits, whereas low level expression or normal expression of Su e/Tim11 was observed in the absence of Su g and Su k, respectively. The prediction underlying this model of F_1F_0-ATP synthase dimerization is that Su e/Tim11 can form a homodimer. Preliminary evidence supporting a dimeric state of the bovine Su e has been reported recently (Belogrudov *et al*., 1996). Investigations are currently in progress to verify a dimeric form of the yeast Su e/Tim11 protein.

What are the functions of Su g and Su k? Su g is essential for the dimerization process. In contrast to Su e/Tim11, but like all other known subunits of the F_0-sector, Su g does not contain a predicted coiled-coil structure. Su g thus appears to play an accessory role to Su e/Tim11 in the dimerization event. The role of Su k remains unclear. Although it is exclusively located in the dimeric form, its presence is not required for stable expression of Su e/Tim11 or Su g, and does not appear to be essential for dimer formation either. However, it is unlikely that Su k represents a contaminant of the ATP synthase. First, its steady-state levels are influenced by those of other ATP synthase subunits, Su k is clearly downregulated in Δ*tim11*, Δ*su g* and rho^0 mitochondria. Secondly, the technique of blue native (BN)–PAGE resolves proteins by their molecular mass. To be a contaminant, Su k would need to have the same native size as the ATP synthase. No other polypeptides which could not be assigned to the ATP synthase were present in the dimer. This result, together with the finding that the abundance of Su k was comparable to that of other F_0-subunits, argues that Su k represents a bona fide subunit of the ATP synthase complex. Su k appears to be peripherally associated with the intermembrane space side of the inner membrane. There, it may interact with the intermembrane space

