A CYTOPLASMIC MOLECULE ACTIVE ON MEMBRANAR Mg^{2+} MOVEMENTS. I – ISOLATION AND PROPERTIES*

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Received 22 July 1971

1. Introduction

The presence, in the cytoplasma of various animal tissues, of a molecule which acts on mitochondrial metabolism has been indicated by Loh et al. in 1968 [1]. This molecule, CMF (cytoplasmic metabolic factor) suppresses totally and specifically the inhibition of the oxidation of glutamate observed in the presence of high concentrations of DNP, an uncoupler of oxidative phosphorylation. We have indicated [2] the role played by this molecule on Mg^{2+} membrane-bound movements. Kun et al. [3] have suggested that this factor could be a small molecule, unstable at acid pH and active at very low concentrations (10^{-9}–10^{-10} M according to estimation made by measuring total organic compounds).

In this paper we present a method of purification of CMF. It is postulated from the first indication on its possible nature that CMF would be a cyclic peptide, with an approximate molecular weight of 2.200, distict by its properties from endogenous ion carrier and certain depsipeptide antibiotics.

Abbreviations:
DNP : 2,4-dinitrophenol;
TPCK trypsin: tosyl-phenylalanine monochloroketone trypsin.

* The present experiments have been performed thanks to a financial support from the CNRS (ERA no. 33) and a grant from the "Ligue Nationale Française contre le Cancer".
** This work will be included in the Doctoral thesis (Doctorat ès-Sciences) of Adrien Binet.
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2. Experimental procedures

Rat liver mitochondria were prepared from Long Evans male rats in 250 mM sucrose + 30 mM tris-HCl (pH 7.4). Activity assays of CMF were performed as indicated by Kun et al. [3]. Mitochondria (6 mg protein) were incubated in 3 ml medium with 0.05 mM DNP and oxygen consumption was followed in a Gilson respirometer. The amino acid composition of the CMF fraction was determined using a Technicon Auto Analyser according to the method of Piez and Morris [4]. The samples were hydrolysed with constant boiling 5.7 N HCl in evacuated sealed tubes at 110° for 18 hr. For quantitative determinations of *N*-terminal amino acids, dansylation was used according to Gros and Labouesse's method [5]. *C*-terminal amino acids were determined as described by Matsuo et al. [6], using the tritium labelling method. Trypsinolysis was performed as follows: 75 μg CMF were incubated in 75 μl 50 mM NH_4HCO_3 with 3 μg TPCK trypsin for 15, 30 and 180 min and samples were taken for *N*-terminal analysis by the "dansyl" procedure.

3. Results

3.1. *Purification of CMF*

5000 g fresh pig liver were homogenized in cold 95° ethanol in a turmix blender and denatured proteins were eliminated by centrifugation. The alcoholic supernatant was evaporated. The residue was dissolved in water and was extracted by chloroform (4 times) in order to eliminate lipids. The aqueous fraction, which retained the total CMF activity was chromato-

