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Identification of a Conserved Calmodulin-Binding Motif in the Sequence of F_0F_1ATPsynthase Inhibitor Protein

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The natural inhibitor proteins IF_1 regulate mitochondrial F_0F_1ATPsynthase in a wide range of species. We characterized the interaction of CaM with purified bovine IF_1, two bovine IF_1 synthetic peptides, as well as two homologous proteins from yeast, namely IF_1 and STF_1. Fluorometric analyses showed that bovine and yeast inhibitors bind CaM with a 1:1 stoichiometry in the pH range between 5 and 8 and that CaM-IF_1 interaction is Ca^{2+}-dependent. Bovine and yeast IF_1 have intermediate binding affinity for CaM, while the K_d (dissociation constant) of the STF_1-CaM interaction is slightly higher. Binding studies of CaM with bovine IF_1 synthetic peptides allowed us to identify bovine IF_1 sequence 33–42 as the putative CaM-binding region. Sequence alignment revealed that this region contains a hydrophobic motif for CaM binding, highly conserved in both yeast IF_1 and STF_1 sequences. In addition, the same region in bovine IF_1 has an IQ motif for CaM binding, conserved as an IQ-like motif in yeast IF_1 but not in STF_1. Based on the pH and Ca^{2+} dependence of IF_1 interaction with CaM, we suggest that the complex can be formed outside mitochondria, where CaM could regulate IF_1 trafficking or additional IF_1 roles not yet clarified.

KEY WORDS: Calmodulin (CaM); inhibitor protein IF_1; CaM-binding motif; target sequence.

INTRODUCTION

IF_1 is the natural inhibitor protein of F_0F_1ATP synthase, the enzyme responsible for the aerobic synthesis of cellular ATP from ADP and Pi using energy derived from the transmembrane proton motive force (Boyer, 1997). IF_1 regulation of F_0F_1 in mitochondria occurs by IF_1 binding to the catalytic sector F_1 in a 1:1 ratio; optimal conditions are a pH below 7.0 and the absence of a proton motive force (Green and Grover, 2000). These conditions occur under energy deficiency, i.e. during myocardial ischemia when IF_1 is responsible for the beneficial inhibition of ATPase activity in mammalian heart, as demonstrated both in vitro and in vivo (Di Pancrazio *et al*., 2004).

Besides the F_0F_1 present in all energy-transducing membranes, several recent reports have shown that at least some F_0F_1 subunits localize in the plasma membrane of endothelial cells (Burwick *et al*., 2005), hepatic cells (Martinez *et al*., 2003; Bae *et al*., 2004) and adipocytes (Kim *et al*., 2004). IF_1 has also been identified on the surface of endothelial cells where it regulates F_0F_1 catalytic activity (Burwick *et al*., 2005). Moreover, exogenous IF_1 modulated the activity of plasma membrane ATPase of hepatic cells (Martinez *et al*., 2003). Considering that IF_1 is encoded by a nuclear gene (Walker *et al*., 1987), these observations suggest that IF_1 is delivered towards both mitochondria and cell surface, at least in some cells. At present, nothing is known about IF_1 cytosolic trafficking; the mechanisms and proteins involved in this process represent an interesting field of study.

There is significant similarity between IF_1 in mitochondria from various species, particularly regarding

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Key to abbreviations: bIF_1, inhibitor protein from bovine; yIF_1, inhibitor protein from yeast; STF_1, product of the STF_1 gene from yeast; CaM, calmodulin; F_1, soluble isolated F_1 domain; K_d, dissociation constant.

the N-terminal region containing the inhibitory sequence (van Raaij *et al*., 1996). Bovine IF_1, a basic protein of 84 amino acids, is present in solution in two oligomeric states, tetramer and dimer, favored by pH values above and below 6.5, respectively (Cabezon *et al*., 2000). In *S. cerevisiae*, two proteins are involved in the regulation of mitochondrial F_0F_1, namely IF_1 and STF_1 (Hashimoto *et al*., 1983), although the precise role of STF_1 remains unclear (Venard *et al*., 2003). Yeast IF_1 and STF_1 are homologous proteins of 63 residues with highly similar sequences (Hashimoto *et al*., 1984) and each has two oligomeric states, dimer and monomer. While yeast IF_1 is dimeric at low pH and monomeric at high pH, the oligomeric pattern of STF_1 is the contrary (dimerization is favored at high pH) (Cabezon *et al*., 2002).

IF_1 is a target of calmodulin (CaM), a ubiquitous and highly conserved protein in all eukaryotes that functions as an intracellular calcium sensor (Chin and Means, 2000). Early studies showed that CaM binds IF_1 purified from rat liver in a Ca^{2+}-dependent fashion in vitro (Pedersen and Hullihen, 1984; Schwerzmann *et al*., 1985). Furthermore, CaM prevented IF_1 from inhibiting F_1-ATPase, both free in solution and membrane-bound, without a direct effect on the catalytic activity (Pedersen and Hullihen, 1984).

CaM is a heat-stable protein with four conserved helix-loop-helix structures (EF-hand motifs) that bind a single calcium ion each. CaM interacts with a large number of structurally and functionally unrelated proteins, including metabolic enzymes, structural proteins, transcription factors, ion channels and pumps, and modulates a wide range of cellular processes in response to calcium (Yamniuk and Vogel, 2004). CaM binds its targets through the recognition of specific sequences and tertiary structures. In particular, CaM-binding motifs consist of approximately 20 amino acids and include critical hydrophobic residues in positions 1 and 10 (or 1 and 14); in some instances, they contain an IQ motif, which is represented by the general sequence [F,I, L,V]Qxxx[R, K]xxxx[R, K] (Calmodulin Target Database: http://calcium.uhnres.utoronto.ca; Rhoads and Friedberg, 1997). These motifs can form a basic amphiphilic α-helix common to a number of Ca^{2+}-CaM binding proteins (O'Neil and DeGrado, 1990). This structural recognition of CaM may explain its ability to bind IF_1, considering that the crystallographic structure of bovine IF_1 indicates that each monomer folds into a single cationic amphiphilic α-helix (Cabezon *et al*., 2001).

