# Modification of the mitochondrial $F_1$-ATPase $\varepsilon$ subunit, enhancement of the ATPase activity of the $IF_1$–$F_1$ complex and $IF_1$-binding dependence of the conformation of the $\varepsilon$ subunit

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Treatment of bovine heart submitochondrial particles with a low concentration of 2-hydroxy-5-nitrobenzyl bromide (HNB), a selective reagent for the Trp residue of the $\epsilon$ subunit [Baracca, Barogi, Lenaz and Solaini (1993) Int. J. Biochem. **25**, 1269–1275], enhances the ATP hydrolytic activity of the particles exclusively when the natural inhibitor protein $IF_1$ is present. Similarly, isolated $F_1$ [the catalytic sector of the mitochondrial $H^+$-ATPase complex (ATP synthase)] treated with the reagent has the ATPase activity enhanced exclusively if $IF_1$ is bound to it. These experiments suggest that the modification of the $\epsilon$ subunit decreases the inhibitory activity of $IF_1$, eliciting the search for a relationship between the $\epsilon$ subunit and the inhibitory protein. Certainly, a reverse relationship exists because HNB binds covalently to the isolated $F_1$ exclusively when the inhibitory protein is present. This finding is consistent with the existence of the $\epsilon$ subunit in different conformational states depending on whether $IF_1$ is bound to $F_1$ or not. Support for this assertion is obtained by measurements of the intrinsic phosphorescence decay rate of $F_1$, a probe of the Trp $\epsilon$ subunit conformation *in situ* [Solaini, Baracca, Parenti-Castelli and Strambini (1993) Eur. J. Biochem. **214**, 729–734]. A significant difference in phosphorescence decay rate is detected when $IF_1$ is added to preparations of $F_1$ previously devoid of the inhibitory protein. These studies indicate that $IF_1$ and the $\epsilon$ subunit of the mitochondrial $F_1$–ATPase complex are related, suggesting a possible role of the $\epsilon$ subunit in the mechanism of regulation of the mitochondrial ATP synthase.

## INTRODUCTION

Proton ATPases of the $F_0F_1$ type [$F_0$ being the membrane sector of the $H^+$-ATPase complex and $F_1$ being the catalytic sector of the mitochondrial $H^+$-ATPase complex (ATP synthase)] found in mitochondria, bacteria and chloroplasts catalyse ATP synthesis from ADP and $P_i$ by using the proton electrochemical gradient generated by respiratory or photosynthetic chains. These ATPases are embedded in membranes by $F_0$, which is capable of $H^+$ translocation, and are linked to $F_1$ through a 'stalk' 4–5 nm long (reviewed in [1,2]). When the two sectors are coupled, the enzyme functions as a reversible $H^+$-transporting ATPase or ATP synthase, whereas isolated $F_1$ is capable only of ATPase activity. The mitochondrial $F_1$ complex has a molecular mass of 370 kDa and is composed of five different subunits $\alpha$ to $\epsilon$ in order of decreasing molecular mass [3]. The $\alpha$, $\beta$, $\gamma$ and $\delta$ subunits are homologous with subunits of bacteria and chloroplast, whereas the mitochondrial $\epsilon$ subunit does not correspond to any bacterial or chloroplast polypeptide [3]. In mitochondria, a sixth polypeptide, a natural inhibitor protein, $IF_1$, first described by Pullman and Monroy [4] can be found associated with $F_1$ during preparation of the enzyme. This polypeptide is considered to take part in the control mechanism *in vivo* proposed to regulate the ATPase complex (reviewed in [5–7]). The binding site of $IF_1$ is thought to be on the $\beta$ subunit of the enzyme and includes the sequence 394–400 [8]. It has also been suggested that the binding site of $IF_1$ could in fact be at an $\alpha/\beta$ interface [9]. Therefore it seems that this binding site does not involve polypeptides belonging to the stalk connecting $F_1$ to the membrane sector, $F_0$. Nevertheless, the inhibition exerted by $IF_1$ on the ATPase activity of the membrane-bound enzyme is sensitive to the membrane potential [10] and to phospholipids [11], suggesting the existence of some type of communication between $F_0$ and the $IF_1$-binding site of $F_1$. In $H^+$-ATPases the importance of long–range conformational changes transmitted from $F_0$ to $F_1$ has been widely described; these conformational changes are considered as the basis of both the mechanism of ATP synthesis [3,12] and the inhibitory action of dicyclohexylcarbodi-imide on the ATP hydrolysis [13] catalysed by the membrane-bound $F_0F_1$ complex.

