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Functional regions of the H^+-ATPase inhibitory protein from ox heart mitochondria

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Abstract

Derivatives of the inhibitor protein (IF_1) of the mitochondrial H^+-ATP synthase, bearing deletions at the N- or C-terminal ends, were tested for their abilities (a) to bind to the synthase, (b) to inhibit its ATPase activity and (c) to respond to energisation of the mitochondrial membrane. Deletion of nine residues from its N-terminus, or ten from its C-terminus had little effect on any of these three properties of IF_1. Further deletions from the N-terminus (up to residue 17) led to an increase in binding affinity but a reduced ability to inhibit ATPase activity and to form a stable ATPase-IF_1 complex. Removal of five more residues from the N-terminus (up to residue 22) reduced these abilities further, but also decreased binding affinity by an order of magnitude. It was concluded that residues 10–17 of IF_1 interact with F_1 in a way which modulates the stability and function of the interaction between F_1 and IF_1.

Keywords: Mitochondrion; H^+-ATP synthase; Inhibitor protein (IF_1); F_1F_0-ATPase; Deletion

1. Introduction

The ATPase inhibitor protein (IF_1) is a protein that inhibits the ATPase activity of the H^+-ATP synthase (F_1F_0-ATP synthase) of mitochondria [1]. It appears to occur solely in these organelles, being found in mitochondria from a wide range of species (mammals, yeasts, plants)[1–4], but with no homologues associated with the related H^+-ATP synthases of chloroplasts or bacteria. IF_1 has been implicated in the regulation of the mitochondrial H^+-ATP synthase in physiological and/or pathological situations (for a review, see [5]).

IF_1 is a small protein, whose molecular weight ranges from 6.5 kDa in potato [6] to around 10 kDa in mammals [7]. Secondary structure predictions and circular dichroism studies [8,9] indicate a high (approx. 80%) α-helical content; probably all amino acids except the extreme N-terminal region lie in an

Abbreviations: F_1F_0, the mitochondrial H^+-ATP synthase; F_1, soluble ATPase fraction of the ATP synthase; IF_1, naturally occurring inhibitor protein of the ATP synthase; [^{125}I]IF_1, radioiodinated inhibitor protein; I$n-m$, peptide containing residues n to m of the ox-heart inhibitory sequence; Mops, 3-(*N*-morpholino)propanesulphonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

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α-helix. Cross linking studies [10] and consideration of the high resolution structure of F_1 [11], are consistent with the binding site of IF_1 being close to the C-terminal region of the catalytic (β) subunit of F_1, which lies on the side of F_1 closest to the membrane.

A number of studies have attempted to identify the 'functional core' of the IF_1 protein. Interspecies comparisons between IF_1 sequences have pointed to its central region (residues 22–46) as crucial to IF_1 function [6]. Experimentally, limited proteolysis studies [12,13] have shown that the extreme N-terminus (residues 1–9 using the ox heart numbering) and a significant portion of the C-terminal region (residues 52–84) of IF_1 are not required for inhibition of F_1-ATPase activity, a conclusion borne out by studies using genetically engineered deletions of parts of the IF_1 molecule [9]. Thus, attention has been focussed on this central region as a key to the function of the IF_1 molecule.

These experimental conclusions, however, involve the measurement of inhibition of ATPase activity as the only arbiter of IF_1 function. In fact, inhibition per se requires IF_1 to have two potentially separable properties: (a) it should bind to F_1; and (b) when bound it should block ATPase turnover. In vivo, a third property of IF_1 is observed: IF_1 is released from its binding site on F_1 in an energy-dependent process [14]. The release of IF_1 is crucial to allow ATP synthesis, because the IF_1-ATP synthase complex is inactive in both ATP synthesis and hydrolysis [15]. For ATP to be made, therefore, IF_1 must be released from this complex under the influence of the membrane potential [16]. Since IF_1 rebinds when the mitochondrial energy state is low, IF_1 provides an effective 'one way valve' allowing ATP synthesis when energy supply is adequate, but preventing ATP hydrolysis under conditions of low energy.

