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The mitochondrial ATP synthase inhibitor protein binds near the C-terminus of the F_1 β-subunit

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The specific, mitochondrial ATP synthase protein (IF_1) was covalently cross-linked to its binding site on the catalytic sector of the enzyme (F_1-ATPase). The cross-linked complex was selectively cleaved, leaving IF_1 intact to facilitate the subsequent purification of the F_1 fragment to which IF_1 was cross-linked. This fragment was identified by sequence analysis as comprising residues 394–459 on the F_1 β-subunit, near the C-terminus. This finding is discussed in the light of secondary structure predictions for both IF_1 and the F_1 β-subunit, and sequence homologies between mitochondrial and other ATP synthases.

Mitochondria; ATP synthase; F_1-ATPase; ATPase inhibitor protein; (Bovine heart)

1. INTRODUCTION

The mitochondrial ATP synthase (F_1-ATPase) is the enzyme responsible for redox-driven ATP synthesis within the mitochondrion. Its activity, in both synthetic and hydrolytic directions, is inhibited by a naturally occurring, intramitochondrial peptide, IF_1 [1], inhibition being complete at 1 mol IF_1/mol F_1 [2]. Since IF_1 is present in a 1:1 molar ratio with F_1 in bovine heart mitochondria [3], it seems likely that a considerable proportion of F_1 exists complexed with IF_1 in vivo. Prior to ATP synthesis, F_1 must be activated by displacing the F_1-IF_1 equilibrium towards free F_1 [1], and this displacement is promoted by the development of an electrical potential across the inner mitochondrial membrane [5].

Like many regulatory peptides (calmodulin [6], troponin C [7], various peptide hormones [8]), IF_1 can assume a highly helical configuration with a superficial hydrophobic patch (an 'amphiphilic helix') [9]. Thermodynamic analysis [2] and differential labelling studies [10] have allowed us to identify the binding site for F_1 as the hydrophobic patch on this amphiphilic helix. The binding site for IF_1 on F_1, however, is less well defined. It appears, from cross-linking experiments, that interaction is with the catalytic (β) subunit of F_1 [11,12], and so presumably this subunit provides a hydrophobic patch complementary to that on IF_1. In the case of the ox heart system, this patch is exposed to the solvent only during ATPase turnover [2], and thus ATP hydrolysis promotes IF_1 association with F_1.

The present work attempts to delineate further the site of F_1 involved in IF_1 binding. A zero-length cross-linker, EEDQ, is used to prepare a covalently linked IF_1-β complex which is then fragmented using CNBr. Since IF_1 itself contains no methionine residues [13], subsequent isolation of all peptides

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Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; HPLC, high-performance liquid chromatography; IF_1, mitochondrial ATP synthase inhibitor protein; PTH, phenylthiohydantoin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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containing IF_1 allows the purification of the single (IF_1-β peptide) cross-linked product, and the localisation of the IF_1-binding site on the penultimate CNBr peptide (residues 394–459) close to the C-terminus of the β-subunit of F_1.

2. MATERIALS AND METHODS

IF_1 and F_1 were prepared from ox heart mitochondria as described [12]. ^{125}I-labelled IF_1 was prepared according to Power et al. [3]. Cross-linking of IF_1 to F_1 was carried out essentially according to Klein et al. [11]. 0.6 mg (60 nmol) IF_1, containing ^{125}I-IF_1, was incubated in 2 ml of 10 mM sodium phosphate buffer (pH 6.7) at 20°C. 15 mM MgATP was added and incubation continued for 20 min to allow complete combination between F_1 and IF_1. 3.75 mM EEDQ was then added, and the cross-linking reaction stopped after a further 30 min by the addition of 450 mM Tris base.

This solution was freeze-dried and the residue dissolved in 10 ml of 80% (w/v) formic acid. After addition of 300 mg solid CNBr and incubation for 4 h [14], the mixture was freeze-dried and the residue dissolved in 2 ml of 6 M guanidine hydrochloride. Peptides were separated by gel-permeation chromatography on a Sephadex G-50 column (1 × 95 cm) equilibrated with 0.5% (w/v) NH_4HCO_3.

Preparative SDS-PAGE was carried out as follows: 6 nmol peptide (estimated from ^{125}I content) was applied as a band across a 150 × 150 × 1 mm slab gel comprising a 5–20% gradient of polyacrylamide. The discontinuous buffer system of Laemmli [15] was used and electrophoresis took place at 150 V for 5 h. After electrophoresis, protein bands were localised by brief staining with Coomassie blue R250 and electroeluted from the gel into a dialysis sac as in [16]. The resulting peptide was dialysed first against 0.1% (w/v) SDS in water (3 × 5 l over 2 days) then against 5 l water (16 h) and freeze-dried.

3. RESULTS

The F_1-IF_1 complex was prepared, and cross-linked using EEDQ, as described in section 2. Inclusion of a small fraction of ^{125}I-IF_1 in this complex served as a tracer for IF_1-containing peptides in subsequent procedures. The cross-linked complex was cleaved using CNBr and the peptide fragments subjected to gel-permeation chromatography. 95% of the radioactivity eluted at molecular masses lower than 20 kDa (fig.1a). Fractions containing the radioactive peak (A) and subsequent ^{125}I-containing fractions (B) were pooled for further analysis.

