Functional domains of the ATPase inhibitor protein from bovine heart mitochondria

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Abstract A study is presented of the activity and temperature dependence of the ATPase inhibitor protein (IF_1) from bovine heart mitochondria and of synthetic partial IF_1 peptides. The results show that the IF_1-(42–58) peptide is the most potent inhibitory domain of IF_1.

Key words: F_1F_0 ATP synthase; Inhibitor protein of mitochondrial ATPase; Synthetic ATPase inhibitor peptide

1. Introduction

The F_1F_0 ATP synthase of coupling membranes catalyzes ATP synthesis driven by the protonmotive force (PMF) generated by respiratory or photoredox systems [1]. In mitochondria, wasteful ATP hydrolysis by the F_1F_0 complex is specifically inhibited by the ATPase inhibitor protein, IF_1 [2–4].

IF_1 associates reversibly in a 1:1 stoichiometry with F_1F_0, from which it is displaced by the PMF, thus IF_1 has no significant effect on ATP synthesis [2]. The ΔpH component of PMF, in particular the matrix pH, on which side IF_1 is associated with the complex, is the critical factor for its binding and inhibitory activity [2,5,6]. In our laboratory it has been found that IF_1 also inhibits passive proton conduction by the F_1F_0 complex [6].

Bovine IF_1 is a basic protein of 84 amino acids [7] and is present in other eukaryotes [8,9]. The amino acid sequence is well conserved in these proteins. Studies from various laboratories have attempted to identify the functional domain(s) of the IF_1 protein. Harris et al. [10,11] first suggested that an α-helical rod encompassing residues 22–79 in bovine heart IF_1 protein represents the functional segment (Fig. 1). Stout et al. [12] showed that the synthetic peptide with the sequence Phe22–Lys46 in bovine IF_1 inhibits the ATPase activity in the soluble F_1. Papa et al. [13] and van Raij et al. [14] found, however, that this peptide has no significant effect on the ATPase activity of the F_1F_0 complex.

Studies with proteolytic fragments of IF_1 [15] and genetic deletions in the recombinant protein [14] indicated that the last 10–20 residues of the C-terminus are not directly essential for the inhibitory activity. The same seems to apply to the first 13 residues of the N-terminus [14]. van Raij et al. [14] concluded from their studies with recombinant products of bovine IF_1 that the minimal inhibitory sequence consists of residues 14–47. Papa et al. [13] have, on the other hand, shown that the synthetic peptide with the Phe42–Lys58 sequence of bovine IF_1 is equally effective, as the natural complete IF_1, in inhibiting the ATPase activity of both the F_1F_0 complex in the membrane and of soluble F_1. The 42–58 segment has at positions 48, 49, 55 and 56 four histidines. Histidine chemical modification [5,6], site directed mutagenesis of His49 [16] and chemical substitution of His48, 49, 55, 56 or of Lys46, 47, 58 in the synthetic 42–58 peptide [13] have shown that these residues are directly responsible for the activity and pH dependence of IF_1.

In this paper a study is presented on the activity and temperature dependence of IF_1 and synthetic IF_1 segments. The results clearly establish that the Phe42–Lys58 segment of IF_1 is the most potent inhibitory domain of IF_1.

2. Materials and methods

2.1. Materials

CM C-25 Sephadex, ATP, phosphoenolpyruvate (PEP), NADH, pyruvate kinase, lactate dehydrogenase, were from Boehringer; chemicals for synthesis and sequence were obtained from Applied Biosystem; thioanisol and ethanediol from Fluka; *tert*-butylether from Carlo Erba. All other chemical reagents were of analytical grade.

2.2. Preparations

Inside-out vesicles of the inner mitochondrial membrane were obtained by exposure of isolated bovine heart mitochondria [17] to ultrasonic energy in the presence of 4 mM EDTA at pH 8.5 (ESMP) or in the presence of 1 mM MgATP (MgATP-SMP) [18]. Sequential treatment of ESMP with Sephadex chromatography produced particles from which IF_1 was removed (SMP) [19]. IF_1 was isolated from MgATP-SMP according to [20]. Recombinant IF_1 was a generous gift of Professor John Walker (Medical Research Council, Cambridge, UK). The protein concentration was determined by the Lowry method [13].

2.3. Assays

The ATP hydrolase activity was measured with an ATP-regenerating system as in [14,21].