In light of this knowledge, the aims of the present study were to characterize the binding interaction between CaM and IF_1 and to identify the CaM-binding motif within the IF_1 primary sequence. Purified bovine IF_1, two bovine IF_1 synthetic peptides, as well as purified recombinant yeast IF_1 and STF_1 were used to localize the binding motif. Our aim was to obtain information on which to base a detailed topological analysis of the CaM-IF_1 complex and to begin to understand the regulatory roles of this complex.

MATERIALS AND METHODS

Materials

Bovine brain CaM and bee venom melittin were purchased from Sigma. Peptides GSESGDNVRSSAGAVRDAGGA and FGKREQAEEERYFRARAKEQLAALK corresponding to residues 1–21 and 22–46, respectively, of bovine IF_1 were purchased from Mimotopes (Clayton Victoria, Australia); these peptides are >95% pure as determined by mass spectrometry. All the other chemicals were commercial products of the purest quality.

Purification and Preparation of Bovine Proteins

Soluble F_1 sector (F_1-ATPase) was isolated from beef heart mitochondria as in (Horstman and Racker, 1970) and subsequently passed through an XK16/40 Superdex 200pg column equilibrated with 20 mM Tris/HCl pH 8.5, 200 mM NaCl, 1 mM ATP, 1 mM EDTA and 5 mM 2-mercaptoethanol buffer according to (Abrahams *et al*., 1994). Inhibitor protein IF_1 was purified from the same source as reported in (Gomez-Fernandez and Harris, 1978). The purity of the preparations was determined by SDS-PAGE (Laemmli, 1970). For binding studies, CaM was fluorescently labeled with dansyl chloride as described previously (Vorherr *et al*., 1990).

Concentrations of F_1-ATPase, IF_1 and dansylated CaM were assayed by the bicinchoninic acid method as in (Smith *et al*., 1985).

Expression and Purification of Recombinant Yeast IF_1 and STF_1

Yeast IF_1 was overexpressed and purified as described in (Corvest *et al*., 2005). The same procedure was developed here to obtain recombinant STF_1. Briefly, the gene encoding STF_1 was amplified by PCR from genomic yeast DNA and the fragment was ligated into plasmid pET-30a(+), which provided an N-terminal (His)6-tag and an enterokinase cleavage site; the resulting plasmid was transformed into *E. coli* BL21 (DE3) by electroporation. Overexpressed STF_1 was purified by

nickel-chelating affinity chromatography as already reported for yeast IF_1 (Corvest *et al*., 2005). Protein concentration was determined by the bicinchoninic acid method as in (Smith *et al*., 1985).

Because of the presence of the N-terminal enterokinase cleavage site, the first three amino acids of the recombinant, processed yeast proteins were Ala-Met-Ala, as compared with the natural IF_1 and STF_1, which starts with Ser. However, the inhibitory activities of recombinant IF_1 and STF_1 were the same as that of the natural yeast inhibitors, as previously reported in (Corvest *et al*., 2005).

IF_1 Binding Assays to Dansylated CaM

Binding of CaM to bovine IF_1 and variants (yeast IF_1 and STF_1; bovine IF_1 peptides) was studied by measuring the changes in fluorescence emission spectrum of dansylated CaM; binding to bee venom melittin served as control (Maulet and Cox, 1983). Fluorescence spectra were recorded in a quartz cuvette (light path, 10 mm), using a Perkin Elmer fluorescence photometer at an excitation wavelength (Ex) of 340 nm and emission wavelength between 400 and 600 nm.

Binding was performed at three different values of pH at 25°C. The reaction contained CaM at 1.4 μM in a solution of 150 mM NaCl, 100 μM $CaCl_2$, and 20 mM pH-specific buffer: Mes pH 5.0, Mops pH 6.5, or Hepes pH 8.0. When indicated, 2 mM EGTA was added to the buffers. Bovine IF_1 and variants were preincubated for several hours at the same pH of the binding assay. After addition of increasing concentrations of bovine IF_1, yeast IF_1, STF_1 (0.28–2.8 μM) or bovine IF_1 peptides (0.28–14 μM) to the CaM-containing buffer, the solution was stirred for 10 min before fluorescent measurements.

Stoichiometry of the interaction between CaM and bovine IF_1 was followed by recording fluorescence intensity at $E_m = 490$ nm averaged over 5 s. The molar ratio of binding protein to CaM was observed when the relative fluorescence intensity (F/F_0) no longer increased with addition of bovine IF_1, where F is the fluorescence intensity of the bound complex and F_0 is the intensity of dansylated CaM.

The dissociation constant (K_d) for the interaction between CaM and bovine IF_1 or variants was determined according to the equation reported in (Vorherr *et al*., 1990). Fluorescence intensity was measured at $E_m = 490$, averaged over 5 s. The fractional degree of saturation of dansylated CaM, α, was calculated from the ratio $(F - F_0)/(F_{max} - F_0)$, where F is the fluorescence intensity of the bound complex, F_{max} is the intensity at saturation, and F_0 is the intensity of dansylated CaM. K_d was obtained by the reciprocal of the slope of the plot of $1/(1 - \alpha)$ versus the total binding protein concentration divided by α.

The effects of IF_1 on the calcium-binding properties of CaM were investigated by measuring changes in relative fluorescence (F/F_0) of dansylated CaM at $E_m = 500$, where F is the measured intensity and F_0 is the intensity without added Ca^{2+}. Measurements were performed at 25°C in a buffer containing 20 mM Mops pH 6.5, 150 mM NaCl, 2 mM EGTA and increasing concentrations of $CaCl_2$ to provide 0.5–4 μM free Ca^{2+}. CaM was present at 1.4 μM, alone or together with 1.4 μM bovine IF_1 or yeast IF_1 or STF_1. Free Ca^{2+} concentration was calculated using WEBMAXCLITE v1.15 program (http://www.stanford.edu/~cpatton/maxc.html).

