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In vitro and in vivo studies of F_0F_1ATP synthase regulation by inhibitor protein IF_1 in goat heart

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Abstract

A method has been developed to allow the level of F_0F_1ATP synthase capacity and the quantity of IF_1 bound to this enzyme be measured in single biopsy samples of goat heart. ATP synthase capacity was determined from the maximal mitochondrial ATP hydrolysis rate and IF_1 content was determined by detergent extraction followed by blue native gel electrophoresis, two-dimensional SDS-PAGE and immunoblotting with anti-IF_1 antibodies.

Anaesthetized open-chest goats were subjected to ischemic preconditioning and/or sudden increases of coronary blood flow (CBF) (reactive hyperemia). When hyperemia was induced before ischemic preconditioning, a steep increase in synthase capacity, followed by a deep decrease, was observed. In contrast, hyperemia did not affect synthase capacity when applied after ischemic preconditioning. Similar effects could be produced in vitro by treatment of heart biopsy samples with anoxia (down-regulation of the ATP synthase) or high-salt or high-pH buffers (up-regulation). We show that both in vitro and in vivo the same close inverse correlation exists between enzyme activity and IF_1 content, demonstrating that under all conditions tested the only significant modulator of the enzyme activity was IF_1. In addition, both in vivo and in vitro, 1.3–1.4 mol of IF_1 was predicted to fully inactivate 1 mol of synthase, thus excluding the existence of significant numbers of non-inhibitory binding sites for IF_1 in the F_0 sector.

Keywords: Inhibitor protein IF_1; Mitochondrial F_0F_1ATP synthase; Ischemic preconditioning; Goat heart; Reactive hyperemia; Blue native polyacrylamide electrophoresis

1. Introduction

The mitochondrial F_0F_1ATP synthase is a universal enzyme, which couples the transport of protons across the mitochondrial inner membrane to the synthesis of the majority of the cell's ATP. The enzyme is made up of two domains, the soluble sector F_1, containing the catalytic sites, and the membrane-spanning F_0, comprising the proton channel, linked by a central and a peripheral stalk (for a review of F_0F_1ATP synthase structure and mechanism, see Ref. [1]). However, the F_0F_1ATP synthase may also be a major consumer of ATP under ischemic conditions, when the electrochemical gradient across the inner membrane collapses and the enzyme switches its catalytic activity from ATP synthesis to ATP hydrolysis [2].

F_0F_1ATP synthase is controlled by different specific regulatory elements, among which the inhibitory protein IF_1 plays a prominent role. IF_1 is a basic protein with a

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significant degree of homology in various species [3]. Reversible binding of IF_1 to the catalytic sector F_1 in a 1:1 stoichiometry is sufficient to fully inhibit the enzyme [4]. IF_1 binds at the interface between α and β subunits [5], and prevents the release of adenine nucleotides during the functioning of either soluble or membrane-bound F_1 [6].

Inhibition of ATP synthase is reversible in vivo, inhibition being favored by a decreased electrical potential across the inner mitochondrial membrane, and by decreased matrix pH, conditions that occur under energy deficiency [7]. Therefore, IF_1 has been proposed to be responsible for the beneficial down-regulation of F_0F_1ATP synthase during ischemia in in vitro and in vivo experimental models, where it acts to reduce ATP depletion [8–11]. Up-regulation of F_0F_1ATP synthase has also been observed in intact rat cardiomyocytes [12,13] and in dog [9] in response to increased ATP demand after stimulation of contraction electrically or with positive inotropic agents, and this has been attributed to IF_1 release. In addition, several studies based on changes in enzyme activity [10,11,14] have suggested that IF_1 could mediate the inhibition of F_0F_1ATP synthase observed during ischemic preconditioning (IP) in rat. IP is defined as a protective effect of a short period of ischemia against cell damage upon subsequent long-lasting ischemia and reperfusion. However, contradictory results have been reported [15–17]. All these data suggest that the binding/release of IF_1 to F_0F_1ATP synthase complex may be a physiological regulator of the enzyme activity, but a direct measurement of IF_1 bound to F_0F_1ATP synthase in different metabolic conditions is still lacking.

The current study develops a methodological approach to correlate F_0F_1ATP synthase capacity with the direct quantification of IF_1 bound to the enzyme in heart, both in vitro and in vivo. The in vitro experiments were performed by subjecting goat heart biopsies to anoxia [8] or to treatments with high-salt or high-pH buffers, which are conditions known to up-regulate the synthase [9,11]. Anaesthetized open-chest goat heart was used as in vivo model. The animals were subjected to IP which resulted in synthase inhibition as reported by Vuorinen et al. [10] and Ylitalo et al. [11], and to sudden increases of the coronary blood flow (CBF) (reactive hyperemia), which induced an increase in synthase capacity. On a small tissue sample, we first measured spectrophotometrically the maximal ATP hydrolysis rate, which represents the fraction of active enzyme molecules, able to either synthesize or hydrolyze ATP depending on mitochondrial energy-state [13]. On the same sample, IF_1 bound to the synthase was measured directly using an electrophoretic approach. Here, the myocardial biopsies were treated with mild detergent and the extracts subjected to blue native gel electrophoresis, followed by two-dimensional SDS-PAGE and immunoblotting with anti-IF_1 antibodies. Over all conditions tested, we showed a close inverse correlation between F_0F_1ATP synthase capacity and IF_1 quantity bound to the enzyme, both in vitro and in vivo. Moreover, for the first time, we obtained direct evidence that IF_1 binding to F_0F_1ATP synthase is responsible for enzyme inhibition after ischemic preconditioning.

2. Materials and methods

2.1. Chemicals

Serva Blue G (Coomassie blue) was supplied by Serva, *n*-dodecylmaltoside by Boehringer, 6-aminocaproic acid by Fluka, acrylamide and bis-acrylamide by Bio-Rad. All other chemicals, dye and enzymes were supplied by Sigma-Aldrich.

2.2. Animal preparation

Goats of either sex weighing 45–60 kg were anesthetized with ketamine hydrochloride (15 mg/kg). Anesthesia was maintained by ventilating the animal with nitrous oxide–oxygen mixture (2:1) and a continuous infusion of ketamine hydrochloride (24 mg/kg/h). Fentanyl was injected hourly in a dose of 1 mg/kg. The chest was opened by left lateral thoracotomy and the heart was removed for in vitro manipulations, or suspended in a pericardial cradle for in vivo experiments. In this latter case, the proximal part of the left circumflex coronary artery was gently isolated and a flow-probe was placed around the artery to record CBF as specified in Ref. [18]. Distal to this flow-probe, a snare was placed around the same artery to perform occlusions. Aortic blood pressure and ECG were recorded as in Ref. [18].

2.3. Preparation of mitochondrial membranes and proteins

The removed heart was immediately processed to obtain goat heart mitochondria, essentially as in Ref. [19]. Submitochondrial particles (Mg-ATP SMP) and mitochondrial matrix fraction (MF) were prepared from goat heart mitochondria as in Refs. [20,21]. Briefly, mitochondria were suspended at 12.5 mg/ml in 0.25 M sucrose, 10 mM Tris/HCl pH 7.4, 1 mM ATP and 15 mM $MgCl_2$ at 4 °C, sonicated (4×30 s) (UP 50H Dr. Hielscher GmbH, Berlin, Germany) and immediately centrifuged at 39 000×*g* for 10 min. An aliquot of the supernatant, representing the total mitochondrial extract, was directly used, and the remainder was centrifuged at 150 000×*g* for 40 min at 4 °C. The supernatant, representing the MF, was conserved, while the pellet, containing the Mg-ATP SMP, was resuspended in the previous buffer and centrifuged again. Then it was suspended in 0.25 M sucrose, 10 mM Tris/HCl pH 7.4 and used.