The $\epsilon$ subunit of the bovine mitochondrial $F_1$ complex is a peptide of 50 amino acid residues [3] that has been cloned, sequenced and studied in several laboratories; however, its functional role is far from clear. It has only been reported that its conformation is sensitive to different nucleotides binding to $F_1$ [14] and that its suppression in a yeast mutant promotes an uncoupling between the two sectors $F_0$ and $F_1$ [15]. Recently we showed that the subunit is located in the stalk region of $F_1$ [16] and was therefore in a position compatible with an involvement in the mechanism of signal transmission between $F_1$ and $F_0$. Preliminary experiments performed with the aim of probing *in situ* the $\epsilon$ subunit by using a selective Trp residue reagent (the sole Trp of $F_1$ is located at position 4 of the chain [3]), revealed that a low concentration (below 0.5 mM) of 2-hydroxy-5-nitrobenzyl bromide (HNB) covalently bound to this residue, affecting the

Abbreviations used: ES-SMP, particles depleted of $IF_1$ by passing EDTA-treated SMP through a Sephadex G-50 column; $F_0$, membrane sector of the $H^+$-ATPase complex; $F_1$, catalytic sector of the $H^+$-ATPase complex (ATP synthase); HNB, 2-hydroxy-5-nitrobenzyl bromide; $IF_1$, naturally occurring inhibitor protein of the $H^+$-ATPase complex; SMP, submitochondrial particles prepared by sonication of mitochondria.

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activity of the crude enzyme preparation assayed [17]. Because this enzyme preparation contained $IF_1$, one proposed interpretation of the results was that on modification of the $\epsilon$ subunit, the $IF_1$–$F_1$ interaction might have changed, resulting in an enhanced ATPase activity of the $IF_1$–$F_1$ complex.

The first purpose of the present study was to demonstrate whether an association of $IF_1$ with $F_1$ is essential in order to observe both the activating effect of the ATP hydrolytic activity by a low concentration of HNB and the covalent binding of the reagent to isolated $F_1$. To accomplish this, two different enzyme preparations, containing or lacking $IF_1$, were examined. The second objective of this study was to determine whether the binding of $IF_1$ to $F_1$ could induce conformational changes in the $\epsilon$ subunit.

## EXPERIMENTAL

### Materials

HNB, NADH (disodium salt), phosphoenolpyruvate (trisodium salt), a 50 % (v/v) glycerol solution of rabbit muscle pyruvate kinase (700 units/ml) and lactate dehydrogenase (1000 units/ml), and ATP (disodium salt) were purchased from Sigma Chemical Co., St. Louis, U.S.A. All chemicals, of reagent grade, were used without further purification.

### Biological preparations

Submitochondrial particles (SMP) were prepared from bovine heart mitochondria by exposure to ultrasonic energy as described by Beyer [18]. The inhibitor-devoid particles (ES-SMP) were prepared by passing EDTA-treated submitochondrial particles through a Sephadex G-50 column as described [19].

Pure inhibitor-free $F_1$ ($IF_1$-free $F_1$) was prepared from bovine heart mitochondria by a modified Penefsky's procedure [13]. Essentially, 10 mg of $F_1$ prepared by the method of Penefsky [20] was passed through a Blue Sepharose CL-6B column (4 cm × 1 cm) in 0.2 M NaCl/0.5 mM EDTA/1mM 2-mercaptoethanol/1 mM ATP/20 mM Tris/HCl (pH 8). Nearly 8 mg of the non-retained protein were concentrated to 15 mg/ml by ultrafiltration with a Diaflo XM-300 (Amicon) membrane. The concentrate was then chromatographed on Sephacryl S-300 (40 cm × 1.6 cm column) in 25 mM Tris/HCl (pH 8)/0.25 M sucrose/1 mM ATP.

