NATURAL PROTEIN ATPase INHIBITOR FROM *CANDIDA UTILIS* MITOCHONDRIA

Binding properties of the radiolabeled inhibitor

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1. Introduction

The energy-transducing ATPases of mitochondria, chloroplasts and bacteria are associated with an endogenous protein inhibitor [1–7]. These naturally occurring inhibitors (IF_1) are low molecular weight and trypsin-sensitive proteins. They are either part of the ATPase molecule in chloroplast and bacterial ATPases [5–7] or a distinct entity in mitochondrial ATPase [8]. They strongly mask the hydrolytic ATPase activity of soluble and membrane-bound ATPase. Studies by Ernster et al. [9,10] and Van de Stadt et al. [11,12] have underlined the regulatory function of IF_1 in oxidative phosphorylation. IF_1 is believed to control the backflow of energy from ATP to ATP-driven reactions. A similar regulatory mechanism has been proposed for chloroplasts [13]. These conclusions were drawn from experiments where the interaction between the natural ATPase inhibitor and the ATPase was measured in terms of inhibition of the ATPase activity, assuming that inhibition of ATPase reflects the binding of IF_1 to ATPase.

IF_1 can be purified from *Candida utilis* mitochondria, allowing the preparation of radioactivity labeled IF_1 by growing *Candida utilis* in amedium supplemented with a radioactive amino acid [3]. This communication describes the use of radioactive IF_1 to study, by means of direct binding assays, the interaction between IF_1 and F_1-ATPase. It is shown that [^{14}C] IF_1 from *Candida utilis* mitochondria binds with high affinity (K_d 8×10^{-8} M) to submitochondrial particles depleted of IF_1 (AS particles). A direct correlation between the binding of IF_1 and the inhibition of the ATPase activity was found. Maximal binding requires a slightly acidic pH and the presence of MgATP. Oligomycin, an inhibitor which binds to the membrane sector of the ATPase complex and AMPPNP, a ligand of the F_1 portion of the ATPase complex both prevent the binding of IF_1 to AS particles.

Abbreviations: F_1-ATPase, coupling factor 1 equivalent to the hydrophilic sector of the ATPase complex; IF_1, natural ATPase inhibitor; AS particles, submitochondrial particles depleted of IF_1 by alkaline treatment and passage on Sephadex

2. Materials and methods

Candida utilis mitochondria, submitochondrial particles depleted of IF_1 (AS particles) and IF_1 were prepared as described [3]. Biosynthetic labeling of IF_1 was achieved by growing yeast cells in the presence of L-[^{14}C]leucine [3]. The specific radioactivity of purified IF_1 was approx. 10^6 dpm/μmol. Beef heart and *C. utilis* F_1-ATPases were purified by the method [14]. Beef heart submitochondrial AS particles and IF_1 were prepared according to [2,15]. The following values of molecular weights were used: F_1-ATPases, 360 000 [8]; beef heart ATPase inhibitor, 10 500 [16]; and *C. utilis* ATPase inhibitor, 7500 [3].

F_1-ATPase and IF_1 activities were assayed as described [3]. Protein of submitochondrial particles

was measured by the biuret method [17]. Soluble proteins were assayed with the Folin reagent [18]. Bovine serum albumin was used as a standard.

Binding assays were carried out with submitochondrial AS particles. Except when indicated in the legends, the standard incubation conditions were as follows. Yeast AS particles (1 mg protein) were incubated at 24°C in 2 ml medium containing 250 mM sucrose, 10 mM 3-(N-morpholino)propane sulfonic acid (MOPS), 0.5 mM ATP, 0.5 mM $MgSO_4$, pH 6.8 with increasing amounts of [^{14}C]IF_1. Incubation was always started by addition of the particles to the medium and was terminated after 25 min by centrifugation at 20 000 × *g* in a Sorvall centrifuge at 4°C. The supernatants were collected and the membrane pellets were dissolved in 1 ml formamide at 180°C. Radioactivities were determined by liquid scintillation.

L-[^{14}C]Leucine (about 50 Ci/mol) was obtained from Commissariat à l'Energie Atomique (CEA), Saclay. Adenylylimidodiphosphate (AMPPNP) and oligomycin were obtained from Boehringer.