These three properties of IF_1 might, in principle, be mediated by different regions of the polypeptide. The work described here investigates this possibility. Genetically truncated derivatives of IF_1 were used in (a) direct binding measurements, (b) inhibition studies and (c) for measurements of energy-dependent responses. It was concluded that suitably truncated IF_1 could bind reversibly to F_1 without inhibiting it completely. Limited truncation of IF_1 at the N-terminal or C-terminal did not significantly affect its energy-dependent release from coupled mitochondrial membranes. A preliminary report of part of this work has been published elsewhere [17]

2. Materials and methods

IF_1 was prepared from ox heart mitochondria and radiolabelled (at a 0.8:1 molar ratio) with ^{125}I as described previously [16]. In some experiments, IF_1 prepared by expression of the cloned gene in *Escherichia coli* was used; the results obtained were independent of the source of IF_1. Cloned IF_1 and the various truncated peptides, prepared as described in [9] were a generous gift of Dr. J. Walker, MRC Laboratory for Molecular Biology, Cambridge. Coupled IF_1-depleted submitochondrial vesicles ('state III particles') [18] and (uncoupled) mitochondrial membranes free of IF_1 ('ammonia-Sephadex particles') [19] were prepared according to published procedures.

Combination of (membrane bound) F_1 with IF_1 or the truncated peptides was carried out in 250 mM sucrose, 20 mM MOPS, 2 mM MgATP, 50 mM KCl, 1 mg/ml cytochrome *c*, pH 6.5 (NaOH) (buffer A) at 37°C essentially as previously [16]. (The last two components are present to minimise non-specific binding of IF_1 (positively charged) to mitochondrial membranes). Unless otherwise indicated, reaction mixtures contained 220 μl of buffer A, 1 μg (0.1nmol) [^{125}I]IF_1 (70 000 cpm/nmol) and 0–20 μg peptide. Binding was initiated by the addition of 180 μg IF_1-free mitochondrial membranes (corresponding to an F_1 content of 0.05nmol) and allowed to proceed for 15 min. An aliquot (5 μl) was then removed for the determination of ATPase activity and the remaining sample used for the determination of membrane bound [^{125}I]IF_1.

Energy-dependent behaviour of the IF_1 derivatives was measured essentially as described in [16]. State III submitochondrial vesicles (ATPase activity approx. 2.5 μmol/min/mg protein) were loaded with IF_1, or the respective peptide, by incubation in buffer A except that the concentration of MgATP was raised to 4 mM. Excess peptide was removed by centrifugation at $100\,000 \times g$ for 20 min. The loaded vesicles showed an ATPase activity of approx. 0.5 μmol/min/mg protein. These particles were then

used for the determination of (a) the extent of ATPase stimulation by NADH and of (b) the exchange of bound inhibitory peptide with radiolabelled, added IF_1 in the presence of succinate and MgATP as described in [16]

ATPase activity was measured continuously by coupling ATP hydrolysis to NADH oxidation through the pyruvate kinase/lactate dehydrogenase system [20]. Where coupled submitochondrial vesicles were to be assayed, 2 μM FCCP was added to the standard reaction medium. [^{125}I]IF_1 bound to mitochondrial membranes was measured by the method of [16]. When the binding reaction (above) was complete, the vesicles were collected by centrifugation in a Heraeus hemofuge (20 min, $18\,000 \times g$) after addition of ammonium sulphate to the binding medium to 10% saturation (to prevent further binding and promote aggregation of the membranes). The pellets were washed once without resuspension and the tubes counted for ^{125}I.

Protein content of particulate preparations was determined by the biuret procedure of Cleland and Slater [21] and soluble protein by the method of Bensadoun and Weinstein [22]. Cytochrome *c* (type III) and FCCP were obtained from Sigma.

3. Results

3.1. Binding properties of IF_1-peptides

Fig. 1 shows that incubation of inhibitor-free mitochondrial membranes with a twofold excess of [^{125}I]IF_1 results in 85% of the available F_1 becoming bound to IF_1 (in agreement with [16]). Inclusion of truncated derivatives of IF_1 in the mixture lead to a decrease in bound label provided that the truncated derivatives can compete with the labelled, complete IF_1 for its binding site.

Such an experiment is shown in Fig. 1. The left-hand curve was obtained with the peptide I18–84, i.e., a derivative of IF_1 lacking residues 1–17. This peptide is clearly able to compete efficiently for the IF_1 binding site on membrane bound F_1. Indeed, at a 1:1 ratio between added IF_1 and peptide, more than half the IF_1 is displaced, indicating that its affinity is even higher than the parent molecule. In contrast, the slightly shorter peptide I23–84, which lacks the first 22 amino acid residues, shows a marked decrease in affinity for F_1 (Fig. 1, right hand curve).

