J Bioenerg Biomembr (2008) 40:257–267
DOI 10.1007/s10863-008-9183-5

Functional and stoichiometric analysis of subunit e in bovine heart mitochondrial F_0F_1ATP synthase

Elena Bisetto · Paola Picotti · Valentina Giorgio · Vera Alverdi · Irene Mavelli · Giovanna Lippe

Received: 1 August 2008 / Accepted: 16 September 2008 / Published online: 29 October 2008

Abstract The role of the integral inner membrane subunit e in self-association of F_0F_1ATP synthase from bovine heart mitochondria was analyzed by *in situ* limited proteolysis, blue native PAGE/iterative SDS-PAGE, and LC-MS/MS. Selective degradation of subunit e, without disrupting membrane integrity or ATPase capacity, altered the oligomeric distribution of F_0F_1ATP synthase, by eliminating oligomers and reducing dimers in favor of monomers. The stoichiometry of subunit e was determined by a quantitative MS-based proteomics approach, using synthetic isotope-labelled reference peptides IAQL*EEVK, VYGVGSL*ALYEK, and ELAEAQEDTIL*K to quantify the b, γ and e subunits, respectively. Accuracy of the method was demonstrated by confirming the 1:1 stoichiometry of subunits γ and b. Altogether, the results indicate that the integrity of a unique copy of subunit e is essential for self-association of mammalian F_0F_1ATP synthase.

Keywords Mammalian F_0F_1ATP synthase · Self-association · Subunit e stoichiometry · LC-MS/MS · AQUA peptides

Elena Bisetto and Paola Picotti contributed equally to this work.

E. Bisetto · P. Picotti · V. Giorgio · V. Alverdi · I. Mavelli · G. Lippe (✉)
Department of Biomedical Sciences and Technologies and M.A.T.I. Centre of Excellence, University of Udine,
Piazzale Kolbe 4,
I-33100 Udine, Italy
e-mail: glippe@makek.dstb.uniud.it

Present address:
P. Picotti
Institute of Molecular Systems Biology,
ETH Zuerich, Wolfgang-Pauli Strasse 16,
CH-8093 Zuerich, Switzerland

Abbreviations

PMF	proton motive force
BN-PAGE	blue native polyacrylamide gel electrophoresis
HBHM	heavy bovine heart mitochondria
AQUA	Absolute Quantification
Vm	F_0F_1ATP synthase monomer
Vd	F_0F_1ATP synthase dimer
Vo	F_0F_1ATP synthase oligomer

Introduction

F_0F_1ATP synthase (complex V) is a rotating nanomotor in prokaryotic and eukaryotic cells that uses energy from the proton motive force (PMF) generated by electron transfer to catalyze the synthesis of ATP. The enzyme consists of a catalytic domain F_1, which lies outside the membrane, and a membrane domain F_0 linked by central and peripheral stalks (Rubinstein et al. 2003). Synthesis of ATP depends upon rotation of the central stalk and a ring of c-subunits buried in F_0, which is driven by the passage of protons via channels located at the interfaces between c-ring and subunit a within F_0 (Adachi et al. 2007; Senior 2007).

The bovine enzyme contains 15 subunits, which are conserved from yeast (Wittig and Schagger 2008). These are the F_1 subunits α–ε and the F_0 subunits a–g, OSCP, A6L (homologous to yeast subunit 8) and F6 (homologous to yeast subunit h). Other subunits are species specific, namely coupling factor B (Belogrudov 2008) and two novel hydrophobic proteins, named MLQ and AGP (Meyer et al. 2007). In addition, the mitochondrial complex can associate with the inhibitor protein IF_1, which reversibly binds to F_1 with a 1:1 stoichiometry at low pH and PMF and fully inhibits the enzyme activity (Di Pancrazio et al. 2004).

High-resolution structures of the F_1 domain (Chen et al. 2006; Bowler et al 2007) and of the peripheral stalk (Dickson et al. 2006) established that in F_1 single copies of subunits γ, δ, ε form the central stalk, which penetrates into a static assembly of three α subunits arranged in alternation with three β subunits and makes extensive contacts with the c-ring. The catalytic sites are located at the interfaces between α and β subunits, mainly on β subunits. In the F_0 sector single copies of subunits b, d, F6 and OSCP form the peripheral stalk which extends from the top of the $\alpha_3\beta_3$ subcomplex along its external surface down into the membrane domain. This stalk is thought to act as a stator that counters the tendency of $\alpha_3\beta_3$ subcomplex to rotate with the central stalk. The so-called minor subunits e, f, g and A6L all span the membrane, but their roles as well as their exact stoichiometries are poorly defined and outline structures are still lacking (Collinson et al. 1994).

In addition to this complex multisubunit structure, it is now widely accepted that ATP synthase from mitochondria is present in the membrane in dimers associated to form long rows of oligomers (Strauss et al. 2008). These oligomers appear to play critical roles in determining cristae morphology (Paumard et al. 2002). In addition, a recent numerical simulation indicates that such supramolecular assembly may favor effective ATP synthesis under proton-limited conditions (Strauss et al. 2008).

The structural properties of dimers/oligomers are best characterized in yeast (Arnold et al. 1998; Thomas et al. 2008). Preferential in-membrane interactions of two ATP synthase monomers became evident from electron microscopic studies (Dudkina et al. 2006), although the contact areas between the individual units have not yet been clearly identified. Self-association of monomeric ATP synthase is independent from IF_1 (Dienhart et al. 2002) and probably occurs through at least six proteins, namely subunits a and b, the so-called dimer-specific subunits g and e, subunit h and the species specific subunit i (Fronzes et al. 2006; Wittig et al. 2008). Of these, subunit e appears to be critical, since the modulation in its amount resulted in modulation of dimer/oligomer stability and mitochondrial morphology (Arselin et al. 2004). In addition, subunit e, together with subunit g and b seems to mediate oligomer formation (Arselin et al. 2003; Bustos and Velours 2005; Fronzes et al. 2006).

Regarding mammalian ATP synthase, electron microscopic analyses evidenced a more complex situation with respect to yeast, as a study showed that dimerization interface includes only the membranous F_0 domain (Strauss et al. 2008), as it is in yeast, while another study reported that both F_0 and F_1/lateral stalk are involved (Minauro-Sanmiguel et al. 2005). In addition, the role of IF_1 in dimerization remains controversial. In fact, exogenous IF_1 promoted the formation of dimeric ATP synthase in different mammalian tissues (Garcìa et al. 2006) and IF_1 overexpression increased ATP synthase dimers in HeLa cells (Campanella et al. 2008), but physical releasing of IF_1 from the inner mitochondrial membrane did not alter the amount of dimers extracted by mild detergent (Tomasetig et al. 2002). Moreover, the subunits involved in oligomer formation are not known.