ATPase Assay

The maximal ATPase activity of isolated F_1-ATPase was determined by coupling the production of ADP to the oxidation of NADH via the pyruvate kinase and lactic dehydrogenase reactions as previously reported (Contessi *et al*., 2004). The assay was performed for F_1-ATPase alone and for F_1-ATPase preincubated with CaM, bovine IF_1 or both. The proteins were combined by sequential additions, at 15-min intervals (at 25°C, in 20 mM Tris/HCl pH 6.7, 1 mM MgATP and 100 μM $CaCl_2$), to concentrations of 0.56 μM F_1-ATPase, 5.5 μM IF_1 and 8–100 μM CaM, prior to dilution 1:100 in assay buffer. The ATPase assay was performed at 37°C in a final volume of 1 ml containing 20 mM Tris/HCl pH 6.7, 1 mM KCl, 3 mM ATP, 4 mM $MgCl_2$, 1.5 mM phosphoenolpyruvate, 200 μM NADH, 2 IU pyruvate kinase and 3 IU lactic dehydrogenase. Activity was measured spectrophotometrically at 340 nm on a Perkin-Elmer Vis–UV Spectrometer Lambda14. The CaM used in these experiments was not dansylated.

Sequence Analyses

The search for CaM binding motifs within the inhibitory sequence of IF_1 was performed by visual inspection for patterns of hydrophobic residues. Potential sequences were tested for CaM binding compatibility using Calmodulin Target Database (http://calcium.uhnres.utoronto.ca; Yap *et al*., 2000), which considers multiple criteria including the net charge of the sequence, level of hydrophilicity, and helical hydrophobic moment. Analysis of conserved residues was performed using CLUSTAL W program, v. 1.8.

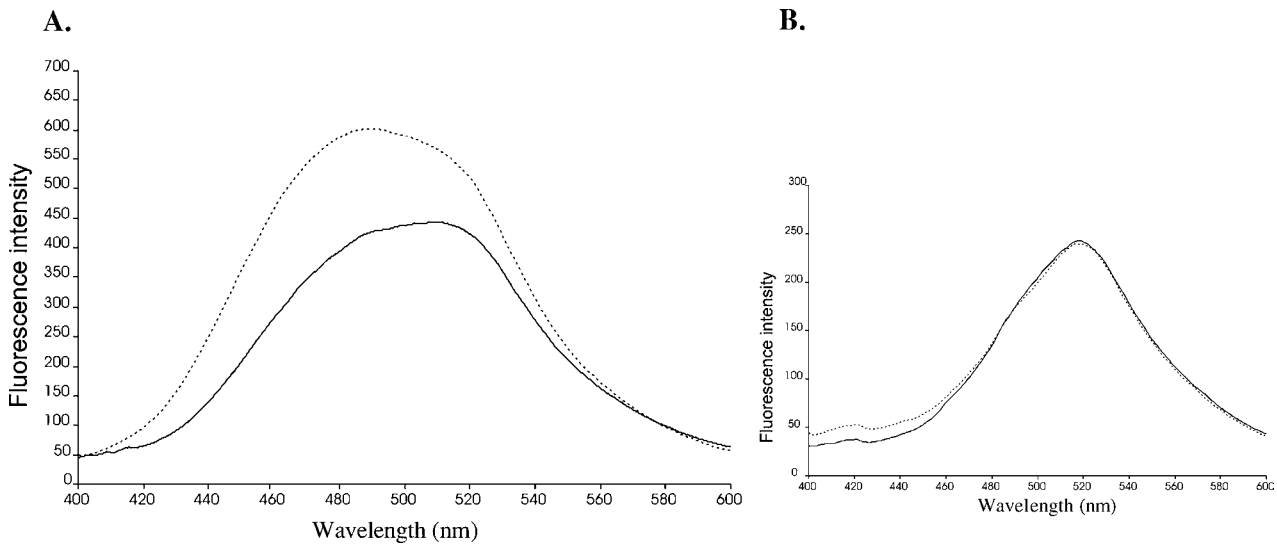

Fig. 1. Changes in fluorescence emission spectrum of dansylated calmodulin upon binding to bovine IF_1, in presence and absence of calcium. Dansylated CaM was diluted to 1.4 μM in 20 mM Mops, 150 mM NaCl and 100 μM $CaCl_2$ in the absence (A) or presence (B) of 2 mM EGTA. Then, bovine IF_1 was added to 2 μM, and the solution was stirred for 10 min at 25°C before measurements. The figure shows the spectra before (*continuous line*) and after (*dotted line*) addition of the saturating concentration of bovine IF_1. The excitation wavelength (Ex) was 340 nm; excitation and emission bandwidths were 10 nm. The spectra are representative of three experiments.

RESULTS

Calmodulin Interaction With Bovine IF_1

The interaction between bovine IF_1 and dansylated CaM was studied using fluorescence spectroscopy. Previous work has shown that the binding of different calmodulin targets were not altered by dansylation (Mori *et al.*, 2000). The maximum in the emission spectrum of dansylated CaM underwent a shift to lower wavelength and an increase in intensity upon complex formation with IF_1 in the presence of calcium, indicating CaM-IF_1 interaction (Fig. 1(A)). In the presence of 2 mM EGTA, the fluorescent intensity of dansylated CaM was less and the peak was sharper; when IF_1 was added, no increase in intensity nor blue shift of the spectrum was observed, showing that binding was Ca^{2+}-dependent (Fig. 1(B)). Titration of 1.4 μM dansylated CaM with increasing concentrations of IF_1 revealed that binding (fluorescence intensity) saturated in the micromolar range in a buffer containing physiologic NaCl concentration (150 mM) and 0.1 mM $CaCl_2$. The fluorescent change was of similar extent in the absence of NaCl, suggesting a prominent role of hydrophobic interactions in CaM-IF_1 binding (data not shown).

