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Primary structure and properties of the inhibitory protein of the mitochondrial ATPase (H^+-ATP synthase) from potato

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Abstract

The primary structure of the inhibitory protein (IF_1) of the potato mitochondrial ATPase has been determined by protein sequencing, and its molecular weight determined by electrospray mass spectrometry. Both are consistent with a 56-residue protein of molecular weight 6697. This protein shows only weak homology with IF_1 sequences from mammals and yeasts, and significant deletions are present compared to these sequences. Homology is strongest in the region between residues 22 and 46 (ox heart numbering), where 5 identities and 6 conserved residues are observed across all five IF_1 species. In addition, this region shows homology with protein inhibitors from ATPases other than mitochondrial F_1. It is suggested that this region might constitute an ATPase 'inhibitory motif'. Functional studies show that, unlike IF_1 from mammals or yeasts, potato IF_1 binds only poorly to ox heart F_1, and does not show the ability to exist in 2 (alternate) stable conformations.

Keywords: Mitochondrion; ATP synthase, H^+-; Inhibitor protein; Sequence homology; (Potato)

1. Introduction

The mitochondrial H^+-ATP synthase (F_1F_o) is that protein responsible for ATP synthesis, linked to transmembrane H^+ transport, in mitochondria. In the absence of supplied energy, this enzyme acts in the reverse direction, as an ATPase. Within the mitochondrion, F_1F_o is associated with a small, basic protein which inhibits its ATPase activity, the ATPase inhibitor protein IF_1 [1]. In coupled membrane preparations, IF_1 has also been shown to inhibit ATP synthesis by F_1F_o [2], and its association with F_1F_o to be modulated according to mitochondrial membrane potential (promotes dissociation) and MgATP (promotes association) [3]. On the basis of these in vitro observations, and other observations on heart muscle mitochondria in vivo, IF_1 has been suggested to play a regulatory role in mammalian mitochondria under physiological [4] and/or pathological [5] conditions.

The sequences of IF_1 from two mammalian species [6,7] and two yeast species [8,9] are known. Homology is strong between IF_1 from ox and rat, with 56/82 identities and a number of conservative substitutions. Homologies are also seen between IF_1 from the two yeast species and, to a lesser extent, between mammalian and yeast IF_1. IF_1 appears to be cross-reactive between species – yeast and rat IF_1, for example will inhibit F_1 from ox heart mitochondria [10,11].

Control of the F_1F_o ATPase has also been demonstrated to occur in plant mitochondria [12,13]. Recently, a protein with the characteristics of IF_1 has been isolated from potato mitochondria [14], although this apparently inhibits ox heart F_1 only poorly. The present work reports the sequence of this protein, which is demonstrated to show only weak homology to the IF_1 species previously isolated. The relationship of this protein to mammalian and yeast IF_1 is considered, together with its relationship to ATPase inhibitory peptides occurring in other systems.

2. Methods

Potato mitochondria were isolated as described by Rickwood et al. [15]. IF_1 was isolated from these mitochondria

Abbreviations: F_1F_o, the mitochondrial H^+-ATP synthase; F_1, soluble ATPase fraction of the ATP synthase; IF_1, naturally occurring inhibitor protein of the H^+-ATP synthase; CM Sephadex, carboxymethyl Sephadez; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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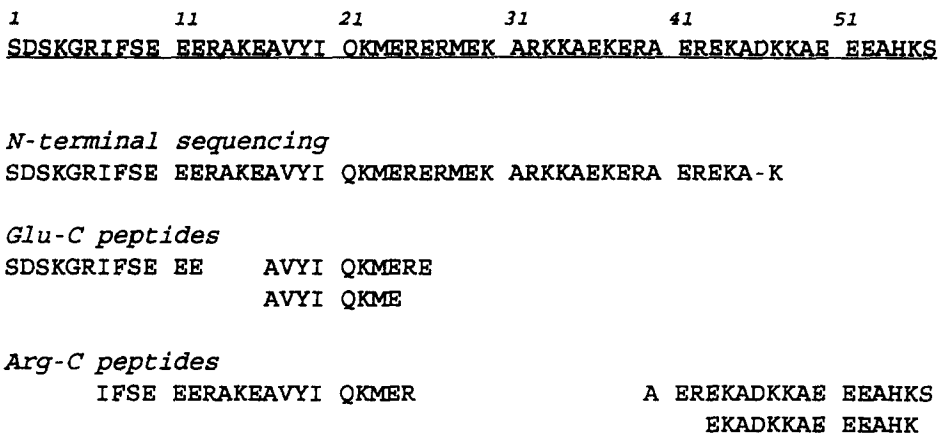

Fig. 1. Primary structure of potato IF_1 and derived peptides.

by heating them to 100° C for 2 min, followed by chromatography of the soluble fraction on CM Sephadex essentially as described for IF_1 from yeast by Ebner and Meier [16]. The major contaminant in this preparation, cytochrome *c*, was removed by chromatography on hydroxyapatite [16]. Typically, 2 g of mitochondria yielded about 1 mg of protein, with specific activity of 3000 U/mg. This is comparable with the method of Norling et al. [14], who used acid/base treatment to release IF_1 from the mitochondria.

