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IMMUNOLOGICAL STUDIES ON THE BEEF HEART NATURAL ATPase INHIBITOR: LOCALIZATION OF AN ANTIGENIC DETERMINANT IN THE INHIBITOR MOLECULE

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Summary. Antibodies were raised against the beef heart mitochondrial ATPase inhibitor. This antiserum prevented the ability of the ATPase inhibitor to inhibit the F_1 ATPase activity. Peptide fragments obtained by enzymatic cleavage of the inhibitor protein were tested by immunoblotting or ELISA for their response to the anti-inhibitor antiserum. An antigenic determinant was located in the sequence spanning His 48 to Lys 58 of the inhibitor molecule.

The natural ATPase inhibitor (IF_1) is a small molecule of Mr ≃ 10.000, which inhibits the hydrolytic activity of the mitochondrial F_1-ATPase. IF_1 from beef heart mitochondria has been extensively studied and the sequence of the 84 residues that compose the IF_1 molecule has been established (1, 2). IF_1 inhibits the F_1-ATPase activity by binding to the β subunit of F_1 (3, 4) ; binding of one IF_1 to one β subunit is sufficient for full inhibition, in spite of the fact that each molecule of F_1 contains 3 β subunits (for review, see 5). Limited enzymatic digestion of beef heart IF_1 showed that a sequence of amino acids corresponding to Mr ≃ 1000 could be removed from the N-terminus of IF_1 without loss of inhibitor activity, whereas removal of a dozen of amino acids from the C-terminus resulted in 50% loss of activity (6). A rabbit antiserum directed against beef heart IF_1 has been prepared. This antiserum prevented the inhibitory activity of IF_1 with respect to F_1-ATPase. With this antiserum, we have undertaken a mapping study of the antigenic determinant(s) in the beef heart IF_1 molecule.

Abbreviations: F_1, catalytic sector of H^+-dependent ATPase ; IF_1, natural ATPase inhibitor ; TPCK-trypsin, trypsin treated by 1-tosyl amido-2 phenylether-chloromethyl ketone ; HPLC, high performance liquid chromatography ; TFA, trifluoroacetic acid ; PITC, phenyl isothiocyanate ; DABITC, dimethyl amino azobenzene isothiocyanate ; ELISA, enzyme-linked immunosorbent assay.

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MATERIALS AND METHODS

Beef heart mitochondria and the derived inside out sonic submitochondrial particles devoid of IF_1 (AS particles) were prepared as described in (7) and (8) respectively. Beef heart IF_1 was purified by the method described in (8), with some modifications (9) concerning the ethanol fractionation. Rabbit antibodies were raised against either native IF_1, or IF_1 cross-linked with glutaraldehyde, mixed with Freund's complete adjuvant. Rabbits were given two intramuscular injections of 0.3 mg IF_1 at two week intervals, followed by two intraveinous injections of 0.1 mg IF_1 and two intradermic injections of 0.3 mg IF_1. Bleeding was performed ten days after the last injection. The collected blood was allowed to clot overnight at 4°C. After centrifugation, and filtration through a 0.45 µ HA-Millex filter, the serum was kept under sterile conditions at 4°C.

For digestion of IF_1 by specific enzymes, the following conditions of incubation at 37°C were adopted ; a) thrombin (EC 3.4.4.13) from ISH laboratories, Paris: incubation for 24 h in 50 mM ammonium acetate and 1% ammonium bicarbonate, pH 8.0 (10) ; b) clostripaïn (EC 3.4.22.8) from Precibio, Paris: incubation for 24 h in 20 mM ammonium bicarbonate pH 7.9 after activation of the enzyme by incubation with 10 mM dithiothreitol (11) for 1 h, using a clostripain/IF_1 ratio of 1/50 (w/w) ; c) TPCK-trypsin from Worthington Biochemicals (EC 3.4.21.4): incubation for 20 h in 100 mM ammonium bicarbonate pH 7.9, using a trypsin/IF_1 ratio of 1/50 (w/w) ; d) **Staphylococcus aureus** V8 protease (EC 3.4.21.19) from Miles ; incubation for 22 h in the same buffer as that used for trypsin incubation with a protease/IF_1 ratio of 1/25 (w/w). The peptides yielded by enzymatic digestion of IF_1 were separated by HPLC on reversed-phase columns (4.6 x 250 mm) with acetonitrile gradients (0 to 50%) in 10 mM TFA. The temperature of the columns was set at 30°C ; the elution was performed at a flow rate of 1 ml/min, and the absorption of the eluates was monitored at 210 nm. Amino acid analysis of the peptides, after total acid hydrolysis (12), was performed by HPLC on a CAT EX column (from Waters). The amino acids were post-derivatized with O-phtalaldehyde and detected by fluorescence at 420 nm at a one nanomol range. The pure peptides were sequenced manually from their N-terminal residue, using double coupling with DABITC and PITC (13). The thiohydantoin derivatives were identified by chromatography on polyamide thin layer (from Schleicher and Schuell) (14). The inhibitory activity of IF_1 and the derived peptides with respect to the ATPase activity of AS particles devoided of endogenous IF_1 was assayed as described in (3).