The interaction between dansylated CaM and bovine IF_1 in the presence of calcium was characterized as having a 1:1 stoichiometry. Figure 2 shows that saturation in the relative fluorescence intensity (F/F_0) was reached when the molar ratio of the two proteins approached unity, although up 2 equivalents of IF_1 were added. Further IF_1 additions up to 10-fold molar excess did not modify F/F_0 ratio (data not shown). The same stoichiometry was observed at pH 5.0 as well as at pH 6.5 and 8.0. These pH values were chosen in consideration of the pH dependence of IF_1 oligomerization (dimer below pH 6.5, tetramer above pH 6.5).

Considering the binding stoichiometry of the IF_1-CaM complex, the equation used by Vorrher *et al.* (Vorherr *et al.*, 1990) was applied to determine K_d values at pH 5.0, 6.5 and 8.0. In fact, these graphs can be utilized only if a 1:1 complex is formed. Figure 3 shows the plots of $1/(1-\alpha)$ against the total IF_1 concentration divided by α, where α is the fractional degree of saturation of dansylated CaM. As expected, the titration points result in straight lines consistent with a 1:1 binding stoichiometry at all values of pH. Mean values of K_d determined from the reciprocal of the slope were 32.75 nM (SD = 6.70 nM) at pH 5.0, 53.50 nM (SD = 3.53 nM) at pH 6.5, and 109.00 nM (SD = 22.60 nM at pH 8.0. Thus, the affinity of the complex is approximately threefold higher at pH 5.0 (when IF_1 is dimeric) than at pH 8.0 (when it is tetrameric).

The effects of IF_1 on the calcium-binding properties of CaM were determined from the change in fluorescence intensity of dansylated CaM at varying concentrations of free Ca^{2+} (Fig. 4). In the absence of IF_1, the relative

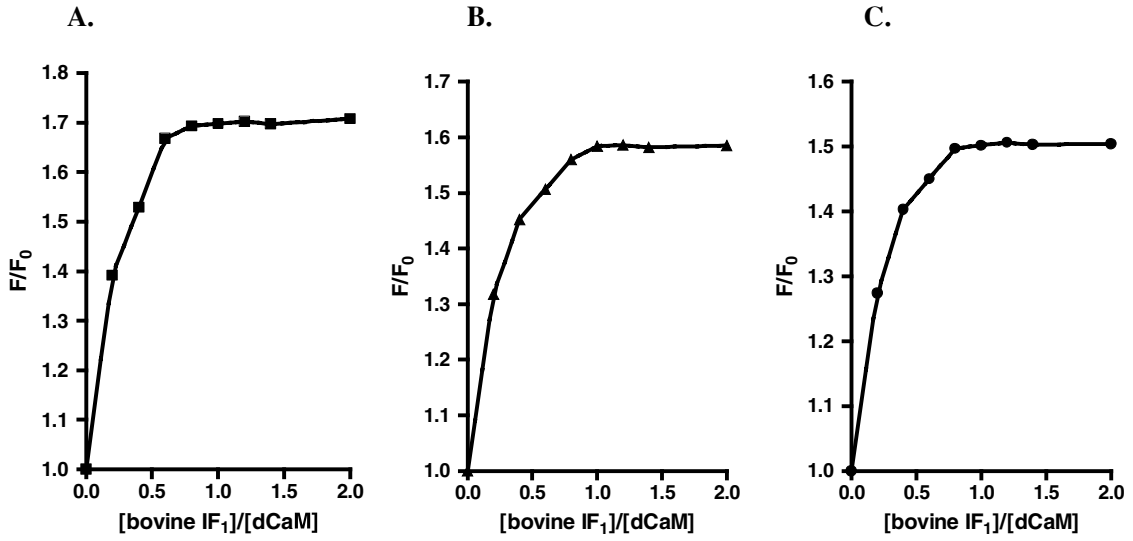

Fig. 2. Calmodulin binding to bovine IF_1 occurs with a 1:1 stoichiometry. Dansylated CaM (dCaM) was diluted to 1.4 μM in solutions containing 150 mM NaCl and 100 μM $CaCl_2$, and 20 mM pH-specific buffer: Mes pH 5.0 (A), Mops pH 6.5 (B), or Hepes pH 8.0 (C). Then, bovine IF_1 was added at 0.28–2.8 μM and the samples were stirred for 10 min at 25°C before measurements. The excitation wavelength (Ex) was 340 nm and the emission wavelength (Em) was 490 nm. *F* is the fluorescence intensity of the bound complex and F_0 is the intensity of dansylated CaM alone. The plots are representative of three experiments.

fluorescence intensity of CaM increased approximately 10-fold in a sigmoid fashion over a range of 1–3 μM free Ca^{2+}. In the presence of an equimolar concentration of IF_1, calcium titration did not noticeably alter the excursion of CaM fluorescence, indicating that IF_1 has no effect on calcium binding by CaM.