An estimated 1 nmol of potato IF_1 was further purified by hplc, using a Brownlee Aquapore RP-300 column (100 × 2 mm), from Applied Biosystems Ltd. The column was equilibrated with 0.1% trifluoroacetic acid, and developed with a gradient of acetonitrile (1%/min).

Table 1
Amino acid composition of potato IF_1

Amino acid	Predicted from sequence (mol/mol IF_1)	Measured content (mol per 1/15 glutamate)
Alanine	8	7.8
Cysteine	0	n.d.
Aspartic acid	2	n.d
Glutamic acid	14	15.0 (Glu + Gln)
Phenylalanine	1	0.9
Glycine	1	4.8
Histidine	1	1.1
Isoleucine	2	2.0
Lysine	11	10.7
Leucine	0	0
Methionine	2	1.9
Asparagine	0	n.d.
Proline	0	0
Glutamine	1	see above
Arginine	7	6.6
Serine	4	4.4
Threonine	0	0
Valine	1	1.3
Tryptophan	0	n.d.
Tyrosine	1	0.9

The predicted composition was taken from Fig. 1. The measured composition was as recorded in Ref. [14], except that the figures are calculated assuming 15 Glx residues in the protein, and not 16 as assumed by Norling et al. n.d. = not determined.

Approximately 200 pmol of the major peak was used for N terminal sequencing, which yielded 46 out of the first 47 residues (see Fig. 1). Proteolysis of the remaining material was performed with either (1) endopeptidase Glu-C (in 100 mM Tris-HCl (pH 8.0) for 4 h at 37° C) or (2) endopeptidase Arg-C (in 40 mM Tris-HCl, 1 mM $CaCl_2$, 2 mM dithiothreitol (pH 7.8) for 16 h at 37° C). Both enzymes were Promega sequencing grade (Promega, UK), and both digestions carried out at a protease/substrate ratio of 1:25 (w/w). Generated peptides were purified by HPLC using a Brownlee Aquapore OD-300 column (100 × 2 mm). Automated N terminal sequencing was performed on all significant peaks in these digests, using an Applied Biosystems gas phase sequencer (Model 470A or 473A).

Electrospray mass spectrometry was performed on the hplc purified material by Dr. Carol Robinson of the Oxford Centre for Molecular Sciences, using a VG Bio-Q triple quadropole atmospheric pressure mass spectrometer equipped with an electrospray interface.

ATPase activity was measured spectrophotometrically, using an ATP regenerating system, as described previously [17]. Inhibition by IF_1 was measured as described by Norling et al. [14]. Binding of IF_1 to F_1F_o was carried out using ^{125}I-labelled IF_1 as described previously [18], except that, in competition experiments, potato IF_1 was included in the incubation medium, as indicated. 1 U ATPase is defined as the amount of enzyme hydrolysing 1 μmol ATP/min; 1 U inhibitor is the amount of that inhibits 0.2 U ATPase by 50%.

3. Results and discussion

3.1. Amino acid sequence of potato IF_1

The primary structure of potato IF_1 is given in Fig. 1. 45/56 residues were determined by N-terminal sequencing

of the intact protein, while the C-terminus was identified from peptides after arg-C endoproteinase digestion. Other peptides, confirming the sequence, were identified as indicated.

The molecular weight of this protein was calculated as 6697. This is considerably lower than that reported by Norling et al. [14], who estimated 8300 on the basis of SDS-PAGE. However, the amino acid composition of this sequence agrees very well with their published amino acid analysis when this is calculated on the basis of 15 [glutamate + glutamine] residues in the protein (Norling et al. [14] assumed 16 such residues). Their analysis does indicate a higher glycine content in their preparation (Table 1); this glycine may, however, represent an artefact of the analysis. It was concluded that both preparations represent the same protein, and that SDS-PAGE overestimates the true molecular weight of such a small protein.

This was confirmed by direct determination of the molecular weight of our preparation, using electrospray mass spectrometry, which yielded an estimate of 6697.9 ± 0.5, in good agreement with the molecular weight calculated from our sequence.