The protein concentration was determined as in (15), using bovine serum albumin as standard. Labeling of arginyl residues with [^{14}C]phenylglyoxal (CEA-Saclay) was performed as in (16). Na Dod sulfate slab gel electrophoresis was performed with 20% acrylamide as separation gel and 10% acrylamide as concentration gel with the buffer system described in (17). Proteins and peptides were stained with Coomassie Brilliant Blue R250. Immunoblotting was carried out according to (18) after electrotransfer to 0.22 µ nitrocellulose sheet (BA 83, from Schleicher and Schuell) (19). The sheet was soaked for 16 h at 4°C in 10 mM sodium phosphate pH 7.4, 150 mM NaCl and 3% bovine serum albumin to saturate additional protein binding sites in nitrocellulose. The sheet was then incubated for 2 h at 20°C with the specific antiserum diluted to 1/200 in the phosphate saline medium and with 3% bovine serum albumin. The presence of antigen-antibody complexes was revealed with diaminobenzidine (Prolabo) after incubation with peroxidase-linked to goat antirabbit serum (Miles-Yeda) (20), in the presence of H_2O_2.

Short peptide fragments were tested for immunological response by ELISA (21) using the indirect mode. Fifty nanog of peptides in 10 mM K phosphate and 150 mM NaCl, pH 7.5 were coated in wells of polystyrene

microtiter plates (from Nunc) and incubated with the anti-IF_1 antisera, using dilutions ranging from 1/100 to 1/3200. The antibody-antigen complexes were then reacted with a peroxidase-linked goat antirabbit serum. The complexes were revealed with 4-amino-antipyrine (Sigma) after addition of H_2O_2. At various times of hydrolysis, absorbance was read at 490 nm in a Dynatech Reader MR600 (22).

RESULTS

Reactivity of IF_1 with anti-IF_1 antisera

Varying amounts of IF_1 (from 0.1 to 1 µg) were incubated for 40 min at room temperature with a fixed amount (10 µl) of antiserum directed against either the glutaraldehyde cross-linked IF_1 or the native IF_1, in 10 mM Mops buffer pH 6.8 and 0.25 M sucrose. A sample of 25 µg AS particles was then added and the incubation was continued at 30°C for 15 min in the same buffer. The ATPase activity was finally tested as described in (3). A control assay carried out in the absence of antiserum showed that 50% of the ATPase activity was inhibited after addition of 0.1 µg IF_1 (Fig. 1). A similar degree of inhibition was obtained when a preimmune serum of rabbit was present. Half maximal inhibition of ATPase activity required 6 times more IF_1 when the particles were preincubated with the antiserum to the glutaraldehyde-treated IF_1 and 10 times more IF_1 when preincubation was performed with the antiserum to the native IF_1. The prevention of the inhibition of ATPase activity was most probably due to the binding of anti-IF_1 antibodies to specific determinant(s) in IF_1.

