Fluorescence resonance energy transfer between coumarin-derived mitochondrial F_1 -ATPase γ subunit and pyrenylmaleimide-labelled fragments of IF_1 and c subunit

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We introduced a reporting group into a critical position of the mitochondrial F_1 -ATPase in order to gain structural information about enzyme–ligand complexes. Incubation of 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) with bovine heart mitochondrial F_1 -ATPase pretreated with 1 mM sodium arsenite modified the only cysteine residue in the γ subunit (γ -Cys⁷⁸), resulting in an enzyme–CPM fluorescent complex (CPM– F_1) with an ATPase activity similar to that of the native enzyme. Transferred fluorescence of F_1 -bound CPM occurred when different peptide fragments of naturally binding polypeptides carrying a pyrenylmaleimide (PM) moiety were bound to the enzyme. Fluorescence resonance energy transfer (RET) from PM bound to cysteine residues associated with Glu⁴⁰, Lys⁴⁷ and Lys⁵⁸ of fragments of the inhibitor protein (IF_1) with CPM– F_1 occurred with an efficiency of approx. 20, 21 and 3 %

respectively. The distance at which the efficiency of energy transfer was 50 %, R_0 , for the CPM and PM donor/acceptor pair was 4.1 nm, indicating that the three IF_1 fragments must be within 6.7 nm of γ -Cys⁷⁸. RET from the PM-bound hydrophilic fragment of c subunit (residues 37–42) of the F_1F_0 -ATPase complex and CPM-bound γ -Cys⁷⁸ occurred with an efficiency of approx. 30 %, indicating a distance of 4.7 nm between the two fluorophores. Based on previous observations and on the present RET measurements, the hydrophilic loop of c subunit was located at the base of the F_1 foot, and the N-terminal region of IF_1 was located on the surface of F_1 in the lower part of the $\alpha_3\beta_3$ hexamer ring.

Key words: F_1F_0 -ATPase, F_1 – IF_1 complex, mitochondria, thiol modification.

INTRODUCTION

Considerable progress has been made in elucidating the structure and mechanism of catalysis of the ubiquitous H^+ -translocating F_1F_0 -ATPase. In mitochondria, chloroplasts and eubacteria, the enzyme consists of a transmembrane proton-transporting domain, F_0 , and a catalytic domain bound at the membrane surface, termed F_1 . The composition of this water-soluble domain is $\alpha_3\beta_3\gamma\delta\epsilon$ [1,2]. The structure of F_1 from bovine mitochondria has been solved at the atomic level by X-ray crystallography [3,4], and remarkable progress has been made in understanding the mechanism of ATP synthesis by a binding-change mechanism involving rotary catalysis [5–7]. The isolated F_1 hydrolyses ATP and induces γ subunit rotation within a hexameric ring of $\alpha_3\beta_3$ subunits, with rotation driven by conformational changes in the catalytic subunits [8]. γ subunit rotation in F_1 should be transmitted to the membrane sector, F_0 , in order to couple the ATP hydrolysis-dependent proton transport by the F_1 – F_0 complex. The detailed mechanism underlying the energy transmission between the γ subunit and F_0 remains unknown, although it has been suggested that γ and ϵ subunits (*Escherichia coli* nomenclature) are involved, since the two subunits may interact with residues in loop regions of the c-ring belonging to F_0 [4]. In eukaryotes, the hydrolytic activity of the enzyme is regulated by a natural inhibitor protein, IF_1 , an 84-residue protein in bovine mitochondria, which is believed to bind and inhibit the enzyme when the pH inside the mitochondria falls [9,10]. IF_1 , and synthetic fragments of it, bind strongly to the ATP synthase with a 1:1 stoichiometry, and more loosely to isolated F_1 [11–13].

Very few experimental reports are available as to the location and mode of binding of IF_1 to F_1 . Certainly, crystallographic studies of the IF_1 – F_1 complex could provide important details, but the complex has not been crystallized as yet, and these studies are difficult to perform over a broad range of experimental conditions. Furthermore, the process of crystallization selects typically only one family of closely related conformers from a potentially diverse population of conformers in solution. Hence, to complement possible X-ray diffraction studies and to provide information regarding F_1 and F_1 – IF_1 complex conformers, the introduction of a fluorescent probe into the domain of the γ subunit that protrudes out of the $\alpha_3\beta_3$ hexagonal ring may be of significant help. Moreover, subtle structural changes and dynamic features of both the catalytic mechanism and its regulation may be achieved.

Bovine heart mitochondrial F_1 -ATPase contains a total of eight half-cystines [2] located at positions 201 and 254 in each α subunit, 78 in the γ subunit and 18 in the ϵ subunit [2]. The cysteine residue of the γ subunit (γ -Cys⁷⁸), located at the interface with the catalytic β subunit and in proximity with the small δ and ϵ subunits [4,14], is an attractive target for being loaded with a reporting probe of F_1 .

7-Diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) is a fluorescent probe, which can be used as a valid candidate to modify F_1 SH groups. Furthermore, it can be used as a fluorescent acceptor in fluorescence resonance energy transfer (RET) measurements in which a pyrene derivative, *N*-(1-pyrenyl)-maleimide (PM), associated with the enzyme in critical positions, acts as the fluorescence donor. The CPM moiety serves as a point

Abbreviations used: CPM, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; γ -Cys⁷⁸, Cys⁷⁸ of the γ subunit; CPM– F_1 , CPM covalently bound to the γ -Cys⁷⁸ of F_1 ; IF_1 , natural inhibitor protein of the mitochondrial F_1F_0 -ATPase; PM, *N*-(1-pyrenyl)maleimide; RET, resonance energy transfer.