$F_1$ preparations containing the inhibitor protein ($IF_1$–$F_1$ complex) were prepared essentially as above, but avoiding the exposure of submitochondrial particles to both alkaline pH (9.2) and EDTA-containing buffers. Figure 1 shows the SDS/PAGE pattern of the $F_1$ preparations studied.

The inhibitor protein was isolated from beef heart mitochondria in pure form by a method of purification that combined the recovery of the supernatant from submitochondrial particles [21] with the procedure described by Horstman and Racker [22]. The inhibitor protein was stored at −80 °C in 0.25 M sucrose/10 mM Tris/$SO_4^{2-}$ (pH 6.8). The binding of the inhibitor (5 $\mu$M) to $F_1$ (1 $\mu$M) was performed by incubating the proteins in 50 mM Mops (pH 6.7)/2 mM $MgCl_2$/1 mM ATP for 15 min [23]. The ATP hydrolytic activity of these preparations was decreased approx. 80 % with respect to $IF_1$-free preparations.

The protein concentration of enzyme solutions was determined by the method of Lowry et al. [24]; particulate protein concentration was determined by the biuret method in the presence of 1 % (w/v) deoxycholate [25]. No correction was made for the contribution of haem groups to the absorption at 450 nm.

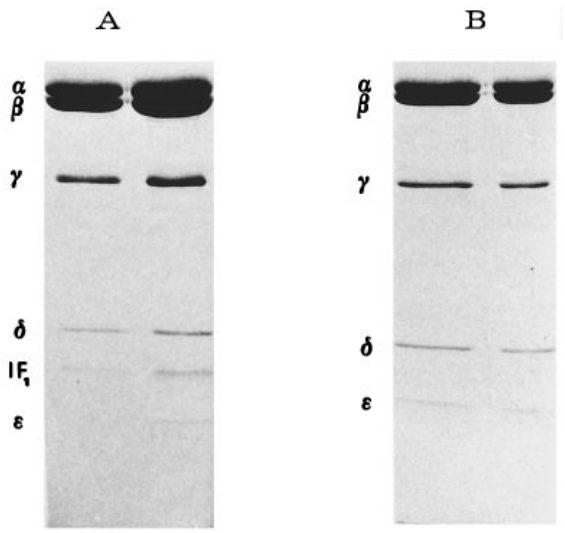


**Figure 1 Purity of the $F_1$ complex**

The samples were analysed by SDS/PAGE and stained with Coomassie Brilliant Blue R250. (**A**) Left lane, 25 $\mu$g of $F_1$–$IF_1$ complex; right lane, 40 $\mu$g of $F_1$–$IF_1$ complex. (**B**) Left lane, 30 $\mu$g of $IF_1$-free $F_1$; right lane, 15 $\mu$g of $IF_1$-free $F_1$.

### Protein modification

The treatment of both submitochondrial particles and $F_1$ with HNB was accomplished in 0.25 M sucrose/1 mM EDTA/0.2 M Tris/HCl (pH 6.7), using the general procedure described by Horton and Koshland [26]. The reagent was dissolved in dry acetone; the final concentration of this solvent in the reaction mixture did not exceed 1 % by volume. Soon after treatment with HNB the pH of each enzyme sample was brought from 6.7 to 7.4. The stoichiometric ratio of HNB to $F_1$ was determined spectrophotometrically soon after excess and non-specifically absorbed HNB had been removed as follows: after 2 min of reaction in the dark at room temperature, the enzyme was precipitated with $(NH_4)_2SO_4$ at 60 % saturation, then solubilized in 0.25 M sucrose/1 mM EDTA/10 mM Tris/HCl (pH 7.4) and exposed to repeated cycles of ultrafiltration on a Centricon microconcentrator (Amicon) and dilution with the buffer described above. Finally it was subjected to chromatography on Sephadex G-25. The HNB concentration of covalently bound reagent was estimated spectrophotometrically at 410 nm ($\epsilon$ 13.5 $mM^{-1} \cdot cm^{-1}$) [27].