F_1 from bovine heart was purified as described in Ref. [22].

IF_1 was purified from bovine heart mitochondria as in Refs. [4,23]. To isolate IF_1 from goat heart, mitochondria were heated to 100 °C for 2 min [24]. Most protein

denatures and precipitates, while IF_1 remains soluble. Then, the suspension was centrifuged at 39 000×*g* for 10 min at room temperature, and the supernatant was collected. It was reported [24] that this procedure allows a recovery greater than 90% of IF_1.

2.4. Experimental protocol of ischemic preconditioning and reactive hyperemia

Experimental maneuvers were commenced after an equilibrating perfusion period of 30 min, during which CBF was 59±3 ml/min. After this time, a biopsy was taken (baseline). Hyperemia was then induced by occluding the left circumflex coronary artery briefly (15 s), followed by release. Biopsies were taken at 15 s, 4 and 6 min after artery reopening. Total hyperemic flow (THF) was defined as the area between the CBF trace and the zero line over the duration of the response, which was defined as the time between the release of the occlusion and the instant the flow returned to no more than 5% from the baseline value [18]. The duration of the first reactive hyperemia was of 150±8 s and THF was 248±8 ml/min.

Ischemic preconditioning was produced by occluding the left circumflex artery for two periods of 2.5 min, separated from each other by 5 min of reperfusion. After an additional period of 10 min of reperfusion, a biopsy was taken to represent the preconditioned heart. IP did not affect CBF (53±3 ml/min). Where indicated, reactive hyperemia was induced (see above) after IP. In this case, the duration of the second reactive hyperemia decreased to 132±5 s and THF decreased to 190±7 ml/min. To assess the effects of IP per se, the second reactive hyperemia was not induced.

The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, DL 116, January 27, 1992, published in the Gazzetta Ufficiale della Repubblica Italiana, issue no. 40, Febrary 18, 1992, and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985). The experiments were also approved by the Ethical Committee of the University of Torino.

2.5. Tissue processing

To assay F_0F_1ATP synthase capacity and IF_1/F_1 ratio, the biopsies were washed in 20 mM HEPES, 1 mM $MgCl_2$, 2 mM EGTA, adjusted to pH 7.2 with NaOH (low-salt buffer) and then chopped in two pieces: one (about 3 mg) was immediately sonicated (3×20 s) in 250 μl of low-salt buffer, to completely expose the F_1 sector of F_0F_1ATP synthase to the solvent, and used for enzyme activity assay (maximal ATPase activity), while the other (about 10–15 mg) was frozen in liquid nitrogen and stored at −80 °C until use for electrophoretic analysis (IF_1/F_1 ratio). The low-salt buffer used [9,13] contained 2 mM EGTA, to minimize Ca^{2+}-dependent ATPase activity, and less than 5 mM Na^+, to minimize Na^+/K^+-dependent ATPase activity. Moreover, sonication was performed at neutral pH to minimize the dissociation of IF_1 from F_0F_1ATP synthase.

When treatments were performed in vitro, fragments of about 1–2 g of left ventricle were taken on, washed in the low-salt buffer, chopped in small fragments of about 30–40 mg and used immediately. Each fragment was cut in two or more pieces, one of which was considered as control while the other(s) was(were) differently treated. When more than two pieces were cut, the same control value was compared to the different treated samples. The fragments were made ischemic by placing it in a sealed Ziploc plastic bag immersed in a circulating water bath at 37 °C for 30 min as described in Ref. [8]. At the same time, control pieces were maintained under O_2 atmosphere. In both cases, the fragments were then chopped in smaller pieces and sonicated in the low-salt buffer at neutral pH as above. To induce a partial release of IF_1 from F_1, fragments were sonicated in the same buffer containing 150 mM KCl (high-salt buffer) [9,11], while almost complete release was obtained by sonicating in 0.25 M sucrose, 2 mM EDTA pH 9.2 with NH_4OH (high-pH buffer) [11]. After sonication, all samples were incubated in the respective buffer for 1 h at 37 °C before further analysis.

2.6. F_0F_1ATP synthase activity assay

The maximal ATP hydrolytic rate (ATP synthase capacity) was assayed spectrophotometrically at 340 nm and 37 °C using an ATP-regenerating system in sonicated samples diluted in the low-salt buffer (see above) [9,13]. Over 90% of the measured maximal ATPase activity was mitochondrial in origin, as judged by sensitivity to oligomycin. The activity was insensitive to uncoupler FCCP, showing that the mitochondria were broken and the F_0F_1ATP synthase was accessible. Moreover, less than 6% of the maximal ATPase activity was sensitive to vanadate or ouabain, showing only minor contributions from plasma membrane and reticular ATPases.

Protein concentration was determined according to Lowry [25].

2.7. Electrophoretic and immunochemical analysis

To selectively extract the oxidative phosphorylation complexes (OXPHOS), frozen samples were rapidly thawed and treated with *n*-dodecylmaltoside in 1 M 6-aminocaproic acid and 50 mM Bis-Tris/HCl pH 7.0 [26]. The in vitro samples were centrifuged at 150 000×*g* for 30 min at 4 °C prior to this treatment. All samples were subjected to one-dimensional blue native electrophoresis (1D BN-PAGE), as previously described in Ref. [26]. The band corresponding to F_0F_1ATP synthase complex was excised and subjected to electrophoresis in SDS (2D SDS-PAGE) using 17% acrylamide in the separating gel and 4% acrylamide in the stacking gel [27]. After electrophoresis, gels were stained

with Coomassie blue G (Serva Blue G) or blotted onto nitrocellulose sheets. Immunological detection of blotted proteins used polyclonal antibodies against α- and β-subunits of F_1, raised in rabbit against beef heart F_1 (generous gift of Prof. F. Dabbeni-Sala University of Padova, Italy), or polyclonal anti-IF_1 antibodies, raised in rabbits against the beef heart IF_1 [28]. For quantitation, F_1 purified from beef heart or IF_1 obtained from goat heart mitochondria was used as standard. The banding patterns were scanned using LKB Ultrascan XL laser densitometer.

Protein concentration of detergent solubilized proteins was determined according to Bradford [29].

2.8. Kinetic analysis and statistics

Dixon analysis of AS particles inhibition by bovine and goat IF_1 was performed by Prism 4.0 (Graphpad Software, USA).

The experimental data are reported as mean±S.D. Alteration of the maximal ATPase activity and IF_1/F_1 ratio in the in vitro experiments were tested by Student's *t* test for paired data. For multiple comparisons, (maximal ATPase activity and IF_1/F_1 ratio) throughout the in vivo experiments, inter-group differences were tested by two-way analysis of variance using the Scheffè multiple-comparison post-test analysis. The relationship between the maximal ATPase activity and IF_1/F_1 ratio was calculated by linear regression model using 20 and 33 pairs of measures for in vitro and in vivo experiments, respectively. The homogeneity of regression slopes was tested by fitting to a model using as measured parameters the maximal ATPase activity and IF_1/F_1 ratio in vitro/in vivo, together with an 'interaction term'. The interaction term provides the test of the null hypothesis of equal slopes. Statistical analyses were performed by Statistical Package for Social Sciences (SPSS). For all analyses, $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Measurement of free and membrane-bound IF_1 in goat heart mitochondria

IF_1 obtained from goat heart by heating the mitochondrial suspension at 100 °C [24] was compared to IF_1 purified from bovine heart mitochondria [4,23] by electrophoretic, immunological and functional analyses. SDS-PAGE (Fig. 1A) shows that goat heart IF_1 has the same electrophoretic mobility as bovine heart IF_1, indicating a similar molecular weight of around 10 kDa. However, IF_1 prepared from goat heart by the single step of heat extraction is only 70±1% pure (Fig. 1A). Attention has been paid to this fact when goat heart IF_1 is used below.