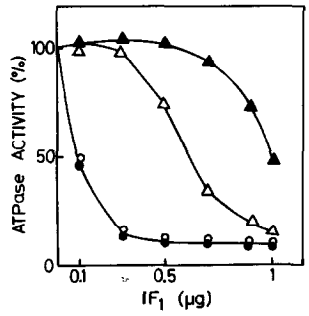

Figure 1. Titration of the inhibitory potency of the anti-IF_1 antiserum on IF_1 against the ATPase activity of AS particles. Experimental conditions were described in Materials and Methods. Increasing amounts of IF_1 were preincubated with 10 µl of antiserum raised against IF_1 cross-linked with glutaraldehyde (Δ—Δ) or 10 µl of antiserum raised against native IF_1 (▲—▲). A control assay was carried out with IF_1 mixed with 10 µl of preimmune serum (●—●) or in the absence of preimmune serum (○—○). After preincubation, AS particles (25 µg protein) were added and let to react with IF_1 and the anti-IF_1 antiserum. Then the ATPase activity of the AS particles was determined as described in (3). The ATPase activity of control particles corresponded to 6 µmol P_i released per min per mg protein at 30°C.

Localization of antigenic determinant(s) in the IF_1 molecule

The amino acid sequence of beef heart IF_1 has been established (1, 2). In the presentation of our results, the peptides released by thrombin, clostripain, trypsin and S. aureus V8 protease will be referred to as Th, Cl, Tr, and V8 followed by two numbers corresponding to the positions of the N and C terminal residues of these peptides in the amino acid sequence of IF_1.

- **Digestion of IF_1 by thrombin.** Cleavage of IF_1 by thrombin (10) yields four peptide fragments, Th 1-9, Th 10-84, Th 10-35/37 and Th 36/38-84, that were separated by HPLC on a C18 μBondapak column (Waters) and characterized by amino acid analysis and N-terminal amino acid sequence. The heterogeneous cleavage at positions 35/37 was due to the presence of the repetitive sequence Arg 35 - Ala - Arg 37 - Ala. Except for Th 1-9, the other three peptides were studied for their inhibitory effect on the ATPase activity of AS particles and their immunological response to IF_1 antisera. Only the peptide Th 10-84 retained the capacity to prevent the activity of mitochondrial ATPase, whereas two peptides, Th 10-84 and Th 36/38-84, were found to react with anti-IF_1 antisera as revealed by blotting ; no reaction was found for the peptide Th 10-35/87 (Fig. 2C). The two rabbit antisera described above, i.e. the antiserum against native IF_1 and the antiserum against glutaraldehyde treated IF_1, gave similar responses. The lack of immuno response of Th 10-35/37 could be due to loss of peptide during electrotransfer to nitrocellulose, in as much as the staining with Ponceau Red was negative. To ascertain this possibility, the peptides Th 10-35/37 and 36/38-84 were labeled by [^{14}C]phenylglyoxal prior to gel electrophoresis. The autoradiography in Fig. 2D shows that after transfer from the gel (Fig. 2A) to nitrocellulose (Fig. 2C), [^{14}C] radioactivity was still present. This result therefore corroborates the conclusion that Th 10-35/37 is not immunoreactive. Peptide Th 1-9 gave no immunoresponse to anti-IF_1 antisera by the ELISA test (not shown). It has been previously shown that this peptide could be removed without alteration of the inhibitory properties of IF_1 (4).

- **Digestion of peptide Th 36/38-84 by clostripaïn.** The peptide Th 36/38-84 was further digested with clostripaïn, which cleaves peptide bonds containing the carboxyl group of arginyl residues. The released peptides Cl 38-62 and Cl 63-84 were separated by HPLC on an Aquapore RP-300 column (Brownlee) (Fig. 3). The material in each peak was characterized by amino acid analysis and N-terminal sequence determination. No cleavage occurred at Arg 69, probably due to the proximity of a number of charged amino acids. In the ELISA test, only peptide Cl 38-62 was recognized by the anti-IF_1 antisera.