### ATPase assay

The ATPase activity was determined with an ATP-regenerating system by following the decrease in NADH absorption at 340 nm in a 7850 model Jasco spectrophotometer as previously reported [28].

### Gel electrophoresis

Electrophoresis in 14–25 % (w/v) polyacrylamide gradient gel containing 0.1 % SDS was performed under reducing conditions as described by Laemmli [29]. After completion of electrophoresis the gel was stained for 1 h with 0.05 % Coomassie Brilliant Blue

R250 in methanol/acetic acid/water (3:1:6, by vol.) and destained with frequent changes of the same solution.

### Phosphorescence decay measurements

Phosphorescence decay in fluid solution at room temperature was monitored with a home-made apparatus suitable for lifetime measurements in the microsecond–millisecond range [30]. The sample, placed in a vacuum-proof quartz cuvette that allowed excitation of the solution, was extensively deoxygenated before analysis. The procedure for the measurements of phosphorescence decays were as described elsewhere [28]. All of the phosphorescence decay signals were digitized and averaged by a computerscope system (EGAA; RC Electronics). Subsequent analysis of decay curves in terms of discrete exponential components was performed by a non-linear least-squares-fitting algorithm, implemented by the program Global Analysis. All the data reported were averages obtained from three or more independent measurements.

For each sample, phosphorescence decay was measured three times and samples were prepared at least four times. The standard error of pre-exponential terms and lifetimes components are better than $\pm 10\%$. It should be noted, however, that the variability of these parameters can be even somewhat greater when one compares different preparations of the protein. Such variability in the decay kinetics can be traced to different amounts of quenching impurities present in buffers and glassware [31]. For this reason, comparisons are always made between samples obtained from the same enzyme preparation.

## RESULTS

### Effect of HNB on the ATP hydrolytic activity of both membrane-bound and soluble purified mitochondrial $F_1$-ATPase

The ATP hydrolytic activity of SMP (i.e. particles containing $IF_1$) and ES-SMP (i.e. $IF_1$-free particles) was assayed in the presence of increasing HNB concentration up to 1 mM (Figure 2A). The SMP activity was first stimulated to a maximum of 30% above the control value at nearly 0.2 mM reagent, then it progressively decreased in a concentration-dependent manner. The ATP hydrolysis rate of ES-SMP particles was markedly different; this showed a steady and progressive decrease as the HNB concentration increased.

To rule out the possibility that the reagent could have enhanced the ATP hydrolysis rate by modifying the Trp residue of the $F_0$ polypeptides, and to verify whether a covalent binding of the reagent had occurred with the $F_1$ moiety of the enzyme, extremely pure $F_1$ either containing or lacking $IF_1$ was prepared and incubated with HNB.

Figure 2(B) shows the concentration-dependent effect of HNB treatment of $F_1$ either lacking or containing endogenous $IF_1$. The ATP hydrolytic activity of the $IF_1$–$F_1$ complex was progressively enhanced with HNB concentration up to 0.2 mM, where a maximum of 25% above the control value was reached. A concentration-dependent decrease in the ATPase activity occurred as the reagent concentration increased above 0.2 mM. The preparation of $F_1$ devoid of the inhibitor did not show any enhancement of activity at low HNB concentration, behaving similarly to the ES-SMP. However, a difference was observed between the two inhibitor-depleted preparations; in fact HNB below 0.5 mM did not significantly affect the ATPase activity of isolated $F_1$, whereas it inhibited the activity of $IF_1$-depleted particles. The inhibition of the ATPase activity at relatively high HNB concentration has been ascribed to non-specific adsorption of the reagent on the enzyme [17], possibly showing a detergent-like effect [32,33]. Therefore it is likely that non-specific interactions of the reagent can inhibit the catalysis of the $F_1$ complex to a greater extent when it is bound to the membrane sector than when it is in the soluble form. Incidentally, to rule out the possibility that the binding of HNB did not dissociate any subunit of $F_1$, or $IF_1$ itself, filtration of the HNB-modified enzyme on Sephacryl S-300 was performed. As a result, a single well-defined peak of protein was eluted (results not shown).