2.4. Synthesis of peptides

Synthetic peptides with conserved or mutated sequences of IF_1 were obtained by a standard solid-phase method with a fully automatic peptide synthesizer (model 431A of Applied Biosystem) using the Fmoc chemistry [22]. After synthesis the peptides were deprotected

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Abbreviations: IF_1, inhibitor protein of mitochondrial ATPase; ESMP, submitochondrial particles prepared in the presence of EDTA; SMP, submitochondrial particles deprived of IF_1; F_0, membrane integral sector of the H^+-ATP synthase; F_1, catalytic sector of the H^+-ATP synthase

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by incubating for 2 h at room temperature in 83% trifluoroacetic acid, 4% thioanisol, 2% ethanediol and 6% crystalline phenol. After cleavage, the peptides were precipitated by 10 vol. of anhydrous *tert*-butylether, pelleted, washed four additional times with ether and lyophilized. The purity of the synthetic peptides was checked by reverse-phase high performance liquid chromatography on a Perkin Elmer C-8/10 column with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The sequence of the synthetic peptides was verified by amino acid analysis performed with a fully automatic peptide sequencer (model 473A of Applied Biosystems). The repetitive yield of sequence analysis was higher than 90%. The solubility in water of the wild and mutant IF_1-(42–58) peptide and of IF_1-(22–46) peptide was >10 mg/ml. The protein concentration of solutions of IF_1 and IF_1 synthetic peptides, prepared on a w/v basis, was checked using the Lowry method against a standard solution of bovine albumin [13].

3. Results

Fig. 2 shows the titrations of the inhibitory effects of the purified native IF_1, recombinant IF_1 and IF_1 synthetic polypeptide segments on the ATPase activity of 'inside-out' submitochondrial particles from bovine heart deprived of the endogenous IF_1 (SMP), measured at 21 and 37°C. It can be observed that the inhibitory affinity of the active peptides increases greatly with temperature. At both temperatures the natural and the recombinant IF_1 proteins exhibited the same inhibitory affinity. The synthetic peptide with the Phe42–Lys58 sequence of the bovine IF_1 showed, both at 21 and 37°C, an inhibitory affinity three times higher than the natural and the recombinant IF_1. Replacement of the four histidines at positions 48, 49, 55 and 56 or of the three lysines at positions 46, 47 and 58 in the IF_1-(42–58) peptide practically abolished its inhibitory activity at both temperatures. The IF_1-(Phe22–Lys46) peptide, which exhibits inhibitory activity on the purified F_1 sector [12], did not exert any significant inhibitory activity on the ATPase activity of the native F_1F_0 complex in SMP (see also [13,14]). It should be noted that,

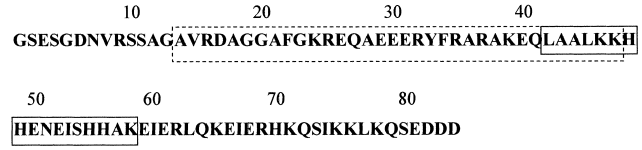

Fig. 1. The amino acid sequence of the bovine heart IF_1. The solid box contains the IF_1-(42–58) peptide, the dotted box the IF_1-(14–47) peptide [14].

like the complete IF_1, the IF_1-(42–58) peptide caused permanent, stable inhibition of the ATPase activity of SMP (Fig. 3). The I_{50} values for natural and recombinant IF_1, when measured in the present study at 37°C, are exactly the same as those obtained at the same temperature by van Raaij et al. for the recombinant IF_1 [14]. Thus, the I_{50} values for the synthetic IF_1 peptides we have synthesized can be directly compared with those obtained for the IF_1 segments used by these authors (see Section 4).

Fig. 4 illustrates the temperature dependence of the inhibitory activities of natural and recombinant IF_1 and of the synthetic IF_1-(42–58) peptide. The I_{50} values of IF_1 and of IF_1-(42–58) peptide decreased as the temperature was increased from 21 to 37°C (Fig. 4). It can be noted that I_{50} values of the IF_1-(42–58) peptide were, in the temperature range examined, always lower than the I_{50} values of IF_1. The latter decreased, however, more steeply as the temperature increased. These results thus show that the IF_1-(42–58) peptide has an inhibitory affinity significantly higher and a binding energy lower than the complete IF_1.

4. Discussion

The I_{50} values for inhibition of the ATPase activity of the F_1F_0 complex in inside-out vesicles of bovine heart inner mi-

