11 S. MINAKAMI, T. CREMONA, R. L. RINGLER AND T. P. SINGER, *J. Biol. Chem.*, 238 (1963) 1529.
12 R. L. PHARO AND D. R. SANADI, *Biochim. Biophys. Acta*, 85 (1964) 346.
13 I. HONJO, H. TAKASAN, K. OZAWA, O. KITAMURA, A. SAKAI AND T. OHSAWA, *J. Biochem. Tokyo*, 63 (1968) 811.
14 I. HONJO, K. OZAWA, O. KITAMURA AND T. OHSAWA, *J. Biochem. Tokyo*, 64 (1968) 311.
15 S. W. EDWARDS AND E. G. BALL, *J. Biol. Chem.*, 209 (1954) 619.
16 H. L. TOOKEY AND A. K. BALLS, *J. Biol. Chem.*, 220 (1956) 15.
17 E. PETRUSHKA, J. H. QUASTEL AND P. G. SCHOLEFIELD, *Can. J. Biochem. Physiol.*, 37 (1959) 975.
18 A. P. NYGAARD AND J. B. SUMNER, *J. Biol. Chem.*, 200 (1953) 723.
19 F. D. ZIEGLER, L. WÁZQUEZ, W. B. ELLIOTT, A. TAUB AND C. GANS, *Biochemistry*, 4 (1965) 555.

Received August 12th, 1968

Biochim. Biophys. Acta, 162 (1968) 624–627

BBA 43211

Some characteristics of cytochrome b_5 from outer membranes of rat liver mitochondria

Outer and inner membranes have been prepared from mitochondria from different sources. The outer membrane of liver mitochondria contains the rotenone insensitive NADH dehydrogenase system and bears some similarities to the endoplasmic reticulum[1,2]. In particular, a hemoprotein with the spectral characteristics of microsomal cytochrome b_5 has also been found in this outer membrane. The existence of a mitochondrial cytochrome b_5 was first proposed by RAW *et al.*[3]. In contrast to the microsomal cytochrome, which can be solubilized only through treatment of microsomes with lipolytic or proteolytic enzymes[4–6], the hemoprotein of outer membranes can be extracted by sonication of swollen and shrunken mitochondria[1]. It is not known whether this dissimilarity is due to differences in the structure of the two cytochromes, of the membranes to which they are attached, or both.

In the present communication, some properties of rat liver mitochondrial cytochrome b_5 are compared with those of the microsomal hemoprotein solubilized with lipase. The two cytochromes are similar in molecular weight, as judged by chromatography on Sephadex G-75, but differ in electrophoretic mobility in acrylamide gels and, particularly, in their solubility in ammonium sulfate solutions.

Cytochrome b_5 from rat liver microsomes was prepared as described for calf liver by STRITTMATTER[7], using lipase (Nutritional Biochem., Type 448) in the presence of trypsin inhibitor as the solubilizing agent. The purity was judged by the ratio of the absorbance at 413 and 280 mμ of the oxidized cytochrome. Preparations with ratios of 4 to 5 were used in different experiments. Mitochondria were obtained by differential centrifugation of the liver homogenate in 0.25 M sucrose. The "soluble" subfraction of the mitochondria containing cytochrome b_5 was prepared according to SOTTOCASA *et al.*[1] using the alternative two-layer gradient system. Other preparations of mitochondrial cytochrome b_5 were obtained by following the above procedure only to the step where mitochondria are swollen for 5 min in Tris–phosphate buffer. They were subsequently centrifuged at 35000 $\times$ g for 20 min. Surprisingly, this supernatant contains about the same amount of cytochrome b_5 as the extract obtained after contraction and sonication of the mitochondria. Generally, our yields were only about 30 % of those described[1]. Extracts obtained by these procedures were passed through