Table 1 shows the behaviour of a range of trun-

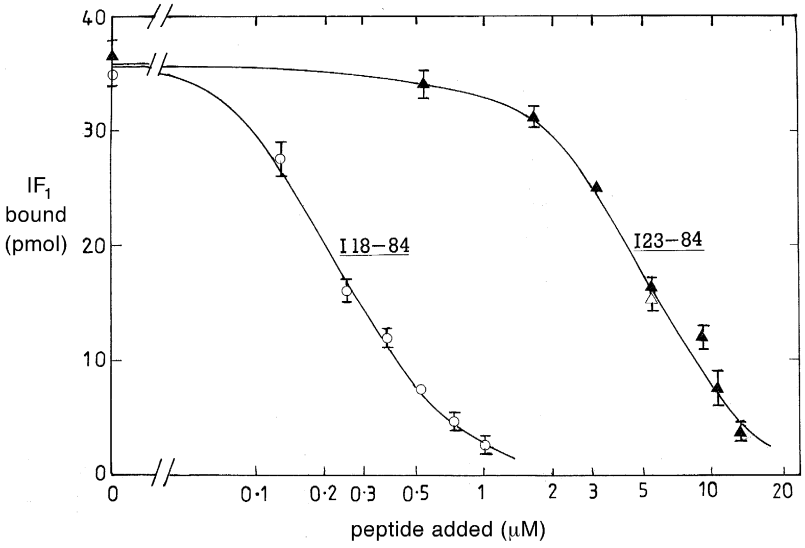

Fig. 1. Competition of truncated IF_1 peptides for IF_1 binding site 180 μg inhibitor-free submitochondrial membranes (bearing 0.05 nmol F_1 [26]) were incubated in a total volume of 250 μl with ^{125}I-labelled IF_1 (0.1 nmol, 7000 cpm) and the indicated amounts of peptide I18–84 (○) or I23–84 (▲). The amount of labelled IF_1 bound to the membranes measured as in Section 2. Where indicated (△), peptide I1–22 (20 μg) was included in the reaction medium in addition to I23–84. The abscissa is plotted on a logarithmic scale in order to display the wide range of concentrations used. Error bars indicate the range of duplicate readings.

Table 1
Relative affinities of IF_1 and its truncated peptides for the IF_1 binding site

Peptide	IF_1	I10–84	I14–84	I18–84	I23–84	I1–22	I1–74	I1–78
$C_{0.5}$ (μM)	0.4	0.38	0.44	0.24	4.7	> 20	0.28	0.31

Competition between radiolabelled IF_1 and the peptides for binding to submitochondrial membranes were measured as in Fig. 1. The values are expressed as $C_{0.5}$, the concentration required to displace half of the labelled IF_1.

cated derivatives in this binding assay. The results are given as $C_{0.5}$, the concentration of peptide required to displace half the bound labelled IF_1. IF_1 has a $C_{0.5}$ of 0.4 μM in this assay, equivalent to the concentration of added, labelled IF_1 (see Section 2). Values of $C_{0.5}$ higher than this indicate reduced binding affinity, while lower values indicate a higher affinity of the truncated peptide.

Table 1 shows that IF_1 truncated at the N-terminal end by up to 17 residues, or at the C-terminal end by up to 10 residues, binds to membrane bound F_1 at least as well as does intact IF_1. The only truncation tested that shows a decreased affinity is that removing the N-terminal 22 amino acids, where $C_{0.5}$ increases by about one order of magnitude. Comparing the results for I18–84 with those for I23–84, it is clear that the affinity of IF_1 binding to F_1 is strongly influenced by the presence of amino acids 18–22 in the IF_1 sequence.