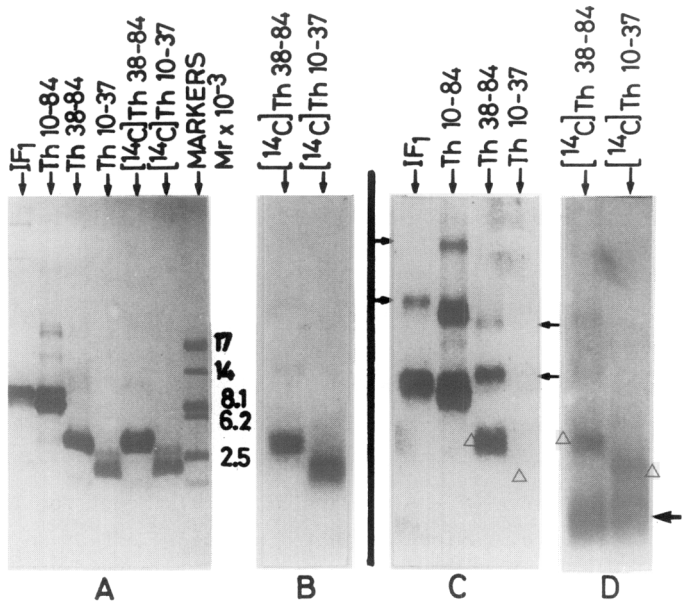

Figure 2. Immunoreactivity of HPLC-purified peptides obtained by digestion of IF_1 with thrombin. A) Na Dod sulfate polyacrylamide gel electrophoresis (SPAGE) and staining of the peptides with Coommassie Blue ; B) autoradiography of the peptides Th 10-37 and Th 38-84 labeled with [^{14}C]phenylglyoxal after SPAGE ; C) immunochemical reaction of the peptides after electrotransfer from polyacrylamide gel to nitrocellulose (arrows designate extra bands of high Mr detected by immunoreaction which correspond to peptide aggregates not detected by Coommassie Blue staining ; open triangles are used to designate the reactive peptide Th 10-37 and the position that would be occupied by Th 10-37, if it were immunoreactive. D) autoradiography of the peptides Th 10-37 and Th 38-84 labeled with [^{14}C]phenylglyoxal after transfer from polyacrylamide gel to nitrocellulose ; the lower ^{14}C spots designated by a thick arrow correspond to free [^{14}C]phenylglyoxal ; the ^{14}C spots designated by triangles correspond to the radioactive peptides Th 10-37 and Th 38-84.

- Digestion of peptide Cl 38-62 by S.a. V8 protease or trypsin. The products of digestion of peptide Cl 38-62 by S.a. V8 protease were not recognized by anti-IF_1 antisera. On the other hand, the tryptic hydrolysate of peptide Cl 38-62 gave a positive response. The fragments released by digestion of Cl 38-62 by S.a. V8 protease and trypsin can be derived from the amino acid sequence of the peptide (Figure 4). Digestion of Cl 38-62 by trypsin yielded four peptides, two of which, Tr 40-46/47 and Tr 48-58, containing 7-8 and 11 residues, were of sufficient length to be potential candidates for an antigenic site (23). The ambiguity about the cleavage at residues 46/47 was due to the two consecutive lysyl residues. As the amino acid sequence of Tr 40-46/47 is shared by V8 41-50, one of the five fragments of the S.a. V8 protease hydrolysate of Cl 38-62, it is inferred that the positive immunoresponse of the tryptic digest of Cl 38-62 is probably due to Tr 48-58. This conclusion was supported by the negative

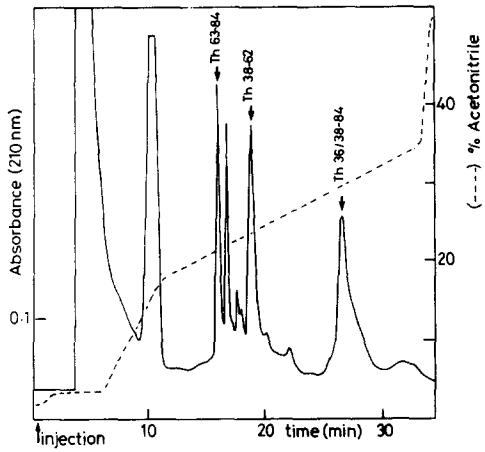

Figure 3. HPLC fractionation of peptide fragments obtained by digestion of Th 36/38-84 with clostripaïn.
Conditions of incubation and fractionation are detailed under Materials and Methods. (----) % of acetonitrile in 10 mM TFA. (——) Absorbance at 210 nm.

immunoresponse obtained in ELISA test with the HPLC-purified peptide Tr 40-47 (data not shown).