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of reference enabling the site of the relative PM-modified residues to be determined.

In the present study, we explore the reactivity of CPM with the bovine F_1 complex, describe the method used to selectively label γ -Cys⁷⁸ and examine the kinetic and stability properties of the modified enzyme. Moreover, we report results of fluorescence RET experiments between γ -Cys⁷⁸-bound CPM and a variety of PM-labelled peptides chosen among fragments of physiologically relevant ligands (IF₁ and c subunit) of the mitochondrial F_1 complex.

EXPERIMENTAL

Materials

The fluorescent thiol reagents CPM and PM were purchased from Molecular Probes (Eugene, OR, U.S.A.). Peptides corresponding to proposed binding domains of both bovine IF₁ [12,15,16] and the c subunit [17,18] were designated as I_{m-n} and c_{m-n}, where m and n are the initial and final residue numbers in the sequence of intact mature bovine IF₁ and c subunit respectively (Table 1). All synthetic peptides containing a C-terminal cysteine residue were supplied by Tecnogen (Caserta, Italy). ATP, phosphoenolpyruvate, Mops, Tris, NADH, pyruvate kinase and lactate dehydrogenase in glycerol-containing buffer were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Enzyme preparation

Bovine heart submitochondrial particles, prepared as described previously [19], were suspended in 10 mM Tris/HCl (pH 9.2) containing 0.25 M sucrose, 4 mM ATP and 1 mM EDTA, and were incubated at a protein concentration of 30 mg/ml for 8 h at 20 °C. The pH was then adjusted to 8.0 with 1 M HCl and crude F_1 -ATPase was released from the particles by chloroform treatment as described by Beechey et al. [20]. The aqueous phase was centrifuged at 105 000 *g* for 30 min at 20 °C and the resulting supernatant was precipitated by adding a 60 %-satd. (NH₄)₂SO₄ solution. After incubation for 10 min, the precipitates were centrifuged at 25 000 *g* for 15 min, and the pelleted enzyme was dissolved in 50 mM Tris/HCl (pH 8) containing 1 mM EDTA and 1 mM ATP. Further purification was achieved by two chromatography steps: 1) gel-filtration on Sephacryl S-300 (40 cm × 1.6 cm) using the above Tris buffer for elution; and 2) affinity chromatography on Blue Sepharose CL-6B (4 cm × 1 cm) equilibrated with 20 mM Tris/HCl (pH 8) containing 0.2 mM NaCl, 0.5 mM EDTA, 1 mM 2-mercaptoethanol and 1 mM ATP to remove minor contaminants from the F_1 preparation [2]. Finally, the enzyme solution was stored at 5 °C as a suspension in 60 %-satd. (NH₄)₂SO₄ containing 4 mM ATP (pH 8). Under this condition, the activity was stable for several weeks.

Chemical modification of F_1

A stock solution of CPM (2.5 mM) was prepared daily by reconstituting the compound in acetonitrile. The labelling of F_1 cysteine residues was carried out essentially as described by Gabellieri et al. [14], but with slight modification. Briefly, the enzyme (1–6 μ M) was incubated with a 5-fold excess of CPM in labelling buffer [20 mM Mops (pH 7)] in the dark for 1 h at 20 °C. Selective modification of the γ -Cys⁷⁸ on F_1 was achieved by preincubating the enzyme with 1 mM sodium arsenite at pH 8.8 as described previously [21]. Subsequently, after removing the excess sodium arsenite by adding a 60 %-satd. (NH₄)₂SO₄ solution, the enzyme was dissolved in the labelling buffer and the reaction with CPM was carried out as above. The unreactive

Table 1 Synthetic peptides

Underlined and bold residues in the amino acid sequences (single amino acid code) indicate helix and coil structures respectively, as predicted from JPRED², EMBL. C, cysteine.

Peptide	Sequence	Mass (Da)	Purity (%)
I ₁₀₋₄₀	SSAGAVRDAGGAFGK <u>REQAEEERYFR</u> AKE-C	3503.81	> 99
I ₁₀₋₂₇	SSAGAVRDAGGAFGK <u>REQ</u> -C	1869.2	> 99
I ₁₁₋₁₉	C-SAGAVRDAG	908.3	> 90.6
I ₁₃₋₁₉	GA <u>VRDAG</u> - C	747.83	> 99
I ₄₂₋₄₇	<u>LAALKK</u> -C	747.5	> 99
I ₄₂₋₅₈	<u>LAALKKH</u> HENEISHHAK - C	2067.0	100
C ₃₇₋₄₂	ARNPSL - C	761.7	96.7

probe was separated from the chemically modified protein by filtration and centrifugation through a Sephadex G-50 column equilibrated with labelling buffer. The CPM concentration of covalently bound reagent was estimated spectrophotometrically at 387 nm ($\epsilon = 30.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) [14].

Chemical modification of inhibitor and c subunit peptide fragments

A stock solution of PM (10 mM) was prepared daily by reconstituting the compound in dimethylformamide. PM derivatives of the peptide fragments were prepared by incubating each peptide (200 μ M) in the presence of a 5-fold excess of PM in 50 mM Mops (pH 6.7) containing 1 mM MgCl₂ overnight in the dark at 25 °C. Unreacted probe was removed by extraction with chloroform. The truncated IF₁ fragments (and the c subunit fragment) used did not aggregate, as determined by light scattering measurements (results not shown).

Steady-state fluorescence measurements

Fluorescence data were collected using a luminescence spectrometer LS50B (PerkinElmer). Emission spectra were recorded from 440–490 and 350–400 nm upon excitation at 390 and 342 nm for CPM and PM respectively.

