Biochimica et Biophysica Acta 816 (1985) 63–67
Elsevier

BBA 72618

The inhibitor peptide of the mitochondrial $F_1 \cdot F_0$-ATPase interacts with calmodulin and stimulates the calmodulin-dependent Ca^{2+}-ATPase of erythrocytes

Klaus Schwerzmann [a,*], Michele Müller [b] and Ernesto Carafoli [c]

[a] *Department of Anatomy, University of Berne, Bühlstrasse 26, CH-3000 Bern 9, and* [b] *Medizinisch-Chemisches Institut, University of Berne, CH-3000 Bern 9 and* [c] *Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH-Z), CH-8092 Zürich (Switzerland)*

(Received February 26th, 1985)

Key words: ATPase inhibitor; $F_1 \cdot F_0$-ATPase; Calmodulin; Ca^{2+}-ATPase; Erythrocyte

The binding of calmodulin to the mitochondrial $F_1 \cdot F_0$-ATPase has been studied. [^{125}I]Iodoazidocalmodulin binds to the ε-subunit and to the endogeneous ATPase inhibitor peptide in a Ca^{2+}-dependent reaction. The effect of the mitochondrial ATPase inhibitor peptide on the purified Ca^{2+}-ATPase of erythrocytes has also been analyzed. The inhibitor peptide stimulates the ATPase when pre-incubated with the enzyme. The activation of the Ca^{2+}-ATPase by calmodulin is not influenced by the inhibitor peptide, indicating that the two mechanisms of activation are different. These in vitro effects of the two regulatory proteins may reflect a common origin of the two ATPases considered and/or of the regulatory proteins.

Introduction

Calmodulin modulates a number of Ca^{2+}-dependent enzymes [1], among them the Ca^{2+}-ATPase of the plasma membrane [1]. This calmodulin-activated ATPase can be classified as $E_1 \cdot E_2$-type, whereby E_1 and E_2 represent two conformational or functional states of the enzyme. This classification sets these ATPases apart from the well-known $F_1 \cdot F_0$-ATPase from mitochondria, chloroplasts and bacteria, where F_1 and F_0 stand for two separate parts of the enzyme. Although the latter type of ATPase has never been found to be modulated by calmodulin [1–3], it has been shown by many authors that drugs known to be calmodulin antagonists interfere with oxidative phosphorylation and ATP-hydrolysis in mitochondria [3–6]. In addition, the isolation of calmodulin-binding proteins from mitochondria has been reported [2,7–9]. These findings could in principle be compatible with a role of calmodulin in the regulation of the $F_1 \cdot F_0$-ATPase. However, it has never been demonstrated convincingly that this is the case, nor that mitochondria contain adequate amounts of calmodulin [1,10]. The $F_1 \cdot F_0$-type ATPase of mitochondria contains its own regulatory subunit instead, the inhibitor peptide first isolated by Pullman and Monroy [11].

The two regulatory proteins, the inhibitor peptide and calmodulin, share some properties like heat stability, low molecular weight, and high content of acidic amino acids. But their net charge is of opposite sign, due to a larger number of basic residues in the inhibitor peptide [12,13]. In order to establish possible functional homologies between these two regulatory proteins and/or between the $E_1 \cdot E_2$- and $F_1 \cdot F_0$-type ATPases themselves, the interaction of calmodulin with the mitochondrial ATPase and the effect of the inhibi-

* To whom correspondence should be addressed.

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tor peptide on the Ca^{2+}-ATPase of erythrocytes have been studied.

Methods and Materials

The inhibitor peptide was isolated from rat liver mitochondria as described by Schwerzmann et al. [14]. Complex V and F_1-ATPase were obtained from bovine heart mitochondria as in Refs. 15 and 16, respectively. The Ca^{2+}-ATPase was purified from erythrocytes by affinity chromatography on a calmodulin-sepharose column by the method of Niggli et al. [17] with the modification described in Ref. 18. The preparation of bovine brain [^{125}I]iodoazidocalmodulin is described in detail in Ref. 19.

ATPase activity was routinely measured using a continuous spectrophotometric coupled-enzyme assay [18]. The assay medium (1 ml) contained 120 mM KCl, 60 mM Hepes-KOH (pH 7.0), 1 mM $MgCl_2$, 0.01 mM $CaCl_2$, 0.2 mM NADH, 0.5 mM phospho*enol*pyruvate, and 1 I.U. of each pyruvate kinase and lactate dehydrogenase. The reaction was started with the addition of the sample to be assayed (1–2 μg of ATPase protein). Calmodulin (1 μg) was added where indicated. The ATP-splitting reaction was followed at 37°C as decrease in absorbance difference between 366 and 550 nm in a dual wavelength spectrophotometer (Aminco DW-2).