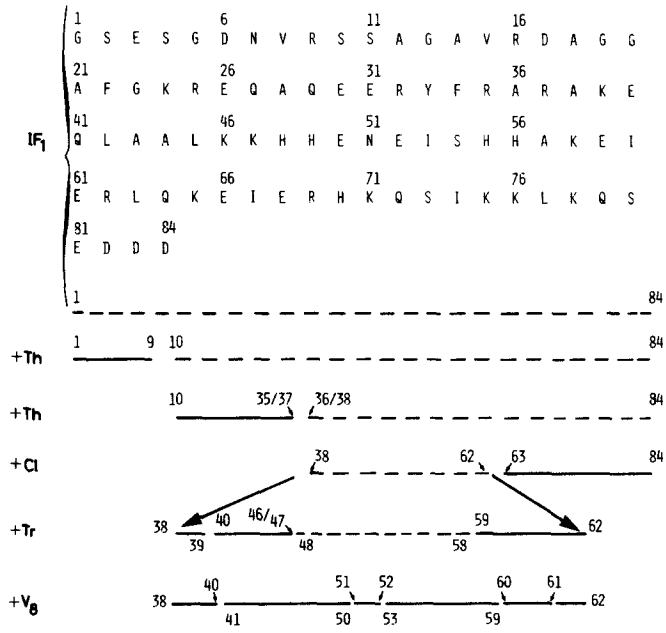

Figure 4. Scheme illustrating the immunoreactivity of peptide fragments obtained by enzymatic cleavage of beef heart mitochondrial IF_1. Th, Cl, Tr and V8 stand for thrombin, clostripaïn, trypsine and **Staphylococcus** V8 protease, respectively. Broken lines (----) correspond to immunoreactive peptides. Plain lines (——) correspond to non immunoreactive peptides. The sequence of IF_1 is given in the upper part of the Figure.

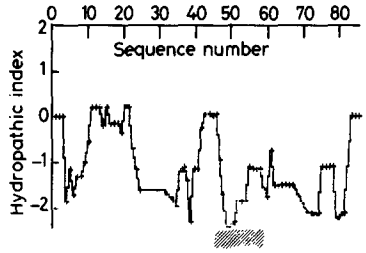

Figure 5. Hydropathic profile of beef heart IF_1. In the method used (28), a negative hydropathic index is assigned to the hydrophilic residues. Note the correspondance between the immunoreactive peptide His 48-Lys 58 denoted by a hatched bar and the presence of a very hydrophilic peak in the profile.

DISCUSSION

Other antibodies directed against beef heart mitochondrial IF_1 have been prepared (24, 25). However, these antibodies could not prevent the ability of IF_1 to inhibit the mitochondrial F_1-ATPase activity. The anti-IF_1 antibodies described in the present paper were able to reduce the inhibitory activity of IF_1. To locate antigenic determinant(s) in the beef heart IF_1, we examined the reactivity of a number of peptide fragments obtained by enzymatic cleavage of IF_1 (Fig. 4). The shortest antigenic determinant revealed by this approach was the peptide fragment spanning His 48-Lys 58. A plot of the hydropathic index versus the sequence number (Fig. 5) as described in (28) shows that the sequence His 48-Lys 58 is particularly hydrophilic in agreement with the general observation that hydrophilic sequences correspond to antigenic determinants (29).

The sequence spanning Lys 47-His 70, which encompasses that of the immunoreactive peptide, His 48-Lys 58, has no equivalent in the IF_1 from the yeast **Candida utilis** (A.C. Dianoux in preparation) or **Saccharomyces cerevisiae** (26). This may explain the lack of immunological cross-reactivity of the anti-beef heart IF_1 antiserum against IF_1 from **C. utilis** (data not shown). Although no common antigenic determinant was found in beef heart IF_1 and **C. utilis** IF_1, **C. utilis** IF_1 is able to inhibit efficiently beef heart F_1-ATPase activity (27). These results suggest that the sequence His 48-Lys 58 in beef heart IF_1 does not play a strategic role in the interaction between IF_1 and F_1.

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