Volume 327, number 3, 294–296 FEBS 12776 August 1993

Mature carnitine palmitoyltransferase I retains the N-terminus of the nascent protein in rat liver

Marian P. Kolodziej and Victor A. Zammit

Hannah Research Institute, Ayr, KA6 5HL, Scotland, UK

Received 25 May 1993

Carnitine palmitoyltransferase I was isolated from octylglucoside extracts of rat liver mitochondrial outer membranes. This native enzyme was digested proteolytically with V8 protease. Five major peptides were obtained all of which were found in the amino acid sequence predicted from the full-length cDNA sequence of the protein. One peptide was found to correspond to the extreme N-terminus of the deduced amino acid sequence. Therefore, the mature protein retains the N-terminus of the nascent protein after import into the mitochondrial membrane. Knowledge of the identity of the N-terminus of the mature protein allows a reappraisal of the role of the two main, N-terminal hydrophobic domains of the protein and of the possible topology of the protein within the membrane.

Carnitine palmitoyltransferase; N-terminus; Import; Outer membrane; Malonyl-CoA; Ketogenesis

1. INTRODUCTION

Major advances have been made in the understanding of the mitochondrial carnitine palmitoyltransferase system in recent years. The overt enzyme (CPT I) was finally correctly localised to the mitochondrial outer membrane [1]; its catalytic activity was shown to reside in a protein that has the same functional size as that labelled by the irreversible inhibitor, tetradecylglycidyl-CoA (TDG-CoA) [2]; specific polyclonal antibodies raised against CPT I were found not to be immunoreactive against latent CPT (CPT II) and vice versa [3]; the cDNA for both CPT I and CPT II have been cloned, sequenced and expressed and, as expected, shown to code for distinct polypeptides [4,5].

The predicted amino acid sequences of the respective N-termini of CPT I and CPT II differ greatly [4,5]. In particular, that of CTP II contains a characteristic mitochondrial inner membrane–matrix targeting sequence containing regularly spaced basic amino acid residues interspaced with hydrophobic ones; this sequence is presumably proteolytically cleaved to produce mature CPT II. No such sequence occurs in the predicted N-terminus of CPT I, as would be expected from its location in the mitochondrial outer membrane [1]. Of particular interest, however, is the presence in the predicted CPT I sequence of two highly hydrophobic stretches of primary sequence in the N-terminal 122 amino acids. Esser et al. [5] have suggested that the shorter (and more C-terminal) of the two sequences constitutes a membrane-spanning anchoring domain and that the more N-terminal sequence (amino acids 48–73) may not be present in the mature protein due to proteolytic cleavage after import of CPT I into the mitochondrial outer membrane. In their experiments, they used a truncated form of CPT I, which may have lacked this N-terminal sequence, for the generation of tryptic peptides.

In the present paper we describe the generation of peptides from intact (non-truncated), mature CPT I and demonstrate that it contains the N-terminal amino acids predicted from the cDNA sequence. The implications for the mechanism of import of CPT I into the mitochondria and for the functions of the N-terminal hydrophobic sequences are discussed.

Correspondence address: M.P. Kolodziej, Hannah Research Institute, Ayr, KA6 5HL, Scotland, UK. Fax: (44) (292) 671 052.

2. EXPERIMENTAL

2.1. *Isolation of intact CPT I*

Mature, intact CPT I was prepared essentially as described previously [3], with minor modifications. Mitochondria were prepared from livers of 48-h starved male Wistar rats and purified on self-forming Percoll gradients (31% in 0.3 M sucrose, 5 mM Tris, 1 mM EGTA, pH 7.4 at 0°C). Outer membranes were prepared from the mitochondria by the swelling and shrinking procedure of Parsons et al. [6] as modified by [2]. The membranes were solubilised by incubation with octylglucoside (37 mM) for 1 h at 0°C. The solubilised proteins of these membranes were incubated with Agarose–concanavalin A to remove the glycoprotein that routinely contaminates CPT I on SDS gels [3]. The resulting supernatant was subjected to SDS-gel electrophoresis and the band corresponding to CPT I (p88, see [3]) was excised and electroeluted. The resultant purified protein was concentrated (Centricon, Amicon, Danvers, MA, USA) and used for proteolytic generation of peptides using Endoprotease Glu-C (*Staphylococcus aureus* V8) by the method of Cleveland et al. [7]. Digestion was allowed to proceed for 45 min at room temperature before separation of the resulting peptides on a 15% polyacrylamide gel. The peptides were transferred to nitrocellulose (Problot Applied Biosystems, Foster City, CA, USA), lightly stained with Amido black, excised, and used directly for microsequencing of the N-termini. The approximate mo-

 Published by Elsevier Science Publishers B.V.

lecular weights of the peptides were determined by concurrent electrophoresis of suitable standards.