Since bovine and yeast subunit e are highly similar (Arnold et al. 1997), this work investigated the role of subunit e of the F_0 sector in enzyme self-association in bovine heart mitochondria. Bovine subunit e is an 8.2 kDa protein with an extramembranal C-terminal that protrudes into the intermembrane space; in mitoplasts (mitochondria devoid of outer membranes which maintain the original polarity of inner membranes), this subunit can be selectively degraded by treatment with low concentrations of proteases without affecting membrane integrity (Belogrudov et al. 1996). We performed *in situ* limited proteolysis of subunit e in mitoplasts and analyzed the supramolecular organization of ATP synthase using the one-step mild detergent extraction of mitochondrial membranes followed by blue native PAGE (BN-PAGE), which separates the different oligomeric states into clearly identifiable bands (Wittig et al. 2006).

There are conflicting results regarding the stoichiometry of subunit e in ATP synthase, but without a precise stoichiometry it is not possible to clarify the role of this subunit in dimers/oligomer formation. Biochemical analyses of purified bovine heart enzyme revealed an apparent stoichiometry of one (Walker et al. 1991), while quantitative immunoblotting of purified and membrane-bound complex from rat liver indicated a stoichiometry of two (Arakaki et al. 2001). In addition, the observations that subunit e expression level is modulated in response to physiological changes or stresses and that a highly homologous protein exists in the nucleus suggest that subunit e may play multifunctional regulatory roles in mammalian cells (Hong and Pedersen 2003). Therefore, we defined the stoichiometry of subunit e in ATP synthase detergent extracts by an MS-based quantitative proteomics approach, which relies on the use of synthetic isotopically labelled reference peptides (Gerber et al. 2003; Barnidge et al. 2003; Gerber et al. 2007). The technique was used to derive the molar ratio of subunit e to two reference subunits (b from F_0 and γ from F_1) of the ATP synthase. Altogether, the results indicate that a unique copy of subunit e functions in the self-association of the ATP synthase in mammalian mitochondria.

Methods

Chemicals

Porcine trypsin, modified, sequencing grade, was purchased from Promega (Madison, WI, USA) and was used for MS

analyses. Chymotrypsin from bovine pancreas was from Sigma. Tris(2-carboxyethyl)phosphine (TCEP) and iodoacetamide were purchased from Fluka (Buchs, Switzerland).

Mitochondrial preparations

Heavy bovine heart mitochondria (HBHM) were isolated as previously described (Ferguson et al. 1977). The final mitochondrial pellet was stored at −80°C and thawed no more than once. Mitoplasts were prepared by incubation of HBHM on ice with digitonin (0.18mg/mg protein) followed by two centrifugations at 35,000 × *g* for 15min at 4°C (Gallet et al. 1999). The quality (membrane integrity) of mitoplasts was judged to be good from the activity of malate dehydrogenase (matrix enzyme), which was 83% of that in HBHM (Gallet et al. 1999).

For *in situ* limited proteolysis, mitoplasts were resuspended to 3 mg/ml in 270 mM sucrose, 10 mM Tris-HCl pH 7.3. Different concentrations of trypsin or chymotrypsin, ranging from 1 to 10 μg/mg protein, were added and incubated for 10 min, 20 min and 30 min at 37°C. Proteolysis was stopped by adding 4mM PMSF and placing samples on ice. Conditions were sought that resulted in maintaining either membrane integrity (malate dehydrogenase assay) or enzyme function (in-solution ATPase capacity assay). Cleavage of subunit e was monitored by separation of Triton X-100-solubilized membranes on 1D BN-PAGE/2D SDS-PAGE followed by LC-MS/MS analysis (see below).

Gel electrophoretic analyses

Mitoplasts (control and protease-treated) and HBHM were solubilized with Triton X-100 at concentrations ranging from 0.8 to 2.5 mg/mg protein. Samples were mixed gently and immediately centrifuged at 100,000 × *g* for 25 min at 4°C. Supernatants were supplemented with Coomassie blue G-250 (Serva), and applied to a first-dimensional 5–11% polyacrylamide native gel (1D BN-PAGE) to separate the different oligomeric forms of ATP synthase as described earlier (Bisetto et al. 2007). Triton X-100 at 1.2 mg/mg protein was chosen as the best concentration for solubilizing the proteins while permitting visualization of monomer (Vm), dimer (Vd) and higher oligomeric forms (Vo) on in-gel activity staining; this concentration was then used for further work. Additional 1D BN-PAGE gels with samples extracted with Triton X-100 at 1.2 mg/mg protein were stained with Coomassie blue (3 h, room temperature) or were used to obtain samples for 2D SDS-PAGE. Coomassie blue-stained gels were quantified by densitometry using ImageQuant software, version 2003.03 (Amersham). The apparent mass of the higher oligomeric form Vo was estimated from a calibration curve obtained by plotting the migration distances of the monomeric forms of complexes I (900 kDa) and V (Vm; 600 kDa) and the dimeric forms of complexes III (480 kDa) and V (Vd; 1200 kDa) of the oxidative phosphorylation system versus their known molecular masses, as described (Wittig and Schagger 2005).

In-gel ATPase activity assay was run via histochemical staining, as previously described (Zerbetto et al. 1997) and recently optimized by us (Bisetto et al. 2007). Briefly, gels were incubated at room temperature in 35 mM Tris-HCl pH 7.4, 270 mM glycine, 14 mM $MgSO_4$. ATPase activity was visualized by adding 0.2% (*w/v*) $Pb(NO_3)_2$ and 8.0 mM ATP.

Bands corresponding to Vm, Vd and Vo were excised from 1D BN-PAGE and subjected to 2D resolution by 17% glycine-SDS-PAGE (2D SDS-PAGE) as described earlier (Di Pancrazio et al. 2006). Proteins in 2D SDS-PAGE were stained with colloidal Coomassie blue (for subsequent LC-MS/MS analyses of subunit e) or with silver, or were transferred to nitrocellulose membranes using Mini Trans-Blot (Bio-Rad). Western blotting was performed with polyclonal anti-αβ F_1 subunit antibody (1:5000) or monoclonal anti-IF_1 antibody (1:2000) in phosphate-buffered saline (PBS) containing 5% (*w/v*) non-fat dry milk at room temperature for 4 h. Immunoreactive bands were detected by enhanced chemiluminescence (Pierce). IF_1 content of Vm, Vd and Vo was expressed as the IF_1/F_1 ratio (Tomasetig et al. 2002; Di Pancrazio et al. 2004).