3. Results

3.1. *Cross-reactions of beef heart and* C. utilis *F_1-ATPases with ATPase inhibitors*

The specificity and the potency of IF_1 from *C. utilis* and beef heart mitochondria were measured by titrating the hydrolytic activity of a constant amount of purified F_1-ATPase from both sources by increasing amounts of IF_1 (fig.1). Data in table 1 show that *C. utilis* IF_1 is seven times more active on *C. utilis* F_1-ATPase than on beef F_1-ATPase (0.32 μg required for half inhibition versus 2.40 μg). Likewise, beef heart IF_1 was four times more active on *C. utilis* F_1-ATPase than on beef heart F_1-ATPase (0.06 μg required for half inhibition versus 0.25 μg). On the other hand, *C. utilis* F_1-ATPase is more efficiently inhibited by beef heart IF_1 than by *C. utilis* IF_1 (0.06 μg required for half inhibition versus 0.32 μg). These results are in agreement with those of previous experiments where bound F_1-ATPase in submitochondrial particles was titrated with IF_1 [3].

Inhibition data shown in fig.1 were replotted according to [19] (fig.2) to calculate the number of

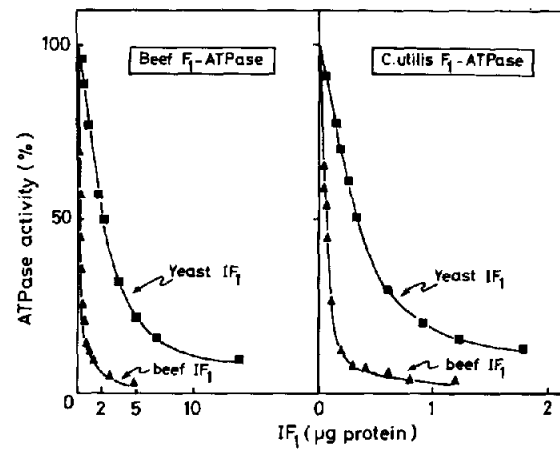

Fig.1. Cross-reaction between beef heart and *C. utilis* F_1-ATPases and their IF_1. Beef heart F_1-ATPase (1.6 μg protein, specific ATPase activity: 76 μmol P_i released/min/mg) and *C. utilis* F_1-ATPase (2.3 μg protein, specific ATPase activity: 45 μmol P_i released/min/mg) were preincubated with increasing amounts of *C. utilis* and beef heart IF_1 for 15 min at 30°C in a medium containing 250 mM sucrose, 2 mM morpholino propane sulfonic acid, 0.5 mM ATP, 0.5 mM $MgSO_4$, pH 6.8, in vol. 345 ml. The incubation was initiated by addition of 0.125 ml of an ATPase assay mixture containing 0.1 M Tris–SO_4, 20 mM ATP, 10 mM $MgSO_4$, 20 mM phosphoenolpyruvate, 30 μg pyruvate kinase, pH 8.0. It was carried out for 10 min at 30°C and stopped with 0.060 ml 2.5 M $HClO_4$. Inorganic phosphate was determined on 0.100 ml aliquots by the method [22].

Table 1
Inhibition parameters for the inhibition of F_1-ATPase by IF_1

System used	K_i[a] (M)	I_{50}[b] (μg)
Beef heart IF_1 versus beef heart F_1-ATPase	2×10^{-8}	0.25
Beef heart IF_1 versus *C. utilis* F_1-ATPase	5×10^{-9}	0.06
C. utilis IF_1 versus beef heart F_1-ATPase	4×10^{-7}	2.40
C. utilis IF_1 versus *C. utilis* F_1-ATPase	6×10^{-8}	0.32

[a] K_i values are calculated from Easson and Stedman plots (fig.2)

[b] Amount of IF_1 (μg) required to inhibit 50% of the ATPase activity in conditions of fig.1