Fraction A contained 84% of the applied radioactivity, while fraction B contained 10% radioactivity but over 50% of the applied peptide (measured at 220 nm). Their peptide compositions were analysed by SDS-PAGE. Fig.1b shows that,

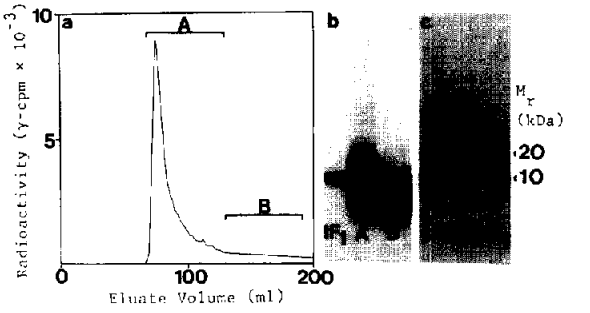

Fig.1. Gel-permeation chromatography of the CNBr-digested F_1-IF_1 cross-linked complex on Sephadex G-50 (see section 2). Fractions A and B, identified on the elution profile (a), were freeze-dried and redissolved in 1% (w/v) SDS. 10 and 20 μl respectively were analysed by SDS-PAGE (5–20% gradient of acrylamide; see section 2) together with 20 μg purified IF_1 for comparison. The gel was stained with Coomassie blue R250 (b), and a corresponding immunoblot probed with rabbit anti-IF_1 antibody/peroxidase-linked goat anti-rabbit IgG (c). The arrows indicate monomeric and dimeric IF_1 on the immunoblot at molecular masses of about 10 and 20 kDa, respectively.

while fraction B contains little peptide material of molecular mass greater than 10 kDa, fraction A also contains 2–3 major bands of molecular mass above 10 kDa. Since IF_1 has a molecular mass of 9.6 kDa [13], any putative IF_1-β conjugate must be present in fraction A.

Immunoblotting [12], followed by staining with anti-rabbit IgG-peroxidase conjugate was used to identify IF_1-containing peptides on this gel (fig.1c). Very little IF_1 monomer was present in either fraction, presumably because excess F_1 over IF_1 was used in the cross-linking step. As expected, fraction B showed little evidence of IF_1-containing peptides. However, two bands in fraction A were stained by this procedure; a faint band at 20 kDa (identified previously as IF_1 dimer [12]) and a single major band at 17 kDa. On the basis of its molecular mass and IF_1 content, this band was assumed to contain IF_1 cross-linked to some peptide derived from F_1, and was taken for further study.

The peptides from fraction A of molecular mass 13–15 kDa do not contain IF_1 (fig.1c). They are presumably derived directly from F_1 by CNBr cleavage. It is notable that these peptides are unlikely to be derived from the β-subunit of F_1, whose largest CNBr peptide has a molecular mass of about 8 kDa; they are presumably derived from

the α-, γ- and δ-subunits, each of which would yield (a) fragment(s) of >11 kDa.

The 17 kDa peptide was purified from fraction A by preparative SDS-PAGE, using a 1 mm thick polyacrylamide slab gel (5–20% gradient) as separating medium (see section 2). After electroelution, exhaustive dialysis and freeze-drying, 0.5 nmol of the peptide (as estimated from ^{125}I present) was taken for solid-phase sequencing.

The results are listed in table 1. Firstly, it is clear that only one peptide sequence is obtained, derived from the F_1 molecule. This is consistent with previous findings that the N-terminus of IF_1 is blocked [9,20]. While this is disputed by some workers [13,21], even these groups report low yields of PTH-amino acids on sequencing IF_1, possibly due to its possessing multiple N-termini [21].

Secondly, the yields of amino acid obtained are consistent with the amount of 17 kDa peptide applied, an estimate of the repetitive yield of the sequenator being about 90% from these values. This suggests that sequencing is indeed of the 17 kDa peptide itself and not of some minor contaminant.

Finally, the sequence obtained can be located unambiguously as starting at residue 394 of the sequence of the β-subunit of bovine F_1, as given in [14]. The cross-linked peptide thus contains that peptide designated CB11 by Runswick and Walker [14], the penultimate CNBr fragment of the β-subunit. This is a 65-residue fragment which, taken with IF_1, would yield a cross-linked peptide of 17–18 kDa as observed in fig.1. It is concluded, therefore, that IF_1 in its bound state can cross-link to (a) residue(s) between residues 394 and 459, close to the C-terminus of the β-subunit of IF_1.