Sample preparation for MS analysis

For recognition of subunit e in 2D SDS-PAGE patterns, the bands stained with Coomassie Colloidal and having the electrophoretic behavior typical of subunit e were excised and subjected to *in situ* trypsin digestion as described elsewhere (de Souza et al. 2006). Peptide mixtures were cleaned using C18 ZipTips (Millipore, Billerica, MA, USA), eluted with 80% acetonitrile, evaporated on a vacuum centrifuge to dryness and resolubilized in 0.1% formic acid for LC-MS/MS analysis.

For determination of subunit e stoichiometry, HBHM were solubilized with Triton X-100 (1.2 mg/mg protein). To remove salts and detergent, Triton extracts were precipitated overnight at −20°C with 4 volumes acetone and centrifuged at 17,000 × *g* for 30 min at 4°C. Pellets were washed with 20% (*v/v*) methanol and were resolubilized, at a final protein concentration of 2 mg/ml, in 0.1 M ammonium bicarbonate, 8 M urea, pH 8.0. Disulfide bonds were reduced with 10 mM TCEP for 30 min at 25°C and the resulting free thiols were alkylated with 20 mM iodoacetamide for 45 min at 25°C. The solution was diluted to 1 M urea with 0.1 M ammonium bicarbonate and the pH was adjusted to 8.0. Samples were digested overnight with trypsin (enzyme:substrate ratio, 1:50) at 37° C. Tryptic digestion was stopped by acidification with formic acid to pH 3.0. Peptide mixtures were cleaned on OASIS HLB

cartridges (Waters, Milford, MA, USA) and eluted with 60% acetonitrile. Cleaned peptide samples were evaporated on a vacuum centrifuge to dryness, resolubilized in 0.1% formic acid and immediately analyzed by LC-MS/MS.

Mass spectrometry analysis

LC-MS/MS analyses were performed on a QSTAR hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with an electrospray ion source. The system also included an Agilent 1100 Series Capillary LC pump (Waldbronn, Germany) and a 150 × 0.5 mm column packed with C18-resin (Phenomenex Jupiter, 5μm particle diameter). Peptides were loaded from a cooled (4°C) Agilent autosampler and separated with a linear gradient of acetonitrile/water (from 5 to 50% acetonitrile in 70 min) containing 0.1% formic acid, at a flow rate of 10 μl/min.

The QSTAR mass spectrometer was operated in the positive-ion mode, using data-dependent acquisition methods, initiated by a 1-s survey MS scan, followed by MS/MS scans on the two highest intensity ions detected in the survey scan. Charge state screening was employed, allowing fragmentation of singly and multiply charged ions, and rejecting ions of unknown charge state. For both MS and MS/MS experiments, two microscans were combined per spectrum. The intensity threshold for triggering an MS/MS attempt was set to 25 ion counts. Ions were dynamically excluded from MS/MS attempts for 120 s after two scans. Acquisition time for tandem MS data was set to 1 s for the highest and 1.2 s for the second highest intensity ion. Quantitation experiments were performed in LC-MS mode (avoiding tandem MS analysis) in order to improve the duty cycle and increase the number of data points collected over time. MS/MS data were searched against a concatenated target (forward orientation) and decoy (reversed orientation) database (Elias and Gygi 2007) of bovine SwissProt/TrEMBL entries (version August 2007) using Mascot (version 2.0, MatrixScience). Precursor and fragment ion tolerances were set to 0.5 Da. Data were searched allowing oxidation of methionine as a variable modification (+15.9949 Da) and carboxyamidomethylation of cysteine residues as a static modification (+57.0215 Da). One internal missed cleavage and one non-tryptic terminus were allowed. A target/decoy database approach was used to estimate false positive rates through distraction of random hits and to establish threshold criteria (Mascot ion score) (Elias and Gygi 2007).

Design of heavy-labelled peptides and quantitative MS-analyses

Among the peptides identified by LC-MS/MS, one peptide each was selected to represent ATP synthase subunits e, b and γ. Candidate peptides were unique to the proteins of interest and contained no chemically unstable residues (M, W, C or N-terminal Q or N) or unstable peptide bonds (e.g. D-P). Among the candidate peptides, one was selected with high ionization efficiency, a good fragmentation pattern with reliable matching of b- and y- ion series, and a length between 7 and 15 amino acid residues. Isotopically labelled synthetic versions of the three selected peptides were custom ordered from Thermo Scientific (AQUA peptides, Thermo Scientific, San Jose, USA). In each peptide a leucine residue was substituted with a heavy leucine containing six ^{13}C and one ^{15}N, for an overall mass shift of +7Da. Labelled peptides were quantified by amino acid analysis. They were also used to optimize the MS parameters such as collision energy and declustering potential. In particular, analytical MS optimization was achieved by direct infusion of the synthetic peptides, at a flow rate of 10 μl/min, using a pump equipped with a 250 μl glass syringe (Hamilton, Bonaduz, Switzerland).

For determination of subunit e stoichiometry, known amounts of each heavy-labelled peptide were added to a freshly prepared Triton extract of HBHM (0.1–0.5 pmol peptide/μg protein). The samples were digested with trypsin as previously described and completeness of digestion was confirmed by SDS-PAGE (12% polyacrylamide Tris-glycine gel, silver-stained). The peptide mixture was analyzed by LC-MS. Peak areas for the labelled and endogenous peptides were calculated from the corresponding extracted ion chromatograms using Analyst QS 1.1 software (Applied Biosystems). Absolute molar amounts of the protein subunits were obtained by multiplying the ratios of the endogenous to labelled peptide peak areas by the amount of labelled peptide added. The ratios between absolute amounts of subunits b and γ, subunits b and e, and subunits γ and e were determined to validate the method (b:γ ratio, 1) and to determine the stoichiometry of subunit e (in relation to subunits b and γ).

Biochemical assays

Protein concentrations of mitochondrial preparations and Triton extracts were determined by the Lowry and Bradford methods, respectively. Malate dehydrogenase was assayed as reported earlier (Gallet et al. 1999). In-solution maximal ATP hydrolysis rate (ATPase capacity) of sonicated mitoplasts was determined spectrophotometrically at 340 nm and at 37°C using an ATP regenerating system at pH 7.4 in the presence or absence of 4 μM oligomycin or 4 μM DCCD (Di Pancrazio et al. 2004).