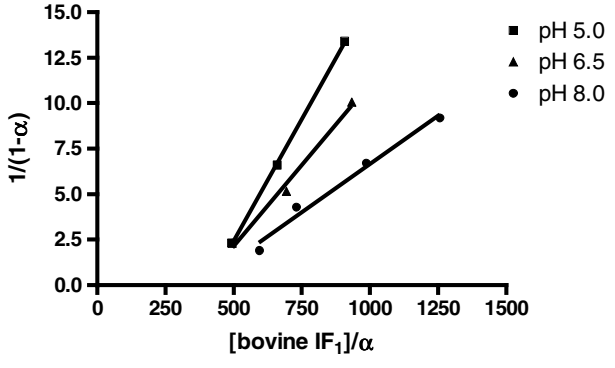

Fig. 3. Determination of the dissociation constant (K_d) for the complex calmodulin-bovine IF_1. Plots of $1/(1-\alpha)$ against the total IF_1 concentration divided by α are reported at the indicated pH values. Experimental conditions were as in Fig. 2. α is the fractional degree of saturation of CaM. The data are means of at least three independent experiments. IF_1 concentration was expressed in micromolar.

Prevention of Bovine IF_1 Binding to F_1-ATPase by Calmodulin

To assess the effects of CaM on the inhibitory activity of bovine IF_1, we measured the ATPase activity of F_1-ATPase alone and in the presence of one or both proteins. The specific activity of F_1-ATPase at 37°C and pH 6.7 was 31.0 ± 0.3 U/mg, which is the expected value at low pH (Contessi *et al.*, 2004). Addition of 100 μM CaM had no effect on the maximal ATPase activity (data not shown). In contrast, addition of 10-fold molar excess of IF_1 reduced the ATPase activity to about 10% of initial values (9.2 ± 0.2 U/mg).

When all three proteins were present, the ability of IF_1 to inhibit F_1-ATPase depended on the temporal order of complex formation (Fig. 5). When the IF_1- F_1-ATPase complex was formed 15 min prior to addition of CaM, IF_1 effectively inhibited F_1-ATPase (to 10% residual ATPase activity) and no amount of CaM reversed this inhibition (up to 18-fold molar excess with respect to IF_1). When CaM was preincubated with IF_1 prior to addition of F_1-ATPase, or if it was preincubated with F_1-ATPase prior to addition of IF_1, the inhibitory effects of IF_1 depended on the concentration of CaM. These observations suggest that CaM's ability to neutralize the action of IF_1 is due to its interaction with the free form of the protein. The modulatory effects of CaM on the activity of IF_1 required the presence of calcium (data not shown).

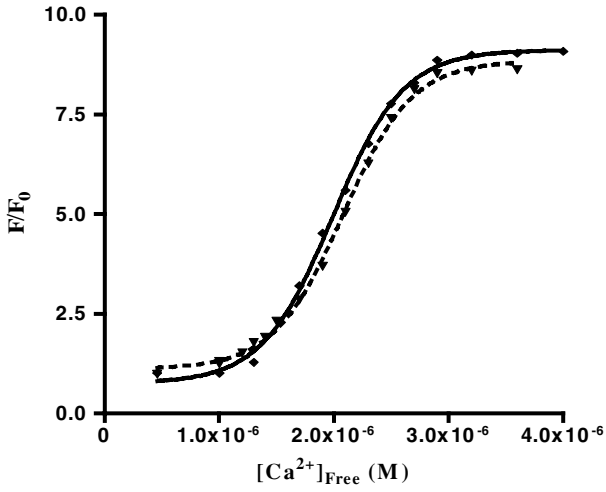

Fig. 4. Ca^{2+}-dependence of dansylated calmodulin fluorescence with or without bovine IF_1. Fluorescence intensity of 1.4 μM dansylated CaM was monitored at Em 500 nm in 20 mM Mops pH 6.5, 150 mM NaCl and 2 mM EGTA, with (*dotted curve*) or without (*solid curve*) 1.4 μM bovine IF_1 and $CaCl_2$. Free Ca^{2+} concentration was calculated using WEBMAXCLITE v. 1.15 program and plotted against F/F_0. F is the fluorescence intensity of the complex and F_0 is the intensity of dansylated CaM in the absence of calcium.

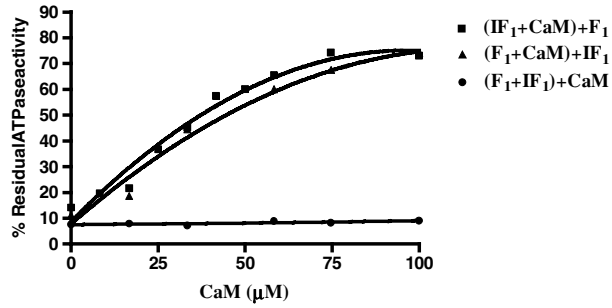

Fig. 5. Calmodulin's capacity to neutralize the inhibitory action of bovine IF_1. The ATPase activity of F_1-ATPase was measured in the presence of bovine IF_1 and increasing concentrations of CaM. Values are expressed as percent residual activity, compared to that of F_1-ATPase alone. The three proteins were combined in varying order by sequential additions, separated by 15-min intervals at 25°C with low MgATP (1 mM); preincubation was performed with F_1-ATPase at 0.56 μM, bovine IF_1 at 5.5 μM, and CaM at 0–100 μM. After the final addition and incubation for 15 min at 37°C, samples were diluted 1:100 in a spectrophotometric cuvette to assay the residual ATPase activity at 37°C.

Interaction of CaM With Peptides From Bovine IF_1 Inhibitory Sequence and With Yeast IF_1 and STF_1

Based on the observation that CaM neutralized bovine IF_1's inhibitory activity when added prior to the formation of the IF_1-F_1-ATPase complex but could not reverse the inhibitory complex, we hypothesized that CaM interacts with the N-terminal inhibitory sequence of IF_1. To test this hypothesis, we studied the ability of CaM to bind synthetic peptides corresponding to residues 1–21 and 22–46 of bovine IF_1 (Fig. 6(A)), as well as to yeast IF_1 and STF_1 proteins, considered as natural mutants.

