Volume 226, number 1, 166–170 FEB 05418 December 1987

In the uncoupling protein from brown adipose tissue the C-terminus protrudes to the c-side of the membrane as shown by tryptic cleavage

Christoph Eckerskorn and Martin Klingenberg

Institute for Physical Biochemistry, University of Munich, Goethestrasse 33, 8000 Munich 2, FRG

Received 5 November 1987

Limited proteolytic digestion of the uncoupling protein (UCP) with trypsin yielded a cleavage product only about 2 kDa smaller than the original UCP (33 kDa). This cleavage can be obtained with the solubilized isolated protein detergent micelle as well as in original brown adipose mitochondria. The cleavage site is identified by C-terminal sequence to be located near the C-terminus at lysine 292. This C-terminus, a 10 residue long peptide, is strongly hydrophilic and can be expected to be localized outside the membrane. In UCP this C-terminal stretch represents a structural difference to the similarly folded ADP/ATP carrier which does not form a corresponding cleavage product. Comparison of tryptic cleavage of UCP in mitochondria with differently broken outer membrane, in sonic particles of mitochondria, as well as in UCP proteoliposomes, indicate that the C-terminus is directed versus the cytosolic site of the membrane. Because of the easy susceptibility to trypsin, the cleavage site must be surface-exposed and the C-terminal section unusually mobile.

Uncoupling protein; C-terminus; Trypsin digestion

1. INTRODUCTION

The uncoupling protein (UCP) from brown adipose tissue mitochondria has a primary structure with a distinct homology to the ADP/ATP carrier [1], although UCP has a transport function widely different from the ADP/ATP carrier, in facilitating transport of H^+ or OH^- instead of ADP and ATP. One peculiar feature revealed by a primary structure comparison of the two proteins is that UCP has an additional 'hydrophilic' C-terminus of about 10 residues. Since in both the UCP and ADP/ATP carrier the last putative membrane-spanning amphipathic α-helix extends to about position 295, it seems reasonable to assume that the additional 10 hydrophilic C-terminal residues are positioned in the extramembranous space. Also, a particular immunogenic quality of the C-terminal of UCP was recently reported, which supports the protruding position of this peptide [2].

An experimental problem is to demonstrate whether this is, in fact, the case and, if so, to which side of the membrane the C-terminal extends into the hydrophilic space. These questions are attacked in this paper by limited proteolysis which turned out to yield highly significant answers.

Correspondence address: M. Klingenberg, Institute for Physical Biochemistry, University of Munich, Goethestrasse 33, 8000 Munich 2, FRG

Abbreviation: UCP, uncoupling protein

2. EXPERIMENTAL PROCEDURES

Trypsin and bovine trypsin inhibitor were obtained from Boehringer. Carboxypeptidase A was

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

obtained from Sigma, carboxypeptidase B was purchased from Boehringer. UCP was prepared from brown adipose tissue mitochondria as described [3]. The UCP proteoliposomes were prepared according to [4]. The SDS slab gel electrophoresis was performed under conditions of Laemmli using a gel with 12% acrylamide and 0.32% bisacrylamide.

The T-1 tryptic product was isolated from a preparative tryptic digestion of UCP, 6 mg UCP were incubated with 0.4 mg trypsin for 90 min. The reaction was terminated by addition of trichloroacetic acid and acetone. The precipitate was washed, dried and dissolved in 80% formic acid. It was chromatographed on a S-200 agarose column. The T-1 peptide appeared in central fractions. For sequencing the C-terminus, isolated T-1 was incubated in a solution of 0.2 M *N*-methylenemorpholine, 0.4% SDS at pH 8.2 and 37°C. At increasing time intervals samples were taken, lyophilized and analyzed in an amino acid analyser.