Results

The supramolecular organization of bovine ATP synthase was analyzed by 1D BN-PAGE, followed by 2D SDS-

PAGE to characterize the subunit composition of the different native complexes. Figure 1a shows a typical Coomassie and in-gel activity staining profile of Triton extracts from HBHM and mitoplasts, separated on 1D BN-PAGE. In both lanes three major bands were evident, the first of which corresponded to monomer (Vm), the second corresponded to dimer (Vd) and was a doublet, as already observed (Paumard et al. 2002); the third band was positioned close to the start of the gel, indicating that it was a higher oligomeric state of ATP synthase (Vo). As Vo had 3.7-times the apparent mass of the monomer, it was tentatively assigned as tetramer. 2D SDS-PAGE resolution of Vm, Vd and Vo bands from mitoplasts demonstrated all to contain the F_1 and F_0 sectors of ATP synthase (Fig. 1b). In particular, all forms contained subunit e, a faintly staining band positioned immediately below the δ subunit. Similar electrophoretic patterns on 2D SDS-PAGE were obtained for Triton extracts of HBHM (data not shown). The identity of subunit e was confirmed in all lanes by in-gel digestion and LC-MS/MS analyses; MASCOT database search revealed a 74% coverage of subunit e sequences (Table 1). These results indicate that, differently from yeast, this subunit remains bound to all oligomeric forms of bovine ATP synthase. Moreover, immunoblotting with anti-αβ F_1 subunits and anti-IF_1 antibodies (Fig. 1c) confirmed that monomers and dimers extracted from mitoplasts contained similar quantities of IF_1 in accordance with our previous results obtained in HBHM (Tomasetig et al. 2002; Di Pancrazio et al. 2004; Bisetto et al. 2007), and showed that IF_1 remains bound to oligomers after Triton extraction in comparable amounts.

Considering that subunit e contains an extramembranal C-terminal that can be degraded by proteases (Belogrudov et al. 1996), we followed the effects of subunit e degradation on enzyme self-association. Among the various proteolytic conditions tested, treatment with trypsin at 1 μg/mg protein gave the best results in terms of enzyme function maintenance and membrane integrity with selective

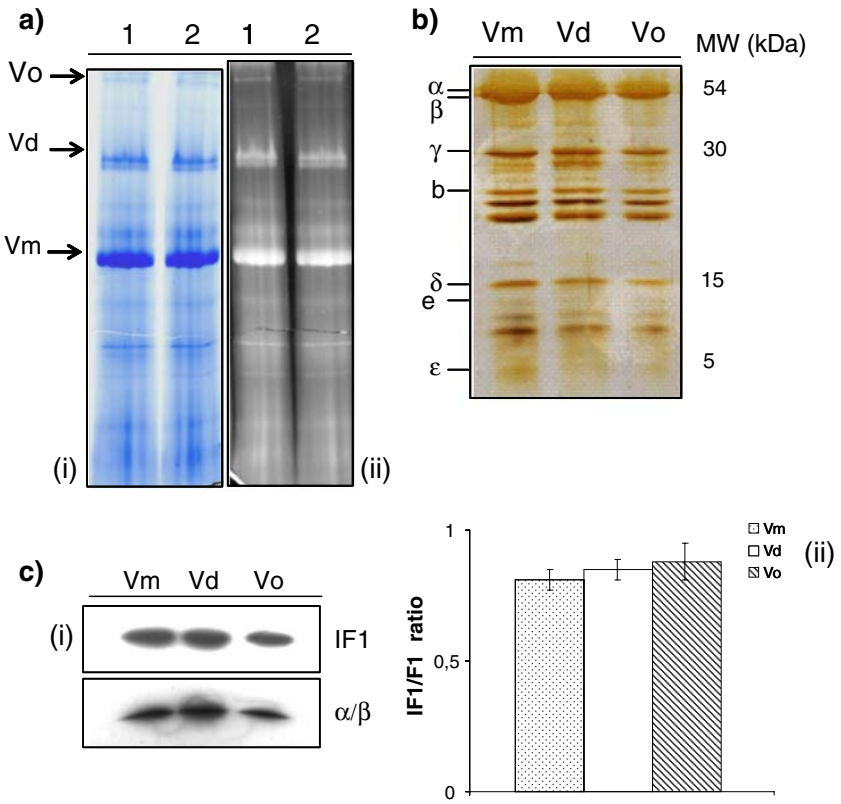

Fig. 1 Electrophoretic analyses of Triton X-100 extracts of heavy bovine heart mitochondria (HBHM) and mitoplasts. (**a**) Triton X-100 extract from HBHM (1) and mitoplasts (2) was subjected to 1D BN-PAGE and stained by Coomassie blue or by in-gel activity staining. Typical appearance of blue stained gel (i) and activity-stained gel (ii) obtained by loading 15 μg protein. The three forms of ATP synthase are indicated by arrows. Vm, ATP synthase monomers; Vd, ATP synthase dimers; Vo, ATP synthase higher oligomers. (**b**) Typical appearance of silver-stained 2D SDS-PAGE gel. The number of Vm, Vd and Vo bands excised from 1D BN-PAGE and loaded per well was adjusted to have similar amounts of Vm, Vd and Vo in 2D SDS-PAGE. The positions of F_1 sector subunit γ and of F_0 sector subunits b and e are indicated. (**c** i) Representative immunoblot after 2D SDS-PAGE performed with anti-αβ subunit antibody (diluted 1:2000 in PBS) and anti-IF_1 antibody (diluted 1:5000 in PBS). C ii) Quantative immunoblot showing the ratio of IF_1 to F_1, data are means ± SD (n=3)

Table 1 LC/MS/MS analysis of the SDS-PAGE band corresponding to the e subunit

Sample	$[M+H]^+$ m/z		Residues	Sequence peptide
	Calculate	Experimental		
e band	1175.73	1175.82	[1–11]	VPPVQVSPLIK
	1406.69	1406.74	[15–27]	YSALFLGMAYGAK
	952.51	952.81	[29–35]	YNYLKPR
	674.36	674.57	[43–48]	LAAEEK
	1756.92	1757.58	[56–70]	IERELAEAQEDTILK
	1359.50	1359.77	[59–70]	ELAEAQEDTILK

The table reports the calculate and experimental mass values and the fragments identified after data processing by Mascot search database corresponding to the e subunit

cleavage of subunit e. Specifically, there were insignificant changes in oligomycin and DCCD inhibition of ATPase capacity at 10 and 20 min of trypsin treatment, compared to controls, whereas at 30 min there was a significant loss of inhibitors sensitivity (Fig. 2a). Moreover, membrane integrity was maintained, as revealed by malate dehydrogenase activity (data not shown). After 20 min of trypsin treatment, subunit e was clearly observed to be degraded on 2D SDS-PAGE, as expected (Fig. 2c). No change in the electrophoretic profile of other ATP synthase subunits was evident. Regarding the oligomeric distribution of ATP synthase, trypsin treatment favored the monomeric state, with a corresponding marked decrease in oligomers and a smaller decrease in dimer abundance (Fig. 2b). Specifically, from the intensities of the Coomas-