Binding, revealed as a change in fluorescence intensity of dansylated CaM, was clearly demonstrated for bovine IF_1 peptide 22–46 and for yeast IF_1 and STF_1 but not for bovine IF_1 peptide 1–21. Binding of peptide 22–46 and yeast proteins to CaM was Ca^{2+}- and pH-dependent, and the effect of pH on binding varied according to the protein studied (Table I). In particular, the K_d of bovine IF_1 peptide 22–46 was lowest at pH 5.0, as previously observed for intact IF_1, and increased more than 10-fold at pH 8.0; compared to intact IF_1, the peptide bound with lower affinity (Table I). Yeast IF_1 bound CaM with affinity similar to that of the bovine protein, but with an opposite pH dependence; STF_1 had a lower affinity for CaM but bound preferentially at low pH. Figure 7 reports the titration plots, obtained as in Fig. 3: Data points of all inhibitors at all values of pH tested resulted in straight lines, thus supporting a 1:1 binding stoichiometry, as already observed for bovine IF_1. As expected, bee venom melittin bound CaM at high affinity and without a major modulation by pH.

Prediction of CaM-Binding Motif

To identify the CaM-binding motif within bovine IF_1 peptide 22–46, we searched for the presence of a CaM-binding motif. We observed that peptide 22–46 contained two instances of hydrophobic amino acids separated by eight residues: one motif was defined by Tyr-33 and Leu-42, while the other was defined by Ala-36 and Leu-45. Moreover, a 1–5–10 hydrophobic motif corresponding to residues 34, 38 and 43 was also found. Alignment of bovine IF_1 peptide 22–46 with yeast IF_1 and STF_1 sequences showed that the only motif whose key bulky residues were conserved was that of sequence 33–42 (bovine numbering) (Fig. 6(B)). These residues are conserved in rat and human IF_1. Analysis of bovine sequence 33–42 using the Calmodulin Target Database confirmed that it is compatible with CaM binding on the basis of a net positive charge, moderate hydrophilicity and high helical hydrophobic moment.

In addition to this hydrophobic motif, we also observed an overlapping IQ motif[4] with Leu and Gln in

[4]C- to N-term direction of IQ motif makes the CaM-binding site reverse.

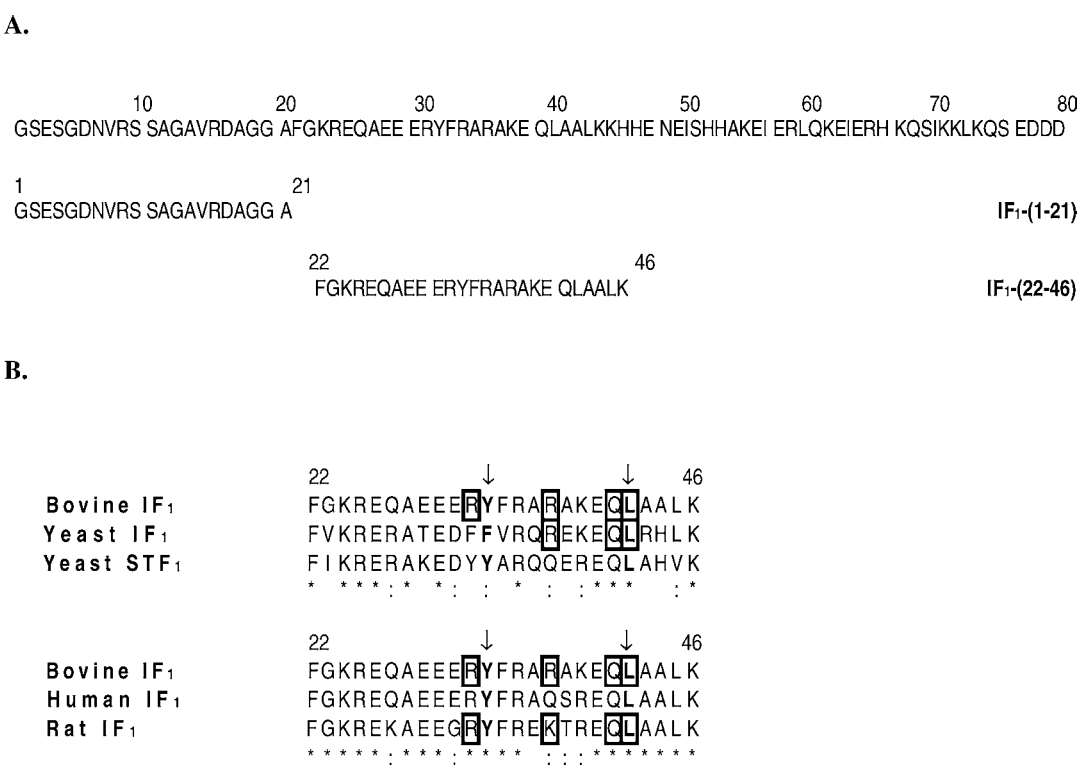

Fig. 6. Sequence analysis for prediction of the calmodulin-binding motif in bovine IF_1. A. Amino acid sequences of bovine IF_1 and synthetic peptides used for binding studies. B. Amino acid sequence alignment of bovine, rat, human, yeast IF_1 and STF_1. Asterisks indicate identical residues and colons denote conserved, as determined using CLUSTAL W (v. 1.8). The residues indicated by arrows form the putative hydrophobic motif, whereas residues marked by boxes form the IQ motif ([F, I, L, V]Qxxx[R, K]xxxx[R, K]).

positions 42 and 41, respectively, and Arg residues in positions 37 and 32 (Fig. 7(B)). This IQ motif is partially conserved in yeast IF_1, although Arg-32 (bovine numbering) is replaced by Phe (IQ-like motif). In contrast, the IQ motif is lost in STF_1. These observations, together with the results from binding studies with bovine and yeast proteins, further support the prediction that the conserved sequence 33–42 of IF_1 is the target of CaM.