Table 1 also shows that peptide I1–22, corresponding to these first 22 amino acids of IF_1, shows no detectable affinity for F_1. While this is perhaps unsur-

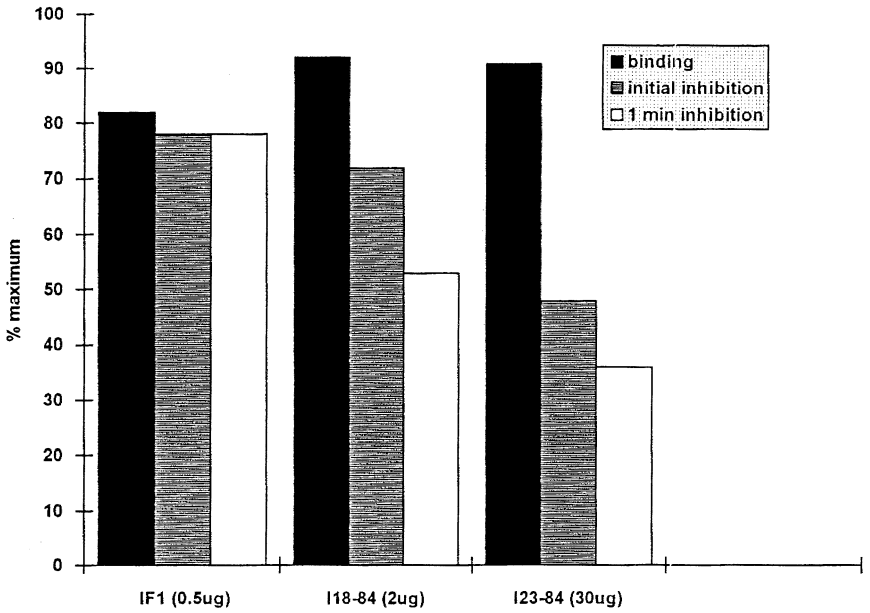

Fig. 2. Relation between binding affinity and ATPase inhibition by IF_1 and truncated peptides. Binding of IF_1 and truncated peptides to IF_1-free mitochondrial membranes was measured as in Fig. 1. The amounts of peptide added in the binding assay are indicated on the figure. % binding is estimated as

$$\frac{\text{counts bound in the absence of added peptide} - \text{counts bound in the presence of added peptide}}{\text{counts bound in the absence of added peptide}}$$

For measurement of ATPase activity, the IF_1 or peptides were incubated with inhibitor–free submitochondrial membranes under the same conditions except that labelled IF_1 was omitted. After incubation, aliquots were removed and added to the ATPase assay medium, and activity followed for 2 min (see Section 2). The initial rate was taken as the initial slope of the curve, and the '1-min' rate taken as the mean slope over the period 60–120 s. % inhibition of ATPase activity = (uninhibited rate − inhibited rate)/uninhibited rate.

prising, it allows the measurement of any synergistic effect between the peptides I1–22 and I23–84, between them comprising the whole IF_1 sequence but 'cleaved' after residue 22. Fig. 1 (open triangle) shows that the addition of 1 μg of I1–22 to a binding assay containing 5 μg I23–84 (sufficient to compete out about 50% of labelled IF_1 binding) has no effect on the ability of I23–84 to bind to F_1, i.e., peptide I1–22 appears not to interact with F_1 either in the absence or present of its conjugate peptide.

As noted above, the data in Table 1 indicate that the affinities of I18–84 and, possibly, the I1–74 peptide are higher than that of intact IF_1. One possible explanation for this observation is that the N and/or C-termini of the IF_1 molecule modulate its binding in some way. This is investigated further below.

3.2. Inhibition of ATPase activity

Inhibition of ATPase activity by IF_1 is measured by (a) preincubating IF_1 with inhibitor-free mitochondrial membranes to form the F_1-IF_1 complex, followed by (b) dilution of an aliquot of the complex into the ATPase assay medium. For IF_1 and other peptides that form a stable complex with F_1, the resulting activity is constant in time and the percent inhibition correlates directly with the amount of bound peptide measured in the binding assay. This behaviour is observed for IF_1 (Fig. 2) and for peptides I10–84, I1–74 and I1–78 (data not shown).

For the remaining three N-terminal deletions, I14–84, I18–84 and I23–84, the situation is more complex. Fig. 2 shows the situation when peptide I18–24 is used. First, the ATPase inhibition measured decreases over the time of the assay from about 75% to 50%, as shown by the mean ATPase activity over the second minute of the assay being some 50% higher that the initial ATPase rate (measured over the first 30 s). This suggests that the peptide, unlike IF_1 itself, forms a complex with F_1that is unstable in the assay medium. Secondly, the amount of bound peptide (as measured in the binding assay) is not linearly correlated with ATPase inhibition – when over 90% of F_1 is bound to the peptide I18–24, the ATPase inhibition initially observed is only 72% (Fig. 2)

The features are less pronounced, but observable, with peptide I14–84 (data not shown) and considerably more pronounced with the shorter peptide I23–84 (Fig. 2). Again the F_1 peptide complex is unstable in the assay mixture, as shown by the increase in ATPase activity in time. And, in this case, sufficient peptide to bind over 90% of F_1 inhibits ATPase activity by less than 50%. It appears, therefore, that complete inhibition of F_1 requires more of the N-terminal region of IF_1 than binding alone.