2.2. *Chemicals*

Agarose–concanavalin A and V8 protease were from Sigma (Poole, Dorset, UK). All materials used for the preparation of the peptides were of sequencing grade.

3. RESULTS AND DISCUSSION

Proteolysis of mature, intact CPT I resulted in the generation of five major peptides with distinct amino-termini (Table I) and multiple other minor peptides which were shorter fragments and had the same N-terminal sequences as those listed in Table I. All the sequences were found to correspond to regions throughout the amino acid sequence predicted from the full-length cDNA sequence obtained for rat liver CPT I [5], indicating that the intact protein prepared by separation of outer membrane proteins on SDS-gels is indeed CPT I. This confirms our earlier findings [3] that a polyclonal antibody raised against this protein removes CPT I activity from octylglucoside extracts of rat liver mitochondria outer membranes. The combination of these observations is significant in that it proves that CPT I activity is not destroyed by octylglucoside, as claimed previously [4]. It also explains the presence of a malonyl-CoA-sensitive CPT activity in octylglucoside extracts of outer membranes [8].

Inspection of the predicted amino acid sequence for CPT I indicated that the specificity of V8 protease (Glu-C) was preserved in the generation of all the peptides. However, there were some differences (about 5%) between the sequences obtained in the present study and those deduced from the cDNA. This low level of discrepancy can be explained by errors arising from sequencing (amino acid or nucleotide) procedures.

Of particular interest was the observation that peptide 1 (Table I) contains the entire N-terminal sequence predicted to result from cleavage at Glu 3 of the protein, at the extreme N-terminus of the predicted sequence [5]. As our starting material was intact, mature CPT I, it is concluded that the N-terminus of the nascent protein is retained after import of CPT I into the mitochondrial outer membrane. Therefore, contrary to the suggestion in [5], the N-terminus of the nascent protein, including the hydrophobic region represented by residues 48–75, is not cleaved upon import and is present in the mature protein. This means that import of CPT I conforms with that of other mitochondrial outer membrane proteins [9].

The knowledge that the N-terminal sequence of nascent CPT I is retained in the mature protein enables direct comparison of the N-terminal sequences of nascent CPT I and CPT II. Of particular interest is the presence of multiple acidic residues in the N-terminal sequence of CPT I which, as expected, are incompatible with matrix targeting. On the other hand it is now possible to suggest that the more N-terminal of the two hydrophobic stretches of the CPT I sequence is not only present in the mature protein but probably acts as a stop–transfer signal and spans the outer membrane. Membrane-spanning domains are usually considered to consist of an uninterrupted series (24 ± 2) of hydrophobic amino acids [10]. A major yeast mitochondrial outer membrane integral protein (OMM 70, [10]) contains such a typical transmembrane sequence of 28 amino acids (although only 19 of these were found to be essential for transmembrane orientation of the protein [12]) in the first N-terminal 38 amino acids of its sequence. Typically, these transmembrane sequences are flanked by highly charged stretches of amino acid sequences [13,14]. It is of interest, therefore, that the more N-terminal of the two hydrophobic sequences of CPT I (residues 48–73) has no fewer than 5 positively charged amino acid residues in the immediate N-terminal flanking region. Similarly, 3 out of the first 5 residues immediately C-terminal to the hydrophobic span are charged (acidic and basic). It is significant, however, that the net positive charge difference between these two charged regions flanking the hydrophobic domain is strongly in favour of the N-terminal region. This suggests that if this hydrophobic domain adopts a transmembrane orientation it is likely that the extreme N-terminus of mature CPT I (this paper) protrudes into the cytosol. This

Table I

Peptides obtained from mature CPT I after V8-protease treatment

Peptide	Approximate mol. wt.	N-Terminal sequence	Position within protein (residues)
1	25 kDa	XHQAVAFQFTVTPDGIXL	4–238
2	15 kDa	MLYITPTDIQAARXGNTI	259–412
3	42 kDa	QQMQQILDDPSEPNGGEAK	361–773
4	20 kDa	VIYASLSSAXLLAND	529–744
5	10 kDa	LFDFEKNPDYVSRGGGFPVAD	696–773