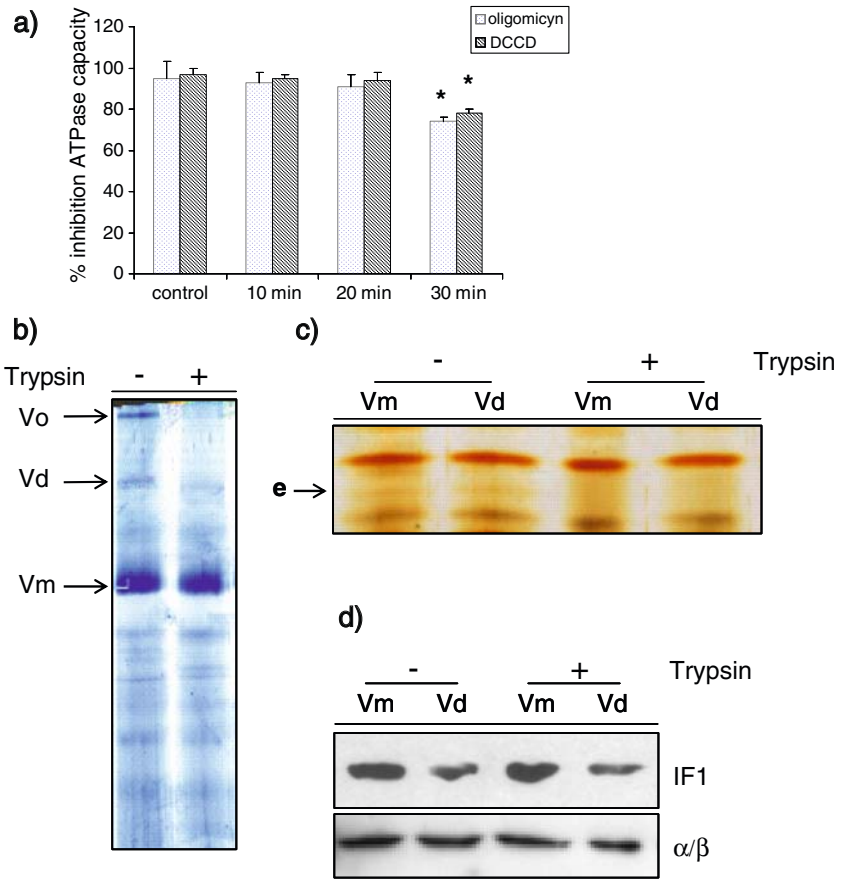

Fig. 2 *In situ* limited proteolysis of mitoplasts. Mitoplasts were treated with trypsin at 37°C for 10–30 min, analyzed for malate dehydrogenase activity as a marker of membrane integrity and for ATPase capacity (A) or extracted with Triton X-100 and subjected to 1D BN-PAGE (B) and 2D SDS-PAGE (C). (**a**) Data are means ± SD (n=3). * p<0.01, Student's t-test. (**b**) Typical appearance of 1D BN-PAGE stained with Coomassie Blue G-250 obtained by loading 15 μg Triton extract from mitoplasts incubated for 20 min in the presence or absence of 1 μg trypsin per milligram protein. (**c**) Silver-stained 2D SDS-PAGE . The number of Vm and Vd bands excised from 1D BN-PAGE and loaded per well was adjusted to ensure equivalent amounts of Vm and Vd in 2D SDS-PAGE**.** The arrow indicates the position of subunit e, whose identity was confirmed by in-gel digestion of control samples and t LC-MS/MS analysis (Table 1). The gel is representative of five independent experiments. (**d**) Representative immunoblot after 2D SDS-PAGE performed with anti-αβ subunit antibody (diluted 1:2000 in PBS) and anti-IF_1 antibody (diluted 1:5000 in PBS)

sie staining of 1D BN-PAGE, in control conditions Vo and Vd represented on average 18.3% (SD = 4.8%) and 20.1% (SD = 5.4%) of the total quantity of ATP synthase (n = 5), while after trypsin treatment Vo was essentially absent and Vd decreased to 12.0% (SD = 3.8%; n = 5). On 2D SDS-PAGE, the gel slices corresponding to the migration position of subunit e were excised and analyzed by LC-MS/MS, which confirmed the presence of subunit e only in trypsin-untreated controls samples as mentioned earlier (Table 1). These results suggest that Vm and Vd lack subunit e after limited tryptic proteolysis. Conversely, immunochemical calculation of the IF_1/F_1 ratios for Vm and Vd established that IF_1 was resistant to trypsin (IF_1/F_1 ratio for Vm, 0.75 and 0.80; for Vd, 0.78 and 0.83) (Fig. 2d). It was not possible to check if subunit e or IF_1 was still present in oligomers due to the low abundance of Vo on 1D BN-PAGE after trypsin digestion. Altogether, these results support the idea that subunit e is not essential for catalysis of bovine ATP synthase, as already postulated in yeast (Arnold et al. 1998), but that its integrity is necessary for oligomerization.

A critical point for elucidating the role of subunit e in ATP synthase self-association is to establish its stoichiometry. For this purpose, we used an MS-based quantitative proteomics approach known as the AQUA workflow. First, an ATP synthase Triton extract from HBHM was trypsinized in solution and the resulting peptide mixture was characterized by LC-MS/MS. Second, among the observed proteotryptic peptides, we chose three peptides–one each from subunits e, b and γ–and synthesized the corresponding isotopically labelled analogues; subunits b and γ were chosen as reference subunits because they are from the two sectors of ATP synthase and their stoichiometries are known. Third, the three synthetic peptides, added in known amounts to the HBHM Triton extracts, were used to quantify the corresponding endogenous peptides generated upon trypsinization and therefore to calculate the molar ratios between the subunits. Figure 3 shows the LC-MS/MS base peak chromatogram of a tryptic digest of Triton-extracted HBHM. The collected MS/MS spectra allowed identification of 13 subunits of ATP synthase, i.e. subunits α, β, γ, δ and ε of F_1, IF_1 and subunits a, b, d, e, f, F6, and OSCP of F_0 sector (Table 2). In addition to ATP synthase-specific proteins, several other proteins of mostly mitochondrial origin were identified (data not shown). Among the identified peptides from subunits b, γ and e of ATP synthase, one from each subunit that met specific selection criteria (Fig. 3b-d) was synthe-