Fig. 1B shows that polyclonal antibodies raised against bovine heart IF_1 cross-react with goat heart IF_1 with roughly

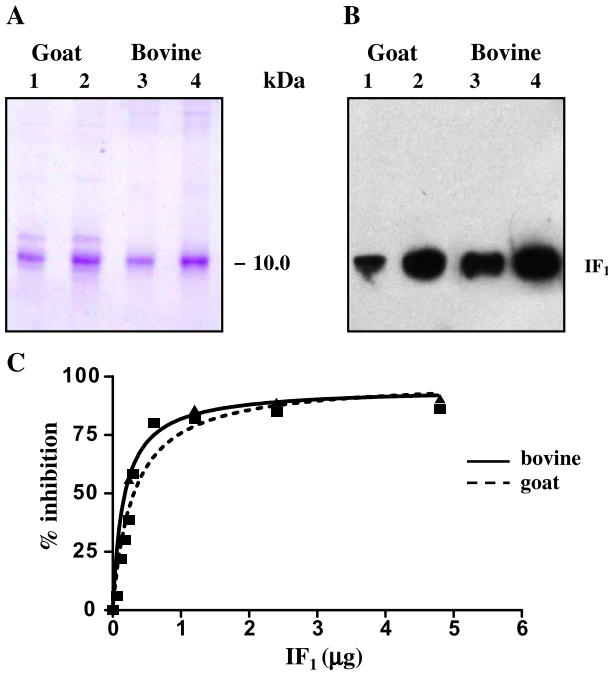

Fig. 1. Electrophoretic, immunological and functional comparison between goat and bovine heart IF_1. IF_1 from goat and bovine heart mitochondria were obtained as reported in Materials and methods. (A) IF_1 (2 and 4 μg) from goat (lanes 1, 2) or from bovine (lanes 3, 4) heart were loaded on SDS-PAGE and stained with Coomassie blue. Based on densitometric analyses of banding patterns, goat heart IF_1 was 70±1.4% pure, where S.D. refers to six different electrophoretic assays of the same IF_1 preparation. (B) IF_1 (0.25 and 0.5 μg) from goat (lanes 1, 2) or from bovine (lanes 3, 4) heart were loaded on SDS-PAGE and blotted onto nitrocellulose sheets for detection with polyclonal anti-IF_1 antibodies as described in Materials and methods. (C) AS particles prepared from bovine heart mitochondria as in Ref. [31] were incubated with different quantities of IF_1 from goat (■) or bovine (▲) heart for 10 min at 37 °C in the presence of 2 mM Mg-ATP. The maximal ATPase activity was measured as reported in Materials and methods. 100% ATP hydrolysis rate represents 8.6±0.5 U/mg. (For colour see online version).

equal sensitivity. The antibodies used, although not monoclonal, selectively bind to the C-terminal region of the bovine inhibitor [30], which is not involved in binding to F_1[5]. It is probable that the same region is recognized in goat heart IF_1, suggesting that the amino acid sequence is conserved in these two proteins. The same region is known to be different in rat [3], and, correspondingly, our antibodies failed to react with IF_1 from rat heart and liver (data not shown).

The functional efficiency of goat heart IF_1 has been tested using IF_1-depleted membranes from beef heart (AS particles) [31]. Fig. 1C shows that goat heart and bovine heart IF_1 inhibit the maximal ATPase activity of AS particles to the same degree. The inhibition was saturable for both proteins. Dixon analysis indicates that the dissociation constant K_d for the combination of goat heart IF_1 and bovine heart IF_1 with membrane bound F_1 is 1.9×10^{-8} and 1.7×10^{-8} M, respectively, the latter value being very similar to that reported by Gomez-Fernandez and Harris [4]. In addition, the inhibition induced by goat heart

IF_1 required ATPase turnover, and was promoted by low pH, just as was observed for bovine heart IF_1 (data not shown). These data strongly suggest that IF_1 from bovine and goat heart mitochondria possess very similar structural and functional properties.

Immunological assays were used to measure the total IF_1 and F_1 content of goat heart mitochondria and the distribution of IF_1 between the inner membrane and matrix fractions. Because of the very high homology between F_1 from different sources [32], polyclonal antibodies raised against bovine heart F_1 could be used to quantify goat heart F_1. Standard curves were drawn using IF_1 from goat heart or purified F_1 from beef heart. Fig. 2 shows that a linear relationship is obtained between the densitometric analysis of protein stain and the amount of either F_1 or IF_1 determined immunologically. According to these standard curves, the total (bound+free) IF_1 present in intact goat heart mitochondria is 8.9±0.9 μg/mg mitochondrial protein and the total F_1 is 134±19 μg/mg mitochondrial protein. Using Mr=380,000 for F_1 [32], goat F_1 is present at 0.35±0.05 nmol/mg mitochondrial protein, similar to the levels recorded in bovine heart mitochondria [19]. Using Mr=10,000 for IF_1 [3], this protein is present at 0.89±0.09 nmol/mg mitochondrial protein in goat heart. IF_1 therefore appears to occur at a 2.5-fold excess over F_1 (IF_1/F_1 ratio=2.5) in intact goat mitochondria.

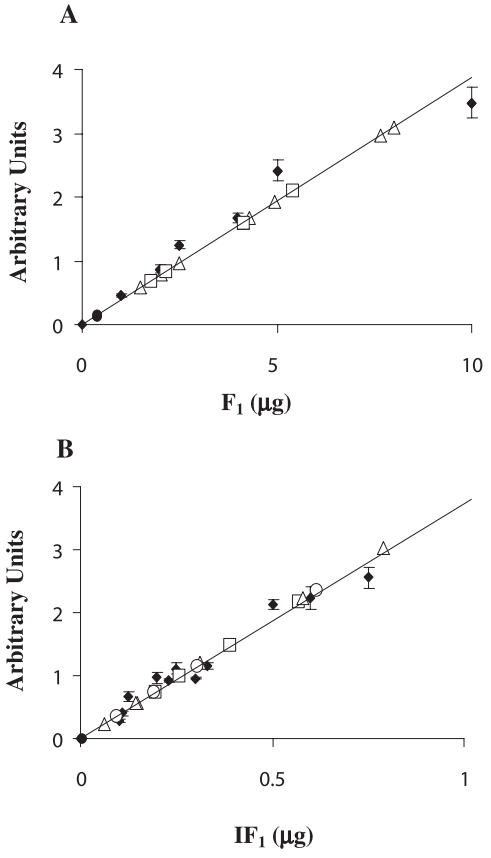

Fig. 2. Titration of F_1(A) and IF_1 (B) in goat heart Mg-ATP SMP, matrix fraction and intact mitochondria. Different quantities of standard proteins and different dilutions of the mitochondrial samples were included on the same SDS-PAGE and blotted onto nitrocellulose sheets for immunodetection as described in Materials and methods. The standard curves (♦) were performed using 2–10 μg of F_1 purified from beef heart (R=0.97) and using 0.1–0.75 μg of IF_1 from goat heart (R=0.99). Data points are as means±S.D. ($n\geq3$). Detection limit (d.l.) was 0.4 μg for F_1 (●) and 0.005 μg for IF_1(●). 10–40 μg of Mg-ATP SMP (△), 50–100 μg of matrix fraction (○) or 10–50 μg of intact mitochondria (□) from goat heart was loaded on the gel to detect F_1. When IF_1 was determined, 15–100 μg of Mg-ATP SMP (△), 20–80 μg of matrix fraction (○) or 20–50 μg of intact mitochondria (□) from goat heart was analyzed. Arbitrary units represent the band area calculated by laser densitometry.