The most important feature of this functional study is that the ATPase activity of the $IF_1$–$F_1$ complex treated with a low

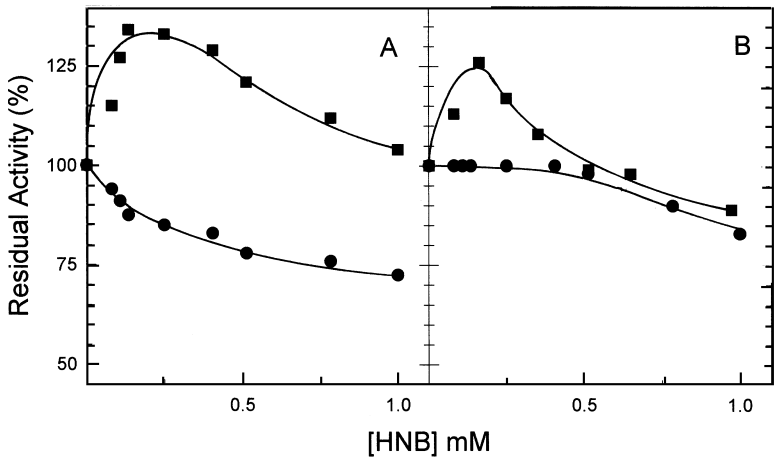


**Figure 2 Effect of HNB on the ATP hydrolytic activity of both submitochondrial particles (A) and $F_1$ (B) either containing (■) or lacking (●) $IF_1$**

The reaction of HNB with either SMP or ES-SMP was accomplished in 0.25 M sucrose/1 mM EDTA/0.2 M Tris/HCl (pH 6.7). After 2 min of reaction in the dark at room temperature, the pH was brought to 7.4 and the particles were then spun down and resuspended; finally 20 $\mu$g of the particles was used to assay the ATPase activity. The specific activities of control SMP and ES-SMP were 1.2 and 3.5 units/mg of protein respectively. The reaction of HNB with the purified enzyme was accomplished under the same conditions used to treat the particles. Soon after the 2 min incubation with the reagent, the pH of each reaction mixture was brought to 7.4 and aliquots of 5 $\mu$g of protein were used to assay the ATPase activity. The specific activity of the $IF_1$–$F_1$ complex was nearly 30 units/mg of protein and that of $IF_1$-free $F_1$ was above 80 units/mg of protein at room temperature.

**Table 1 Binding of HNB to $F_1$-ATPase in the absence and presence of $IF_1$**

The purified enzyme (4 $\mu$M), either in the presence or in the absence of $IF_1$, was treated with 0.2 mM HNB in 0.25 M sucrose/1 mM EDTA/0.2 M Tris/HCl (pH 6.7). Excess reagent was removed by repeated cycles of ultrafiltration on a Centricon-30 microconcentrator (Amicon) and dilution with 0.25 M sucrose/1 mM EDTA/25 mM Tris/HCl (pH 7.4) followed by gel filtration through Sephadex G-25. The concentration of covalently bound HNB was estimated spectrophotometrically at 410 nm ($\epsilon$ 13.5 $mM^{-1} \cdot cm^{-1}$). Numbers in parentheses indicate the number of determinations.

| Enzyme preparation | ATPase activity ($\mu$mol/min per mg) | HNB bound (mol/mol) |
|---|---|---|
| $IF_1$–$F_1$ complex | 32 ± 4 (5) | 0.62 ± 0.10 (3) |
| $IF_1$-free $F_1$ | 85 ± 5 (5) | 0.08 ± 0.01 (2) |
| $IF_1$-free $F_1$ + $IF_1$ | 10 ± 2 (2) | 0.55 ± 0.11 (2) |

concentration of HNB (below 0.5 mM) was enhanced, suggesting that HNB can affect $IF_1$ inhibition to some degree.