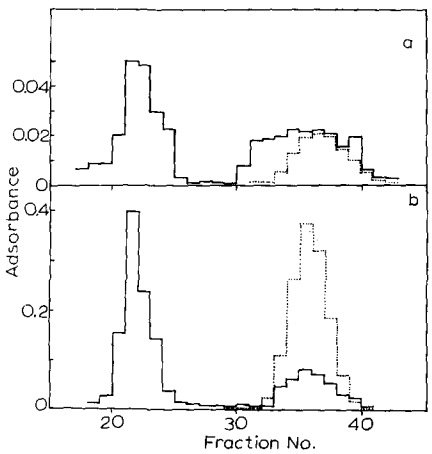
a column of DEAE-cellulose (1 cm × 8 cm) equilibrated with 20 mM phosphate buffer (pH 7.2). Cytochrome b_5 from mitochondria, as well as from microsomes, is retained under these conditions and can be eluted with 0.2 M potassium phosphate–0.1 M KCl buffer (pH 7.0). The eluate was adjusted to pH 7.8–8.0 and fractionated with ammonium sulfate. In the case of mitochondrial cytochrome b_5, the material precipitating between 35 and 65 % saturation was collected, dissolved in 20 mM phosphate buffer, dialyzed over-night and freeze-dried. The content of cytochrome b_5 in different solutions was estimated from the difference spectrum between reduced and oxidized aliquots[8]. Careful comparison of the spectra of mitochondrial and microsomal cytochrome b_5 at room temperature did not reveal any differences. However, the low-temperature difference spectrum of an isolated outer membrane fraction from liver mitochondria has been shown to differ from that of microsomal cytochrome b_5 in the position of the α_1 and α_2 peaks[10].

Approx. 85 % of the partially purified mitochondrial cytochrome b_5 was found to precipitate between 35 and 65 % saturation with ammonium sulfate at pH 7.8–8.0. Under these conditions, all of the microsomal cytochrome b_5 remains in solution. In accordance with STRITTMATTER AND VELICK[4], over 95 % of the microsomal hemoprotein was found to precipitate only at pH 4.2 and 85 % saturation. The crude ammonium sulfate fraction containing the bulk of the mitochondrial cytochrome b_5 was treated with agents known to solubilize the microsomal species. It was incubated with (a) trypsin at 4° for 16 h, (b) pancreatin (Merck) at 4° for 48 h, (c) lipase at 37° for 3 h. After any of these treatments, mitochondrial cytochrome b_5 could still be precipitated with ammonium sulfate at pH 8 and 35–65 % saturation. No significant conversion to a species fractionating like microsomal cytochrome b_5 could be observed.

Liver microsomal cytochrome b_5 has a molecular weight of the order of 10000 (refs. 5, 6, 9). It is not known to which extent this hemoprotein is degraded during the isolation. It was therefore of interest to compare the molecular weights of microsomal and mitochondrial cytochrome b_5, since the latter can be isolated under conditions where the action of hydrolytic enzymes should be negligible. In Fig. 1 the elution patterns of the two cytochromes from a column of Sephadex G-75 are compared. They are both retarded to the same extent and any large differences in the molecular weight can therefore be excluded. In the case of mitochondrial cytochrome b_5, this Sephadex chromatography results in a considerable purification and the ratio of absorbance coefficients at 413 mμ and 280 mμ increases to about one. This step did not alter its behaviour in the presence of ammonium sulfate. The two cytochromes were also compared with respect to their electrophoretic mobility in acrylamide gels. Rat liver microsomal cytochrome b_5 thereby separates into one major component and two very minor bands on either side (Fig. 2b). Under the same conditions, the mitochondrial cytochrome migrates considerably slower, but a minor component, with a mobility similar to the microsomal species, was observed (Fig. 2a).