This analysis was performed on preparations of the matrix fraction (MF) and vesicles of the (inverted) inner membrane (Mg-ATP SMP). Care was taken to minimize IF_1 release from membrane during preparation of these vesicles (see Materials and methods). The molar amount of F_1 in Mg-ATP SMP is 0.48±0.10 nmol/mg membrane protein, in accordance with previous data [19,33], while the molar amount of IF_1 is 0.43±0.03 nmol/mg. Consequently, the IF_1/F_1 ratio in these vesicles is 0.89. A comparable value was recently shown in bovine heart Mg-ATP SMP [19]. There is no detectable F_1 (<0.01 nmol/mg MF protein) in the matrix fraction, but 0.49±0.09 nmol IF_1/mg MF protein is found. Such IF_1 could be either loosely bound to membranes (being lost upon sonication) or free in the matrix space of goat heart mitochondria.

3.2. In vitro correlation between the changes of enzyme activity and IF_1/F_1 ratio

Although activity changes in F_0F_1 in different metabolic states have been well documented, previous analyses of membrane protein content were insufficiently precise to detect changes in the IF_1/F_1 ratio directly. In the current work, these problems have been overcome by separating the complex from the other inner mitochondrial membrane proteins before determining the IF_1/F_1 ratio by immunological assay. Fragments of goat heart were sonicated and then treated with mild detergent under conditions that, in bovine heart fragments, are known to extract selectively and quantitatively the complexes of the oxidative phosphorylation (OXPHOS) [26,34]. The complexes were then separated by blue-native gel electrophoresis (1D BN-PAGE) of the detergent extract. The F_0F_1ATP synthase band was then analyzed by SDS-PAGE and quantified by immunoblotting with anti-F_1 and IF_1 antibodies.

Fig. 3A (lanes 1–5) shows a 1D BN-PAGE pattern obtained from goat heart fragments in comparison with that of bovine heart preparations. Densitometry indicates that, in both cases, OXPHOS complexes comprise 77±5% of stained proteins, as previously reported for bovine heart tissue by Zerbetto et al. [26]. Moreover, complex V, as a percentage of the total areas of OXPHOS complexes (I+III+V), is similar in goat (50±3%) and bovine heart (51±2%), the latter value having been reported previously by Schagger and Pfeiffer [34]. These values indicate an F_1 concentration of 1.1±0.1 nmol/mg extract protein. This

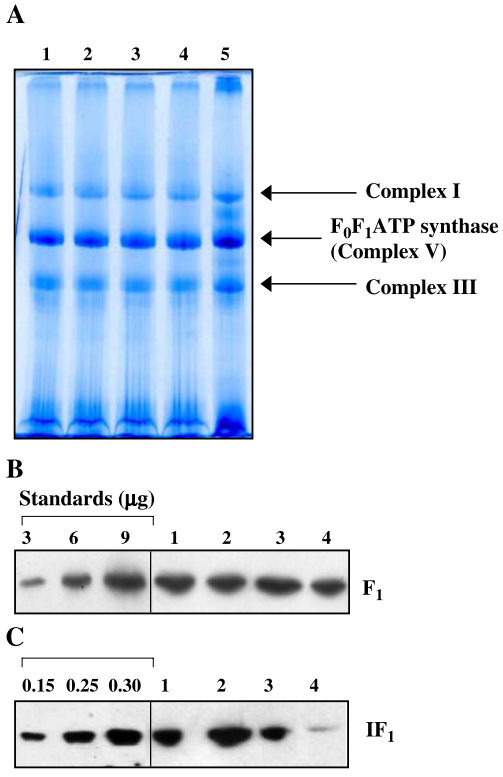

Fig. 3. 1D BN-PAGE of OXPHOS complexes (A) and immunodetection of F_1 (B) and IF_1 (C) in goat heart tissue exposed to different in vitro conditions. Goat heart tissue fragments were collected and treated as indicated in Materials and methods. *N*-Dodecylmaltoside extracts were separated by 1D BN-PAGE stained with Coomassie blue G. Lanes 1–4 were loaded with 15 μg of protein. As standard, lane 5 was loaded with 22 μg *n*-dodecylmaltoside protein extracts from bovine heart fragments. The bands corresponding to F_0F_1ATP synthase were excised from 1D BN-PAGE and analyzed, after 2D SDS-PAGE, by immunoblotting with anti-F_1 and anti-IF_1 antibodies as described in Materials and methods. Standards were as in Fig. 2. 1.—control; 2—ischemia; 3—high-salt buffer; 4—high-pH buffer; 5—bovine heart. This figure reports BN-PAGE and immunoblotting of a representative set of experiments of which five sets were performed. (For colour see online version).

figure compared with the above, and reports of high levels of protein recovery from beef heart [34], suggests that this extraction leads to virtually complete recovery of complex V from the membranes.

After SDS-PAGE in a second dimension, immunoblotting was able to detect F_1 at essentially the same level (1.2±0.3 nmol/mg extract protein) (Fig. 3B, lane 1). This indicates that proteins are also quantitatively recovered after the SDS-PAGE procedure.

Fig. 3C, lane 1, shows the IF_1 content of this complex. Densitometry estimates the IF_1 content as 1.0±0.2 nmol/mg extract protein. Thus, the molar ratio between IF_1 and F_1 in this extract is 0.86±0.05, comparable with the value obtained in Mg-ATP SMP from goat heart (Fig. 2).

All these data support the idea that (i) detergent extraction of F_0F_1ATP synthase from biopsies is complete in goat heart tissue, and (ii) that the procedure did not alter the IF_1 content of the enzyme. Similar results have been obtained in bovine heart [19]. The similarity of the IF_1/F_1 ratio in mitochondrial vesicles and membrane extracts of complex V also indicates that Mg-ATP SMP do not contain IF_1 bound to proteins other than F_0F_1ATP synthase. There does not appear to be any tight binding site for IF_1 on mitochondrial membranes other than that on F_1.

To investigate whether this method could detect changes in IF_1 content with changes in F_0F_1ATP synthase capacity, cardiac muscle biopsies were exposed to different in vitro conditions. To down-modulate the enzyme, the tissue fragments were exposed for 30 min at 37 °C to anoxic condition [8]. To up-modulate the enzyme, the tissue samples were sonicated in a high-salt or high-pH buffer to induce a partial or the almost complete release of IF_1 from F_1, respectively [9,11]. Table 1 shows the maximal ATP hydrolysis rate (F_0F_1ATP synthase capacity) of muscle samples treated in this way. After anoxia, the maximal ATPase activity decreases by 46%, as compared to biopsies maintained under O_2 atmosphere and sonicated in low-salt buffer at neutral pH. When high-salt or high-pH sonication was used, the activity increases by 24% and 156%, respectively.

Fig. 3A (lanes 1–4) shows that 1D BN-PAGE pattern was unaltered under all these conditions, suggesting that the changes in activity were not due to modifications of the enzyme content. The F_1 content determined after 2D SDS-PAGE and immunoblotting with anti-F_1 (Fig. 3B, lanes 1–4) was similar in all samples. However, changes were seen in the IF_1 content, which varied significantly after the different treatments (Fig. 3C, lanes 1–4). After ischemia, the IF_1/F_1 ratio increased from 0.85±0.05 to 1.07±0.04 (P<0.001 vs. control), while the ratio decreased to 0.71±0.01 and to 0.066±0.003 (for both P<0.001 vs. control) in the samples sonicated in the high-salt or high-pH buffer, respectively.