3.3. Energy-dependent response of IF_1

In coupled submitochondrial vesicles, energisation causes IF_1 to dissociate from its complex with the ATP synthase [16]. This can be observed as an

Table 2
Energy-linked responses of IF_1 and truncated peptides

	ATPase after energisation (U/mg)	ATPase before energisation (U/mg)	^{125}I-exchange during energisation (nmol IF_1/mg)	^{125}I-exchange without energisation (nmol IF_1/mg)
IF_1-loaded vesicles	2.08	0.51	0.13	0.05
I10–84-loaded vesicles	1.63	0.43	0.12	n.d.
I1–74-loaded vesicles	2.34	0.69	0.15	n.d.

Coupled, inhibitor-depleted submitochondrial vesicles were prepared and loaded with IF_1, I10–84 or I1–74 as described in Section 2. For studying the NADH stimulation of ATPase activity, vesicles were diluted to a concentration of 2.7 mg/ml protein in buffer containing 20 mM Hepes, 10 mM potassium phosphate, 5 mM $MgCl_2$, pH 7.5 (NaOH) and incubated at 37°C. Where indicated, NADH was added to a final concentration of 1mM, and aliquots taken after 2 min for ATPase assay. For studying energy-dependent exchange of [^{125}I]IF_1, 10 mg of loaded vesicles were incubated for 5 min at 30°C (with shaking) in 4 ml of the same buffer containing, in addition, 2 mM AMP, 0.5 mg/ml bovine serum albumin and 25 μg [^{125}I]IF_1 (5000 cpm/μg), with or without the addition of 25 mM succinate and 4 mM MgATP (see [14]). The vesicles were then washed by centrifugation 3 times, and samples taken for ^{125}I and protein measurement. Results are expressed per mg membrane protein. n.d., not determined

increase in ATPase activity of the vesicles [23], or a release of bound, labelled IF_1 from the vesicles [16], induced by adding an oxidisable substrate (NADH or succinate). Alternatively, if free radiolabelled IF_1 is added to vesicles, electron transport in the presence of MgATP promotes an exchange of bound (unlabelled) IF_1 for free IF_1 and this can be detected as an energy-dependent increase in bound label [24]. If the bound IF_1 (or its derivative) cannot respond to energisation, neither of these phenomena will be observed.

In the experiments described here, coupled submitochondrial vesicles were loaded with IF_1 itself, peptide I10–84 or peptide I1–74. The response of these loaded vesicles to energisation was monitored by (a) monitoring their ATPase activity before and after incubation with NADH and by (b) following the exchange of bound peptide with labelled IF_1 in the presence or absence of succinate (Table 2). Similar experiments could not be performed using the remaining N-truncated peptides (I14–84, I18–84 or I23–84) because these did not form a stable complex with the ATP synthase (see above) and thus vesicles 'loaded' with these peptides could not be prepared.

Table 2 shows that vesicles loaded with peptides I10–84 or peptide I1–74 show a similar increase in ATPase activity after energisation (about fourfold) to that observed with particles loaded with IF_1 itself, suggesting that the truncated peptides respond to energisation much as does the native species of IF_1. This is confirmed by the exchange results (Table 2), which show that 0.12–0.15 nmol labelled IF_1/mg membrane protein (about 30%) of bound peptide exchanges with added (labelled) IF_1 in energised vesicles, irrespective of whether the vesicles were loaded with native IF_1 or either of the truncated peptides.

These results suggest that the truncated peptide (I10–84, I1–74) respond to energisation very much as does the native IF_1. This conclusion may be questioned in that 'loading' the vesicles with IF_1 or peptide will not displace all the IF_1 preexisting on the membrane – typically only 30–50% of F_1 becomes associated with the added peptide [16]. (Unfortunately, it is not possible to remove all endogenous IF_1 from mitochondrial membranes and still retain coupling.) It could be, therefore, that the energy-linked response involves only that IF_1 originally bound to the vesicles and not the added peptides at all. However, we have shown previously that this is not the case when IF_1 itself is bound to the vesicles during loading [16] and the similarity between the observed responses of particles loaded with IF_1 or with the truncated peptides suggests that the latter respond very much as does IF_1 itself. Thus it appears that neither the extreme N-terminal nor the extreme C-terminal of ox-heart IF_1 is important in modulating the response of this protein to membrane energisation.