For energy transfer studies, 1–3 μ M CPM- F_1 and variable amounts of labelled peptides (up to a 1:5 molar ratio) were mixed in 150 μ l of a buffer containing 50 mM Mops, 1 mM ATP and 2 mM MgCl₂ at either pH 6.7 or pH 7.5 for the inhibitor and c subunit fragments respectively. After incubation for 5 min in the dark at 20 °C, 100 μ l of the mixture was applied on to a Sephadex G-25 centrifuge column to separate excess peptide. The eluted solution was used directly in fluorescence measurements and subsequent determination of the protein concentration. Separation of excess PM-bound peptides could be omitted provided that careful subtraction of the contribution of excess PM fluorescence to CPM fluorescence (475 nm) was performed.

Energy-transfer efficiencies (E_T) were measured by comparing the fluorescence intensities of F_1 -bound CPM after the binding of donor-labelled peptides. Transferred fluorescence was evaluated as a function of mol peptide/mol F_1 and extrapolated to 1 mol/mol as described previously [14,22] in order to obtain maximal energy transfer, which was then used to calculate the distance, *r*, between donor and acceptor. As described by Forster [23]:

$$E_T = R_0^6 / (R_0^6 + r^6)$$

where R_0 is the distance for 50% energy transfer, which is evaluated to be 4.1 nm for the couple CPM/PM from absorption and corrected emission spectra of the protein-bound fluorophore, as described previously [14].

Enzyme activity

ATPase activity was determined with an ATP-regenerating system by following the decrease of NADH absorption at 340 nm in a Lambda 6 spectrophotometer (PerkinElmer) as previously reported [24]. Inhibition of IF_1 fragments was assayed as described by Solaini et al. [25]. Protein concentration was determined by the method of Lowry et al. [26] using BSA as a standard.

Electrophoresis analysis

SDS/PAGE on a 14% (w/v) polyacrylamide gel was carried out using a Bio-Rad Mini-Protein dual slab cell as described by Laemmli [27]. Proteins were detected by silver staining. Densitometric analysis was performed by scanning the gels and analysing the density pattern with gel software (Sigma).

RESULTS

Reactivity of F_1 with CPM

The F_1 preparations used in this study had a specific activity of 50–70 μ mol of ATP hydrolysed/min per mg of protein at 20 °C, and were free of IF_1 . The enzyme showed the typical pattern of five bands after SDS/PAGE separation of the subunits followed by silver staining (Figure 1, lanes a–c).

The labelling of F_1 (0.37–1 mg/ml) with 5–12 μ M CPM at pH 7 was relatively rapid over the first 10 min, and became gradually slower thereafter (Figure 2). Samples of the incubation mixture obtained at different times indicated that the probe was incorporated into two subunits, as revealed by the exposure of the modified enzyme to UV light following SDS/PAGE of the subunits under denaturing conditions (Figure 1, lanes d and e). The fluorescent bands corresponded to the α and γ subunits. Since both γ -Cys⁷⁸ and the reactive cysteine residue of the α subunit are accessible to the reagent, the residues are likely to be found in an aqueous environment that can be reached from the surface of the protein. Moreover, the ϵ subunit band was not fluorescent when excited to induce coumarin fluorescence

Figure 2 Time-course of CPM labelling of isolated mitochondrial F_1

The reaction was initiated by adding 3 μ M CPM to 1 μ M F_1 in 20 mM Mops (pH 7). At the times indicated, 50 μ l aliquots were diluted into 120 μ l of 20 mM Mops (pH 7) and the sample passed through a 1 cm $\times$ 6 cm Sephadex G-50 column to remove unbound CPM. The fluorescence of bound CPM was measured at 475 nm with excitation at 387 nm.

emission, indicating that the ϵ -Cys residue had not reacted with the coumarin maleimide. The lack of reactivity of the ϵ -SH was expected, since a previous study on ϵ subunit conformation [25], performed on the basis of analysis of intrinsic tryptophan phosphorescence, indicated that the N-terminal segment of the subunit, where the cysteine residue is present, is located in a hydrophobic environment within a tight, rigid core of the protein structure.

An estimation of the binding stoichiometry of CPM to F_1 was obtained by exploiting the fluorescence of CPM at 475 nm (λ_{ex} = 387 nm) upon the binding to F_1 ; unbound CPM has no appreciable fluorescence. Figure 3(a) shows that, at concentrations of CPM below that of F_1 (1 μ M), the titration curve is linear, suggesting that all the CPM added was bound to the enzyme. A plateau was reached at approx. 4 μ M CPM. An extrapolation of the linear portion of the titration curve (at low CPM concentrations) intersected an extrapolation of the plateau value at a CPM: F_1 molar ratio of approx. 2, indicating that there are two binding sites on F_1 for CPM under the above conditions. We also observed that incubation of the enzyme with the reagent in the presence of the substrates ATP or ADP (Mg^{2+} present) induced a slightly decreased fluorescence of both α and γ subunits, demonstrating that saturation of the nucleotide-binding sites of the enzyme results in hindered exposure of the α - and γ -SH groups to the external medium (results not shown).

Specific labelling of the subunit γ -SH with CPM

Since the α - and γ -SH groups had similar reactivities towards CPM, in order to selectively modify the γ -SH group of F_1 we induced the formation of a bridge between cysteine residues in the α subunit by preincubating the enzyme with arsenite, which reacts with vicinal thiols forming a dithioarsenite [21], followed by the reaction with the coumarin derivative. SDS/PAGE of the modified enzyme revealed a single fluorescent band corresponding to the γ subunit (Figure 1, lanes f and g).