3. RESULTS AND DISCUSSION

3.1. *Limited proteolysis by trypsin*

Proteolytic studies were performed first with the isolated solubilized UCP. In this state UCP exists as a mixed protein-detergent micelle with some residual phospholipid. In the non-ionic detergent Triton X-100 UCP forms a dimer and can be assumed to exist in a conformation largely similar to that in the original membrane [5]. The protein is visualized to form the central core within a detergent annulus which has replaced the phospholipid of the membrane. This implies that the hydrophilic sections protrude on both sides of the micelle, just as in the original membrane.

Exploratory proteolysis experiments on the isolated UCP have been performed with a variety of proteases such as trypsin, chymotrypsin, bromelain, papain, subtilisin, elastase and thermolysin. Only trypsin and chymotrypsin produced reasonably large peptides of >5 kDa in size, whereas the other proteases degraded the proteins to too many peptides which were not defined in the SDS gel electrophoresis. We shall confine the following report only to the proteolysis by trypsin, which produces a well defined cleavage.

The limited digestion of isolated UCP by trypsin is shown in fig.1. This proteolysis occurs at the relatively low trypsin/UCP ratio of 1:20. At the time indicated the trypsin was inactivated by addition of excess bovine trypsin inhibitor. The most striking result is the degradation of the UCP to a product T-1 with a molecular mass only slightly lower. Whereas this interconversion occurs relatively fast, with a half-time of about 30 min under these conditions, the further degradation is much slower. No further defined peptides can be found during the continuing degradation, indicating that all the products have molecular masses <3 kDa.

In mitochondria the UCP can also be degraded by trypsin to the same cleavage product. The UCP and its major cleavage products were isolated from mitochondria by chromatography on hydroxyapatite before being applied to the SDS gel. With conditions such as temperature and amount of trypsin the same, the degradation rate was slightly slower than for the solubilized UCP. Fig.1B shows that, in addition to the primary product T-1, another peptide T-2 with M_r = 25 kDa appears. The time delay in the emergence of this peptide indicates that it is produced by cleavage from T-1.

3.2. *Protection by nucleotide binding*

Of great interest is the possible influence of nucleotide binding on the proteolysis of UCP. Nucleotides, such as GTP, are inhibitors of the H^+ transport activity of UCP [6] and bind with relatively high affinity [7] ($K_d = 10^{-6}$ M at pH 7). It can be assumed that under these conditions UCP is nearly exclusively present as a UCP–GTP complex with a ratio UCP–GTP/UCP = 10^3. As shown in fig.2, there is a clear inhibition of the UCP degradation by GTP. In the presence of GTP, 45% UCP are still undigested after 30 min, whereas without GTP only 10% are left. The level of the cleavage product T-1 is largely suppressed in the presence of GTP. This suggests that the subsequent degradation of T-1 is not inhibited by GTP binding since the first step degradation of UCP is so slow that the level of T-1 remains small. It can be concluded from these results that GTP binding at least partially protects UCP against proteolysis by trypsin.

The influence of GTP on the proteolytic sensitivity of UCP suggests that the inhibition of the