SDS gel electrophoresis was performed on 10% polyacrylamide slab gels using the buffer system of Lämmli [20] with minor modifications [21]. The samples were mixed with equal volumes of a buffer containing 20 mM sodium phosphate (pH 7.0), 6.5 mM dithiothreitol, 20% (v/v) glycerol and 10% (w/v) SDS, and heated for 3 min at 95°C. Under these conditions, calmodulin from bovine brain had an apparent molecular weight of 19000 determined with the molecular weight standards of Bio-Rad (Richmond, CA, U.S.A.). After electrophoresis, the gels were either stained with Coomassie blue or subjected to autoradiography with Kodak X-Omat AR film. The sensitivity was increased with an intensifying screen.

All chemicals were of the highest purity available. Carrier-free Na^{125}I was obtained from EIR (Würenlingen, Switzerland). R24571 (calmidazolium) was a kind gift of Janssen Pharmaceutical Research Laboratories (Beerse, Belgium).

Results

It has been shown by others that calmodulin, whether added to mitochondria [3], inverted submitochondrial particles [2] or isolated F_1-ATPase [2], has no effect on the ATPase activity, but can prevent exogeneous inhibitor peptide from exerting its inhibitory activity [2]. Based on this and the similarities between the two regulatory proteins the binding of radiolabeled azidocalmodulin to the isolated F_1-part, as well as to the whole $F_1 \cdot F_0$-ATPase was investigated. The enzymes were incubated in presence of Ca^{2+} or Ca^{2+} + EGTA with the labeled azidocalmodulin. Then, the azidogroup was activated by ultraviolet light and the whole mixture subjected to electrophoresis. The usual protein pattern of the F_1-ATPase and whole complex V is seen upon staining with Coomassie blue (Fig. 1A). Autoradiography of the gel of F_1-ATPase incubated with calmodulin revealed in addition to the unreacted calmodulin (M_r approx. 19000) a labeled protein with a M_r of approx. 25000 (Fig.

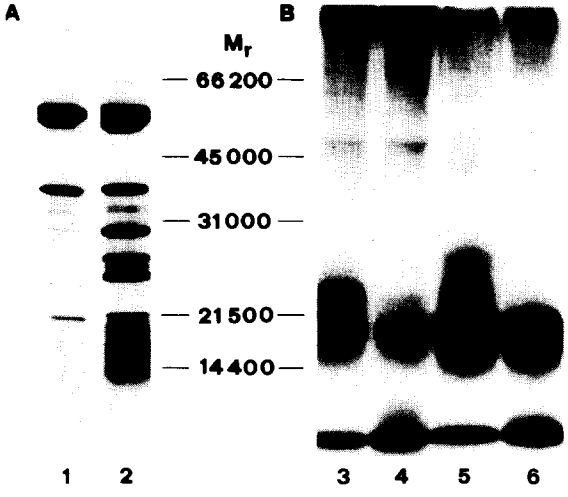

Fig. 1. Binding of [^{125}I]iodoazidocalmodulin to purified F_1-ATPase and complex V from bovine heart mitochondria. (A) SDS gel electrophoresis of mitochondrial F_1-ATPase (lane 1) and complex V (lane 2). The gels were stained with Coomassie blue (0.025%). (B) 1 μg of [^{125}I]iodoazidocalmodulin was incubated with F_1-ATPase (40 μg) and complex V (10 μg), respectively, in presence of either 0.1 mM $CaCl_2$ (lanes 3 and 5) or 2 mM EGTA (lanes 4 and 6) for 1 min. After photolysis (UV-mineral light, type UVS-11, 1 min), the samples were mixed with sample buffer and subjected to electrophoresis and autoradiography as indicated in Materials and Methods. F_1-ATPase (lanes 3 and 4), complex V (lanes 5 and 6).

1B). Its molecular weight after subtracting the contribution by calmodulin was estimated at approx. 6000, corresponding to the ε-subunit of bovine heart F_1-ATPase [16]. When the whole complex V was subjected to the same treatment an additional band with an estimated M_r of 28000 was detected. After correcting for calmodulin its approximate molecular weight was 9000. The only protein in bovine heart complex V corresponding to this M_r is the endogeneous inhibitor peptide [15,22]. In either case, no binding occurred when an excess of EGTA was present during the incubation with calmodulin. Thus, these experiments show that calmodulin binds in a Ca^{2+}-dependent fashion to two hydrophilic subunits of the mitochondrial $F_1 \cdot F_0$-ATPase complex.