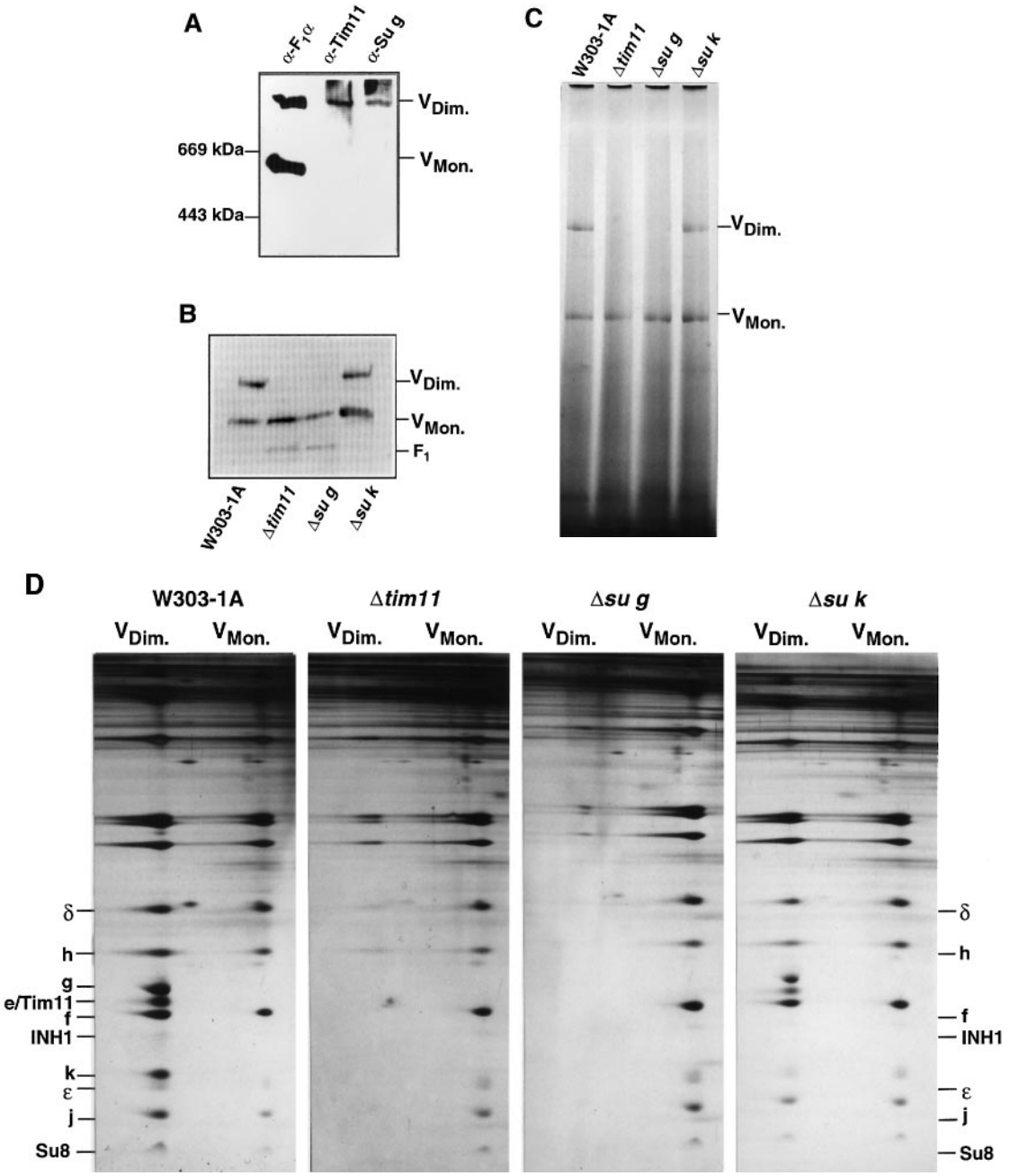

Fig. 5. Expression of Su e/Tim11 and Su g are essential for the formation of dimeric F_1F_0-ATPase. (**A**) Wild-type mitochondria were solubilized with digitonin, as described in Materials and methods and analysed by BN–PAGE followed by Western blotting. Antisera specific for the α-subunit of the F_1-ATPase, Su e/Tim11 (α-Tim11) and Su g (α-Su g) were used for immunodecoration. The positions of the dimeric and monomeric forms of the F_1F_0-ATPase, are indicated by $V_{Dim.}$ and $V_{Mon.}$, respectively, as well as those of the molecular mass standards thyroglobulin (669 kDa) and apoferritin (443 kDa). (**B**) Mitochondria isolated from wild-type yeast (W303-1A) or from yeast mutants deficient in either Su e/Tim11 (Δ*tim11*), Su g (Δ*su g*) or Su k (Δ*su k*) were solubilized in digitonin, as described above. The dimeric state of the F_1F_0-ATPase was analysed by BN–PAGE followed by Western blotting and decoration with antiserum specific for the α-subunit of the F_1-sector. The positions of dimeric ($V_{Dim.}$) and monomeric ($V_{Mon.}$) ATP synthase and free F_1-sector (F_1), are indicated. (**C** and **D**) Dimeric and monomeric forms of the ATP synthase in wild-type, Δ*tim11*, Δ*su g* and Δ*su k* strains. Mitochondria were solubilized at a low Triton X-100/protein ratio (0.6 g/g) and BN–PAGE was performed (C). BN–PAGE samples were processed in a second dimension by Tricine-SDS–PAGE (D).

exposed C-termini of Su e/Tim11 and/or Su g. An investigation into the possible interactions of Su e, Su g and Su k with each other is currently being undertaken.

Why does the mitochondrial F_1F_0-ATP synthase from yeast form a dimer? The specific activity of the ATP synthase did not appear to be adversely affected in the Δ*tim11* or Δ*su g* mutant mitochondria, indicating that the monomer is as active as the dimer found in wild type. The dimerization, however, appears to confer a stability advantage on the ATP synthase. During preparations of the ATP synthase, we observed that the monomer form is more labile and susceptible to proteolysis than the dimeric form. The decreased levels of the ATP synthase complex in the Δ*tim11* or Δ*su g* mutant mitochondria probably reflects this increased lability. Could the dimeric ATP synthase complex display a dynamic relationship with the