The titration curve (Figure 3b) obtained by monitoring the fluorescence enhancement of CPM when it was added to the arsenite-treated F_1 shows a 1:1 molar ratio of CPM: F_1 , indicating that arsenite pretreatment reduced the number of CPM-binding sites on the enzyme to only one. This binding

Figure 1 Binding of CPM to F_1 as analysed by SDS/PAGE

Labelling was performed by incubating CPM (5 μ M) with untreated (lanes d and e) and arsenite-treated enzyme (1 μ M) (lanes f and g). Labelled protein (1–5 ng) was loaded on to a 14% (w/v) polyacrylamide gel. Following electrophoresis, the unstained gel was photographed under UV light to visualize fluorophores (lanes d–g) and subsequently silver stained (lanes a–c).

Figure 3 Titration of F_1 cysteine residues with CPM

The cell contained 1 μ M F_1 and the indicated concentration of CPM in 150 μ l of 20 mM Mops (pH 7) under the conditions described in the Experimental section. Relative fluorescence emission intensity of CPM was monitored at 475 nm upon excitation at 387 nm (**a**). (**b**) CPM was added to 1 μ M arsenite-treated F_1 and fluorescence emission intensity determined as above. Dotted lines indicate the linear extrapolation of the curves as described in the text.

Table 2 Time-dependent ATPase activity of CPM-labelled and unlabelled F_1

The enzyme (2 mg/ml) was incubated at 20 °C in 10 mM Tris/HCl (pH 7.5), 0.25 M sucrose and 1 mM EDTA and, at the indicated times, samples were withdrawn and tested for ATPase activity under steady-state conditions by adding 4 mM Mg-ATP to the reaction mixture.

Time (h)	ATPase activity (%)	
	F_1	CPM- F_1
0	100	100
1	109	105
2	98.5	98.5
3	95	92
4	90	94
5	87	87
6	85	85

stoichiometry was confirmed by comparing the protein concentration with that of bound CPM, as evaluated spectrophotometrically at 387 nm ($\epsilon_{\text{mM}} = 30.2$) after the removal of excess reagent.

According to the crystal structure of F_1 [3], γ -Cys⁷⁸ is located in a small α -helix segment interacting with the β_{TP} subunit. The β/γ interaction appears to be critical for enzyme catalysis, whereas the α subunit cysteine residues are located in protein domains of structural importance, but not thought to be involved in catalysis. Therefore it could be expected that modification of the γ -SH group would affect enzyme activity. Functional investi-

Figure 4 Spectral overlap between CPM and PM

The emission spectrum of PM bound to the I_{10-40} inhibitor segment is shown as a solid line. Acceptor absorption spectrum of CPM-labelled F_1 is shown as a broken line. In order to visualize the spectral overlap, the fluorescence spectrum has been normalized to fit the absorption spectrum magnitude and the overlap integral, and the resulting R_0 distance has been obtained as in [14]. Almost identical emission spectra for the other PM-bound protein fragments were recorded (results not shown).

gation of the CPM- F_1 complex showed that neither the reaction of the reagent with both α and γ subunits nor with γ -SH groups alone resulted in changes in the kinetic parameters. In a typical observation the following parameters were found: a K_m of 250 μ M for both the modified and unmodified enzyme; and a V_{max} of approx. 60 μ mol/min per mg of protein ($k_{\text{cat}} = 371 \text{ s}^{-1}$) for the untreated enzyme and 55 μ mol per min/mg ($k_{\text{cat}} = 340 \text{ s}^{-1}$) for the CPM- F_1 complex. The stability of the CPM- F_1 complex was also similar to that of the native enzyme. Table 2 shows the time-dependent ATPase activity of both the untreated and γ Cys-modified enzyme at 20 °C. Full catalytic efficiency was preserved for approx. 2 h; afterwards, a significant time-dependent decay in activity occurred, but it did not exceed 15% after 6 h for both enzyme preparations. However, it has to be noted that, whereas the native enzyme can be stored as an ammonium sulphate precipitate, the modified enzyme quickly loses activity in the precipitate, suggesting that the bulky and relatively hydrophobic coumarin derivative might irreversibly bind to amino acid residues upon exposure to high-ionic-strength media where hydrophobic interactions are favoured.

Fluorescence RET between γ subunit-bound CPM and PM-labelled inhibitor peptides bound to F_1

The very high specificity for cysteine residues and the spectral properties of pyrene make it a useful probe for fluorescence RET measurements. The emission spectrum of PM overlaps the absorption spectrum of CPM, resulting in a donor-acceptor pair having an R_0 of 4.1 nm (Figure 4). Because of these features, if PM is in proximity to CPM, RET is possible from PM to CPM, causing a quenching of PM fluorescence and an enhancement of CPM fluorescence. Several potential ligands of F_1 were used to carry the PM fluorophore in critical positions on the F_1 surface. Labelling of I_{F_1} amino acid segments (I_{10-40} , I_{10-27} , I_{13-19} , I_{11-19} , I_{42-47} and I_{42-58}) with PM at their terminal cysteine residues (Table 1) was carried out overnight at pH 6.7 to favour the reaction of maleimide with the SH groups. Excess reagent was removed to avoid its possible reaction with nucleophilic groups of F_1 . Virtually all of the peptide molecules were completely labelled with PM by this procedure.

The PM-labelled peptides were assayed for inhibition of F_1 . Only PM- I_{10-40} reduced the ATPase activity to 80% of the

Table 3 Fluorescence RET between PM-peptides and CPM- F_1

r , distance (in nm) between donor and acceptor; E_T , energy-transfer efficiency. n.d., not detectable.