Table I. Binding Affinity (K_d) of Calmodulin for Bovine IF_1, Bovine IF_1 Peptide 22–46, Yeast IF_1 and STF_1 at Different Values of pH in the Presence of Calcium

Protein	pH 5.0	pH 6.5	pH 8.0
Bovine IF_1	32.8 ± 6.7	53.5 ± 3.5	109.1 ± 22.6
Bovine IF_1 Peptide 22–46	140.1 ± 9.6	422.6 ± 34.3	1602.3 ± 57.1
Yeast IF_1	118.2 ± 17.3	65.1 ± 5.9	31.2 ± 4.7
Yeast STF_1	213.3 ± 25.2	310.2 ± 1.2	458.9 ± 36.1
Melittin	13.2 ± 0.9	13.8 ± 3.4	16.2 ± 3.9

Note. Values are expressed in units of nanomolar. Data are mean and SD of at least three independent experiments.

DISCUSSION

This study demonstrated that purified bovine and yeast F_1 inhibitory proteins bind calmodulin (CaM) with a 1:1 stoichiometry in the pH range between 5 and 8. In addition, CaM-binding blocks bovine IF_1's inhibitory activity on F_1-ATPase, in accordance with previous reports (Pedersen and Hullihen, 1984). CaM-IF_1 interaction was Ca^{2+}-dependent, and of intermediate affinity, i.e. 10 nM $< K_d <$ 100 nM (Persechini and Cronk, 1999).

Binding studies of CaM with synthetic peptides corresponding to part of the inhibitory region of bovine IF_1 and with yeast IF_1 and STF_1 allowed us to predict sequence 33–42 (bovine numbering) as the CaM target. This sequence contains a hydrophobic motif conserved in bovine, yeast, rat and human, supporting the importance of its role in binding. The presence of an overlapping IQ motif in yeast IF_1 but not in STF_1 may explain greater CaM-binding affinity of bovine and yeast IF_1 with respect to STF_1. The lack of one arginine in the yeast IF_1 sequence gives rise to an "IQ-like" motif that is still suitable for CaM binding (Rhoads and Friedberg, 1997). IQ motif

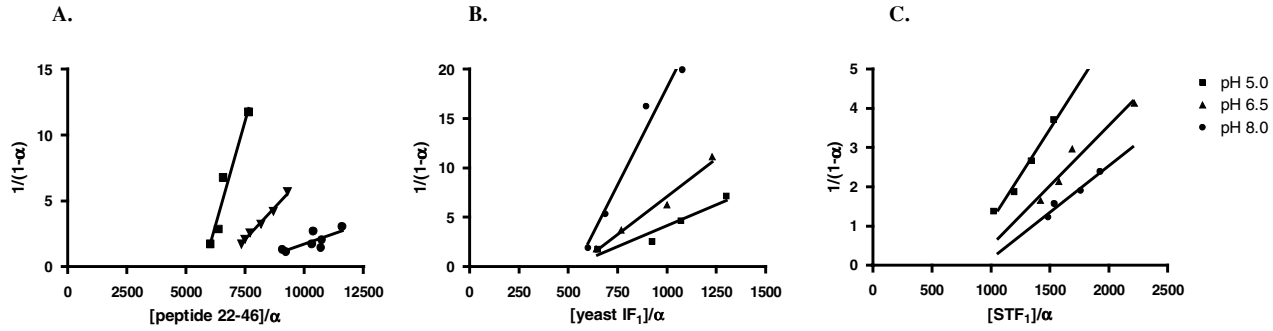

Fig. 7. Titration plots of bovine IF_1 peptide 22–46, yeast IF_1 and STF_1 at different values of pH in the presence of calcium. Plots of $1/1 - \alpha$ against the total IF_1 concentration divided by α are reported at the indicated pH values for bovine IF_1 peptide 22–46 (A), yeast IF_1 (B) and STF_1 (C). Experimental conditions were as in Fig. 2. α is the fractional degree of saturation of CaM. The data are means of at least three independent experiments.

is conserved in rat and not in human, but the corresponding K_d values for CaM are not yet available. These observations place IF_1 among the CaM-binding proteins whose target sequences contain an IQ motif as well as a hydrophobic motif (Rhoads and Friedberg, 1997). The presence of two overlapping CaM-binding motifs within the same sequence is not unusual (Yamniuk and Vogel, 2004), but its significance remains to be determined.

Relatively high concentrations of H^+ ions induce conformational changes in CaM targets that make CaM-binding domains more accessible (Huang *et al.*, 1994). This appears to be valid also for bovine IF_1 which has higher affinity for CaM at pH 5.0 than at pH 6.5 or 8.0; at pH 5.0 the protein is in dimeric form (Cabezon *et al.*, 2000). Dimerization of bovine IF_1 occurs by formation of an antiparallel α-helical coiled-coil between the C-terminal regions of monomers (Cabezon *et al.*, 2001; Gordon-Smith *et al.*, 2001). This arrangement places the N-terminal sequences containing the CaM-binding motif accessible to solvent and in opposition (Cabezon *et al.*, 2000). When bovine IF_1 forms tetramers at high pH, this motif is less accessible: according to the solution structure of IF_1 residues 35–47 are involved in protein-protein contacts within the tetramer (Cabezon *et al.*, 2000); according to the crystal structure this sequence is partially hidden in the tetramer (Cabezon *et al.*, 2001). Considering that our data show that bovine IF_1 binding to CaM occurs with the same stoichiometry at pH 8 and pH 5, we suggest that CaM binding is able to shift the IF_1 equilibrium towards the dimeric form in which the CaM-binding target becomes accessible. Therefore, the CaM-IF_1 complex could be formed by dimeric IF_1 associated with two CaM molecules simultaneously or by one molecule of each protein; both kinds of CaM-IF_1 complexes could coexist in solution, as already reported for the complex of bovine IF_1 with F_1-ATPase (Dominguez-Ramirez *et al.*, 2004).