The assignments of the amino acid residues were made by a combination of comparison of the N-terminal amino acid sequences of the peptides with that deduced from the full-length cDNA sequence for the protein [5], the approximate molecular weights of the peptides, and the Glu-C specificity of the protease. Residues for which a definitive assignment could not be made are indicated by X. Differences from the cDNA-deduced sequence are indicated by an asterisk.

is because a strong correlation has been found, for other transmembrane proteins (microsomal and mitochondrial outer membrane), between the difference in the charges of the 10–15 residues flanking signal–anchor domains of the proteins in the membranes with their orientation within the membrane. In general, the more positive portion faces the cytosol, with the orientation of all subsequent transmembrane segments determined by the most N-terminal one [15]. It is noteworthy that the more C-terminal of CPT I's two putative transmembrane domains does not have such a charge difference across it. Current evidence about the topology of CPT I in the mitochondrial outer membrane is controversial, as the work of Murthy and Pande [1] suggested that the malonyl-CoA-binding and catalytic sites of the protein are differentially exposed to proteases in intact mitochondria, whereas the data of McGarry and coworkers [17] suggest that such differential sensitivity is not due to membrane topology but to the tertiary structure of the protein which responds to malonyl-CoA-induced changes in conformation [18,19]. It is anticipated that the present results on the identity of the N-terminus of the mature protein (e.g. through the use of specific anti-peptide antibodies) can provide an important first step in the resolution of the topology of CPT I within the mitochondrial outer membrane.

Acknowledgements: We thank Mr. C.G. Corstorphine for invaluable assistance and Mr. B. Dunbar (SERC Protein Sequencing Facility, Department of Molecular and Cell Biology, University of Aberdeen) for sequencing of the peptides. The work was funded by the British Diabetic Association and supported by the Scottish Office Agriculture and Fisheries Department.

REFERENCES

[1] Murthy, M.S.R. and Pande, S.V. (1987) Proc. Natl. Acad. Sci. USA 84, 378–382.
[2] Zammit, V.A., Corstorphine, C.G. and Kolodziej, M.P. (1989) Biochem. J. 263, 89–95.
[3] Kolodziej, M.P., Crilly, P., Corstorphine, C.G. and Zammit, V.A. (1992) Biochem. J. 282, 415–421.
[4] Woeltje, K.F., Esser, V., Weis, B.C., Sen, A., Cox, W.F., McPhaul, M.J., Slaughter, C.A., Foster, D.W. and McGarry, J.D. (1990) J. Biol. Chem. 265, 10720–10725.
[5] Esser, V., Kuwagima, M., Britton, C.H., Krishnan, K., Foster, D.W. and McGarry, J.D. (1993) J. Biol. Chem. 268, 5810–5816.
[6] Parsons, D.F., Williams, G.R. and Chance, B. (1966) Ann. NY Acad. Sci. 137, 643–666.
[7] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[8] Murthy, M.J.R. and Pande, S.V. (1990) Biochem. J. 268, 599–604.
[9] Glover, L.A. and Lindsay, J.G. (1992) Biochem. J. 284, 609–620.
[10] Gierasch, L.M. (1989) Biochemistry 28, 923–930.
[11] Hse, T., Riezman, H., Suda, K. and Schatz, G. (1983) EMBO J. 2, 2169–2172.
[12] Li, J.-M. and Shore, G.C. (1992) Science 256, 1815–1817.
[13] von Heinje, G. (1985) J. Mol. Biol. 184, 99–105.
[14] von Heinje, G. (1986) J. Mol. Biol. 189, 239–242.
[15] Hartmann, E., Rapoport, T.A. and Lodish, H.F. (1989) Proc. Natl. Acad. Sci. USA 86, 5786–5790.
[16] Parks, C.D. and Lamb, R.A. (1991) Cell 64, 777–789.
[17] McGarry, J.D., Brown, N.F., Inthanousay, P.P., Park, D.I., Cook, B.A. and Foster, D.W. (1992) in: New Developments in Fatty Acid Oxidation (Coates, P.M. and Tanaka, K. eds.) pp. 47–61, Wiley-Liss, NY.
[18] Zammit, V.A. (1984) Biochem. J. 218, 379–386.
[19] Zammit, V.A., Corstorphine, C.G. and Gray, S.R. (1984) Biochem. J. 222, 335–342.