The relationship between IF_1/F_1 ratio and the maximal ATPase activity measured in the different in vitro samples is reported in Fig. 4. An inverse linear plot between IF_1/F_1 ratio and enzyme activity was obtained with a highly significant (R=0.99; n=20) correlation between the two parameters. These data clearly indicate that the modulation of F_0F_1ATP synthase activity obtained by the different treatments was due to IF_1 binding to or release from the F_1 sector of the whole complex. From Fig. 4, the maximal ATPase activity of the enzyme completely free of IF_1,

Table 1
Maximal ATPase activity in goat heart tissue exposed to different in vitro conditions: ischemia, high-salt or high-pH buffer

Maximal ATPase activity (U/mg)			
Control	Ischemia	High-salt buffer	High-pH buffer
0.97±0.11	0.52±0.07*	1.20±0.08*	2.48±0.10*

Goat heart tissue fragments were maintained under O_2 atmosphere and sonicated in low-salt (control), high-salt or high-pH buffer. The fragments were made ischemic by placing them in a sealed Ziploc plastic bag for 30 min at 37 °C. The maximal ATPase activity was assayed as described in Materials and methods.
Data were from five different experiments and are as means±S.D. *P<0.001 vs. control.

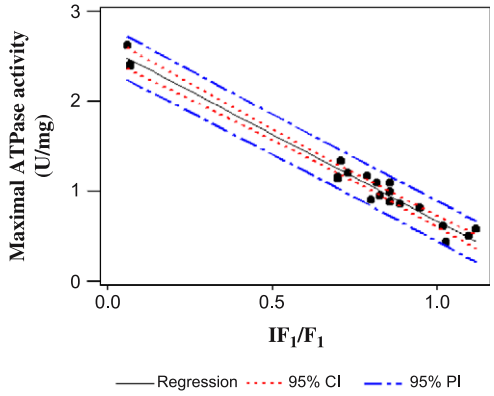

Fig. 4. In vitro correlation between the changes of enzyme activity and IF_1/F_1 ratio. The individual in vitro values of maximal ATPase activity (U/mg) (mean values reported in Table 1) and the corresponding IF_1/F_1 ratio, obtained as shown in Fig. 3, were plotted. The regression line shown was calculated using the individual values obtained from nine tissue fragments maintained under O_2 atmosphere (controls), four fragments made ischemic, four and three fragments sonicated in high-salt or high-pH buffer, respectively. $y=-1.936x+2.601$. CI is confidence interval; PI is prediction interval. (For colour see online version).

corresponding to 100% of active protein molecules, can be estimated as 2.60 U/mg sonicated protein. The maximal IF_1/F_1 ratio, i.e., the ratio when 100% of enzyme molecules were inhibited (ATPase=0 U/mg), is predicted to be 1.34 IF_1/F_1. Comparing this latter value with the ratio obtained in control biopsies (0.85 ± 0.05), it can be deduced that 63% of F_0F_1ATP synthase complexes in the control were inactive as a consequence of IF_1 binding. This value agrees well with the ratio found between the enzyme activity in control conditions and in high-pH buffer (see Table 1), which suggests that only 39% of F_0F_1ATP synthase complexes are active (and hence 61% inhibited) under control conditions. Harris and Das [7] have also reported that only a low percentage of F_0F_1ATP synthase complexes is active in resting rat heart cells.

3.3. In vivo correlation between the changes in the enzyme activity and IF_1/F_1 ratio

This approach could now be extended to investigate changes in the IF_1/F_1 ratio during metabolic transitions in goat heart in vivo. In this series of experiments, we measured the IF_1/F_1 ratio and the maximal ATPase activity in biopsies obtained from anaesthetized open-chest goat heart subjected to hyperemia (which provoked a marked increase of the THF), and in ischemic preconditioning. The combined effect of hyperemia and ischemic preconditioning was also investigated.

Fig. 5A–B shows the mean values of the maximal ATPase activity obtained in the different biopsy samples. In hyperemia, enzyme activity significantly increased to 121% of the baseline within 15 s of artery reopening. After 4 min, this value had decreased markedly, to 72% of the baseline level. The ATPase returned to baseline levels at 6 min (Fig. 5A). In contrast, IP induced a 13% decrease in the maximal ATPase activity as compared to baseline value (ΔATPase= -0.13 ± 0.07 U/mg, $P<0.05$). In two additional animals in which we assessed the effects of ischemic preconditioning per se, the enzyme inhibition persisted at least for 30 min, thereby allowing us to observe the combined effect of hyperemia and ischemic preconditioning. During the second hyperemia (after preconditioning) the maximal ATPase activity rose much less, and the change was statistically insignificant (Fig. 5B). After 4 min, the activity remained unchanged.

Recovery of OXPHOS complexes by detergent extraction and the percentage of complex V with respect to the

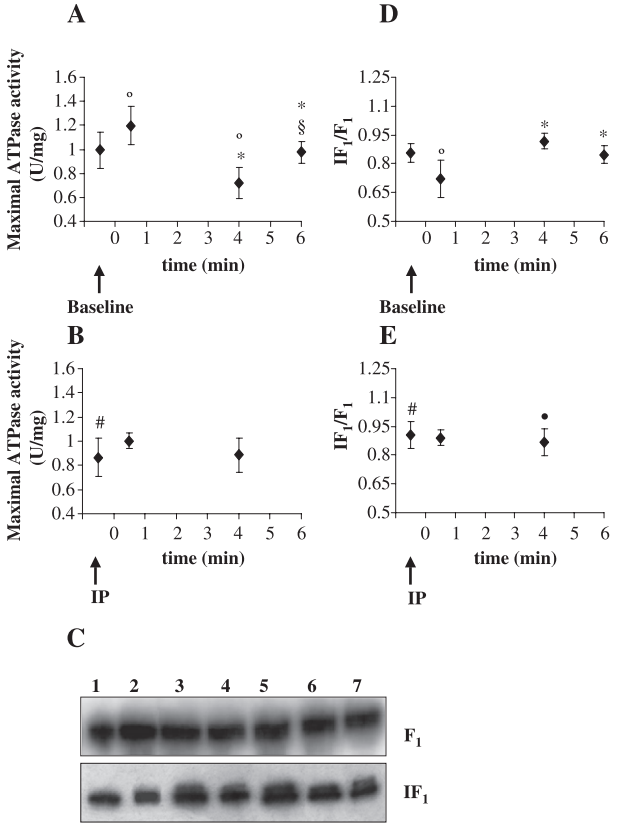

Fig. 5. Changes in the enzyme activity and IF_1/F_1 ratio during metabolic transitions in goat heart biopsies. Biopsies were collected, treated and analyzed as indicated in Materials and methods. (A) Maximal ATPase activity during the first reactive hyperemia. Baseline refers to biopsy taken after 30-min equilibrating perfusion. (B) Maximal ATPase activity during the second reactive hyperemia (after preconditioning). IP was considered as basal value vs. the second hyperemia. (C) Immunoblotting with anti-F_1 and anti-IF_1 antibodies. First reactive hyperemia: 1—baseline; 2—15 s after artery reopening; 3—4 min after artery reopening; 4—6 min after artery reopening; Second reactive hyperemia (after preconditioning): 5—IP; 6—15 s after artery reopening; 7—4 min after artery reopening. (D) IF_1/F_1 ratio during the first reactive hyperemia. Baseline refers to biopsy taken after 30-min equilibrating perfusion. (E) IF_1/F_1 ratio during the second reactive hyperemia (after preconditioning). IP was considered as basal value vs. the second hyperemia. At t=0 artery occlusion was performed. All data were from eight animals and are expressed as means±S.D. (○) $P<0.05$ vs. baseline; (*) $P<0.05$ vs. 15 s after artery reopening; (§) $P<0.05$ vs. 4 min after artery reopening; (●) $P<0.05$ vs. IP; (#) $P<0.05$ IP vs. baseline (Student's t test).

total areas of OXPHOS complexes I, V and III proved to be the same in all biopsy samples (data not shown) and were similar to that of the in vitro samples (above). Immunoblotting (Fig. 5C) also confirmed that the F1 content was similar in all biopsy samples, confirming that this method of protein analysis was applicable to in vivo experimentation.