In the course of this work, we also sequenced IF_1 from rat liver mitochondria, and determined its molecular weight by electrospray mass spectrometry (data not shown). This work confirmed the sequence deduced from the DNA sequence by Lebowitz and Pedersen [7], and also showed that, like potato IF_1 – but in contrast to ox heart IF_1 [19] – the mature rat protein has an unblocked N-terminus.

3.2. Comparison with homologous proteins

Fig. 2 shows the sequence of potato IF_1 aligned with the known sequences of IF_1 from mammals and yeasts. Like these proteins, it is a basic protein ($pI \approx 10$), with a high content of both positively and negatively charged amino acids. None of the 5 proteins contain cysteine; however, potato IF_1 is particularly parsimonious in lacking five other amino acids, leucine, asparagine, proline, threonine and tryptophan.

Considering their similarity in function, these five proteins show surprisingly weak homology compared to the other subunits of F_1 – which are highly conserved between species [24]. Alignment of the sequences shows some conservation of the protein at the extreme N-terminal end, and a more conserved region in the centre, where IF_1 is predicted to be α-helical in structure [6,7,19]. Fig. 2 shows that, between residues 22 and 46 (numbering according to ox IF_1), there are 5 positions of identity and 6 conservative substitutions between the 5 homologues (8 identities and 6 conserved positions between the potato and *Candida* IF_1), suggesting that this region may have functional importance. The major differences in potato IF_1 lie in a deletion of residues 8–21 (relative to ox IF_1), and a deletion after residue 50. Similar deletions occur in yeast IF_1 species, except that the N-terminal deletion is some 9 amino acids shorter, and a second deletion occurs near the C-terminus.

Several groups [6,8,9,19] have pointed out the presence of internal repeats in the IF_1 sequence. For example, the sequence KEIER in ox heart IF_1 (residues 58–62) is followed by KEIER (residues 65–69). Similar repeats are observed in potato IF_1; residues KAEKE (34–38) are followed by RAERE (39–43), for example (Fig. 1). However, Fig. 2 shows that such repeats are not conserved between species. Notably, residues 58–62 in ox heart IF_1 which corresponds to the first repeat mentioned above, are covered by a deletion in the potato and yeast proteins. We conclude that such repeats are non-functional and may well be a statistical consequence of the limited repertoire of amino acids used in constructing these proteins.

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      1          11          21          31          41
ox    GSESGDNVRS SAGAVRDAGG AFGKREQAEE ERYFRARAKE QLAALKKHHE
rat   GSDSSESMDS GAGSIREAGG AFGKREKAEE DRYFREKTRE QLAALKKHHE
pot   .SDSKGRI.. .......... .FSEEERAKE AVYIQKMERE RMEKAR....
C.u   .TAGATGATR QDGS.TDA.. .FEKREKAQE DLYIRQHEKE QLEALKES..
S.c   .SEGSTGTPR GSGS.EDS.. .FVKRERATE DFFVRQREKE QLRHLKEQ..
      △△          ○○ ○○    ▲  ▲▲▲  △△   △▲ △  △△

      51          61          71          81
ox    NEISHHAKEI ERLQKEIERH KQSIKKLKQS EDD
rat   DEIDHHSKEI ERLQKQIERH KKKIKYLKNS EH
pot   .......... ....KKAEKE RAERKKADKK AEEEAHKS
C.u   .........L KKQKKSLDD. ......LEEK IDDLTK
S.c   .........L EKQRKKIDS. ......LENK IDSMTK
            ○  ○  ▲ △△   ○     △         ○
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Fig. 2. Sequence alignments for IF_1 species. ▲ indicates residues identical in all 5 sequences. △ indicates homologous replacements between all 5 species. ○ indicates conservation of properties in regions of deletions. Data taken from Refs. [7–9,22] and Fig. 1.

3.3. Properties of potato IF_1

Like other mitochondrial IF_1 species [20], potato IF_1 shows a slow ($t_{0.5} \approx 30$ s) ATP dependent inhibition of its corresponding F_1-ATPase. This inhibition is measured by preincubating F_1 with IF_1, followed by dilution of the mixture into an ATPase assay system; if F_1 complexes with IF_1 in the preincubation, its activity is inhibited in the subsequent assay. However, this procedure requires that a stable (slowly dissociating) complex be formed in the preincubation.