The pattern of pH dependence of IF_1 binding was also observed for bovine IF_1 peptide 22–46, but the peptide had a markedly reduced affinity for CaM at pH 8.0 (K_d was reduced by more than one order of magnitude). Calculation of the titration point of the residues in the region 22–46 suggests that the lateral chains of Glu residues 26, 29, 30, 31, 40 and of Phe22 could be responsible for the affinity decrease at pH 6.5 and 8.0, respectively (data not shown). In particular, the change in charge of the N-terminal Phe22 group appears to strongly affect the interaction with CaM.

Contrary to what has been observed for bovine IF_1 and for its peptide 22–46, a low pH dependence characterizes the binding to CaM of melittin, a canonical high affinity CaM target peptide ($K_d \sim 10$ nM). These differences may be due to the different amino acid compositions, since melittin does not contain acidic residues. Moreover, our data show that IF_1 contains critical hydrophobic residues for CaM binding in different positions with respect to melittin, i.e. 1 and 10 rather than 1 and 14. The peptides also exhibit different conformational behaviors upon binding with CaM. In fact, although melittin is completely unstructured in solution, it adopts a cationic, amphiphilic α-helical structure on binding to CaM (Mori *et al.*, 2000), as generally occurs when target peptides form a complex with Ca^{2+}-CaM. In contrast, using circular dichroism, we did not note any increase in α-helical content upon complex formation between bovine IF_1 peptide 22–46 and CaM in the pH range between 5 and 8 (data not shown). This data is in agreement with observations that the CaM-binding motif is already in helical structure in bovine IF_1, in accordance with the presence of helical determinants in the peptide backbone at each pH tested (de Chiara *et al.*, 2002). Interestingly, melittin's adoption of an alpha helical structure is also important for its binding to F_1-ATPase, which occurs at the same inhibitory site

of IF_1, as recently reported (Gledhill and Walker, 2004). However, a different affinity and pH dependence characterize the binding of melittin to F_1-ATPase with respect to the IF_1-F_1-ATPase interaction (Gledhill and Walker, 2004; Bullough *et al*., 1989).

The affinity of yeast IF_1 and STF_1 for CaM are also influenced by pH. The K_d of the STF_1-CaM complex increases with pH, as is true also for bovine IF_1, while the K_d of the yeast IF_1-CaM interaction decreases with pH. This may be the consequence of the peculiar influence of pH on the oligomerization state of yeast IF_1, which is monomeric at pH 5 and dimeric at pH 8 (Cabezon *et al*., 2002). On the contrary, STF_1 oligomerizes in an opposite direction (Cabezon *et al*., 2002). Then, both proteins show a higher affinity for CaM as monomers. If the yeast proteins dimerize through the C-terminal segment homologous to the bovine sequence, this behavior could be due to a wider accessibility for CaM of the monomers, since the putative CaM-binding motif is located close to the dimerization segment (residues 44–84, bovine numbering). Although the dimerization regions in the yeast proteins have not yet been identified, our data are consistent with a direct involvement of CaM-binding motif in dimer formation.

Our data clearly show that the interaction between bovine IF_1 and CaM is regulated by low micromolar Ca^{2+}. Similarly, yeast IF_1 and STF_1 bind CaM in a comparable Ca^{2+} range (data not shown). This suggests that IF_1-CaM binding is at least partially controlled by local changes in intracellular Ca^{2+} concentration, which are particularly high in the microdomains around the mouths of Ca^{2+} channels of the internal Ca^{2+} stores such as mitochondria (Carafoli, 2003). Interestingly, a significant proportion of the total cellular CaM has been found in the cytosol in association with mitochondria (Pardue *et al*., 1981), the organelle into which IF_1 is transported after synthesis (Walker *et al*., 1987). The presence of CaM in the mitochondrial matrix, however, seems to be excluded at present, after several controversial results (Hatase *et al*., 1982; Schnaitman and Greenawalt, 1968; Moriya *et al*., 1993; Nakazawa, 2001; Milikan and Bolsover, 2000; Lopez *et al*., 2000). Mitochondrial inorganic phosphate sequesters and precipitates Ca^{2+} in the matrix (Carafoli, 2003), making Ca^{2+} regulation by Ca^{2+}-binding proteins unlikely. In light of these considerations, the hypothesis of intramitochondrial CaM binding to IF_1 and a consequent regulation of ATPsynthase activity, already proposed (Harris and Das, 1991) appears weak, although intriguing, to explain Ca^{2+}-mediated enzyme activation (Territo *et al*., 2001). Nonetheless, CaM binding to IF_1 is likely to occur outside mitochondria where IF_1 has been recently documented by confocal microscopy (Cortes-Hernandez *et al*., 2005). CaM may regulate IF_1 import into mitochondria or IF_1 trafficking towards the cell surface (Burwick *et al*., 2005; Martinez *et al*., 2003); CaM may also maintain IF_1 in an inactive form until it is at its site of action (Pedersen and Hullihen, 1984) or modulate additional IF_1 functions not yet demonstrated but already suggested (Cortes-Hernandez *et al*., 2005). Interestingly, multifunctional roles have also been proposed for e subunit (Hong and Pedersen, 2003). In agreement with this hypothesis, our data show that CaM binding to IF_1 occurs at neutral pH, which is observed in the cytosol under aerobic conditions (Porcelli *et al*., 2005). Moreover, the helical determinants found in the peptide backbone of IF_1 binding region for CaM suggest that the presence of the pre-sequence for import into mitochondria is compatible with CaM binding.

We conclude that the presence of a conserved CaM-binding site in eukaryotic IF_1 sequences, responsible for the intermediate affinity, Ca^{2+}-dependent binding with CaM, argues for the physiological relevance of the complex. Limited proteolysis experiments are underway in order to describe the topology of the IF_1-CaM complex. *In vivo* analyses are required to clarify the localization and the biological roles of this interaction.

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