ON THE SITE OF SYNTHESIS OF ENZYMES TIGHTLY BOUND TO MITOCHONDRIAL STRUCTURE IN RAT LIVER

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1. Introduction

Isolated mitochondria can incorporate amino acids into their insoluble structural proteins [1–3] by a mechanism apparently similar to that of protein synthesis in the hyaloplasm [4,5]. The soluble mitochondrial proteins, like cytochrome *c* [1,3] and malate dehydrogenase [1] are not labelled, when isolated mitochondria are incubated in the presence of labelled AA *. These mitochondrial proteins are synthesized on the extramitochondrial ribosomes [3,6,7]. On the other hand, the present evidence does not exclude the possibility that enzymes which are tightly bound to mitochondrial structure, like GDH and SDH, are synthesized inside the organelles.

It has been shown that CAC is a potent inhibitor of AA incorporation into mitochondrial protein [8,9]. In contrast, the incorporation of AA into protein by mammalian microsomal systems is normally not affected by this antibiotic [10,11]. Therefore, CAC may be a useful tool for finding out which mitochondrial proteins are synthesized in mammals by the mitochondrial protein synthesizing system and which are synthesized outside of the mitochondria and thereafter transferred into the organelles.

Since it was suggested that increases in GDH and SDH activities in rat liver mitochondria induced by thyroid hormones are due to enhanced *de novo* synthesis of the enzymes [12–14], we have tried to ascertain whether this process is affected by CAC, hoping in this way to obtain a clue concerning the site of synthesis of these enzymes. Our results suggest, in accord with the findings of Kadenbach [3], that these enzymes are synthesized outside the mitochondria.

* *Abbreviations*

GDH	α-glycerophosphate dehydrogenase
SDH	succinate dehydrogenase
CAC	chloramphenicol
T_3	3, 3′, 5-triiodo-L-thyronine
AA	amino acid
cpm	counts per minute

2. Materials and methods

Male Wistar rats (Potsdam-Rehbrücke) about 4 months old and weighing 180 to 200 g were used. CAC (monosuccinate-Na salt, VEB Berlin-Chemie) and T_3 (Calbiochem) were administered intraperitoneally in the doses indicated in the legends of the tables. The rats were killed by a blow on the head and liver mitochondria were isolated at 0° in a 0.25 M sucrose-0.002 M EDTA solution of pH 7.4 by a procedure described in detail elsewhere [15]. The mitochondria, resuspended in 0.25 M sucrose solution of pH 7.4 to a final concentration of about 10 mg protein/ml, were disrupted by a 30-sec treatment at 0° with a high-speed Ultra Turrax homogenizer (Jahnke and Kunkel, Staufen/Br.). They were frozen overnight at −20° and thawed, and the activities of GDH (EC 1.1.2.1) and SDH (EC 1.3.99.1) were estimated spectrophotometrically at 25° and 600 nm according to Ells [16], except that the concentrations of phenazine methosulfate and dichlorphenolindophenol were 2.28×10^{-4} M and 6×10^{-5} M, respectively.

Experiments with labelled AA were performed with U-^{14}C-labelled algal protein hydrolysate (UVVR,

Prague) having a specific activity of 769 mC/g. Radioactivity was measured in TCA-insoluble proteins in a Nuclear Chicago Mark I liquid scintillation spectrometer. The labelled protein samples were placed in amounts up to 100 μl on filter paper discs (Whatman 3MM, 22 mm in diameter), which were treated according to Mans et al. [17] and placed into vials filled with 10 ml scintillation solution. This solution contained, per 1000 ml toluene, 5 g of 2, 5-diphenyloxazole and 0.2 g of 1, 4-bis [2(5-phenyloxazyl)] benzene.

Structural protein of rat liver mitochondria was prepared according to Richardson et al. [18] without the final step of acetone treatment.

CAC was estimated colorimetrically at 414 nm with 1-naphthol according to Masterson [19] in blood plasma and in a 10000 × *g* supernatant of a liver homogenate in water (1 g liver + 1 ml water), prepared by using a teflon-glass homogenizer. To eliminate any turbidity CAC was extracted before estimation with ethylacetate from aliquots of the liver supernatant and the plasma by the method of Glazko et al. [20]. Aliquots of the organic phase were dried in a water bath at 100°C and the residue was dissolved in water.

According to Worth et al. [21] the LD_{50} for CAC is 1800 mg/kg body weight, when given i.p. in a single dose to adult rats. In our experiments all rats were still surviving on the 3rd day after having received 2100 mg CAC/kg i.p. in 4 successive doses within 2 days, but, when the dose was increased to 3500 mg/kg more than 50% of the animals had died within this time. Therefore the former dose was used in the experiments with T_3.