### HNB binding to $F_1$ in the presence and in the absence of $IF_1$

It has been shown that the target of HNB on crude $F_1$ preparations (i.e. with $IF_1$ present) is the Trp residue of the $\epsilon$ subunit [17]. To verify whether the inhibitory protein has a role in the binding of HNB to $F_1$, the stoichiometry of bound HNB to $IF_1$–$F_1$ and to $IF_1$-free $F_1$ was determined.

Results presented in Table 1 show that 1 mol of bovine heart $F_1$ bound tightly more than 0.6 mol of HNB when the reagent was added to the enzyme at low concentration, provided that the inhibitor protein was present. This tightly bound HNB could not be removed by a variety of procedures including $(NH_4)_2SO_4$ precipitation, filtration on a Sephadex G-25 column, repeated cycles of filtration and dilution under high and low ionic strength, and dialysis. In addition, attempts to extract this tightly bound HNB with chloroform failed. Because bound HNB could not be removed by these procedures, it must be covalently bound to the Trp of the $\epsilon$ subunit as previously demonstrated [17]. In the absence of $IF_1$ the covalently bound HNB-to-$F_1$ ratio is almost nil; therefore one has to conclude that HNB binds covalently to the $\epsilon$-Trp of $F_1$ exclusively in the presence of the inhibitor protein.

Because the $F_1$ molecule contains one Trp residue, one could have expected an HNB-to-$F_1$ ratio near 1; however, this did not occur, possibly because the rate of HNB reaction with the $F_1$ Trp is too slow with respect to the hydrolysis rate of the reagent in aqueous media [26]. Considering that only the fraction of enzyme molecules associated with the inhibitor could react with HNB, a second possibility might be that in our enzyme preparations, $IF_1$ was substoichiometric with respect to $F_1$, as inferred by both the relatively high ATPase activity of the $IF_1$–$F_1$ preparations (Table 1) and the lower intensity of the $IF_1$ band with respect to the $\delta$-subunit of $F_1$ in the SDS/PAGE slab gel (Figure 1). However, preincubation of excess $IF_1$ with $IF_1$-free $F_1$ under conditions of maximal binding (the ATPase activity was markedly decreased) did not result in an HNB-to-$F_1$ ratio of 1, suggesting that this possibility could be ruled out. A third hypothesis might be that the Trp-bearing polypeptide was substoichiometric in our enzyme preparations, as has been supposed by Abrahams et al. [34] in their own crystallized $F_1$. However, this possibility is unlikely because densitometric analysis of protein bands of our $F_1$ preparation after SDS/PAGE [14] revealed an 80 % volume of the $\epsilon$ subunit with respect to its expected volume on the basis of the subunit's molecular mass reported by Walker et al. [3] and on the basis of a 3:3:1:1:1 subunits stoichiometry.