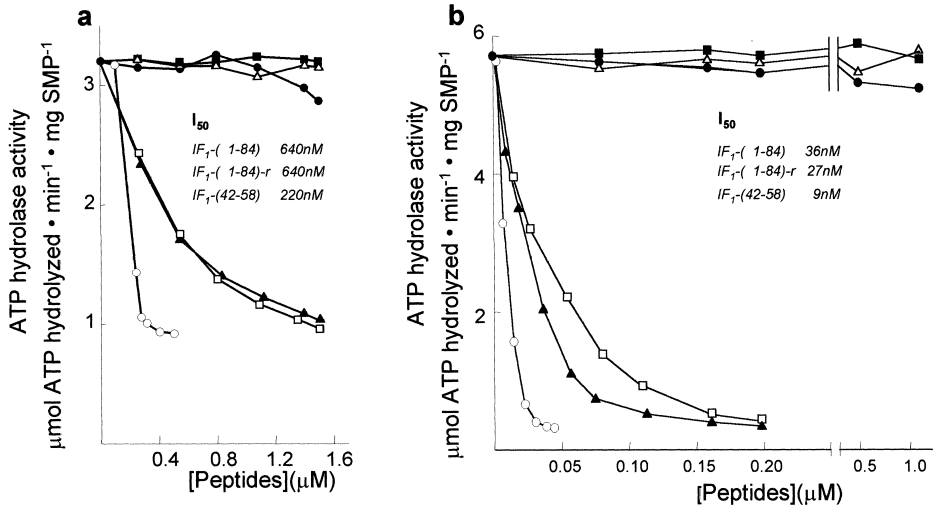

Fig. 2. Titration at two temperatures of the inhibitory effect of natural IF_1, recombinant IF_1 and synthetic, conserved and mutant, IF_1 segments on ATP hydrolase activity in SMP. SMP (1 mg/ml) were incubated in a reaction mixture containing: 200 mM sucrose, 10 mM Tris/acetate, 1 mM K-EDTA, 6 mM $MgCl_2$, 1 mM MgATP, pH 6.7 in the presence of IF_1 or IF_1 partial peptides in the concentration range reported in the figure. The mixture was incubated at 21°C (a) or 37°C (b) for 10 min and then an aliquot of the suspension containing 50 µg particle protein was added to 1 ml of the ATPase assay mixture containing: 200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 10 U lactate dehydrogenase, 20 mM Tris/HCl, 0.1 mM NADH, 1 mM PEP, 4 U pyruvate kinase and 1 mM rotenone, pH 8.0. The ATPase activity, started by the addition of 1 mM MgATP, was followed by monitoring the oxidation of NADH at 340 nm at the temperature of 21°C (a) or 37°C (b). Submitochondrial particles deprived of IF_1 were incubated in the presence of: (□) natural IF_1; (▲) recombinant IF_1; (○) IF_1-(42–58) synthetic peptide; (△) IF_1-(22–46) synthetic peptide; (●) [Ala48,49,55,56]-IF_1-(42–58) synthetic peptide; (■) [Ala46,47,58]-IF_1-(42–58) synthetic peptide.

tochondrial membrane show that, compared to the complete IF_1 from the same source or produced by heterologous expression, the synthetic IF_1-(42–58) peptide, with an I_{50} of 220 nM at 21°C and 9 nM at 37°C, is the most potent inhibitory IF_1 partial segment among all those so far tested in our [13] and other laboratories [14,23]. The inhibitory affinity of the IF_1-(42–58) peptide has, in fact, an inhibitory affinity for the ATPase activity of the F_1F_0 complex even higher than that of the complete IF_1. The inhibitory activity of the IF_1-(42–58) peptide is permanent, i.e. the peptide–F_1F_0 complex is as stable as that formed by the complete IF_1 (Fig. 3), exhibits the same pH dependence, with an optimum at a pH around 6.5 [13], and a marked temperature dependence as the complete IF_1. The marked enhancement with temperature of the inhibitory affinity of IF_1 for the ATP synthase, could play an important role in promoting effective inhibition of wasteful ATP hydrolysis, which, under conditions of respiratory deficiency, might be quite relevant at body temperatures around 37°C.

No equivalent data are available on the pH and temperature dependence of the inhibition of the ATPase activity of the F_1F_0 complex exerted by the partial IF_1 peptides tested by van Raaij et al. [14]. Of the various IF_1 peptides used by these authors, the IF_1 (10–47) synthetic peptide was reported to be the minimal effective inhibitory peptide. With an I_{50} of 45 nM at 37°C and pH 6.5 it is, however, five-fold less active than the IF_1-(42–58) peptide. Furthermore, at difference with the IF_1-(42–58) peptide which produced a stable inhibition, the inhibition of the ATPase activity by the IF_1-(10–47) peptide was reported to decrease during the assay period, this indicating that the peptide–F_1F_0 complex was unstable [14]. Van Raaij et al. [14] have proposed that the four histidines in the 42–58 segment increase the stability of the IF_1–F_1F_0 complex. A truncated IF_1 peptide, missing up to 24 residues from the