Using this procedure, potato IF_1 was to be a poor inhibitor of mammalian F_1 [14]. This lack of inhibition might reflect (a) an inability of potato IF_1 to bind to mammalian F_1, (b) an inability of this IF_1 to inhibit once bound, or (c) formation of an unstable F_1-potato IF_1 complex, which rapidly dissociates in the assay mixture.

To distinguish between these possibilities, we investigated the binding of potato IF_1 directly, by measuring its ability to compete with (radiolabelled) mammalian IF_1 for its binding site on mammalian F_1F_0. The results are shown in Fig. 3. While unlabelled mammalian IF_1 competes effectively for the binding site, even a 25-fold excess of potato IF_1 has no detectable effect on the binding of

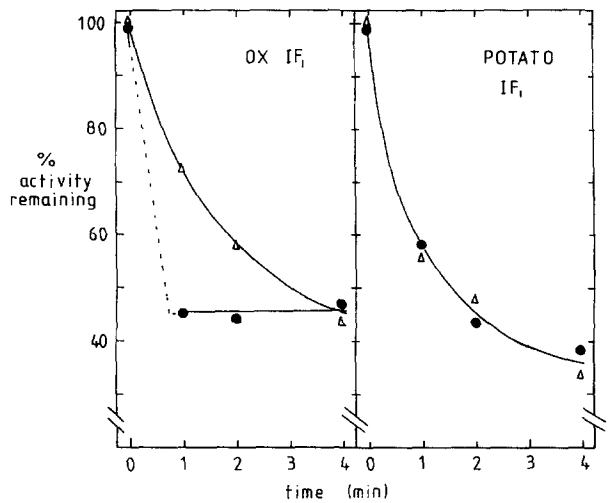

Fig. 4. pH-dependent conformational states in IF_1 20U IF_1 from ox (left-hand panel) or potato (right-hand panel) was incubated for 10 min, 25° C at either pH 4.8 (●) or pH 8.2 (△) in 0.1 ml buffer containing 50 mM glycine brought to the relevant pH with Tris base. Aliquots containing 2 U IF_1 were then added to 0.2 ml 250 mM sucrose, 50 mM Mops (pH 6.7, 25° C) containing respectively inhibitor-depleted ox heart submitochondrial vesicles or potato F_1 (1 U ATPase in each case). Binding was initiated by the addition of 2 mM MgATP, and samples taken for assay of ATPase activity at intervals as indicated.

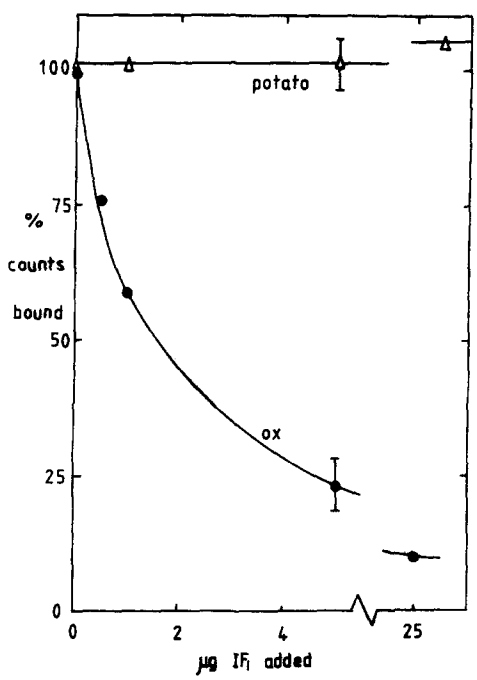

Fig. 3. Competition by potato IF_1 for IF_1-binding site on submitochondrial vesicles. To 220 μl buffer containing 250 mM sucrose, 50 mM KCl, 20 mM Mops, 1 mg/ml cytochrome *c* (pH 6.7) was added 1 μg ox [^{125}I]IF_1 (4000 cpm/μg), 1–25 μg unlabelled ox IF_1 or potato IF_1 (as indicated), and 2 mM MgATP. Binding was initiated by addition of 180 μg inhibitor-depleted ox heart submitochondrial vesicles. The total volume was 250 μl. Binding was terminated by addition of ammonium sulfate solution to 10% saturation followed by centrifugation, and the washed pellet was then counted for bound radioactivity [19]. 100% value ≈ 1600 cpm bound, which is equivalent to about 85% of all the available binding sites occupied by [^{125}I]IF_1.

mammalian IF_1. It can be concluded that potato IF_1 has a very low affinity (< 1/100th that of mammalian IF_1) for this binding site, and its inability to inhibit mammalian F_1 relects its lack of binding, presumably because of the low degree of homology between two proteins.