4. Discussion

4.1. The C-terminal region

The ATPase inhibitor from ox heart is a relatively small protein, only 84 amino acids long. The above results are consistent with the view that, nonetheless, its inhibitory function requires only a portion of the whole protein. In particular, deletions of up to 10 amino acids from the C-terminus affect neither binding nor inhibition by IF_1. This is consistent with the activity studies of Hashimoto et al. [13] using a proteolytic fragment of IF_1 and Van Raaij et al. [9] using genetic deletions. These workers showed that truncation back to residue 51 [13] or 45 [9] – removing nearly half of the total peptide – still yielded a protein with normal inhibitory powers.

A recent study by Papa et al. [27] has, however, shown contrasting results. These workers showed that a (synthetic) peptide I42–58 inhibited ATP hydrolysis by F_1 as least as efficiently as IF_1 itself. This observation cannot easily be reconciled on a simple model with the ability of peptide I1–45 [9], lacking most of I42–58 and the inability of peptide I23–84 (above), containing I42–58, to inhibit F_1. One possibility might be that peptide I42–58 binds to a separate inhibitory site on F_1. Competitive binding studies, such as those carried out above, would be necessary to investigate this hypothesis.

The apparent lack of function of at least the extreme C-terminal region in the inhibitory action of IF_1 led us to consider a possible role of this region in the energy-linked responses of this protein. Such a role seemed feasible since (a) IF_1 appears to respond to the membrane potential ($\Delta\psi$) component of mem-

brane energisation [25] and (b) IF_1 terminates in a cluster of 4 acidic residues (^{81}EDDD^{84}), immediately preceded by a cluster of 3 basic residues (^{75}KKLK^{78}). However, as shown above (Table 2), the truncated peptide lacking all of these residues (I1–74) showed energy linked responses identical to that of native IF_1. It was concluded that this region did not participate in the responses of IF_1 to the energy state of the membrane.

4.2. The N-terminal region

Truncations from the N-terminal end reveal more interesting behaviour. The smallest deletion, I10–84, reveals no change in binding to F_1, inhibition of ATPase activity, or energy linked behaviour (Tables 1 and 2). The unaltered inhibitory activity of peptide I10–84 has been demonstrated previously [12]. Truncation past residue 10 does not decrease the binding affinity of IF_1 until residue 18 is reached, as shown by the fact that peptide I18–84 binds well to F_1 while peptide I23–84 shows a binding affinity decreased by an order of magnitude (Fig. 1). Residues 18–22 seem therefore to be important in binding. Interestingly these residues are absent from potato IF_1 [6] a homologue which is unable to inhibit [4] or even bind to [6] ox heart IF_1. This said, peptide I23–84 can clearly bind to F_1 with significant affinity ($C_{0.5} = 4.7$ μM); the change of an order of magnitude in affinity is equivalent in energy terms to approximately one hydrogen bonding interaction and thus most of the binding energy is provided by residues subsequent to phe22 in the sequence.

The properties of I18–84 reveal additional features of the IF_1 interaction. First, its binding affinity appears greater than IF_1 itself (Table 1) While this observation may be explained trivially by changes in the kinetics of binding of the smaller peptide, it is also possible that residues 10–17 might interact with F_1 in the bound state and modulate the F_1–IF_1 interaction in some way (perhaps in response to energisation). An acidic residue (asp17 in ox heart IF_1) is conserved between mammalian and yeast homologues in this region [6]

Secondly, despite this higher binding affinity, the complex formed between peptide I18–84 and F_1 is less stable than the IF_1-F_1 complex since the former, but not the latter, dissociates during the course of the ATPase assay (Fig. 2). This is not possible on a simple 2-state equilibrium binding model, where increased stability must always follow increased affinity. Instead, this behaviour supports the two-step model for IF_1 binding previously proposed [26]. Taken together, these data suggest that the initial (reversible) interaction between IF_1 and F_1 involves the conserved helix starting at around residue 22 (and the 3–4 immediately preceding residues) and that this is followed by further stabilising interactions with residues 10–17. The instability of the F_1 complexes with peptides I18–84 and I23–84 presumably accounts for the lower inhibition by these peptides observed by Van Raaij et al. [9] – who measured ATPase over a 5-min period – compared to the inhibition reported here, where rates over 0–0.5 min and 1–2 min are measured.