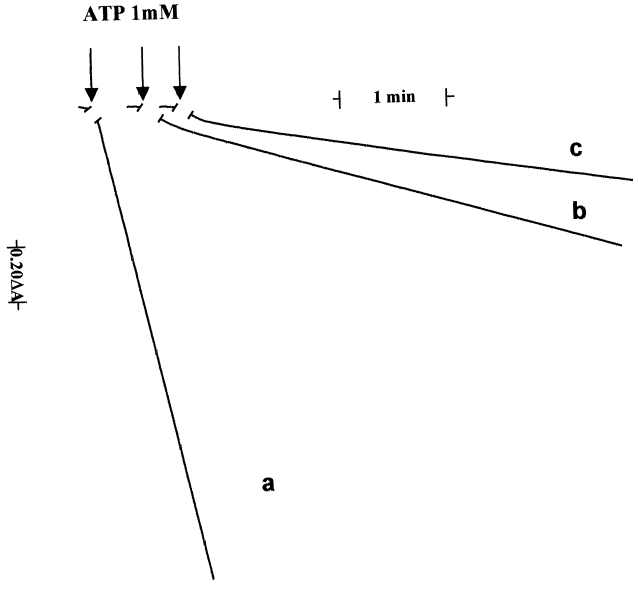

Fig. 3. Inhibitory effect of recombinant IF_1 and IF_1-(42–58) synthetic peptide on the ATP hydrolase activity of SMP. SMP (1 mg/ml) were incubated in the same reaction mixture reported in the legend to Fig. 1 at 37°C in the absence (a) or in the presence of 0.075 μM recombinant IF_1 (b), or in the presence of 0.025 μM IF_1-(42–58) synthetic peptide (c). For other details see Section 2 and the legend to Fig. 2.

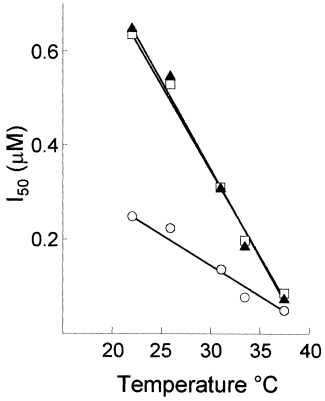

Fig. 4. Temperature dependence of the inhibitory effect of natural IF_1, recombinant IF_1 and synthetic IF_1 (42–58) peptide on the ATP hydrolase activity of SMP. The I_{50} for the inhibition of the ATP hydrolase activity of SMP were obtained by incubating the particles with (□) natural IF_1, (▲) recombinant IF_1 and (○) IF_1-(42–58) synthetic peptide in a range of concentrations between 0 and 2 μM for 10 min at the temperatures reported in the figure. The ATPase activity was then started and measured as reported in the legend to Fig. 2. For other details, see the legend to Fig. 2 and Section 2.

C-terminus, i.e. the IF_1-(1–60) peptide containing the IF_1-(42–58) segment, exhibited practically the same inhibitory affinity and stability as the complete IF_1 and the IF_1-(42–58) peptide [14]. It seems possible to conclude that the 42–58 segment represents the primary critical domain of IF_1. The inability of the IF_1-(23–84) peptide [14,23] and the IF_1-(44–84) peptide [14] to inhibit the ATPase activity of the F_1F_0 complex, could result from an occlusion of the active 42–58 segment by the C-terminal region in the absence of the N-terminus. The IF_1-(42–58) peptide contains the residues K46 and K47 which are shared by the 10–47 peptide, the H49, which has been found to be responsible for the pH dependence of the inhibitory activity of IF_1 [16], three additional histidines and one more lysine, which are likely to increase the stability of the inhibitor–F_1F_0 complex. The pH dependence of the inhibitory activity of IF_1 and the IF_1-(42–58) peptide with optimum around 6.5 [13], indicates that positive charges on protonated histidine imidazole groups in addition to those of the lysines are involved in the binding of IF_1 to negatively charged dicarboxylic residues in the F_1F_0 complex. The sequence K46K47H48 could specifically interact with the last three residues of the conserved DELSEED sequence [24] at position β 394–400 in the C-terminal domain of the β subunit at the bottom of the F_1 spherical body facing the F_0 sector in the membrane [24,25].

Recently, Cabezon et al. [26] have proposed that protonation of His49 induces the IF_1 to acquire a dimeric active state which inhibits the ATPase activity of dimeric F_1F_0 complexes [27]. Deprotonation of His49 results, according to these authors [26] in the formation of an inactive IF_1 tetramer. If the dimer–tetramer interconversion of IF_1, observed for the isolated IF_1, also takes place in the interaction of IF_1 with the F_1F_0 complex in the membrane, this interconversion could represent an additional consequence of the histidine protonation in the IF_1-(42–58) domain.

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