It became interesting at this point to study whether the inhibitor peptide could cross-react with a calmodulin-sensitive ATPase, such as the Ca^{2+}-ATPase of erythrocytes [1]. Indeed, it was found that the mitochondrial inhibitor peptide could mimic the effect of calmodulin. When the purified Ca^{2+}-ATPase was pre-incubated with the inhibitor peptide the basal hydrolysis rate became activated as compared to a control without inhibitor peptide (Fig. 2A). Addition of calmodulin produced an additional stimulation resulting in an elevated maximal activity (Fig. 2A). A large excess of inhibitor peptide was required to produce a substantial activation of the basal rate (Fig. 2B). When the inhibitor peptide was added directly to the assay medium no effect on the basal nor the calmodulin-stimulated rate was seen (Fig. 2B). This was not surprising, since pre-incubation with the inhibitor peptide is also necessary to achieve inhibition of the mitochondrial ATPase [11]. However, unlike the binding of calmodulin, the stimulation of the Ca^{2+}-ATPase by the inhibitor peptide occurred also in the absence of free Ca^{2+} (Table I) nor did it require MgATP (Table I) as does inhibition of the mitochondrial ATPase [11]. In this respect, the effect of the inhibitor peptide resembled the stimulation of the Ca^{2+}-ATPase by acidic phospholipids [1].

To elucidate the site of action of the inhibitor peptide on the Ca^{2+}-ATPase, the sensitivity of the inhibitor-stimulated activity to R24571, a calmodulin antagonist [23,24], was studied. The erythrocyte ATPase was first incubated with the

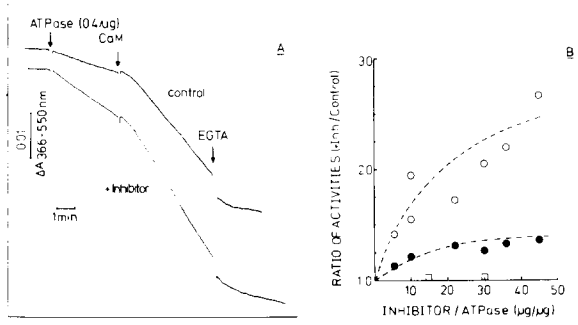

Fig. 2. Effect of mitochondrial inhibitor peptide on the activity of erythrocyte Ca^{2+}-ATPase. (A) Purified Ca^{2+}-ATPase (0.55 µg) was incubated at room temperature with or without (control) 20 µg of inhibitor peptide in 0.10 ml buffer containing 25 mM potassium phosphate (pH 7.0), 0.5 mM $MgCl_2$, 0.5 mM K_2ATP and 0.05 mM $CaCl_2$. After 10 min, aliquots (75 µl) were withdrawn and the ATPase activity measured as indicated in Materials and Methods. Calmodulin (1 µg) and EGTA (1 mM) were added to the cuvette where indicated. (B) Purified Ca^{2+}-ATPase (0.55–1.50 µg) was incubated as in (A) with increasing amounts of inhibitor peptide. ATPase activity was measured before and after addition of calmodulin. The ratio of the activity of inhibitor-treated to control ATPase is plotted against the ratio (w/w) of inhibitor peptide to Ca^{2+}-ATPase in the incubation medium. ○, basal rate; ●, with calmodulin; □, control with inhibitor added to the assay medium.

inhibitor peptide, and the activity measured before and after addition of calmodulin. The Ca^{2+}-ATPase activity was then measured at different

TABLE I

CAPACITY OF INHIBITOR PEPTIDE TO STIMULATE THE ERYTHROCYTE Ca^{2+}-ATPase UNDER DIFFERENT INCUBATION CONDITIONS

Purified Ca^{2+}-ATPase (1.5 µg) was incubated at room temperature in 0.1 ml of 25 mM potassium phosphate containing one or more of the following ingredients: 33 µg inhibitor peptide, 0.5 mM $MgCl_2$, 0.5 mM K_2ATP, 0.1 mM $CaCl_2$, 5 mM EGTA (pH 7.4). After 10 min, an aliquot was assayed for ATPase activity before and after adding 1 µg calmodulin.

Incubation conditions	ATPase activity (nmol/min per mg protein)	
	basal rate	+ calmodulin
Control, Mg, Ca, ATP [a]	0.79 (0.06)	2.58 (0.25)
Inhibitor, Mg, Ca, ATP [a]	1.15 (0.13)	3.45 (0.07)
Inhibitor, EGTA, ATP [a]	1.32 (0.31)	2.67 (0.78)
Inhibitor, Ca	1.28	3.62
Inhibitor	1.15	3.40

[a] Numbers are means of two experiments (in parenthesis standard error).