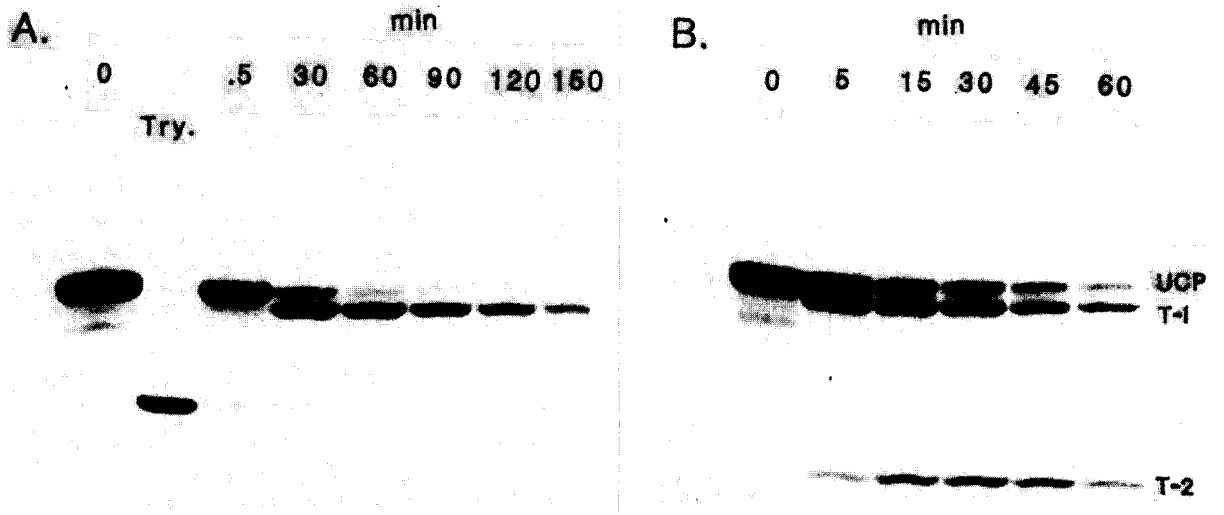

Fig.1. Limited tryptic proteolysis of uncoupling protein (UCP). (A) Isolated UCP was subjected to trypsin at a protein ratio of 20:1 UCP/trypsin at 0.5 mg UCP/ml, 37°C, pH 6.7. At the time intervals indicated excess trypsin inhibitor was added. (B) Isolated brown adipose mitochondria were frozen-thawed before exposure to trypsin under conditions similar to those given in A. T-1, T-2, first, second tryptic peptide; Try, trypsin.

H^+ transport activity is the result of a conformational change induced by the nucleotide binding. As a result of this conformational tightening the cleavage site becomes inaccessible. In order to further examine this finding, the influence of a nucleotide analogue was tested which was found to bind but not to inhibit the H^+ transport activity (unpublished). As shown in earlier studies [8], the fluorescent ATP derivative 1.3 dimethylaminonaphthoyl-3′-*O*′-ATP (DAN–ATP) binds rapidly and effectively to UCP, emitting a strong fluorescent signal. Kinetic analysis of fluorescence also suggested that the DAN-nucleotide can only form an encounter complex with UCP, but does not induce the subsequent slower conformation exchange leading to the inhibition, as does ATP or GTP.

Isolated UCP was exposed to near saturating concentration of DAN–ATP, where UCP exists largely as a DAN–ATP–UCP complex at a ratio of approximately 50:1. No inhibition of the rate of tryptic digestion of UCP to T-1 and further products was observed (not shown). These results strongly support the contention that not the nucleotide binding per se, but the conformational change induced by the nucleotide binding is responsible for the inhibition of transport, as well as for the inhibition of proteolysis.

3.3. *Localization of the specific cleavage site*

The formation of a cleavage product differing only by 1 to 2 kDa from the original UCP indicates that the cleavage site is either close to the N- or C-terminus. In order to elucidate this site, the cleavage product T-1 was isolated from trypsinolytic preparations in which there was a relatively high content of T-1 and chromatographed in 80% formic acid on a Sephacryl S-200 column.

The N-terminus of the isolated T-1 product was found to be identical to that in UCP. Therefore the cleavage site must be close to the C-terminus. The isolated T-1 was therefore cleaved with carboxypeptidases A and B. The time-dependent appearance of the cleaved amino acids is shown in fig.3. First, with carboxypeptidase B, lysine appears as a major product and then, after addition of carboxypeptidase A, leucine, glutamine and glutamic acid are released, as well as valine, threonine and arginine. The results can be interpreted to indicate that the C-terminus of the T-1 is