The IF_1 content of the complexes was then investigated by immunoblotting (Fig. 5C). Densitometry showed significant changes in the ratio between the band area of IF_1 and that of F_1, indicating significant differences in the quantity of IF_1 bound to F_0F_1ATP synthase in line with the measured maximal ATPase activity. Mean values from a number of experiments are plotted in Fig. 5D and E. The changes in IF_1/F_1 ratio, shown in Fig. 5D, indicate that, 15 s after artery reopening, the ATPase capacity has increased while the ratio of IF_1 bound to F_0F_1ATP synthase significantly decreased from 0.86±0.05 to 0.72±0.09. After 4 min, maximal ATPase activity has declined (above) and the IF_1/F_1 ratio has significantly increased to 0.92±0.04. Both ATPase (above) and IF_1/F_1 ratio had returned to baseline levels (0.85±0.04) 6 min after reactive hyperemia (Fig. 5D).

After preconditioning, the IF_1/F_1 ratio was significantly higher than baseline (0.91±0.07), showing that the preconditioning maneuvers induced the binding of IF_1 to F_0F_1ATP synthase. Under these conditions, there was little significant change during a second hyperemia in either the maximal ATPase activity or the IF_1/F_1 ratio (Fig. 5B and E).

Fig. 6 shows that, when IF_1/F_1 ratio was plotted against maximal ATPase activity, an inverse correlation (R=0.83; n=33) was found. The slope of the linear regression obtained in vivo was not significantly different from the slope obtained in vitro (see Fig. 4). From Fig. 6, the value of IF_1/F_1 ratio when the enzyme was fully inhibited (maximal ATPase activity=0) is estimated as 1.43, and the enzyme activity when no IF_1 was bound to F_0F_1ATP synthase (IF_1/F_1 ratio=0) as 2.37 U/mg. These values correlate closely with those obtained in the in vitro experiments.

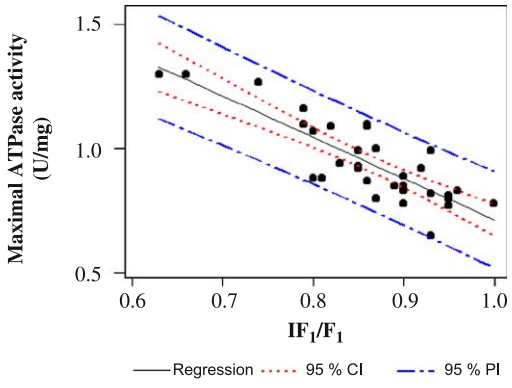

Fig. 6. In vivo correlation between the changes in the enzyme activity and IF_1/F_1 ratio during metabolic transitions. The individual in vivo values of maximal ATPase activity (U/mg) and IF_1/F_1 ratio (mean values given in Fig. 5A/B and D/E, respectively) were plotted. The regression line shown was calculated using the individual values obtained by: first reactive hyperemia—seven biopsies taken at baseline, four taken 15 s after artery reopening, four taken 4 min after artery reopening, four taken 6 min after artery reopening; second reactive hyperemia (after preconditioning)—six taken in IP, four taken 15 s after artery reopening, four taken 4 min after artery reopening. $y=-1.660x+2.373$. CI is confidence interval, PI is prediction interval. (For colour see online version).

4. Discussion

4.1. Modulation of F_0F_1ATP synthase in goat heart

The above describes the development of novel procedures for the investigation of F_0F_1ATP synthase capacity and IF_1/F_1 association in a single biopsy sample from cardiac tissue. Assay conditions were optimized for small tissue samples (a minimum of 3 mg for enzyme activity and 10 mg for IF_1 content) without subjecting them to multiple processing steps, assuring minimal perturbation of the IF_1/F_1 interaction. Biopsy samples could be obtained with minimal disruption to cardiac function. This allowed us to follow ATP synthase capacity and IF_1/F_1 association in a single working goat heart over time.

The work demonstrates that activity of the F_0F_1ATP synthase is modulated in goat heart in response to metabolic conditions. Ischemia in isolated tissue samples leads to a decrease in the maximal ATP hydrolytic activity from 0.97 U/mg in oxygenated tissue to 0.52 U/mg after ischemia (Table 1). In intact heart, hyperemia induces a rise in the maximal ATPase activity from 0.99 to 1.2 U/mg (Fig. 5A). Several lines of evidence have shown that the ATP synthase can be modulated in other organisms (rat, dog, humans) [2,9,11–13]. In common with these workers, an increase in ATP synthase capacity in high work conditions and a decrease in ischemia are observed in the goat system. It was suggested that the ability to switch off the ATP synthase was beneficial to maintaining ATP levels in ischemia, preventing hydrolysis by the ATP synthase working in reverse.

These findings have been disputed by other workers. Neither Green et al. [15] nor Kobara et al. [16] working on rat heart nor Vander Heide et al. [17] working on dog heart were able to observe such transitions. However, these negative results could be explained by the experimental procedures employed by these workers. F_0F_1ATP synthase capacity in these reports was measured in submitochondrial particles after a prolonged mitochondrial isolation. Moreover, the samples were diluted [16] in high-pH buffer or the heart was homogenized in high-salt buffer [17], both of which conditions are known to increase maximal ATPase activity by F_0F_1. It seems likely, therefore, that ATP synthase modulation is a common feature in vivo, at least in heart tissue.

The observations on ATP synthase levels after ischemic preconditioning are interesting. IP in goat heart leads to a fall in maximal ATPase activity from 0.99 to 0.86 U/mg, which is mirrored in a rise of IF_1 content. These changes seem to persist throughout the second hyperemia, the final

ATP synthase capacity in Fig. 5B being 0.89 U/mg. Other workers have reported a reversible fall in mitochondrial ATPase activity after IP in other systems. Vourinen et al. [10] and Ylitalo et al. [11] showed that in Langendorff-perfused rat hearts, IP induced an inhibition of F_0F_1ATP synthase capacity which persisted in the early stages of prolonged ischemia but vanished after 30 min. The brief nature of this effect does not rule out the possibility that changes in the ATP synthase may contribute to the protective effects of ischemic preconditioning.

4.2. Modulation is due to IF_1

Several workers have suggested that such modulation is due to variation of the interaction of F_1 with a naturally occurring inhibitor protein, IF_1. Before the current work, for example, the down-regulation of F_0F_1ATP synthase during ischemia was attributed to IF_1 binding, based on the observation that ischemia-induced inhibition was reversed by exposure of processed tissue to high-salt concentrations and/or high pH [9,11]. This evidence, however, is circumstantial, and confirmation awaited the direct demonstration of a correlation between ATP synthase capacity and IF_1 content in in vivo systems. The methods used in the present study allow the direct quantification of IF_1 bound to F_0F_1ATP synthase and the measurement of enzyme activity in single myocardial samples from goat.