Peptides	Parameters		
	E_T (%)	r (nm)	F_1 -ATPase inhibition (%)
I ₁₀₋₂₇	n.d.	—	n.d.
I ₁₀₋₄₀	21	5.1	20
I ₁₁₋₁₉	n.d.	—	n.d.
I ₁₃₋₁₉	n.d.	—	n.d.
I ₄₂₋₄₇	20	5.2	n.d.
I ₄₂₋₅₈	3	6.7	n.d.
C ₃₇₋₄₂	30	4.7	n.d.

Figure 5 Titration of coumarin-labelled F_1 with the PM-labelled I₁₀₋₄₀ inhibitor segment

Fluorescence of CPM- F_1 ($\lambda_{ex} = 342$ nm) was measured in different samples with increasing molar ratio of PM-labelled inhibitor fragment over CPM- F_1 . Unlabelled F_1 and isolated PM peptide contributions were subtracted. The numbers in the spectra indicate the molar ratio of PM-I₁₀₋₄₀ to F_1 (1 μ M).

control, whereas none of the other peptides significantly inhibited the enzyme (Table 3).

Upon incubation of the peptides with CPM-bound F_1 , fluorescence intensity ($\lambda_{ex} = 342$ nm) of PM associated with both I₁₀₋₄₀ and I₄₂₋₄₇ decreased (results not shown). In parallel,

transferred fluorescence due to sensitized CPM was observed. Figure 5 shows the emission spectra ($\lambda_{ex} = 342$ nm) of CPM- F_1 when the enzyme was exposed to increasing PM-I₁₀₋₄₀ concentrations.

A concentration-dependent study of the efficiency of energy transfer was quantified (Table 4). With PM-I₁₀₋₄₀, a maximal efficiency of 20 % was observed at 9 μ M, whereas with PM-I₄₂₋₄₇ a maximal efficiency of 21 % was found at 10 μ M. The distance between CPM-bound γ -Cys⁷⁸ and PM bound to the inhibitor segments was measured at peptide concentrations inducing maximal efficiency. The results are shown in Table 3. The r values were calculated as 5.1 and 5.2 nm for the I₁₀₋₄₀ and I₄₂₋₄₇ peptides respectively. I₄₂₋₅₈ showed a very low RET with a maximal efficiency of 3 % and an r of 6.7 nm.

When the same experiments were performed with PM bound to I₁₁₋₁₉, I₁₃₋₁₉ or I₁₀₋₂₇ as the fluorescence donor, no energy transfer was observed. This was due to the lack of binding between the inhibitor segments and F_1 under the conditions used (results not shown). However, one cannot exclude that, at higher concentrations, the fragments could bind to F_1 , but at higher concentrations used in the fluorescence RET experiments, values could not be reliably interpreted.

Fluorescence RET between the γ subunit-bound CPM and PM-labelled c subunit polar loop bound to F_1

The electron density map of crystals of the yeast F_1 -c₁₀ complex has recently been reported [28]. In this immobilized system, the polar loop of six or seven c subunits of F_0 interact with δ and γ subunits of F_1 , lying at distances of approx. 5 nm from the base of the $\alpha_3\beta_3$ hexagonal ring. To determine the distance between the F_1 -bound polar loop region of c subunits with respect to γ -Cys⁷⁸ of F_1 in solution, fluorescence RET was used. The addition of increasing amounts of PM-labelled c subunit segments to CPM- F_1 revealed increased fluorescence upon excitation at 342 nm originating from F_1 -bound CPM (Table 4). A maximum of fluorescence intensity was achieved at approx. 8 μ M PM-peptide added, and the best fitting ($R^2 = 0.993$) of the data was obtained by an hyperbola (results not shown). This provides strong evidence for energy transfer between a donor-acceptor couple, from which a donor-acceptor separation was calculated as 4.7 nm.

It should be noted that all of the PM-peptides bound to F_1 gave distances close to R_0 , where errors in E_T led to small errors in distance.

Table 4 CPM- F_1 fluorescence enhancement by bound PM-peptides

In typical experiments, CPM- F_1 was 1 μ M in 50 mM Mops (pH 6.7) and 2 mM MgCl₂. Phe³⁴² (F^{342}) and Phe³⁸⁷ (F^{387}) are the coumarin fluorescence intensities ($\lambda_{em} = 475$ nm) of CPM- F_1 /PM-peptides obtained using $\lambda_{ex} = 342$ and 387 nm respectively. Unlabelled F_1 and isolated PM-peptide contributions were subtracted. F_0^{342} and F_0^{387} are the fluorescence intensities of isolated CPM- F_1 with unlabelled F_1 subtracted. Results are expressed as the ratios of the fluorescence intensities obtained.