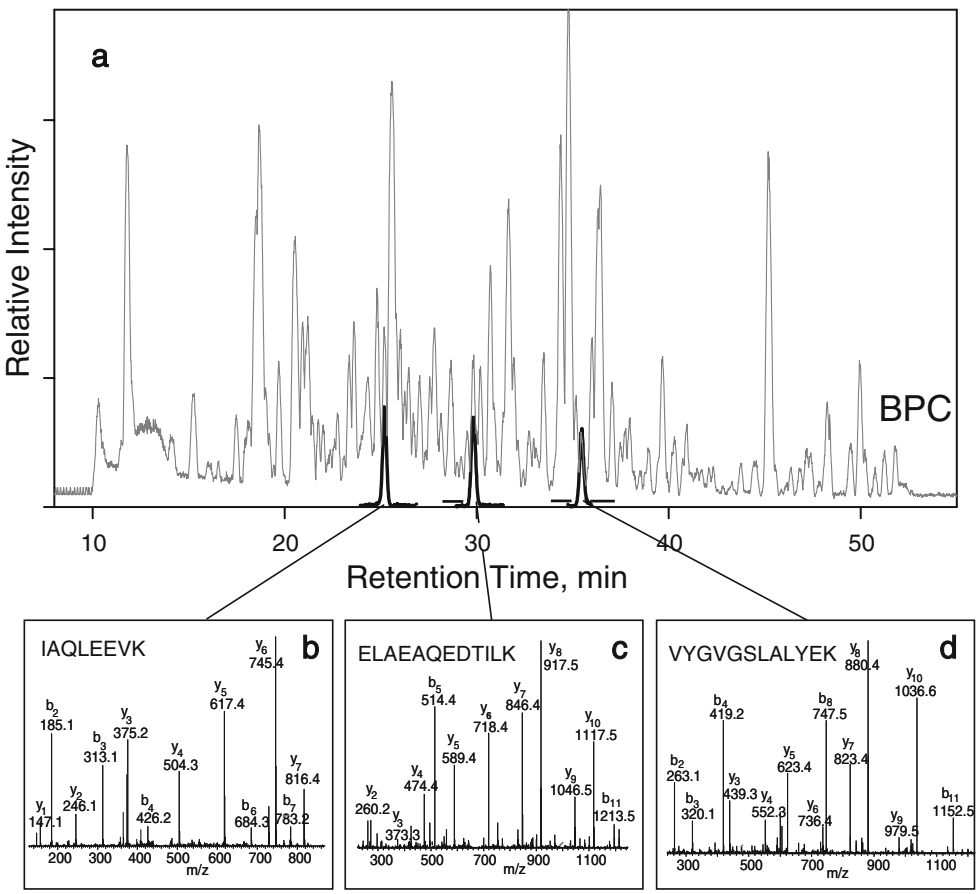

Fig. 3 LC-MS/MS analysis of a HBHM tryptic digest and selection of peptides for quantitative analyses. (**a**) Base peak chromatogram (BPC). Extracted ion chromatograms at m/z 465.26, 680.35 and 649.85, corresponding to peptides IAQLEEVK, ELAEAQEDTILK, and VYGVGSLALYEK from subunits b, e and γ, respectively, are shown with thick lines. (**b-d**) Tandem mass spectra that allowed identification of the three peptides

Table 2 Subunits of the ATP synthase complex for which tryptic peptides were identified in the HBHM Triton extract by LC-MS/MS. Accession numbers are according to the SwissProt/TrEMBL nomenclature

	Accession number	Protein	Coverage (%)
F_1	P19483	ATP synthase α subunit, heart isoform	76.7
	P00829	ATP synthase β subunit	87.7
	P05631	ATP synthase γ subunit	23.8
	P05630	ATP synthase δ subunit	23.3
	P05632	ATP synthase ε subunit	32.0
F_0	P13621	ATP synthase OSCP subunit	11.1
	P13619	ATP synthase b subunit	40.2
	P13620	ATP synthase d subunit	56.3
	P13618	ATP synthase f6 subunit	67.1
	Q28851	ATP synthase f subunit	27.6
	Q00361	ATP synthase e subunit	32.9
	P00847	ATP synthase a subunit	7.1
	P01096	ATPase inhibitor IF_1	31.0

sized in an isotopically labelled form. These were IAQ-L*EEVK, VYGVGSL*ALYEK, and ELAEAQEDTIL*K, where * indicates the heavy labelled amino acid, for subunits b, γ and e, respectively. After analytical optimization of the MS parameters with heavy peptides, a linear signal response in LC-MS mode was observed from 200 to 1nmol.

In quantitation experiments, known amounts of each synthetic peptide were added to the HBHM Triton extracts prior to tryptic digestion. Completeness of tryptic digestion, crucial for accurate protein quantitation using synthetic labelled peptides, was confirmed by SDS-PAGE (data not shown). The tryptic digest was analyzed by LC-MS and absolute quantitation of the three subunits was achieved by comparison of the AUC of the extracted ion chromatograms of the endogenous and labelled peptides. Overall, five instrumental, three technical (different extractions from the same HBHM preparation) and two biological (different HBHM preparations) replicates were performed. Mean absolute amounts of subunits e, γ and b in 1 μg HBHM Triton extract were 251 fmol, 263 fmol and 230 fmol. Average molar ratio for subunits b:γ was 1.03; since ATP synthase contains single copies of these two subunits, this result validated the quantitation method. Average molar ratios for subunits e:b and e:γ were 1.04 and 0.99, respectively. Coefficients of variation were <3% for instrumental replicates and <12% for technical and biological replicates (Table 3). Figure 4 shows a representative experiment in which equimolar amounts of the three labeled peptides were used. Altogether these data indicate that subunit e is present in bovine ATP synthase with a stoichiometry of one.

Discussion

This study documented that the selective tryptic degradation of subunit e in mitoplasts altered the distribution of the ATP synthase oligomeric forms in favour of monomers. In particular, degradation of subunit e reduced the abundance of dimers substantially and essentially eliminated the higher oligomeric form. This finding suggests that subunit e contributes to the enzyme's ability to maintain the dimeric and especially the higher oligomeric forms.

In addition, this study determined that ATP synthase contains a single copy of subunit e using an MS-based quantitative proteomics approach, i.e. AQUA workflow.