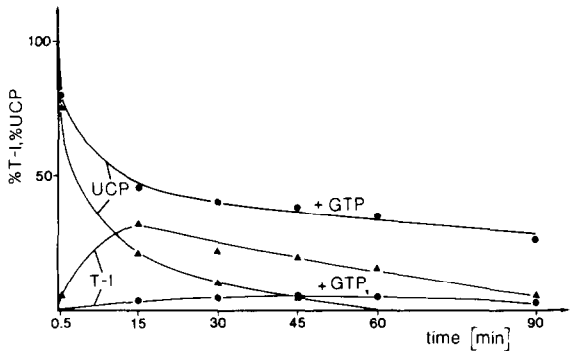

Fig.2. GTP binding protects UCP against proteolysis by trypsin. Isolated UCP was incubated with and without 0.5 mM GTP under conditions similar to those described for fig.1A. T-1, first tryptic peptide. The degree of proteolysis is obtained by integration of the color of the slab gel electrophoreses by a laser scanner.

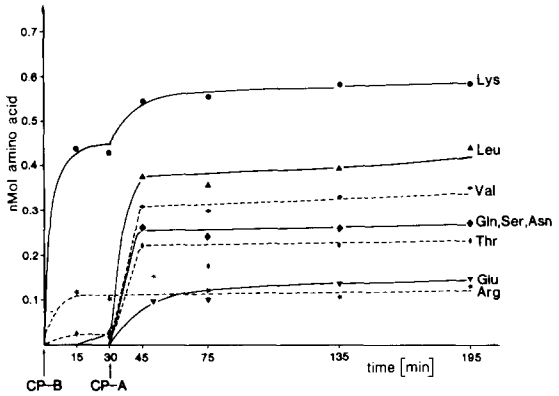

Fig.3. Identification of C-terminal sequence of the T-1 tryptic peptide by carboxypeptidase digestion. Time dependence of the amino acid appearance. For experimental details see text.

Lys-292. This is the first cleavage by the carboxypeptidase B, with a residual lysine at position 293.

290
-Phe-Glu-Gln-Leu-Lys-Lys-Glu-Leu-Ser-Lys-Ser-Arg-

300 306
Glu-Thr-Val-Asp-Cys-Thr-Thr

The cleavage by carboxypeptidase A is in line with this position since it releases Leu, Gln and Glu. The parallel release of Val, Thr and Arg can be interpreted to originate from other cleavage products, in particular the 25 kDa product, if we assume that the C-terminal is Arg-238 followed by Thr and two Val. For the present purpose the most important conclusion is that the first step of the limited tryptic proteolysis of UCP leads to the cleavage of the last 14 residues at the C-terminus.

3.4. *Membrane sidedness of the C-terminus*

The distinct cleavage by trypsin at position 292 near the C-terminus intact UCP both in the isolated Triton micelle and in the mitochondrial membrane suggests that the C-terminus extends outside the membrane confines. This can be rationalized in view of the hydrophilic composition of the C-terminal 17 residues. Therefore also the membrane sidedness of the protruding C-terminus was investigated. For this purpose the degradation of UCP in sonic particles, in mitochondria and in frozen mitochondria was compared. In intact mitochondria the outer membrane will prevent the access of trypsin to the inner membrane. In frozen mitochondria the outer membrane would be largely broken. In sonic particles the inner membrane should be inverted, at least to a major extent. These parameters, controlling the sidedness and accessibility of UCP, are present in the three mitochondrial preparations only to a varying degree and not exclusively. Therefore only relative differences in the trypsin accessibility can be expected. Fig.4 shows that the degradation is slowest

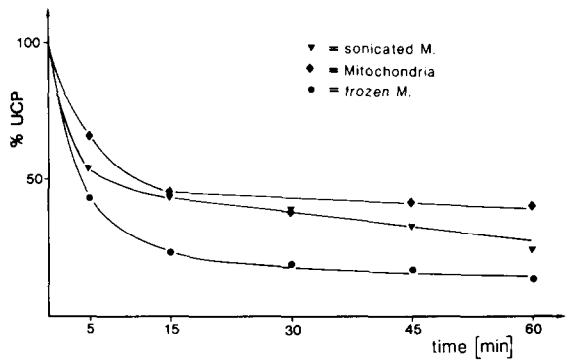

Fig.4. Accessibility to trypsin of UCP in various mitochondrial membrane preparations. Brown adipose mitochondria, fresh, frozen-thawed, and sonicated were exposed to trypsin under the conditions given in fig.1. The proteolysis was evaluated from SDS-gels with an integrating laser scanner.

in sonic particles and is most rapid in frozen mitochondria. These results are best explained by assuming that the C-terminus protrudes to the cytosolic side of the inner mitochondrial membrane.