[PM-I ₁₀₋₄₀]/ [CPM- F_1]	(F_0^{342}/F_0^{387})/ (F^{342}/F^{387})	[PM-I ₄₂₋₄₇]/ [CPM- F_1]	(F_0^{342}/F_0^{387})/ (F^{342}/F^{387})	[PM-I ₄₂₋₅₈]/ [CPM- F_1]	(F_0^{342}/F_0^{387})/ (F^{342}/F^{387})	[PM-C ₃₇₋₄₂]/ [CPM- F_1]	(F_0^{342}/F_0^{387})/ (F^{342}/F^{387})
1.19	1.19	0.75	0.83	0.62	1.01	0.71	1.07
2.4	1.34	1.26	1.13	1.2	1	1.4	1.18
3.6	1.5	2.24	1.2	2.4	0.98	2.1	1.36
5.4	1.64	3.48	1.31	3.06	0.98	2.81	1.52
6.7	1.72	4.48	1.39	4.31	1.06	3.47	1.64
7.8	1.79	6.02	1.47	5.5	0.94	4.92	1.84
8.9	1.81	7.06	1.53	8	1.16	6.28	1.98
10.2	1.83	8.54	1.8	11.23	1.2	7.7	2.19
		12.5	1.86			11.22	2.22

DISCUSSION

Important structural information on the mitochondrial F_1F_0 -ATPase, necessary to fully understand the subtle functional mechanism of coupling F_1 to F_0 and the regulation of enzyme activity, are still elusive. Among these, the exact location of the proteins bound to F_1 under physiological conditions, including IF_1 and c subunit, are of primary interest. By means of fluorescence RET using CPM as a reference group bound to a well defined and critical position of the γ subunit, γ -Cys⁷⁸, its distance from the fluorophore PM, bound to both IF_1 fragments and the hydrophilic loop of c subunit, has been calculated.

Arrangement of the subunits in mitochondrial F_1 is derived from X-ray crystallography data [3]. The structure of the F_1 - IF_1 complex can be inferred from results of cross-linking reactions and topological considerations on the basis of both prediction and functional studies [9,29,30]. Recent X-ray diffraction studies [3,4] revealed the whole structure of the bovine mitochondrial F_1 , as shown schematically in Figure 6. The γ

Figure 6 Three-dimensional structure of bovine mitochondrial F_1 -ATPase

The α , β , γ , δ and ϵ subunits are yellow, blue, green, orange and black respectively. Part of the c subunit is indicated in blue at the bottom of the Figure. I_{10-59} is shown in red. The black circle includes the β DELSEED segment and the small yellow circle within indicates Lys²⁴ of the inhibitor protein. The red circles represent cysteine residues on the inhibitor protein and the γ and c subunits. Graphic representation of the inhibitor protein segment is on the basis of the SWISS-PDB Viewer v. 3.7b2 program, and structure prediction was from JPRED², EMBL. These figures were prepared with the graphics program Explorer 1.85 Beta (www.proteinexplorer.org) using atomic co-ordinates and structure factors deposited in the Brookhaven Protein Data Bank for both bovine mitochondrial F_1 -ATPase (reference 1E79) and subunit c (reference 1A91).

subunit forms a left-handed coiled-coil which runs along the axis of pseudosymmetry of the $\alpha_3\beta_3$ subcomplex, protruding from it by about 5 nm and, in association with both the δ and ϵ subunits, forms the central stalk connecting F_1 to the membrane bound sector F_0 . Each α subunit contains two vicinal SH groups (Cys²⁰¹ and Cys²⁵⁴), and the γ subunit has an SH group in the short inclined helix of the polypeptide. The eighth cysteine residue is at position 18 of the ϵ subunit.

In the first result of the present study, we found that, among the eight SH groups, only two are accessible to CPM: one in the α subunit and the other in the γ subunit. Most importantly, we found that pretreatment of the protein with arsenite induced the modification of the only γ subunit SH group (Figure 1).

The presence of energy transfer between CPM bound to γ -Cys⁷⁸ and PM bound to c-Cys⁴³ demonstrates directly the interaction of the hydrophilic loop region of the c subunit with F_1 . It shows that in solution the mitochondrial F_1 -ATPase complex binds to the polar loop of the F_0 c subunit at a distance of 4.7 nm from γ -Cys⁷⁸, thus at the base of the foot of F_1 comprising part of the γ , δ and ϵ subunits (Figure 6). Under our experimental conditions, only one c loop bound F_1 , but it is believed that several c subunits interact with the F_1 δ and γ subunits in the complete F_1F_0 -ATPase complex [28]. In our model, the c subunit polar loop has been located in contact with the δ subunit; more precisely, δ -Glu¹³⁹ has tentatively been suggested to associate with c-Arg³⁸, as the two amino acids could form a polar interaction. However, other locations on the surface of the δ or γ subunits, at approx. 4.7 nm from CPM at the foot of F_1 , could be possible. Our findings are in general agreement with data from X-ray crystallographic studies on the F_1 -c₁₀ subcomplex of yeast F_1F_0 -ATPase [28], indicating that mitochondrial F_1 in its active form in solution has the foot at a similar distance from the $\alpha_3\beta_3$ domain as that found in crystals.

The γ -Cys⁷⁸-bound fluorescent coumarin could also be used to gather information on the location of regions of IF_1 when bound to F_1 at acidic pH. Our RET studies, combined with previous studies of cross-linking between IF_1 and F_1 by Jackson and Harris [31], have provided the model of the I_{10-59} - F_1 complex as shown in Figure 6. The model locates the positively charged residues Lys²⁴, Arg²⁵ and Gln²⁷ of IF_1 on the surface of F_1 in close proximity with the negative microenvironment of the DELSEED region (residues 394–400; single amino acid code) of the β_E subunit, and extends the helical segment of IF_1 outward from the γ subunit. This results in the amino acid residues at positions 41 and 48 of IF_1 being a distance of 5.1 and 5.2 nm respectively from γ -Cys⁷⁸. Other models are unlikely. Orientation of the IF_1 helix in directions other than that chosen would result in both Cys⁴¹ and Cys⁴⁸ of IF_1 being much closer to γ -Cys⁷⁸ than the 5.1 and 5.2 nm distances measured. Moreover, of the three β subunits, only the binding of IF_1 to β_E is consistent with the distances found in our experiments between the C α of γ -Cys⁷⁸ and C α of β subunits Asp³⁹⁴. Indeed, from the X-ray structure of F_1 , the distances between γ -Cys⁷⁸ C α and of β_E , β_{DP} and β_{TP} Asp³⁹⁴ C α are 2.8, 1.3, and 1.1 nm respectively. Since the helical segment of IF_1 extends nearly 2.8 nm (Lys²⁴ C α to Cys⁴¹ C α) along the helical axis, the C α of the IF_1 Cys⁴¹ bound to β_E , β_{DP} , and β_{TP} would give a separation of 5.6, 4.1 or 3.9 nm respectively from γ -Cys⁷⁸ C α . Therefore the distances between Cys⁴¹ of IF_1 and either Asp³⁹⁴ of β_{DP} or β_{TP} appear to be too short with respect to the distances measured in our experiments. The location of IF_1 on the surface of the β_E subunit, but not on the surface of one of the other two homologous subunits β_{DP} and β_{TP} , might be explained if one considers the closer vicinity of both α_{TP} to β_{TP} and α_{DP} to β_{DP} with respect to α_E and β_E ; an effect that might be sterically unfavourable to the binding of IF_1 .