Using these approaches, we obtained evidence that F_0F_1ATP synthase is directly modulated by IF_1 in a variety of in vitro and in vivo functional states. In ischemic tissue samples, a decrease in the maximal ATPase activity is accompanied by an increase in bound $IF_1$from 0.85 mol/mol (control) to 1.07 mol/mol. In the beating heart, hyperemia leads to a series of changes in ATP synthase capacity (rise, fall, return to baseline) which is mirrored in changes in bound IF_1 (fall, rise, return to baseline) (Fig. 5A and D). Effects on both the activity and on IF_1 content are largely abolished after ischemic preconditioning (Fig. 5B and E).

The effects observed show directly that IF_1 is responsible for the modulation of ATP synthase capacity in goat heart. To investigate whether other factors might be involved, the relationship between ATP synthase capacity and IF_1/F_1 ratio was plotted over the entire range of results obtained. Investigation with both in vitro systems and working heart samples showed an inverse linear correlation between IF_1/F_1 ratio and enzyme activity (Figs. 4 and 6) with similar slopes and intercepts. There was a highly significant statistical correlation between the two parameters in both systems. It could therefore be concluded that the only significant parameter modulating the ATP synthase during these transitions was its interaction with IF_1. This statement can only be made in confidence over a limited range of ATP synthase activities for the in vivo samples, since our physiological manipulations could only vary the activity over a twofold change in ATPase. However, the similarity between the lines obtained with in vivo samples and with manipulated tissue certainly does not indicate that any other factors are important in the intact heart.

It is possible of course that other control factors act by modulating the IF_1/F_1 interaction. It is plausible that up-down modulation of F_0F_1ATP synthase during hyperemia may be related to changes of oxygen demand associated with the changes of coronary flow (see Materials and methods), although how IF_1 senses oxygen demand is unknown. Likely candidates are mitochondrial matrix Ca^{2+} and/or $\Delta\Psi$, as suggested by Harris and Das [7], where in vivo ATP synthase transitions were first demonstrated. In hyperemia, the increase of the synthase capacity at 15 s after artery reopening may be due to mitochondrial matrix Ca^{2+}. In fact, a Ca^{2+}-dependent up-regulation of F_0F_1ATP synthase has been recently observed in pig upon increased cardiac work, which induced increase in oxygen consumption [35,36]. Conversely, the down-modulation at 4 min after artery reopening appears to be a compensatory response to overshoot, and might be mediated by $\Delta\Psi$ or ΔpH decrease consequent to the lowered oxygen uptake observed in response to hyperemia [37]. After IP, a reduction of THF during the second hyperemia occurred (see Materials and methods), probably accompanied by reduced myocardial oxygen demand [38] and decreased mitochondrial matrix Ca^{2+} increase [39], both of which would mediate against activation. The current work indicates that, whatever the primary signal, the factor directly responsible for modulating the ATP synthase capacity is IF_1.

4.3. IF_1 levels in mitochondria

Extrapolation of the linear relationship between IF_1 and inhibition of maximal ATPase activity shows that about 1 mol of IF_1 is sufficient to fully inactivate 1 mol of enzyme both in vitro and in vivo experiments. The same value was previously obtained in isolated membrane preparations and purified proteins [4]. This result suggests that the scheme of IF_1 action proposed for the isolated IF_1–F_1 complex [5] probably holds in vivo and is responsible for the F_0F_1ATP synthase modulation in response to metabolic changes.

The current work estimates the ratio of IF_1 to F_0F_1ATP synthase in goat heart mitochondria to be 2.5. This represents both bound and unbound IF_1 and suggests that this protein is in excess of F_1 in goat heart. Different values for the IF_1/F_1 ratio, depending on tissue and species, have been reported. A value similar to the above was estimated in pig heart mitochondria by Lopez-Mediavilla et al. [33], determined by immunoblotting. However, Power et al. [24] estimated an IF_1/F_1 ratio of approximately 1 in bovine heart mitochondria, using activity determinations. The explanation of this discrepancy could be species-related—but it may well relate to the conditions for purification and storage of the mitochondria.

In the current work, the time between sampling and assay is relatively short, and thus these results may better represent the in vivo content of IF_1.

The role of the excess IF_1 is uncertain. Lopez-Mediavilla et al. [33] suggested the existence of a non-inhibitory binding site in the inner mitochondrial membrane, which could be a subunit of F_0 sector of F_0F_1ATP synthase. The data presented here, however, allow us to exclude the existence of a binding non-inhibitory site for IF_1 on F_0 sector since the IF_1/F_1 ratio was about 1 when the enzyme was fully inhibited both in vitro and in vivo, and the activity varied linearly with IF_1 content. We cannot rule out a loose binding site for IF_1 on the mitochondrial membrane but IF_1 unbound to F_1 is certainly not found in detergent preparations of F_0F_1. Possibly, free IF_1 in the mitochondrial matrix—as implied in the studies on goat and pig heart—could increase the rate of interaction between F_0F_1ATP synthase and IF_1, which is characterised by a site with high affinity (K_d=1–2×10^{-8} M) (above) but with slow binding kinetics ($t_{1/2}$=5–10 s) [40].

These data also confirm the suggestion [7,24] that in heart, under most conditions, most (70–80%) of F_1 is present in mitochondrial membranes in its inhibited form, with bound IF_1. Regulation consists of variation of the percentage of active F_1 around this level.

In conclusion, all these findings are consistent with an in vivo model in which IF_1 is a physiological regulator of F_0F_1ATP synthase, which rapidly responds to metabolic alterations affecting the fraction of active enzyme molecules. The active molecules are able to either synthesize or hydrolyze ATP depending on mitochondrial energy state, as previously proposed in the in vitro and in vivo studies [8–14].