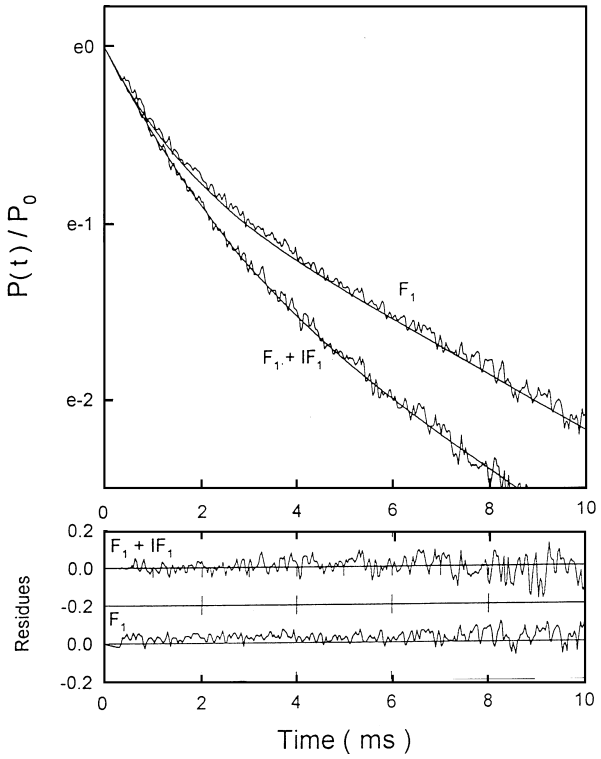


**Figure 3 Effect of $IF_1$ binding on $F_1$ phosphorescence decay at 20 °C**

Both samples, the control (2 $\mu$M $IF_1$-free $F_1$) and 2 $\mu$M $IF_1$-free $F_1$ with 5 $\mu$M $IF_1$ added, were in 50 mM Mops (pH 6.7)/2 mM $MgCl_2$/1 mM ATP; $\lambda_{ex} = 295$ nm; $\lambda_{em} = 440$ nm. Experimental data were fitted with the biexponential equation $P(t) = \alpha_1 e^{-t/\tau 1} + \alpha_2 e^{-t/\tau 2}$. The average lifetime ($\tau_{av}$) was obtained as $\tau_{av} = \Sigma \alpha_i \tau_i$. The plot of residues, shown in the lower panel, displays the differences between experimental data and fitting results. Abbreviations: e0, 1; e−1, 0.1; e−2, 0.01.

Thus the present binding data confirm and support previous observations, where it was shown that HNB binds covalently to $F_1$ in the presence of $IF_1$ [17]. In its absence, and this is first described here, the covalent binding of HNB to isolated $F_1$ is almost zero, suggesting that the conformation of the $\epsilon$ subunit allows the access of HNB to the Trp residue only when $IF_1$ is bound to $F_1$.

### Effect of the inhibitor protein on the phosphorescence decay of $F_1$

The luminescence of aromatic amino acids in proteins can provide unique structural information on the macromolecule. In particular, the rate of phosphorescence decay is a very sensitive indicator of the physicochemical properties of the Trp microenvironment of mitochondrial $F_1$; therefore it provides information on the structure of the polypeptide around it [28]. Figure 3 shows a typical phosphorescence decay curve of purified $IF_1$-free $F_1$ either with or without preincubation with $IF_1$ at 20 °C under conditions of maximal binding [23]. Both of the decays seem heterogeneous, as discussed earlier [28], and the presence of the inhibitor increases the phosphorescence decay rate. The average phosphorescence lifetime ($\tau_{av}$) in the presence of the inhibitor decreases by more than 25 % from 3.9 to 2.9 ms (Table 2). The correlation between $\tau_{av}$ and local viscosity [35] indicates

**Table 2 Intrinsic phosphorescence lifetime of $F_1$ in the presence of $IF_1$**

Data obtained in a typical observation are reported. Experimental conditions and parameters are detailed in the legend to Figure 3. The phosphorescence decay parameters of the control were the same at both pH values.

| Sample | $\tau_1$ (ms) | $\tau_2$ (ms) | $\alpha_1$ | $\alpha_2$ | $\tau_{av}$ (ms) |
|---|---|---|---|---|---|
| Control | 1.0 | 6.4 | 0.47 | 0.53 | 3.9 |
| + $IF_1$ (pH 6.7) | 1.3 | 5.4 | 0.61 | 0.39 | 2.9 |
| + $IF_1$ (pH 8.0) | 0.9 | 6.6 | 0.50 | 0.50 | 3.7 |

that the formation of the $IF_1$–$F_1$ complex results in an increase in flexibility of the $\epsilon$ subunit. Addition of $IF_1$ to $F_1$ at pH 8, a condition that does not correspond to a functional binding of the proteins [23], did not result in any significant change in the $F_1$ intrinsic phosphorescence decay parameters, clearly indicating the need of a functional $IF_1$ binding to $F_1$ to observe an effect of the inhibitor on the conformation of the $\epsilon$ subunit.