Other observations support the proposed model. Energy transfer results indicate that PM bound to IF₁ Cys⁵⁹ is at the furthest limit of the range of RET (3 % efficiency), a finding consistent with a separation between PM and γ -Cys⁷⁸ of nearly 6.7 nm. In the model, Arg²⁶, Lys⁴⁶ and Lys⁴⁷ of IF₁ are in close proximity with compatible (i.e. to form potentially favourable interactions) residues from subunit β_e , namely His⁴⁵¹, Glu⁴⁶⁴ and Glu⁴⁶⁵ respectively. Moreover, the suggested location of the IF₁ fragment is consistent with other observations previously reported [25,32–34]. First, ϵ subunit senses binding of the inhibitor to F₁, as detected by phosphorescence decay measurements [25], which might indicate an interaction between the two polypeptide chains. In the present model ϵ -Lys²⁷ and ϵ -Thr²⁸ could potentially form hydrogen bonds with Ser¹⁰ and Ser¹¹ of IF₁ and stabilize the F₁–IF₁ complex, as previously suggested for residues 10–17 of IF₁ [32]. This binding might at least be responsible in part for inhibition of catalysis (i.e. 20 % by I_{10–40} in the present study), since rotation of γ , δ , and ϵ with respect to the $\alpha_3\beta_3$ ring would require a higher activation energy than in the absence of the inhibitor protein. Indeed, Harris [32] showed that amino acids residues 10–17 are necessary to inhibit F₁. Second, residues 48–58 of IF₁, rich in histidine residues, are not bound to F₁, therefore dimer or tetramer formation of IF₁ is possible, as suggested by Walker and co-workers [33–35]. The proposed location of IF₁ could allow dimeric IF₁ formation and active binding to two F₁ complexes, as suggested by the Cabezon et al. [34].

The implications of the present results for regulation of catalysis are that the full inhibitory activity of IF₁ must require the presence of the essential C-terminal domain of IF₁ either to bind other additional points of contact of the protein with the enzyme [36], or to induce an active dimer form [34].