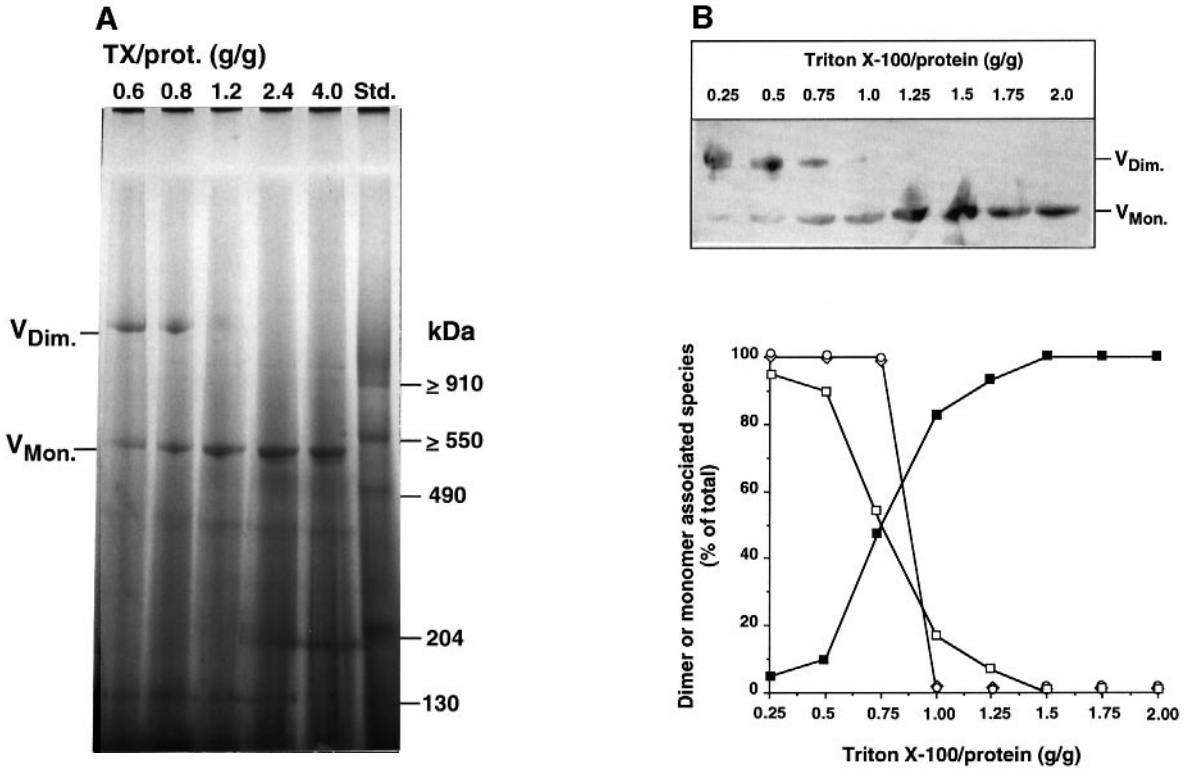

Fig. 6. Release of Su e/Tim11 and Su g from the dimeric form of the F_1F_0-ATPase upon its dissociation into the monomeric form. (**A**) BN–PAGE of mitochondria solubilized at varying Triton X-100:protein ratios. The positions of the dimeric and monomeric forms of the F_1F_0-ATPase, are indicated by $V_{Dim.}$ and $V_{Mon.}$, respectively. Complexes I–V from bovine heart mitochondria were used as molecular mass standards (Std.; see Figure 1B). (**B**) Isolated wild-type mitochondria were solubilized with increasing amounts of Triton X-100. The mitochondrial lysates were applied to three gels in parallel, and the dimeric and monomeric forms of the F_1F_0-ATPase were analysed by BN–PAGE and Western blotting. The three blots were decorated with antisera specific for either the $F_1\alpha$-subunit, Su e/Tim11 or Su g. Upper panel: decoration with $F_1\alpha$-antiserum. Lower panel: the levels of $F_1\alpha$, Su e/Tim11 and Su g associated with the dimer and monomer were quantified by densitometry. The levels of $F_1\alpha$ associated with the dimer (open squares) and monomer (filled squares) is expressed as a percentage of the sum dimer and monomer signals at each Triton X-100 concentration. No Su e/Tim11 or Su g was observed associated with the monomeric form, therefore only the dimer-associated species could be quantified for each point; Su e/Tim11 (open circles), Su g (open triangles).

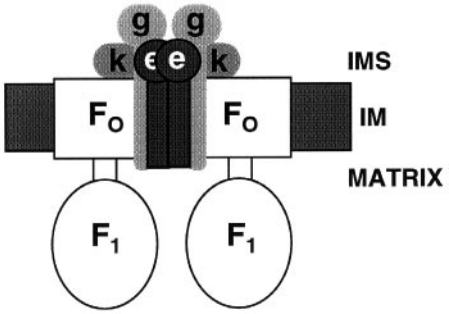

Fig. 7. Schematic representation of the F_1F_0-ATPase dimer. Dimerization of two monomeric F_1F_0-ATPase complexes occurs through their F_0-sectors and involves three subunits, Su e/Tim11, Su g and Su k. Abbreviations: e, Su e/Tim11; g, Su g; k, Su k; IM, inner membrane; IMS, intermembrane space.