## DISCUSSION

The results of the experiments described here show that a relationship between $IF_1$ and the $\epsilon$ subunit of the mitochondrial $F_1$ complex exists. It cannot be conclusively established at present whether the relationship has a functional implication or whether it is merely an unspecific transmission of conformational changes between different domains of the protein, although the data more probably support the first hypothesis. It has been clearly shown that the binding of $IF_1$ to $F_1$ affects the conformation of the $\epsilon$ subunit of mitochondrial $F_1$. In fact the Trp reagent HNB binds covalently to the $\epsilon$ subunit of the purified enzyme exclusively when the inhibitory protein is associated with the complex; moreover, the addition of $IF_1$ to $F_1$ previously lacking $IF_1$ induces conformational changes in the $\epsilon$ subunit, as revealed by an increase in the protein phosphorescence decay rate.

It has been reported by several authors that the small subunits of mitochondrial $F_1$ complexes are strongly associated [36–38]; the binding of the inhibitor to $F_1$ might loosen their interaction, making the Trp residue accessible to HNB. This is supported by the observation of an increased flexibility of the N-terminal segment of the $\epsilon$ subunit when $IF_1$ binds to the enzyme (Figure 3). A reverse relationship (i.e. a perturbation of the $\epsilon$ subunit changes the action of the inhibitory protein) seems also to exist because modification of the $\epsilon$-Trp residue likewise affects the inhibitory function of $IF_1$, suggesting that conformational changes transmitted from the $\epsilon$ subunit to $IF_1$ and/or to its binding domain on $F_1$ have a role in modulating the ATPase activity of the enzyme. It should be of interest to determine whether the above conformational changes are transmitted directly through a physical interaction of the two polypeptides or indirectly via other $F_1$ subunits. The inhibitory protein binds to the $\beta$ subunit of the bovine enzyme [8]; in related systems such as $F_1$ of both pig and yeast it seems unlikely that it binds to the low-molecular-mass polypeptides of the enzyme ($\gamma$, $\delta$ and $\epsilon$ subunits) [39,40] because the inhibitory protein did not cross-link with them. However, when cross-linked products are found, one can consider the cross-linked polypeptides to be closely associated but cannot rule out the possibility that other, non-cross-linked, polypeptides in fact interact with each other. Moreover, Harris [41] and Van Raaij et al. [42] have recently suggested that residues 10–17 and 10–13 respectively of the inhibitor might be involved in the stabilization of the $F_1$–$IF_1$ complex via some secondary interaction. Whether $IF_1$ and the $\epsilon$ subunit interact *in situ* can only be a matter for speculation until a crystal structure of the $IF_1$–$F_1$ complex is available.

The functional implications of the $\epsilon$ subunit conformational changes induced by $IF_1$ binding to $F_1$ can at present only be speculative: these changes might contribute to the regulation of the $F_0F_1$–ATPase complex *in vivo*. On binding of $IF_1$ to $F_1$, the $\epsilon$ subunit undergoes conformational changes that in turn might be transmitted to $F_0$ components to inhibit (or contribute to inhibiting) $H^+$ transport. This is consistent with the location of the $\epsilon$ subunit in the stem region of the $F_1$ complex [16] and it might contribute to envisaging a molecular mechanism to explain several observations reported in the literature. Guerrieri et al. [43] showed that $IF_1$ not only inhibits ATP hydrolysis but also inhibits the passive proton conductivity of the $F_0F_1$ complex. These authors suggested that the inhibitory protein could be involved in the gate of the $H^+$-ATPase. Vàzquez-Contreras et al. [44] showed that the $F_0F_1$–$IF_1$ complex reconstituted in vesicles is capable of a tighter coupling than $IF_1$-devoid $F_0F_1$ between hydrolytic and synthetic reactions, and suggested that $IF_1$ could act as a coupling device that increases the efficiency of energy transduction. The $\epsilon$ subunit might also be involved in the reverse process: polypeptides of the $F_0$ sector (or stalk) might change their conformation on de-energization of the membrane; this change might in turn be transmitted through the $\epsilon$ subunit to the $IF_1$-binding domain of $F_1$, contributing to the functional binding of the inhibitor. This might explain both the functional binding of $IF_1$ to $F_1$ after a decrease in membrane potential [10] and the sensitivity of $IF_1$ to the phospholipid microenvironment of the membrane $F_0$ sector [11].

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