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REFERENCES

- Senior, A. E. (1988) ATP synthesis by oxidative phosphorylation. *Physiol. Rev.* **68**, 177–231
- Walker, J. E., Fearnley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M. and Tybulewicz, V. L. J. (1985) Primary structure and subunit stoichiometry of F₁-ATPase from bovine mitochondria. *J. Mol. Biol.* **184**, 677–701
- Abrahams, J. P., Leslie, A. G. W., Lutter, R. and Walker, J. E. (1994) Structure at 2.8 Å resolution of F₁-ATPase from bovine heart mitochondria. *Nature (London)* **370**, 621–628
- Gibbons, C., Montgomery, M. G., Leslie, A. G. W. and Walker, J. E. (2000) The structure of the central stalk in bovine F₁-ATPase at 2.4 Å resolution. *Nat. Struct. Biol.* **7**, 1055–1061
- Boyer, P. D. (2000) Catalytic site forms and controls in ATP synthase catalysis. *Biochim. Biophys. Acta* **1458**, 252–262
- Noji, H., Yasuda, R., Yoshida, M. and Kinoshita, K. (1997) Direct observation of the rotation of F₁-ATPase. *Nature (London)* **386**, 299–302
- Sambongi, Y., Iko, Y., Tanabe, M., Omote, H., Iwamoto-Kihara, A., Ueda, I., Yanagida, T., Wada, Y. and Futai, M. (1999) Mechanical rotation of the c subunit oligomer in ATP synthase (F₀F₁): direct observation. *Science (Washington, D.C.)* **286**, 1722–1724
- Wang, H. and Oster, G. (1998) Energy transduction in the F₁ motor of ATP synthase. *Nature (London)* **396**, 279–282
- Harris, D. A. and Das, A. M. (1991) Control of mitochondrial ATP synthesis in the heart. *Biochem. J.* **176**, 967–975
- Green, D. W. and Grover, G. J. (2000) The IF₁ inhibitor protein of the mitochondrial F₁F₀-ATPase. *Biochim. Biophys. Acta* **1458**, 343–355
- Stout, J. S., Partridge, B. E., Dibbern, D. A. and Schuster, S. M. (1993) Peptide analogs of the beef heart mitochondrial F₁-ATPase inhibitor protein. *Biochemistry* **32**, 7496–7502
- Van Raaij, M. J., Orriss, G. L., Montgomery, M. G., Runswick, M. J., Fearnley, I. M., Skehel, J. M. and Walker, J. E. (1996) The ATPase inhibitor protein from bovine heart mitochondria: the minimal inhibitory sequence. *Biochemistry* **35**, 15618–15625
- Zanotti, F., Raho, G., Vuolo, R., Gaballo, A., Papa, F. and Papa, S. (2000) Functional domains of the ATPase inhibitor protein from bovine heart mitochondria. *FEBS Lett.* **482**, 163–166
- Gabellieri, E., Strambini, G. B., Baracca, A. and Solaini, G. (1997) Structural mapping of the ϵ -subunit of mitochondrial H⁺-ATPase complex F₁. *Biophys. J.* **72**, 1818–1827
- Hashimoto, T., Yamamoto, Y., Yoshida, Y. and Tagawa, K. (1995) Cleavage of bovine mitochondrial ATPase inhibitor with endopeptidases, and binding of the resulting peptides to the interface between the α - and β -subunits of F₁-ATPase. *J. Biochem. (Tokyo)* **117**, 641–647
- Papa, S., Zanotti, F., Cocco, T., Perrucci, C. and Minuto, M. (1996) Identification of functional domains and critical residues in the adenosinetriphosphatase inhibitor protein of mitochondrial F₀F₁ ATP synthase. *Eur. J. Biochem.* **240**, 461–467
- Fillingame, R. H. (1992) Subunit c of F₀F₁ ATP synthase: structure and role in transmembrane energy transduction. *Biochim. Biophys. Acta* **1101**, 240–243
- Groth, G. and Walker, J. E. (1997) Model of the c-subunit oligomer in the membrane domain of F-ATPases. *FEBS Lett.* **410**, 117–123
- Lee, C. P. and Ernster, L. (1968) Studies of the energy-transfer system of submitochondrial particles. Effects of oligomycin and aurovertin. *Eur. J. Biochem.* **3**, 391–400
- Beechey, R. B., Hubbard, S. A., Linnett, P. E., Mitchell, A. D. and Munn, E. A. (1975) A simple and rapid method for the preparation of adenosine triphosphatase from submitochondrial particles. *Biochem. J.* **148**, 533–537
- Stoney-Simons, S., Chakraborti, P. K. and Cavanaugh, A. H. (1989) Arsenite and cadmium (II) as probes of glucocorticoid receptor structure and function. *J. Biol. Chem.* **265**, 1938–1945
- Shapiro, A. B., Gibson, K. D., Scheraga, H. A. and McCarty, R. E. (1991) Fluorescence resonance energy transfer mapping of the fourth of six nucleotide binding sites of chloroplast coupling factor 1. *J. Biol. Chem.* **266**, 17276–17285
- Forster, T. (1959) Transfer mechanisms of electronic excitation. *Discuss. Faraday Soc.* **27**, 7–17
- Solaini, G., Baracca, A., Parenti Castelli, G. and Strambini, G. (1993) Tryptophan phosphorescence as a structural probe of mitochondrial F₁-ATPase ϵ -subunit. *Eur. J. Biochem.* **214**, 729–734
- Solaini, G., Baracca, A., Gabellieri, E. and Lenaz, G. (1997) Modification of the mitochondrial F₁-ATPase ϵ subunit, enhancement of the ATPase activity of the IF₁-F₁ complex and IF₁-binding dependence of the conformation of the ϵ subunit. *Biochem. J.* **327**, 443–448
- Lowry, O. H., Rosebrough, A. L., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (London)* **227**, 680–685
- Stock, D., Leslie, A. G. W. and Walker, J. E. (1999) Molecular architecture of the rotary motor in ATP synthase. *Science (Washington, D.C.)* **286**, 1700–1705
- Mimura, H., Hashimoto, T., Yoshida, Y., Ichikawa, N. and Tagawa, K. (1993) Binding of an intrinsic ATPase inhibitor to the interface between α - and β -subunits of F₁F₀-ATPase upon de-energization of mitochondria. *J. Biochem. (Tokyo)* **113**, 350–354
- Papa, S., Zanotti, F. and Gaballo, A. (2000) The structural and functional connection between the catalytic and proton translocating sectors of the mitochondrial F₁F₀-ATP synthase. *J. Bioenerg. Biomembr.* **32**, 401–411
- Jackson, P. J. and Harris, D. A. (1988) The mitochondrial ATP synthase inhibitor protein binds near the C-terminus of the F₁ β -subunit. *FEBS Lett.* **229**, 224–228
- Harris, D. A. (1997) Functional regions of the H⁺-ATPase inhibitor protein from ox heart mitochondria. *Biochim. Biophys. Acta* **1320**, 8–16
- Cabezon, E., Butler, P. J., Runswick, M. J. and Walker, J. E. (2000) Modulation of the oligomerization state of the bovine F₁-ATPase inhibitor protein, IF₁, by pH. *J. Biol. Chem.* **275**, 25460–25464
- Cabezon, E., Arechaga, I., Jonathan, P., Butler, G. and Walker, J. E. (2000) Dimerization of bovine F₁-ATPase by binding the inhibitor protein, IF₁. *J. Biol. Chem.* **275**, 28353–28355
- Gordon-Smith, D. J., Carbajo, R. J., Yang, J.-C., Videler, H., Runswick, M. J., Walker, J. E. and Neuhaus, D. (2001) Solution structure of a C-terminal coiled-coil domain from bovine IF₁: the inhibitor protein of F₁-ATPase. *J. Mol. Biol.* **308**, 325–339
- Harris, D. A. (1989) Azide as a probe of co-operative interactions in the mitochondrial F₁-ATPase. *Biochim. Biophys. Acta* **974**, 156–162