Finally, even when initial rates of ATPase activity are estimated, there still appears a discrepancy between the amount of bound peptide and ATPase inhibition using peptides I18–84 and, more markedly, I23–84. In both cases, $> 90\%$ occupancy of their binding site on IF_1 yielded a much smaller percentage of ATPase inhibition (70% and 50% respectively, Fig. 2). This indicates that binding of the truncated inhibitor protein can occur without complete inhibition of ATPase activity – inhibition requires the interaction of F_1 with the region of IF_1 from residue 10–17. Thus, on a simple model, the stabilising interaction between these residues and F_1 may also be responsible for full development of ATPase inhibition.

Investigations with smaller peptides are consistent with these conclusions. Hashimoto et al. [13] have shown that peptide I52–84 appears to bind to F_1, in the IF_1 binding side, without inhibiting it, while Schuster et al. [28] have shown that a peptide I22–46 – corresponding to the central region of IF_1 – can inhibit F_1, but only if present at high concentration (> 10 μM) in the assay medium. The present work shows that N-terminal peptide I1–22 showed no detectable affinity for IF_1, either alone or in combination with peptide I23–84 (Fig. 1). While this might seem surprising, the high binding affinity of the I18–84 peptide noted above suggests that the N-terminal region contributes relatively little to the affinity of IF_1 for F_1.

In conclusion, this work confirms the view, de-

rived from sequence comparisons [6], that reversible binding of IF_1 to F_1 is largely mediated by residues 22–46 in the ox heart IF_1 sequence. These residues are believed to lie on an amphipathic helix [8] and to interact hydrophobically with F_1 [26]. Residues 18–21 contribute a further 10x increase in binding affinity, corresponding to about 5kJ/mol extra binding energy. Residues 10–17 (predicted to lie in an unstructured region) contribute no additional binding energy, but are required for the formation of a stable F_1-IF_1 complex and complete inhibition of ATPase activity. It has been shown, indeed, that the truncated peptide I10–45 stably inhibits ATPase activity, whereas smaller peptides do not [9].

This work also shows that the response of IF_1 to membrane energisation in mitochondria does not involve its 9 N-terminal amino acids nor its 10 C-terminal amino acids. It may be that residues 10–17 are involved in this function of IF_1 too; their presence decreases the affinity of IF_1 for F_1 to some extent and also modulates stability of the F_1-IF_1 complex. A conserved acidic residue exists at position 17 and could mediate a response to membrane potential. However, in the absence of further experiments, the involvement of residues closer to the C-terminus (residues 46–75) in energy-linked responses cannot be ruled out. These conclusions are summarised in Fig. 3.

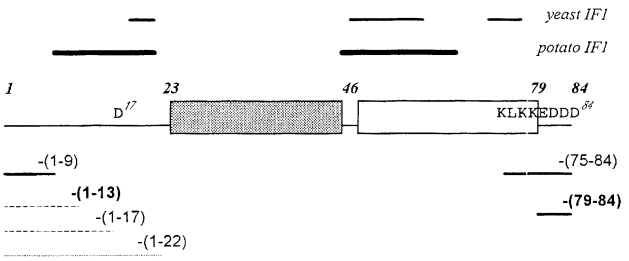

Fig. 3. Importance of the various regions of IF_1 sequence. The primary structure of ox heart IF_1 is represented. The solid boxes indicate regions predicted to be α helical in [8]; the region conserved between species [6] is shaded. The residues missing in potato and yeast IF_1 are indicated above the diagram [6]. The residues which have been deleted from the ox heart sequence in the current work are indicated below the diagram. Solid lines indicate no observed change in activities, the dashed line indicates a deletion showing no loss of affinity for F_1 but a loss of stability in the F_1–IF_1 complex, and the dotted line indicates a deletion with reduced affinity for F_1 and a loss of stability in the F_1-IF_1 complex (see text). Residues investigated as possibly involved in energy linked responses (see Section 3) are indicated.

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