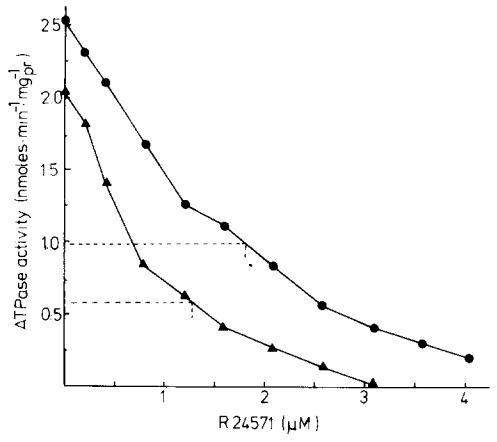

Fig. 3. Effect of the anti-calmodulin drug R24571 on the Ca^{2+}-ATPase activity incubated with inhibitor peptide. Purified Ca^{2+}-ATPase (0.55 μg) was incubated with 20 μg of inhibitor peptide as in Fig. 2A. ATPase activity was measured as indicated in Materials and Methods in presence of 1 μg of calmodulin. Increasing amounts of R24571 were added and the ATPase activity determined. A control was treated identically, but omitting inhibitor peptide in the incubation medium. ▲, control; ●, with inhibitor peptide. The dotted lines indicate the basal activities (before addition of calmodulin).

concentrations of antagonist and compared to the activity of ATPase treated identically but without the inhibitor peptide in the incubation medium (Fig. 3). Whereas similar concentrations of R24571 were needed to reverse the activation induced by calmodulin, the residual activity was much less sensitive to R24571 when the Ca^{2+}-ATPase had been pre-incubated with inhibitor peptide before. This experiment, and the fact that the effects of inhibitor peptide and of calmodulin are cumulative indicate that the two peptides act on different sites of the Ca^{2+}-ATPase.

Discussion

The experiments presented here have shown that the mitochondrial ATPase inhibitor peptide and calmodulin interact with one another and with each other's natural target ATPase. Thus, in spite of fundamental difference in physiological role, the two ATPases apparently contain binding sites for either of the two regulatory proteins. Exogeneous calmodulin bound to the ε-subunit and to the endogeneous inhibitor peptide of the $F_1 \cdot F_0$-type mitochondrial ATPase, without influencing the ATPase activity [2]. On the other hand, the inhibitor peptide isolated from mitochondria activated the $E_1 \cdot E_2$-type Ca^{2+}-ATPase of erythrocytes without interfering with the effect of calmodulin on this ATPase.

Whereas these data may represent laboratory curiosities only and bear no relation to cell biochemistry, it is still tempting to suggest that calmodulin may play a role in the regulation of the mitochondrial energy transducing ATPase. This is made somewhat more appealing by the findings that calmodulin-binding proteins have been isolated from mitochondria [7–9] and that calmodulin drugs also interfere with oxidative phosphorylation [3–6]. Further evidence comes from the work of Pedersen and Hullihen [2] which shows that the binding of calmodulin to the inhibitor peptide prevents its inhibition of the ATPase activity in submitochondrial and isolated F_1-ATPase. However, the facts that calmodulin has no direct effect on the mitochondrial ATPase activity [1–3] and that calmodulin is present in mitochondria only in minute amounts [10], if not absent at all [1], make a physiological role of calmodulin in the regulation of the mitochondrial $F_1 \cdot F_0$-ATPase highly questionable.

As for the stimulation of the red blood cell Ca^{2+}-ATPase by the mitochondrial ATPase inhibitor peptide, this is evidently not a physiological effect. But the observation that this regulatory peptide of mitochondrial origin is capable to mimic the action of calmodulin is interesting nonetheless. The relative insensitivity of this effect to the calmodulin-antagonist R25471 indicates that the inhibitor peptide either stabilizes the ATPase activity in a calmodulin-insensitive fashion or that it unmasks a new active site on the enzyme. A similar observation has been reported recently by De Meis et al. [25] who found that the mitochondrial inhibitor peptide increased the ATP-P_i exchange rate of the sarcoplasmic reticulum Ca^{2+}-ATPase, without affecting other activities of the enzyme. Interestingly, also this $E_1 \cdot E_2$-type ATPase is sensitive to calmodulin [1].

A comparison of the primary structures of the two modulator proteins [26,27] may help understanding their 'cross-reactivity'. Both have a similar sequence of positively charged amino acids at the amino terminal. Furthermore, the 74–84 se-

quence in calmodulin, containing a series of five acidic amino acids preceded by four basic residues [26] is almost identical to a sequence near to the carboxy terminal of the inhibitor peptide [27].

The homologies of the primary structure, the mutually exclusive localization and the 'cross-reactivity' could suggest that the two modulator proteins have a common ancestor. In the cytoplasm it developed into a Ca^{2+}-sensitive, universal regulator of many enzymic activities, in the mitochondria where free Ca^{2+} is constantly at a relatively high level [28] it specialized as regulator of the energy transducing ATPase.

Acknowledgments

The authors would like to thank Drs. Mauro Zurini, Arthur Clark and Paolo Gazzotti for the preparations of erythrocyte ATPase and calmodulin, Professor Peter L. Pedersen for making available a manuscript of a paper prior to its publication, and to Rose-Marie Fankhauser for typing the manuscript.

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