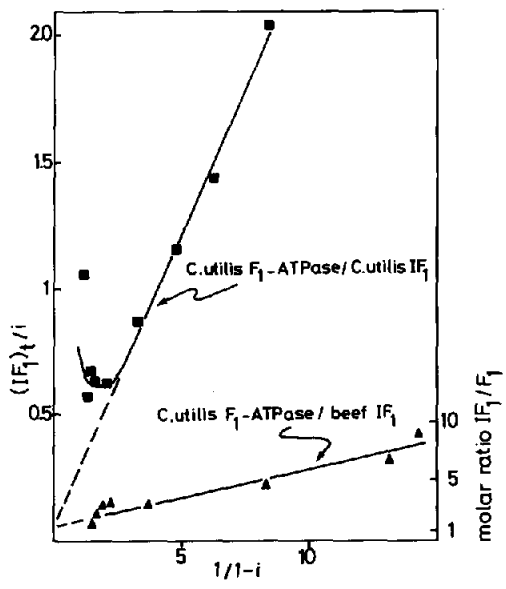

Fig.2. Easson and Stedman plots of *C. utilis* F_1-ATPase inhibition by IF_1. The inhibition data shown in fig.1 (right part) were plotted as described in the text. The value extrapolated on the ordinate axis gives the number N of binding sites and the slope of the linear portion the apparent inhibitor constant, K_i. $(IF_1)_t$ is given in μg; i represents the percentage of inhibition. The ratio IF_1/F_1 is given in mol/mol.

binding sites, N and the inhibitor constant, K_i. Assuming that one molecule of IF_1 reacts with one active site of ATPase according to a simple equilibrium and that the ATPase activity is proportional to the amount of free F_1-ATPase, then a plot of $(IF_1)_t/i$ against $1/1-i$, should be a straight line corresponding to the equation $(IF_1)_t/i = N + K_i/1-i$, where $(IF_1)_t$ is the total amount of IF_1 added and i the degree of inhibition. Inhibitor constants (K_i) estimated from the slopes of the linear portions of the curves are collected in table 1. The lowest value (5×10^{-9} M) was observed for the inhibition of *C. utilis* F_1-ATPase with beef IF_1. The highest value (4×10^{-7} M) was obtained for the reverse system, i.e., beef F_1-ATPase and *C. utilis* IF_1. Intermediary K_i values ($2-6 \times 10^{-8}$ M) were observed for the inhibition of F_1-ATPases by their proper inhibitors. The number of binding sites N in F_1-ATPase can be deduced from the values extrapolated on the ordinate axis. In the case of the titration of *C. utilis* F_1-ATPase either by beef IF_1 or by *C. utilis* IF_1, N was slightly higher than 1; when beef F_1-ATPase was titrated by its own inhibitor, N was closer to 4. It must however be pointed out that the scattering of the data precludes an accurate determination of the number of binding sites and that the above values are only tentatitve estimates.

3.2. *Binding parameters of* C. utilis *$[^{14}C]IF_1$ to* C. utilis *AS particles*

IF_1 binding sites in *C. utilis* submitochondrial particles depleted of their endogenous inhibitor (AS particles) were titrated with increasing amounts of $[^{14}C]IF_1$ and the subsequent decrease in ATPase activity of the particles was determined. In fig.3, the percentage of inhibition of ATPase activity is compared to the fractional saturation of the particles with $[^{14}C]IF_1$. The two curves are virtually superimposable, which indicates that there is a direct relationship between the binding of IF_1 to AS particles and its inhibitory effect on the ATPase activity of the same particles. In four independent experiments, the number of binding sites was between 66 and 84 pmol IF_1/mg protein and the K_d was $8.4 \pm 1.9 \times 10^{-8}$ M. This K_d value, deduced from $[^{14}C]IF_1$ binding assays, is practically similar to the K_i value (6×10^{-8} M) deduced from inhibition data (table 1 and fig.1).

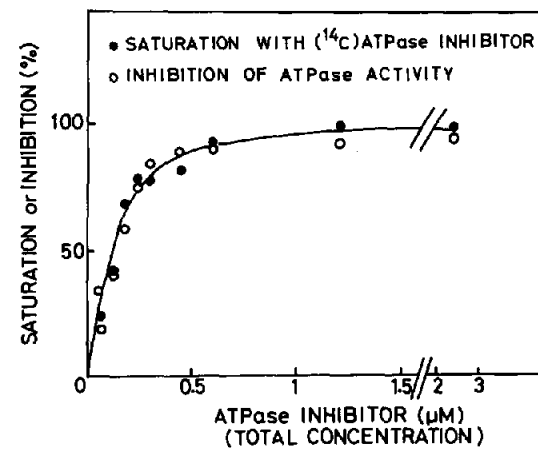

Fig.3. Correlation between binding of *C. utilis* $[^{14}C]IF_1$ to *C. utilis* AS particles and inhibition of ATPase activity. *C. utilis* AS particles were incubated with increasing amounts of $[^{14}C]IF_1$ as described under Materials and methods. Before centrifugation aliquot samples were taken (100 μg protein) to measure ATPase activity. The specific activity of ATPase was 1.4 μmol P_i released/min/mg and the maximal extent of $[^{14}C]IF_1$ binding was 70 pmol/mg protein.