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References

[1] A.E. Senior, S. Nadanaciva, J. Weber, The molecular mechanism of ATP synthesis by F_1F_0-ATP synthase, Biochim. Biophys. Acta 1553 (2002) 188–211.
[2] W. Rouslin, Protonic inhibition of the mitochondrial oligomycin-sensitive adenosine 5′ -triphosphatase in ischemic and autolyzing cardiac muscle. Possible mechanism for the mitigation of ATP hydrolysis under non-energizing conditions, J. Biol. Chem. 258 (1983) 9657–9661.
[3] D.W. Green, G.J. Grover, The IF_1 inhibitor protein of the mitochondrial F_1F_0-ATPase, Biochim. Biophys. Acta 1458 (2000) 343–355.
[4] J.C. Gomez-Fernandez, D.A. Harris, A thermodynamic analysis of the interaction between the mitochondrial coupling adenosine triphosphatase and its naturally occurring inhibitor protein, Biochem. J. 176 (1978) 967–975.
[5] E. Cabezon, M.G. Montgomery, A.G.W. Leslie, J.E. Walker, The structure of bovine F_1-ATPase in complex with its regulatory protein IF_1, Nat. Struct. Biol. 10 (2003) 744–750.
[6] F. Penin, A. Di Pietro, C. Godinot, D.C. Gautheron, Fate of nucleotides bound to reconstituted F_0-F_1 during adenosine 5′ -triphosphate synthesis activation or hydrolysis: role of protein inhibitor and hysteretic inhibition, Biochemistry 27 (1988) 8969–8974.
[7] D.A. Harris, A.M. Das, Control of mitochondrial ATP synthesis in the heart, Biochem. J. 280 (1991) 561–573.
[8] W. Rouslin, M.E. Pullman, Protonic inhibition of the mitochondrial adenosine 5'-triphosphatase in ischemic cardiac muscle. Reversible binding of the ATPase inhibitor protein to the mitochondrial ATPase during ischemia, J. Mol. Cell. Cardiol. 19 (1987) 661–668.
[9] T.D. Scholz, R.S. Balaban, Mitochondrial F_1-ATPase activity of canine myocardium: effects of hypoxia and stimulation, Am. J. Physiol. 266 (1994) H2396–H2403.
[10] K. Vuorinen, K. Ylitalo, K. Peuhkurinen, P. Raatikainen, A. Ala-Rämi, IE. Hassinen, Mechanisms of ischemic preconditioning in rat myocardium. Roles of adenosine, cellular energy state, and mitochondrial F_1F_0-ATPase, Circulation 91 (1995) 2810–2818.
[11] K. Ylitalo, A. Ala-Rämi, K. Vuorinen, K. Peuhkurinen, M. Lepojarvi, P. Kaukoranta, K. Kiviluoma, I. Hassinen, Reversible ischemic inhibition of F_1F_0-ATPase in rat and human myocardium, Biochim. Biophys. Acta 1504 (2001) 329–339.
[12] A.M. Das, D.A. Harris, Control of mitochondrial ATP synthase in heart cells: inactive to active transitions caused by beating or positive inotropic agents, Cardiovasc. Res. 24 (1990) 411–417.
[13] A.M. Das, D.A. Harris, Regulation of the mitochondrial ATP synthase in intact rat cardiomyocytes, Biochem. J. 266 (1990) 355–361.
[14] F. Bosetti, G. Yu, R. Zucchi, S. Ronca-Testoni, G. Solaini, Myocardial ischemic preconditioning and mitochondrial F_1F_0-ATPase activity, Mol. Cell. Biochem. 215 (2000) 31–38.
[15] D.W. Green, H.N. Murray, P.G. Sleph, F.L. Wang, A.J. Baird, W.L. Rogers, G.J. Grover, Preconditioning in rat hearts is independent of mitochondrial F_1F_0 ATPase inhibition, Am. J. Physiol. 274 (1998) H90–H97.
[16] M. Kobara, T. Tatsumi, S. Matoba, Y. Yamahara, C. Nakagawa, B. Otha, T. Matsumoto, D. Inoue, J. Asayama, M. Nakagawa, Effect of ischemic preconditioning on mitochondrial oxidative phosphorylation and high energy phosphates in rat hearts, J. Mol. Cell. Cardiol. 28 (1996) 417–428.
[17] R.S. Vander Heide, M.L. Hill, K.A. Reimer, R.B. Jennings, Effect of reversible ischemia on the activity of the mitochondrial ATPase: relationship to ischemic preconditioning, J. Mol. Cell. Cardiol. 28 (1996) 103–112.
[18] D. Gattullo, R.J. Linden, G. Losano, P. Pagliaro, N. Westerhof, Ischaemic preconditioning changes the pattern of coronary reactive hyperaemia in the goat: role of adenosine and nitric oxide, Cardiovasc. Res. 42 (1999) 57–64.
[19] L. Tomasetig, F. Di Pancrazio, D.A. Harris, I. Mavelli, G. Lippe, Dimerization of F_0F_1ATP synthase from bovine heart is independent from the binding of the inhibitor protein IF1, Biochim. Biophys. Acta 1556 (2002) 133–141.
[20] S.J. Ferguson, D.A. Harris, G.K. Radda, The adenosine triphosphatase-inhibitor content of bovine heart submitochondrial particles. Influence of the inhibitor on adenosine triphosphate-dependent reactions, Biochem. J. 162 (1977) 351–357.
[21] Z. Technikova-Dobrova, A.M. Sardanelli, S. Papa, Phosphorylation of mitochondrial proteins in bovine heart. Characterization of kinases and substrates, FEBS Lett. 322 (1993) 51–55.
[22] L.L. Horstman, E. Racker, Partial resolution of the enzymes catalyzing oxidative phosphorylation, J. Biol. Chem. 245 (1970) 1336–1344.
[23] N. Nelson, H. Nelson, E. Racker, Partial resolution of the enzymes catalyzing photophosphorylation: XII. Purification and properties of an inhibitor isolated from chloroplast coupling factor 1, J. Biol. Chem. 247 (1972) 7657–7662.
[24] J. Power, R.L. Cross, D.A. Harris, Interaction of F_1-ATPase, from ox heart mitochondria with its naturally occurring inhibitor protein. Studies using radio-iodinated inhibitor protein, Biochim. Biophys. Acta 724 (1983) 128–141.

[25] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[26] E. Zerbetto, L. Vergani, F. Dabbeni-Sala, Quantification of muscle mitochondrial oxidative phosphorylation enzymes via histochemical staining of blue native polyacrylamide gels, Electrophoresis 18 (1997) 2059–2064.
[27] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[28] I. Husain, P.J. Jackson, D.A. Harris, Interaction between F_1-ATPase and its naturally occurring inhibitor protein. Studies using a specific anti-inhibitor antibody, Biochim. Biophys. Acta 767 (1985) 64–74.
[29] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 74 (1970) 248–254.
[30] P.J. Jackson, D.A. Harris, Sites of protein–protein interaction on the mitochondrial F_1-ATPase inhibitor protein, Biochem. J. 235 (1986) 577–583.
[31] A. Vadineanu, J.A. Berden, E.C. Slater, Proteins required for the binding of mitochondrial ATPase to the mitochondrial inner membrane, Biochim. Biophys. Acta 449 (1976) 468–479.
[32] J.E. Walker, I.M. Fearnley, N.J. Gay, B.W. Gibson, F.D. Northrop, S.J. Powell, M.J. Runswick, M. Saraste, V.L.J. Tybulewics, Primary structure and subunit stoichiometry of F_1-ATPase from bovine mitochondria, J. Mol. Biol. 184 (1985) 677–701.
[33] C. Lopez-Mediavilla, H. Vigny, C. Godinot, Docking the mitochondrial inhibitor protein IF1 to a membrane receptor different from the F_1-ATPase β subunit, Eur. J. Biochem. 215 (1993) 487–496.
[34] H. Schagger, K. Pfeiffer, The ratio of oxidative phosphorylation complexes I–V in bovine heart mitochondria, and the composition of respiratory chain supercomplexes, J. Biol. Chem. 276 (2001) 37861–37867.
[35] P.R. Territo, V.K. Mootha, S.A. French, R.S. Balaban, Ca^{2+} activation of heart mitochondrial oxidative phosphorylation: role of the F_0/F_1-ATPase, Am. J. Physiol. 278 (2000) C423–C435.
[36] P.R. Territo, S.A. French, R.S. Balaban, Simulation of cardiac work transitions, in vitro: effects of simultaneous Ca^{2+} and ATPase additions on isolated porcine heart mitochondria, Cell Calcium 30 (2001) 19–27.
[37] J.H. Ruiter, J.A. Spaan, J.D. Laird, Transient oxygen uptake during myocardial reactive hyperemia in the dog, Am. J. Physiol. 235 (1978) H87–H94.
[38] Z.L. Xu, H. Endoh, A. Ishihata, E. Takahashi, K. Doi, Effect of ischemic preconditioning on myocardial oxygen consumption during ischemia, J. Mol. Cell. Cardiol. 30 (1998) 2165–2174.
[39] K.V. Ylitalo, A. Ala-Rämi, E.V. Liimatta, K.J. Peuhkurinen, I.E. Hassinen, Intracellular free calcium and mitochondrial membrane potential in ischemia/reperfusion and preconditioning, J. Mol. Cell. Cardiol. 32 (2000) 1223–1238.
[40] G. Lippe, M.C. Sorgato, D.A. Harris, Kinetics of the release of the mitochondrial inhibitor protein. Correlation with synthesis and hydrolysis of ATP, Biochim. Biophys. Acta 933 (1988) 1–11.