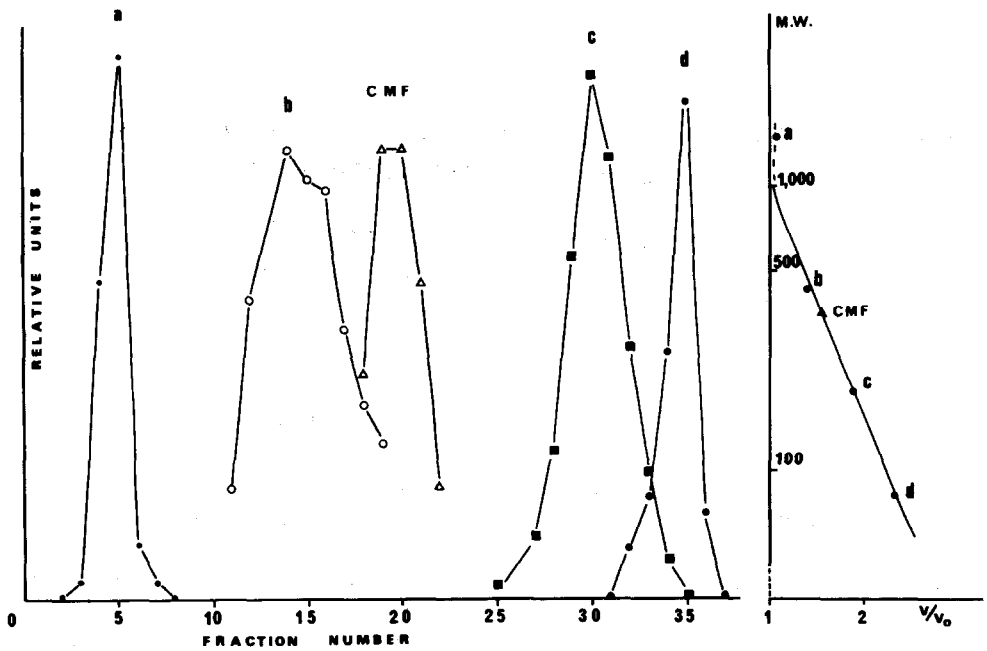

Fig. 1. Elution pattern of CMF and various standards on a Biogel P_2 100–200 mesh column 90 × 2.5 cm. a = albumine; b = synthetic peptide of M.W. 450; c = glucose; d = ions as K^+ of Mg^{2+}. V_0 = 150 ml.

graphed on a Biogel P_2 column (90 × 2.5 cm) and the elution pattern obtained is shown in fig. 1. According to these results CMF would have an approximate molecular weight of 500. CMF was rechromatographed on a cation-exchange CM-Sephadex column, equilibrated with 50 mM Tris-HCl pH 7.0, and was eluted without retention. Finally CMF was purified on two successive DEAE-Sephadex A 25 columns as shown in fig. 2. On the first one, equilibrated with 50 mM tris-HCl pH 7.0, it was eluted by a linear KCl gradient (0 to 300 mM) at an ionic strength approximately 120 mM. On the second one, equilibrated with 20 mM phtalate buffer pH 4.5, it was eluted by the same gradient but this time at an approximate ionic strength of 75 mM. After each chromatography, the active fraction was desalted on a Biogel P_2 column. At the last step the 1.5 mg of the active fraction we obtained were submitted to further analysis.

3.2. *Identification of CMF*

Table 1 shows the amino acid composition of the CMF fraction. Data are presented first in μmoles of each amino acid for 1 mg of the CMF fraction then as relative contents of each amino acid as compared to

Table 1
Amino acid composition of the CMF fraction.[a]

Amino acid	μmoles/1.00 mg	Molar ratio of amino acid relative to lysine	Nearest integer
Lys	0.55	1.00	1
His*	0.25	0.45	1
Arg	0.50	0.90	1
Asp	0.90	1.60	2
Thr*	0.50	0.90	1
Ser*	0.50	0.90	1
Glu	1.20	2.15	2
Pro	0.35	0.65	1
Gly	1.40	2.50	2
Ala	0.90	1.60	2
Val	0.60	1.05	1
Cys	–	–	–
Met*	0.25	0.45	1
Ileu	0.45	0.80	1
Leu	0.70	1.25	1
Tyr*	0.30	0.55	1
Phe	0.35	0.65	1

M.W. 2220; n.b. residues: 20

[a] Each value is the average of four determinations where hydrolysis time at 110° varies from 18 hr to 48 hr except for amino acids with * which are calculated by extrapolation to zero time.