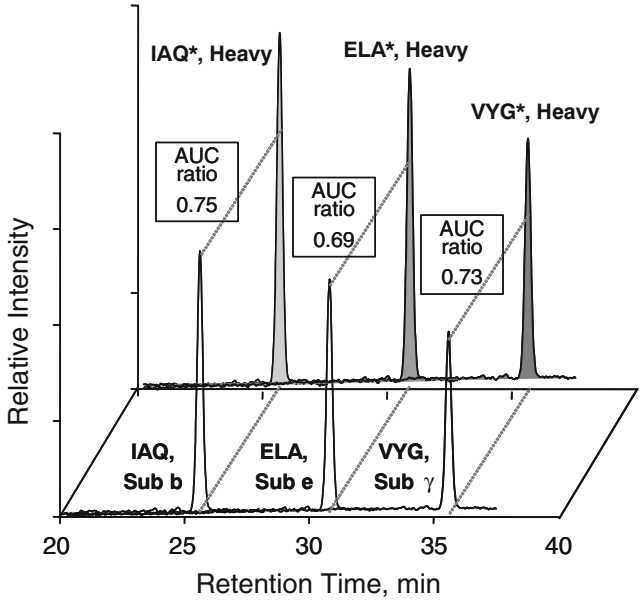

Fig. 4 Stoichiometry determination of ATP synthase subunits b, γ and e. An equimolar mixture of the three isotopically labelled peptides was added to the HBHM Triton extract prior to tryptic digestion and analysis by LC-MS/MS. Shown are the extracted ion chromatograms of the endogenous peptides IAQLEEVK (IAQ, m/z 465.26), ELAEAQEDTILK (ELA, m/z 680.35) and VYGVGSLALYEK (VYG, m/z 649.85) and of each of the coeluting heavy-labelled analogues (IAQ*, m/z 468.78; ELA*, m/z 683.86; and VYG*, m/z 653.36). The AUC ratio of each endogenous peptide to its corresponding AQUA peptide is indicated. Stoichiometry of the three protein subunits was derived by comparison of the three AUC ratios for the three corresponding peptides

This method allows for absolute quantitation of proteins of interest for which no standards are available. The approach is a variation of isotope dilution MS techniques used for decades for quantitation of small molecules. The technique has been successfully applied in the proteomics context to the absolute quantification of proteins in whole cell lysates (Gerber et al. 2003). Although compatible with 2D SDS-PAGE separation and in-gel digestion, in our application to determine subunit e stoichiometry the method was coupled to a gel-free, shotgun proteomics workflow, in order to avoid errors due to incomplete peptide extraction from the gel or impaired protein digestion within the gel matrix. This allowed the identification by LC-MS/MS of most of the known subunits of intra- and extramembranal parts of ATP synthase in the peptide mixture obtained after in-solution trypsinization of Triton extracts. The proteotypic synthetic peptides were added to the samples prior to tryptic digestion, in order to account for any factor modifying the peptide concentration, introduced during enzymatic digestion or peptide clean-up steps. This protocol allowed us to obtain low coefficients of variation for instrumental and technical replicates, confirming the validity of this method even for quantitation of integral membrane proteins like subunit e, in accordance with a previous report (Cheng et al. 2006). In addition, the two biological replicates gave the same stoichiometry of one, confirming the earlier result for bovine heart enzyme deduced by densitometric analysis of Coomassie-stained bands (Walker et al. 1991). These results contrast with that determined for rat liver enzyme (two subunits e per ATP synthase complex) by immunodetection, in absence of a statistical analysis (Arakaki et al. 2001). A possible explanation for these contrasting results is the tissue studied, especially considering that subunit e expression in liver is influenced by dietary conditions, stresses and carcinogens (Hong and Pedersen 2003).

The particular ability to study oligomeric ATP synthase on 1D BN-PAGE, in this study but not in our previous work (Tomasetig et al. 2002; Di Pancrazio et al. 2006; Bisetto et al. 2007) is attributed to the lower concentration of Triton X-100 used (1.2 vs. 2.5 mg/mg protein), since all other conditions were identical. In the present study, Vo + Vd comprised approximately 40% of total ATP synthase protein, whereas in previous work Vd alone represented approximately the same percentage of total protein (Tomasetig et al. 2002; Di Pancrazio et al. 2006). Thus, it is possible that the higher concentration of Triton X-100 destabilized Vo into Vd, because detergents in general destabilize protein complexes (Wittig et al. 2006). The concentration used in this study produced two types of dimers, as it was observed in yeast, which may correspond to the so-called "true-dimers" and "pseudo-dimers". These latter are thought to consist of two neighbor monomers

Table 3 Experimentally derived molar ratios between protein subunits

	Ratio sub e/γ			Ratio sub e/b			Ratio sub γ/b		
	Av	StDev	CV%	Av	StDev	CV%	Av	StDev	CV%
Instrumental	0.96	0.01	1.02	1.1	0.02	1.86	1.15	0.03	2.7
Technical	1.04	0.08	7.57	1.15	0.05	4.2	1.09	0.05	4.88
Biological	0.99	0.05	4.66	1.04	0.12	11.18	1.03	0.06	5.98

Values obtained from instrumental, technical (replicated extractions) and biological replicates are reported, as well as average (*Av*), standard deviation (*StDev*) and coefficient of variation (*CV%*) for each type of replicated analyses

from two broken adjacent true dimers in the oligomers (Dudkina et al. 2006; Thomas et al. 2008). Although speculative, the observation that the lower-mobility species is more susceptible to trypsin treatment suggests it may correspond to "pseudo-dimers".

Based on the present findings that ATP synthase contains one subunit e per monomer and that limited proteolysis of this subunit was more destabilizing to Vo than to Vd, we suggest that its prominent role is to stabilize dimer association into higher oligomers. These results are in accordance with the observations that, in yeast, oligomers are stabilized by e-e homodimers (Arselin et al. 2003) and that the GXXXG motif in the N-terminal membrane-embedded domain of subunit e, which mediates e-e homodimer association in yeast, is conserved in mammals (Arnold et al. 1997). In addition, the extramembranal C-terminal of mammalian subunit e protrudes into the intermembrane space (Belogrudov et al. 1996), as it does in yeast where it strengthens the oligomeric interaction (Everard-Gigot et al. 2005). Thus, both parts of subunit e may be involved in the interactions among dimers in the oligomers.

Since different experimental evidences support IF_1 involvement in ATP synthase dimer stabilization (Campanella et al. 2008), in this study the IF_1 content before and after trypsin treatment was determined. In addition, as a single particle electron microscopy analysis showed that, in bovine heart, monomeric ATP synthase formed dimers not only through F_0 membrane-embedded subunits, but also through extramembranal subunits, which might be of the peripheral stalks (Minauro-Sanmiguel et al. 2005), the trypsin effect on the integrity of the lateral stalks was evaluated by measuring the oligomycin or DCCD sensitivity of the mitoplasts' ATPase capacity. As trypsin treatment neither altered the IF_1 content in the residual monomers and dimers nor the enzyme inhibitors sensitivity, it is unlikely that IF_1 or lateral stalk subunits are involved in dimer destabilization. These findings rather suggest that this is related to subunit e degradation, supporting the idea that the dimeric and oligomeric interfaces are interdependent, as already observed in yeast.