3.3. *pH-Dependence and MgATP requirement for binding of* C. utilis *[^{14}C]IF_1 to* C. utilis *AS particles*

As shown previously, beef heart IF_1 and *C. utilis* IF_1 inhibit more efficiently the activity of F_1-ATPase at acidic pH values [1–3] and in the presence of MgATP [2,3]. The following data pertain to the effect of pH and MgATP on the binding of [^{14}C]IF_1 to AS particles. As shown in fig.4, addition of $MgSO_4$ together with ATP resulted in a high affinity binding of IF_1 with a saturation plateau; replacement of $MgSO_4$ by EDTA led to the loss of the high affinity binding. On the other hand, when the pH was raised from 6.8 to 8.3, IF_1 binding was almost abolished (fig.5).

3.4. *Effect of adenylylimidodiphosphate (AMPPNP) and oligomycin on the binding of* C. utilis *[^{14}C]IF_1 to* C. utilis *AS particles*

As shown above, binding of IF_1 to F_1-ATPase can be deduced from ATPase activity measurements (fig.2). However this is an indirect method which cannot be easily applied when other molecules which act per se as ATPase inhibitors are also present in the incubation medium. In this case, a choice method is the direct binding assay of [^{14}C]IF_1 to F_1-ATPase. The [^{14}C] IF_1

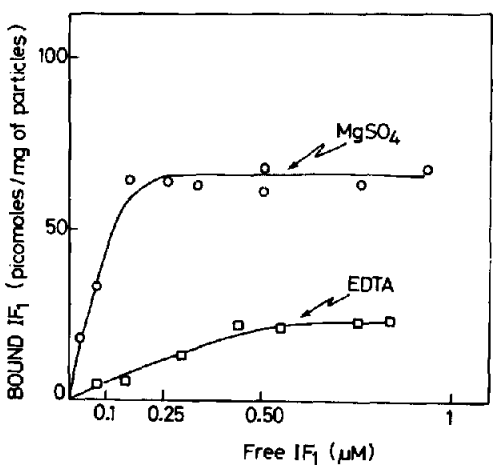

Fig.4. Mg requirement for binding of *C. utilis* [^{14}C]IF_1 to *C. utilis* AS particles. Incubation conditions were as described under Materials and methods for the $MgSO_4$ control curve. For the EDTA curve, 0.5 mM $MgSO_4$ was replaced by 2 mM EDTA. IF_1 bound to AS particles is given in pmol/mg protein.

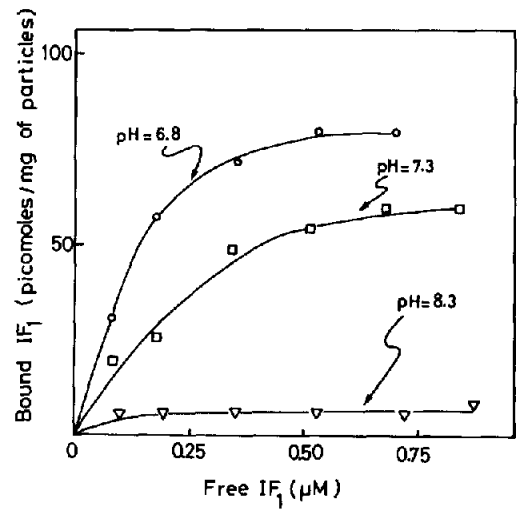

Fig.5. Effect of pH on binding of *C. utilis* [^{14}C]IF_1 to *C. utilis* AS particles. Incubation conditions were as described under Materials and methods. The pH of the incubation medium was adjusted to 6.8, 7.3 or 8.3. IF_1 bound to AS particles is given in pmol/mg protein.