Calf liver microsomal cytochrome b_5 solubilized with lipase contains 85 amino acids and its sequence has recently been determined[9]. Preparations of cytochrome b_5 isolated from rat and rabbit liver microsomes by treatment with trypsin were found to contain about 78 and 100 amino acids, respectively[5,6]. Moreover, in each instance at least two components were observed upon electrophoresis. In light of this unusual variability in the chain length of a homologous protein, it has been proposed that *in vivo* this cytochrome may be a larger protein and that only "hemopeptides" are

actually isolated. The present study shows that rat liver mitochondrial cytochrome b_5, although extracted under completely different conditions, is similar in size to the microsomal species solubilized with lipase. The difference in electrophoretic mobility and ammonium sulfate fractionation could have two explanations: (1) the two cytochromes differ in primary structure, (2) the microsomal and mitochondrial hemoprotein are identical *in vivo*. However, during preparation of the microsomal cytochrome b_5 some fragment(s) is removed. This difference, too small to be detected by chromatography on Sephadex G-75, markedly changes the electrophoretic mobility and the solubility in ammonium sulfate solutions. Only a detailed comparison of the two cytochrome b_5 species, requiring much larger amounts of the mitochondrial hemoprotein than presently available, would allow one to distinguish between these possibilities.



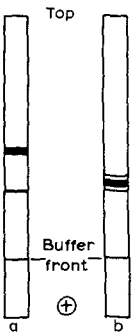


Fig. 1. Fractionation of mitochondrial (a) and microsomal (b) cytochrome b_5 on Sephadex G-75. Samples of cytochrome b_5, previously purified on Sephadex G-75, were dialyzed against H_2O, lyophilized and dissolved in a small volume of buffer containing blue dextran (Type 2000, Pharmacia) to indicate the void volume and a trace of $(NH_4)_2SO_4$. Column size: 1.5 cm × 80 cm. Buffer: 20 mM potassium phosphate (pH 7.2). Fractions of 2.1 ml were collected. Blue dextran and protein were detected at 280 mμ (full line) and cytochrome b_5 at 413 mμ (dotted line). Sulfate ions first appeared in Tube 54 in Run a and in Tube 53 in Run b.

Fig. 2. Disc electrophoresis of cytochrome b_5 from mitochondria (a) and microsomes (b). The origin is at the top and the anode at the bottom. Cytochrome b_5 could be observed as red bands and the buffer front is indicated. Samples were run at pH 7 in Tris–veronal buffer in gels containing 15% acrylamide.

K.A.D. is grateful to the Muscular Dystrophy Association of Canada for a post-doctoral research fellowship.

Institute for Molecular Biology,
Austrian Academy of Sciences,
Vienna (Austria)

K. A. DAVIS
G. KREIL

1 G. L. SOTTOCASA, B. KUYLENSTIERNA, L. ERNSTER AND A. BERGSTRAND, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. X, Academic Press, New York, 1967, p. 448.
2 D. F. PARSONS AND G. R. WILLIAMS, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. X, Academic Press, New York, 1967, p. 443.

3 I. RAW, R. MOLINARI, D. F. DO AMARAL AND H. R. MAHLER, *J. Biol. Chem.*, 233 (1958) 225.
4 P. STRITTMATTER AND S. F. VELICK, *J. Biol. Chem.*, 221 (1956) 253.
5 J. R. SARGENT AND B. P. VADLAMUDI, *Biochem. J.*, 107 (1968) 839.
6 T. KAJIHARA AND B. HAGIHARA, *J. Biochem.*, 63 (1968) 453.
7 P. STRITTMATTER, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. X, Academic Press, New York, 1967, p. 553.
8 M. KLINGENBERG, *Arch. Biochem. Biophys.*, 75 (1958) 376.
9 J. OZOLS AND P. STRITTMATTER, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 264.
10 D. F. PARSONS, G. R. WILLIAMS, W. THOMPSON, D. F. WILSON AND B. CHANGE, in E. QUAGLIARIELLO, S. PAPA, E. C. SLATER AND J. M. TAGER, *Round Table Discussion on Mitochondrial Structure and Compartmentation*, Adriatica Editrice, Bari, 1967, p. 23.

Received July 8th, 1968