In conclusion, based on results from biochemical and state-of-the-art MS-based proteomic methods, we propose that the unique copy of subunit e functions in the self-association of bovine heart F_0F_1ATP synthase to form oligomers. This appears to be a common mechanism in yeast and mammals, and is compatible with the electron microscopy images of the enzyme from both organisms.

Acknowledgements We thank Dr. Bruno Domon (Institute of Molecular Systems Biology, ETH Zuerich, Switzerland) for fruitful discussions and Prof. Gennaro Esposito (Department of Biomedical Sciences and Technologies, University of Udine, Italy) for useful comments on LC-MS/MS analyses. This work was supported by MIUR (PRIN 2007) and by the University of Udine. Valerie Matarese provided scientific editing.

References

Adachi K, Oiwa K, Nishizaka T, Furuike S, Noji H, Itoh H, Yoshida Y, Kinosita K (2007) Cell 130:309–321
Arakaki N, Ueyama Y, Hirose M, Himeda T, Shibata H, Futaki S, Kitagawa K, Higuti T (2001) Biochim. Biophys. Acta 1504:220–228
Arnold I, Bauer MF, Brunner M, Neupert W, Stuart RA (1997) FEBS Lett. 411:195–200
Arnold I, Pfeiffer K, Neupert W, Stuart RA, Schagger H (1998) EMBO J. 17:7170–7178
Arselin G, Giraud MF, Dautant A, Vaillier J, Brethes D, Coulary-Salin B, Schaeffer J, Velours J (2003) Eur. J. Biochem. 270:1875–1884
Arselin G, Vaillier J, Salin B, Schaeffer J, Giraud MF, Dautant A, Brèthes D, Velours J (2004) J Biol Chem. 279:40392–40399
Barnidge DR, Dratz EA, Martin T, Bonilla LE, Moran LB, Lindall A (2003) Anal. Chem. 75:445–451
Belogrudov GI (2008) Arch Biochem Biophys. 473:76–87
Belogrudov GI, Tomich JM, Hatefi Y (1996) J. Biol. Chem. 271:20340–20345
Bisetto E, Di Pancrazio F, Simula MP, Mavelli I, Lippe G (2007) Electrophoresis 28:3178–3185
Bowler MW, Montgomery MG, Braig K, Leslie AGW, Walker JE (2007) J. Biol. 282:14238–14342
Bustos D, Velours J (2005) J. Biol. Chem. 280:29004–29010
Campanella M, Casswell E, Chong S, Farah Z, Wieckowski MR, Abramov AY, Tinker A, Duchen MR (2008) Cell Metab. 8:13–25
Chen C, Safena AK, Simcoke WN, Garboczi DN, Pedersen PL, Ko YH (2006) J. Biol. Chem. 281:13777–13783
Cheng D, Hoogenraad CC, Rush J, Ramm E, Schlager MA, Duong DM, Xu P, Wijayawardana SR, Hanfelt J, Nakagawa T, Sheng M, Peng J (2006) Mol. Cell. Proteomics 5:1158–1170
Collinson IR, Runswick MJ, Buchanan SK, Fearnley IM, Skehel JM, van Raaij MJ, Griffiths DE, Walker EJ (1994) Biochemistry 33:7971–7978
de Souza GA, Godoy LM, Mann M (2006) Genome Biol. 7:R72
Di Pancrazio F, Mavelli I, Isola M, Losano G, Pagliaro P, Harris DA, Lippe G (2004) Biochim. Biophys. Acta 1659:52–62
Di Pancrazio F, Bisetto E, Alverdi V, Mavelli I, Esposito E, Lippe G (2006) Proteomics 6:921–926
Dickson VK, Silvester JA, Fearnley IM, Leslie AG, Walker JE (2006) EMBO J. 25:2911–2918
Dienhart M, Pfeiffer K, Schagger H, Stuart RA (2002) J. Biol. Chem. 277:39289–39295
Dudkina NV, Sunderhaus S, Braun HP, Boekema EJ (2006) FEBS Lett. 580:3427–3432
Elias JE, Gygi SP (2007) Nat Methods 4:207–214
Everard-Gigot V, Dunn CD, Dolan BM, Brunner S, Jensen RE, Stuart RA (2005) Eukaryot. Cell. 4:346–355
Ferguson SJ, Harris DA, Radda GK (1977) Biochem. J. 162:351–357
Fronzes R, Weinmann T, Vaillier J, Velours J, Brethes D (2006) Biochemistry 45:6715–6723
Gallet PF, Zachowski A, Julien R, Fellmann P, Devaux PF, Maftah A (1999) Biochim. Biophys. Acta 1418:61–70
Garcìa JJ, Morales-Rios E, Cortés-Hernandez P, Rodrìguez-Zavala JS (2006) Biochemistry 45:12695–12703
Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP (2003) Proc. Natl. Acad. Sci. USA 100:6940–6945
Gerber SA, Kettenbach AN, Rush J, Gygi SP (2007) Methods Mol. Biol. 359:71–86
Hong S, Pedersen PL (2003) Proteins 51:155–161
Meyer B, Wittig I, Trifilieff E, Karas M, Schagger H (2007) Mol Cell Proteomics 6:1690–1699
Minauro-Sanmiguel F, Wilkens S, García JJ (2005) Proc Natl Acad Sci U S A. 102:12356–12358

Paumard P, Vaillier J, Coulary B, Schaeffer J, Soubannier V, Mueller DM, Brethes D, di Rago JP, Velours J (2002) EMBO J. 21:221–230
Rubinstein JL, Walker JE, Henderson R (2003) EMBO J. 22:6182–6192
Senior A (2007) Cell 130:220–221
Strauss M, Hofhaus G, Schroder R, Kuhlbrandt W (2008) EMBO J. 27:1154–1160
Thomas D, Bron P, Weimann T, Dautant A, Giraud MF, Paumard P, Salin B, Cavalier A, Velours J, Brethes D (2008) Biol Cell
Tomasetig L, Di Pancrazio F, Harris DA, Mavelli I, Lippe G (2002) Biochim. Biophys. Acta 1556:133–141
Walker EJ, Lutter R, Dupuis A, Runswick MJ (1991) Biochemistry 30:5369–5378
Wittig I, Schagger H (2005) Proteomics 5:4338–4346
Wittig I, Schägger H (2008) Biochim Biophys Acta. 1777:592–598
Wittig I, Braun HP, Schägger H (2006) Nature Protocols 1:418–428
Wittig I, Velours J, Stuart R, Schägger H (2008) Mol Cell Proteomics 5:995–1004
Zerbetto E, Vergani L, Dabbeni-Sala F (1997) Electrophoresis 18:2059–2064