monomeric form, perhaps regulated by the energetic state of the mitochondria? We tested this possibility *in vitro*, either by inhibiting of the enzymatic activity by oligomycin, depleting the membrane potential or altering the ATP:ADP:AMP ratios and the pH. None of these treatments caused alterations in the dimer:monomer ratios (results not shown). We conclude therefore that the dimeric form of the F_1F_0-ATP synthase seems to be a stable rather than a dynamic structure in isolated mitochondria. Whether or not the dimerization enables an allosteric regulation of the enzymatic activity of the F_1F_0-ATP synthase remains an open question. Furthermore, the dimeric form of the ATP synthase may confer the ability, *in vivo*, to modulate the activity or levels of the ATP synthase under different growth conditions, such as aerobic and anaerobic conditions. Finally, the mitochondrial inner membrane is a protein-rich membrane. Formation of dimers of respiratory chain complexes could serve to accommodate more protein in the membrane, as less lipid to surround the protein complex would be required. Interestingly, not only the ATP synthase, but also other complexes of the respiratory chain, such the cytochrome bc_1 complex and cytochrome oxidase complex are known to form stable dimers.

Materials and methods

Yeast strains and growth conditions

Construction of the Δ*tim11*::HIS3 (Δ*tim11*), Δ*su g::HIS3* (Δ*su g*) and Δ*su k::HIS3* (Δ*su k*) yeast strains is described below. The introduction of the *HIS3* gene resulting in a partial deletion and disruption of the Su e, Su g and Su k genes was performed as follows: the *HIS3* gene was amplified from the plasmid pFA6a-HIS3MX6 (Wach *et al*., 1994) using the following primers:

For Su e, Δtim11::HIS3 (Δtim11). S1: 5′-CGGAACATAACGTATATA-GGAACTAGCTGAGTGAGTTAAAGGATGCGTACGCTGCAGGT-CGAC-3′ [corresponding to nucleotides –42 to +3 of the *TIM11* gene (*ATP21*) locus and 18 nucleotides of the multiple cloning site (MCS) of

pFA6a-HIS3MX6 from the 5′ flanking region of the *HIS3* gene] and S2: 5′-GTGTAGCTTGGCGTATTCCTTCTTTGCCTCCTCTACCAGCTT-CAAATCGATGAATTCGAGCTCG-3′ (corresponding to nucleotides +171 to +127 of the *TIM11* gene (*ATP21*) and 19 nucleotides of the MCS of pFA6a-HIS3MX6 from the 3′ flanking region of the *HIS3* gene).

For Su g, Δsu g::HIS3 (Δsu g). S1: 5′-ACCTGCCGATAAATCATATTT-CAGAACGCTAATCAATTCATCATGCGTACGCTGCAGGTCGAC-3′ [corresponding to nucleotides –42 to +3 of the Su g gene locus (*ATP20*) and 18 nucleotides of the MCS of pFA6a-HIS3MX6 from the 5′ flanking region of the *HIS3* gene] and S2: 5′ACGAATATACAAGGGTTTTGCG-AATAGATAGAATTAAAAAGCTTAATCGATGAATTCGAGCTCG-3′ [corresponding to nucleotides +406 to +450 of the Su g gene (*ATP20*) and 19 nucleotides of the MCS of pFA6a-HIS3MX6 from the 3′ flanking region of the *HIS3* gene].

For Su k, Δsu k::HIS3 (Δsu k). S1: 5′-ATAAGGCCAGGTGACTT-CAAATAATAATTTATAACTACGACATGCGTACGCTGCAGGTCG-AC-3′ [corresponding to nucleotides –42 to +3 of the Su k gene locus (*ATP19*) and 18 nucleotides of the MCS of pFA6a-HIS3MX6 from the 5′ flanking region of the *HIS3* gene] and S2: 5′-TATGTACAAAAGAT-CTTCAACCGCGCAGCAATCAAGCTATATTTAATCGATGAATTCG-AGCTCG-3′ [corresponding to nucleotides +205 to +249 of the Su k gene (*ATP19*) and 19 nucleotides of the MCS of pFA6a-HIS3MX6 from the 3′ flanking region of the *HIS3* gene].