One unusual feature of mammalian IF_1 is its ability to exist in two stable conformational forms – a form induced by low pH (active) and a form induced by high pH (inactive). These forms interconvert slowly – preincubation of IF_1 at low pH leads to an active state which is retained for several minutes during assay at a higher pH [21]. This interconversion may be due to ionisations of histidine residues around residues 48–56 (Fig. 2), and may be responsible for the dissociation of IF_1 from F_1 in response to the transmembrane H^+ gradient [22].

Fig. 2 shows that these histidine residues are not conserved in potato IF_1. We therefore investigated whether this IF_1 could exist in two such conformations. Fig. 4 shows that, as shown previously, inhibition of mammalian F_1 by IF_1 pretreated at pH 4.8 is faster than that by IF_1 pretreated at pH 8.3; the low pH conformation is active and maximal inhibition is achieved within 1 min, compared to about 4 min for the high pH conformation (Fig. 4, left hand panel). In contrast, incubation of potato IF_1 at either pH has no effect on the rate of inhibition of potato F_1 (right hand panel). It is likely, therefore, that potato IF_1, unlike mammalian IF_1, cannot exist in 2 stable conformational forms.

This may indicate a role of the histidine cluster in the conformational transition, but in view of other differences

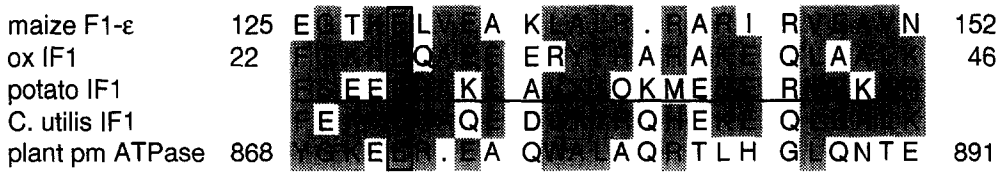

Fig. 5. Interprotein homologies with the highly conserved region of IF_1. Homologous substitutions between three or more of the sequences are boxed. The numbering systems used are from [23] (plant plasma membrane ATPase autoinhibitory peptide) and [24] (F_1-ϵ subunit).

in the sequence of potato IF_1, this deduction cannot be taken as conclusive. More importantly, however, it means that – unless the mammalian system has a unique regulatory mechanism – this conformational transition cannot be essential for regulating F_1–IF_1 interactions in general.

3.4. Comparison with non-homologous proteins

Comparison of the sequence of potato IF_1 with a protein sequence database revealed a number of weakly homologous sequences. Interestingly, mammalian IF_1's were not revealed as homologous by this search, although the IF_1 species from yeast were shown to be related. The only other similarities detected were with proteins with long stretches of glutamic acid and lysine/arginine residues (such as the malaria antigen from *Plasmodium*, which contains a 50-amino-acid stretch containing only Glu and Lys), and any functional significance of such similarities is uncertain. However, Realini et al. [25] have recently implicated Glu/Lys rich regions ('KEKE motifs') in mediating protein–protein interactions in a variety of systems, such as chaperonins and MAP/microtubule binding. It is possible that the Glu/Lys rich regions in the various IF_1 species function in promoting the F_1–IF_1 interaction in this general manner.

An alternative approach was to look for homologies between other proteins of similar function. Other classes of protein have been identified as inhibiting ATP hydrolysing enzymes. For example, IF_1 is found associated with only mitochondrial F_1-ATPases. In chloroplasts and bacteria, its inhibitory role is taken over by part of an intrinsic subunit, the ϵ-subunit of the F_1-ATPases. Matsubara et al. [8] have pointed out sequence similarities between the F_1-ϵ subunit of *E. coli* and yeast IF_1. Fig. 5 shows such a homology for chloroplast F_1, and demonstrates that such homologies involve the conserved region of IF_1, residues 22–46, which we have suggested to have functional importance. (Other ϵ-subunit sequences are given in Ref. [24].)

Recently, Palmgren and co-workers [23] have identified a related situation on the plasma membrane ATPase from plants (*Avena, Arabdopsis*). Here, ATP hydrolysis at the active site of the enzyme is inhibited by a sequence close to the C-terminus of the same enzyme polypeptide. These workers have identified the autoinhibitory peptide by partial proteolysis, and by peptide synthesis, and its concensus sequence is also given in Fig. 5. It can be seen that this peptide, too, bears some homology to the conserved region of IF_1. It is possible to suggest, therefore, that the conserved region noted in the various homologues of IF_1 may represent a general ATPase 'inhibitory motif' which can be more widely utilised – either by duplication or by convergence – in regulating a variety of enzymes.

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