Table 1

PTH-amino acid yields from the sequence analysis of the 17 kDa, IF_1-containing peptide

Cycle	PTH-amino acid	Yield (pmol)	Position in F_1 β-subunit [14]
1	Asp	219	394
2	Glu	466	395
3	Leu	309	396
4	Ser	178	397
5	Glu	379	398
6	Glu	366	399
7	Asp	279	400
8	Lys	149	401
9	Leu	221	402
10	Thr	151	403
11	Val	163	404
12	Ser	73	405

0.5 nmol of the cross-linked peptide (17 kDa) was coupled to *p*-phenylenediisothiocyanate-glass, and its sequence determined [18]. PTH-amino acids were identified by reverse-phase HPLC as in [19]

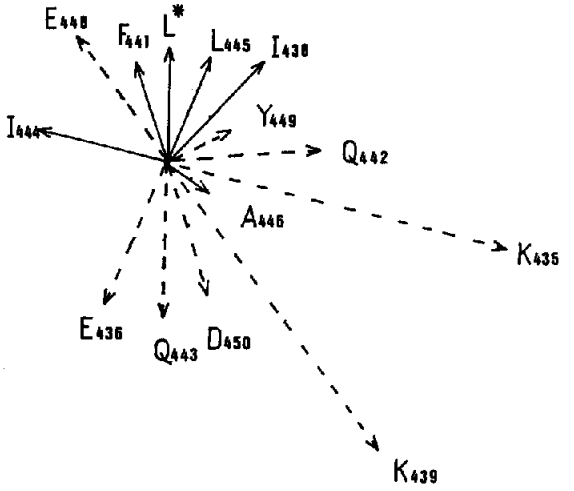

Fig.2. Helical plots of residues in the IF_1-binding peptide. The spatial arrangement of residues 434–450 (LKETIKGFQQILAG EYD) on the bovine F_1 β-subunit, plotted as in [23]. Leu 434 is denoted by the asterisk and subsequent amino acids are spaced at 100° intervals. The hydrophobicity of a residue is represented by the length of the corresponding vector, positive hydrophobicities being indicated by continuous lines and negative hydrophobicities by broken lines. Only residues that contribute significantly to the amphiphilicity are shown. The hydrophobic moment of this helical segment is equal to 0.28.

4. DISCUSSION

The results described above indicate that, in the F_1-IF_1 complex, IF_1 lies in close proximity to (at least part of) the peptide comprising residues 394–459 of the bovine β-subunit. The use of substoichiometric amounts of IF_1, and the unambiguity of the sequence obtained (table 1), indicate that the interaction is specific and, indeed, that this region of F_1 constitutes (at least part of) the binding site for IF_1.

Secondary structure prediction [9] suggests that the C-terminal two-thirds of IF_1 exists as an amphiphilic helix, with a high hydrophobic moment. Application of the same techniques (using software provided by Dr E. Eliopoulos, Astbury Depart-

ment of Biophysics, University of Leeds) to peptide 394–459 of bovine F_1-β (Geddes, A.J. and Jackson, P.J., unpublished) suggests that, as shown for the *E. coli* enzyme [22], the C-terminal region of this peptide also very probably adopts a helical conformation, and a helical plot [23] indicates that the segment Leu 434–Ala 446 would possess a high hydrophobic moment (fig.2). No other section of this peptide shows both helical and amphiphilic properties (not shown). It is therefore proposed that the amphiphilic helix of IF_1 packs against the amphiphilic helix corresponding to residues 430–450, near the C-terminus of the F_1 β-subunit. This mode of packing has been proposed for a variety of inter-protein interactions [24].

The precise mode of action of IF_1 is at present unknown. It may exert its effect by (i) sterically blocking the ATP-binding site of F_1, (ii) (allosterically) blocking turnover on the subunit to which it binds or (less obviously) (iii) preventing the cooperative interactions between the three catalytic sites. From its site of binding (above residue 400), it seems unlikely that IF_1 does interfere directly with nucleotide binding, which is thought to involve residues 156–162, 249–253, 301–311, 199 and 368 [17]. Furthermore, the requirement of IF_1 binding for ATP makes it unlikely that both bind to the same residues on the enzyme. However, since its unstructured N-terminal third (not considered in the model above) is required for inhibition by IF_1 [25], an interaction of this protein with the active site of F_1 cannot be completely ruled out.

One surprising feature of our localisation of the IF_1-binding site in the peptide 394–459 of the bovine β-subunit is that this region is very highly conserved between species. *E. coli* and the bovine enzyme show 53 out of 66 identities (and numerous conservative replacements) in this region [17]. In contrast, IF_1, while inhibiting most species of mitochondrial F_1, has no effect on bacterial or chloroplast systems ([26] and unpublished). The lack of action of IF_1 on non-mitochondrial F_1 led Walker et al. [22] to propose that IF_1 bound at the N-terminus of the bovine β-subunit, a region which has no homologous segment in bacteria, in contrast to the conclusions here. The two proposals might be reconciled if the N-terminus of the β-subunit (known to be on the enzyme surface [17]) lies near the C-terminus in space. More likely, however, is that our proposed IF_1-binding site is present in all species, but that the IF_1-F_1 interaction is blocked in non-mitochondrial species by the (poorly conserved) δ- or ϵ-subunit. This view is supported by the demonstration [27] that the ϵ-subunit of *E. coli* (which inhibits ATP hydrolysis by this enzyme [28]) also binds close to the C-terminus of the β-subunit.

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