The resulting PCR products were transformed into the haploid yeast strain W303-1A (Rothstein and Sherman, 1980) using the lithium acetate method (Gietz *et al*., 1992) and *HIS3*-positive clones were selected. Correct integration of the *HIS3* marker into the Su e/Tim11 (*ATP21*), Su g (*ATP20*) and Su k gene (ATP19) loci were confirmed by PCR using oligonucleotides which primed upstream and downstream of the respective disrupted genes.

Mitochondria were isolated from the resulting yeast strains Δ*su g*, Δ*su k* and Δ*tim11*, and the corresponding wild-type yeast, W303-1A which had been grown in YPGal medium supplemented with 0.5% lactate at 30°C (Herrmann *et al*., 1994).

Isolation of mitochondrial membranes for BN–PAGE

Yeast cells were harvested by centrifugation at 1800 *g* and washed three times with sucrose buffer (250 mM sucrose, 5 mM 6-aminohexanoic acid and 10 mM Tris–HCl pH 7.0). Five grams of sedimented cells, 5 ml of glass beads (0.25–0.5 mm) and 5 ml of sucrose buffer were vortexed for 10 min in a 50 ml tube. After dilution with 10 ml sucrose buffer, the sedimented glass beads were removed and the supernatant was centrifuged for 20 min at 1250 *g*. Mitochondrial membranes were collected by 30 min centrifugation at 18 000 *g* and stored in sucrose buffer at –80°C.

Isolation of F_1F_0-ATPase

Dimeric and monomeric forms of F_1F_0-ATPase were isolated by BN–PAGE (Schägger *et al*., 1994) with the following modifications: mitochondrial membranes (200 μg protein for the analytical scale) were sedimented by 10 min centrifugation at 100 000 *g*. The pellet was resuspended in 20 μl 50 mM NaCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA, 50 mM imidazole-HCl pH 7.0, 5 mM PMSF. Solubilization of the F_1F_0-ATP synthase was achieved by the addition of Triton X-100 [6 μl from a 2% (w/v) stock solution] and incubated on ice for 20 min. After centrifugation at 100 000 *g* the supernatant was supplemented with 1 μl Coomassie-dye suspension (5% Serva Blue G in 750 mM 6-aminohexanoic acid) and immediately applied to an acrylamide gradient gel (linear 4–13% gradient, overlaid with a 4% sample gel). Two-dimensional resolution of the lanes from the BN–PAGE was performed using Tricine-SDS–PAGE, as described previously (Schägger *et al*., 1987), except that 14% T, 6% C gels (containing 6 M urea) were used, where 'T' stands for the total concentration of acrylamide and cross-linker and 'C' for the percentage of cross-linker relative to T.

Solubilization in digitonin was achieved by resuspending the mitochondrial pellet in 20 μl of 30 mM HEPES pH 7.4, 150 mM K-acetate, 10% (w/v) glycerol, 1 mM PMSF, 1% (w/v) digitonin and incubated on ice for 30 min. The samples were subjected to a clarifying spin and further treated for BN–PAGE as described above.

Isolation of subunits and partial protein sequencing

The above protocol for solubilization of the F_1F_0-ATPase by Triton X-100 was scaled up 40-fold and an acrylamide gradient gel with the dimensions 0.3×14×14 cm was used for the preparative BN–PAGE. The blue bands of the dimeric and monomeric F_1F_0-ATPase visible following BN–PAGE were excised from the preparative gel and cut into four pieces. These pieces were loaded as a stack of four slices onto a Tricine-SDS gel. Following electrophoresis, the proteins were blotted onto Immobilon P (Schägger, 1994) and sequenced directly using a 473A protein sequencer (Applied Biosystems) or after incubation in a 1:1 (v/v) mixture of trifluoroacetic acid and methanol (24 h at 37°C for deformylation; 57 h at 37°C for partial deacylation) (Gheorghe *et al*., 1997).

Antibody production

Antisera against the C-terminal region of both Su g and Su k were raised in rabbits against chemically synthesized peptides (CIGRRKLVGYKHH) (Su g) and (CENYLKKHSDKQDA) (Su k) which had been coupled to activated ovalbumin (Pierce).

Miscellaneous

Hypotonic swelling and carbonate extraction of mitochondria were performed as described previously (Pfanner *et al*., 1988; Fölsch *et al*., 1996). Protein determination and SDS–PAGE were performed according to the published methods of Bradford (1976) and Laemmli (1970), respectively.

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