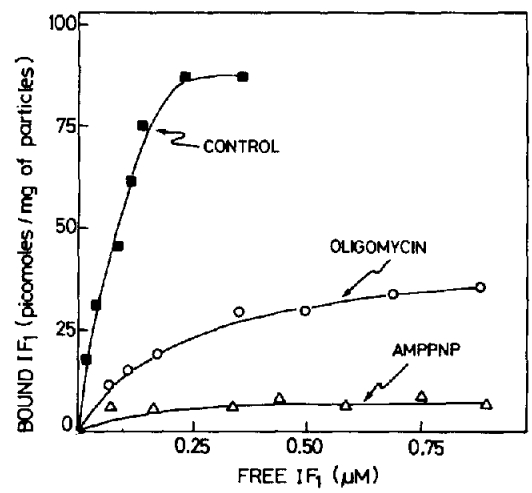

Fig.6. Effect of oligomycin and AMPPNP on binding of *C. utilis* [^{14}C]IF_1 to *C. utilis* AS particles. Incubation conditions were as described under Materials and methods for the control curve. Where indicated 5 μg oligomycin/mg protein was added to the incubation medium and ATP was replaced by 0.5 mM AMPPNP. IF_1 bound to AS particles is given in pmol/mg protein.

binding assay has been used to determine whether AMPPNP and oligomycin, two inhibitors of F_1-ATPase, interfere with the binding of IF_1 to bound F_1-ATPase in AS particles. AMPPNP is an ATP analog which is not hydrolysed by ATPase. It has the ability to bind tightly to F_1-ATPase (K_d 1.3 μM) and to inhibit strongly ATP hydrolysis [20]. Figure 6 shows that AMPPNP cannot replace ATP for the binding of IF_1 in a $MgSO_4$ medium. In another experiment (not shown), it was found that AMPPNP even competes with ATP and abolishes the stimulatory effect of ATP on the binding of IF_1 to AS particles.

Oligomycin inhibits the membrane-bound ATPase; its binding site is located in the membrane sector of the ATPase complex [21]. Addition of oligomycin, at a concentration which inhibited about 75% of the ATPase activity, resulted in a 70% decrease of the binding of IF_1 promoted by MgATP (fig.6). As oligomycin binds to the membrane sector of the ATPase complex, its inhibitory effect on the binding of IF_1 to AS particles is not the result of a direct competition with IF_1.

4. Discussion

The present work deals with the binding of [^{14}C]-IF_1 to bound F_1-ATPase in submitochondrial AS particles. [^{14}C]IF_1 was obtained from the yeast *Candida utilis* grown in a medium supplemented with L-[^{14}C]leucine. That [^{14}C]IF_1 binds to F_1-ATPase in AS particles is shown by the observation that the degree of saturation of IF_1 binding sites strictly parallels the degree of inhibition of ATPase activity by IF_1. Furthermore using the yeast system, the K_d value relative to the binding of [^{14}C]IF_1 to AS particles was found to be virtually similar to the K_i value relative to the inhibition of the ATPase activity of purified F_1-ATPase by IF_1 (8×10^{-8} M versus 6×10^{-8} M).

Optimal conditions for binding of [^{14}C]IF_1 include a slightly acidic pH and the presence of MgATP. The effect of two ATPase inhibitors, oligomycin and AMPPNP, on the binding of IF_1 to F_1-ATPase has been determined, using the direct binding assay. Oligomycin and AMPPNP were chosen as representative compounds which act at different levels of the ATPase complex, the hydrophobic membrane sector and the hydrophilic sector F_1 respectively. The fact that [^{14}C]IF_1 binding to AS particles requires MgATP and that it is inhibited by oligomycin, an inhibitor which binds to a site of the membrane sector distant from F_1-ATPase, strongly suggests that the turnover of the ATPase or some specific conformation are required for the binding of IF_1 to F_1-ATPase. This is in agreement with the suggestion formulated [11] based on other experimental data. The inhibitory effect of AMPPNP on the binding of [^{14}C]IF_1 to AS particles may depend on a direct competition of IF_1 and AMPPNP on the F_1-ATPase itself. Experiments are in progress to test this hypothesis.

Acknowledgements

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