For further elucidation of the sidedness, the influence of trypsin on UCP incorporated into artificial phospholipid vesicles was studied. As shown previously, more than 85% of the UCP molecules in these vesicles are incorporated right-side-out i.e., in the same direction as in mitochondria [4]. Thus in this preparation the membrane sidedness of UCP is defined more clearly than in the mitochondrial preparations. In the phospholipid vesicles, UCP was partially degraded to form T-1. However, the degradation was strongly accelerated by trypsin digestion.

4. CONCLUSIONS

Limited proteolysis applied to membrane proteins has often yielded valuable information concerning the orientation of certain lysine groups and of membrane-protruding protein domains, provided that well defined and relatively large peptides can be identified. As shown previously, no definite peptides can be obtained by digestion with the ADP/ATP carrier either in the solubilized or membrane-bound state [9]. In view of these results it is not surprising that only one intermediary product can be distinctly obtained with the UCP, by amputation of a small C-terminal peptide which in the UCP represents an 'addition' to the membrane insertion of these two homologous proteins. The fact that the cleavage site, lysine 292 is much more accessible to trypsin than the other lysines or arginines can be attributed to an unusual position at the surface of the protein. It may also reflect a mobility of the C-terminus, which is required for the insertion of the cleavage site into the trypsin binding cleft. In accordance, also the hydrophilic nature indicates that this mobile C-terminus protrudes out of the membrane. The results support a location directed towards the cytosolic side of the inner mitochondrial membrane. This location determines the sidedness of the folding arrangement, of UCP as previously shown in fig.7 of [1].

In view of the homology to the ADP/ATP carrier, it can be expected that C-terminus also of the AAC protrudes to the cytosolic side, with only five residues emerging from the last amphipathic membrane-spanning α-helix. These results are in line with the observation that the C-terminus in UCP has strong antigenic properties, as demonstrated by cloning C-terminal containing fragments of the UCP [2]. Recently the homology has also been extended to the phosphate carrier (P_iC) from mitochondria. The primary structure indicates that this carrier has an even longer hydrophilic C-terminus of more than twenty residues [10,11].

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft. We like to thank H. Aquila for advice on the sequence work.

REFERENCES

[1] Aquila, H., Link, T.A. and Klingenberg, M. (1985) EMBO J. 4, 2369–2376.
[2] Ridley, R.G., Patel, H.V., Parfett, C.L.J., Olynyk, K.A., Reichling, S. and Freeman, K.B. (1986) Biosci. Rep. 6, 87–94.
[3] Lin, C.S. and Klingenberg, M. (1980) FEBS Lett. 113, 299–303.
[4] Klingenberg, M. and Winkler, E. (1986) Methods Enzymol. 127, 772–779.
[5] Lin, C.S., Hackenberg, H. and Klingenberg, M. (1980) FEBS Lett. 113, 304–306.
[6] Nicholls, D.G. (1976) Eur. J. Biochem. 62, 223–228.
[7] Lin, C.S. and Klingenberg, M. (1982) Biochemistry 21, 2950–2956.
[8] Klingenberg, M. (1984) Biochem. Soc. Trans. 12, 390–393.
[9] Klingenberg, M., Riccio, P. and Aquila, H. (1978) Biochim. Biophys. Acta 503, 193–210.
[10] Aquila, H., Link, T.A. and Klingenberg, M. (1987) FEBS Lett. 212, 1–9.
[11] Runswick, M.J., Powell, S.J., Nyren, P. and Walker, J.E. (1987) EMBO J. 6, 1367–1373.