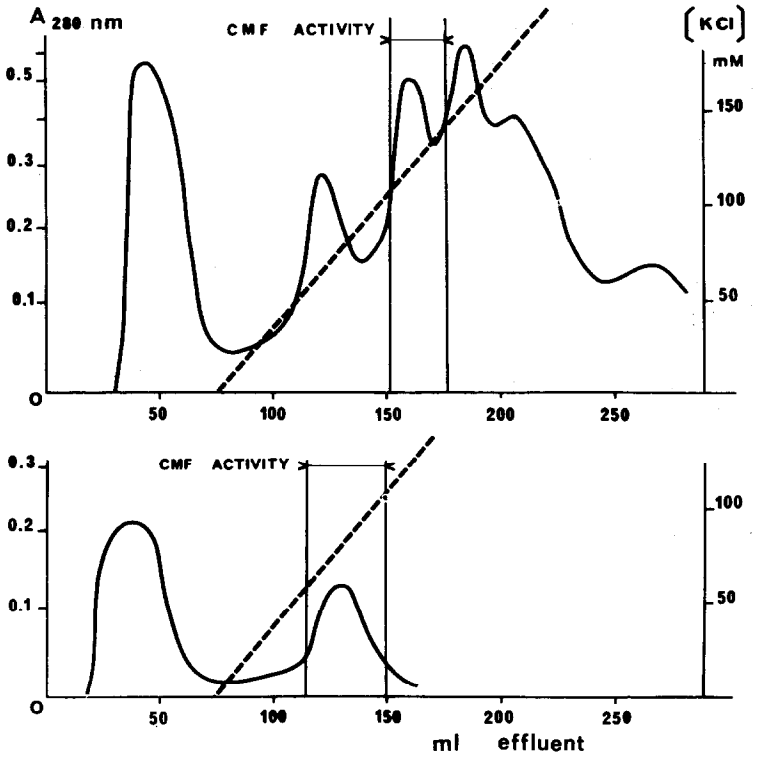

Fig. 2. Elution pattern of CMF on a DEAE-Sephadex A 25 column (12 × 12 cm). Elution was obtained by a linear gradient of KCl (0 to 300 mM). Upper figure shows the pattern obtained with a resin equilibrated with 50 mM tris-HCl buffer pH 7.0 ———; absorbance at 280 nm of effluent and - - - - -; KCl concentration of the gradient. Lower figure shows purification obtained with the same gradient on a column equilibrated with 20 mM phtalate buffer pH 4.5.

lysine taken as unity. Further purification by thin layer chromatography was attempted in order to check the purity of the product. All tentatives were unsuccessful using several solvent systems: CMF did not migrate giving a spot on the start line undetectable by the ninhydrin test but sensitive to the chlorine–tolidine–iodide test. The dansylation technique used for *N*-terminal analysis showed the absence of free α-amino group and the presence of a fluorescence spot of ε-DNS lysine stoechiometricaly to the lysine content of CMF. No *N*-terminal groups were detected even after 3 hr trypsinolysis except ε-DNS-lysine in a quantitative recovery. The results obtained with the technique of selective tritium labelling of *C*-terminal amino acids showed the presence of only one *C*-terminal residue which is glutamic acid or glutamine. According to these results, CMF could be a peptide of 20 AA residues having a molecular weight of 2220.

4. Discussion

The fact that CMF was present in mammalian cells only at extremely low concentration approximately 10^{-8} M, prevented, until now, isolation of a sufficient amount of this material to carry out successful identification analysis. However, the present results give positive information since we have obtained after several chromatographic procedures 1.5 mg of an active compound which seems highly purified. The approximate molecular weight of 500, calculated from the elution pattern of CMF and various markers from the Biogel P_2 column, is in contradiction with the molecular weight of 2220 calculated from the results of the amino acid and *C*-terminal analysis. The probaility for CMF to be a cyclic peptide, as is strongly suggested by the absence of free terminal amino group (dansylation and negative ninhydrin tests) and

its resistance to a prolonged trypsinolysis, may explain the abnormal behaviour on gel filtration. The mechanism of its action on the Mg^{2+} movements at the mitochondrial membrane level may be to prevent the leakage of membrane-bound Mg^{2+} [2].

CMF share several structural characteristics with alamethicin a cyclopeptide antibiotic from *Trichoderma viride*: 18 AA residues no free terminal amino group but a glutamic terminal group [7]. CMF however is hydrosoluble and negatively charged. Its acid character, which may be explained by the high amount of aspartic and glutamic residues in its amino acid composition, its solubility and its activity are in sharp contrast with the properties of depsipeptide or membranar ionophore which induce the active transport of cations in mitochondria and synthetic membranes [7, 8]. CMF may act specifically on a Mg^{2+} shuttle mechanism of the mitochondrial membrane and the finding of a more universal role for this factor [2] may be interesting for further studies on natural as well as artificial membrane systems.

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