Protein was estimated according to Lowry et al. [22], using egg albumin as a standard.

3. Results and discussion

At first the influence of CAC on the T_3-induced elevation of GDH and SDH activities in rat liver mitochondria was investigated. Twelve rats were divided into 4 groups of 3 animals each, of which the 1st group served as control, while the others were treated with T_3 and CAC in doses indicated in the legend of table 1, in which the results are summarized. It can be seen that CAC has no inhibitory effect on the activity of either enzymes in liver mitochondria and that the percentage increase in the activity of both enzymes induced by T_3 is the same in the presence of CAC as in its absence.

The uptake of AA into mitochondrial protein *in vitro* is inhibited by 50 to 90% by a CAC concentration of 50 μg/ml [9,23,24]. Three and a half hours after the animals had received 53 mg of the antibiotic per 100 g body weight the levels of CAC in liver and blood plasma of 3 rats were 206 ± 49 μg/g and 77 ± 0.0 μg/ml, respectively. Because these rats were given CAC in 4 successive doses of 53 mg/100 g each, one would have expected that the T_3-induced increases in the activities of GDH and SDH in the liver mitochondria would have been inhibited by the antibiotic if these enzymes are synthesized by the mitochondrial protein synthesizing machinery. Such inhibitions did not occur (table 1), even though the CAC concentration in the liver attained levels much higher than those required to prevent the AA uptake into mitochondrial protein *in vitro*.

We then tried to determine to what extent the incorporation of AA into total and structural protein of rat liver mitochondria is inhibited *in vivo* by CAC, when the tissue concentration is raised above 200 μg/g. CAC-treated and control rats were given ^{14}C-labelled algal protein hydrolysate 1 hour prior to death and the cpm/mg protein of the whole mitochondria and of mitochondrial structural proteins were estimated. The results are shown in table 2. As can be seen, no specific inhibition of AA incorporation into either structural or total mitochondrial protein took place and the ratio of structural to total protein cpm remained unaltered. Because in isolated mitochondria labelled AA are incorporated into structural proteins [3] and because their incorporation is very sensitive to CAC [8,9], we expected to see a strong inhibition by CAC of the AA uptake into structural proteins of liver mitochondria *in vivo* and a decrease in the ratio of structural to total protein cpm. Surprisingly, this was not the case even in the presence of a high CAC concentration in the liver.

The most plausible explanation of the results of the experiments presented in table 2 is that mitochondria are capable of synthesizing only a very small fraction of their structural proteins in a process sensitive to CAC and that the bulk of their structural

Table 1

Influence of chloramphenicol on the T_3-induced stimulation of the activities of GDH and SDH in rat liver mitochondria.

(Means ± S.E.) Number of animals	Treatment	Enzyme activities μmoles/min/g mitochondrial protein at 25° GDH	Increase (%)	SDH	Increase (%)
3	–	8.4 ± 0.9		21.2 ± 4.9	
3	+ T_3	17.9 ± 4.9	213	31.9 ± 5.2	151
3	+ CAC	9.1 ± 1.7		29.7 ± 9.8	
3	+ T_3,CAC	20.2 ± 2.7	222	48.5 ± 3.3	163

CAC was given in 4 successive doses of 53 mg/100 g body wt. i.p., dissolved in 0.15 ml H_2O, 47, 40, 25, and 16 h prior to death, while T_3 was given in a single dose of 100 μg/100 g body wt. i.p., dissolved in 0.1 ml of alkaline 0.9% NaCl solution, 44 h prior to death.

Table 2

Influence of chloramphenicol on the amino acid incorporation into protein of rat liver mitochondria and into structural protein of the mitochondria

Number of animals	Treatment	Incorporation of labelled amino acids CPM/mg protein Mitochondrial protein 1	Structural protein 2	Ratio of 2 to 1
2	^{14}C-AA	240	166	0.69
2	^{14}C-AA + CAC	369	249	0.67

^{14}C-AA: rats (200 g body wt.) received 1 hour prior to death i.p. 10 μC algal protein hydrolysate (U-^{14}C-labelled), dissolved in 0.4 ml physiological NaCl-solution.

CAC: 4 h prior to death rats were given 53 mg CAC/100 g body wt. i.p., dissolved in 0.15 ml H_2O.

proteins is synthesized in a CAC-insensitive process, presumably outside the organelles. More specifically, the present results make it appear very unlikely that the mitochondrial enzymes GDH and SDH are synthesized in rat liver inside the mitochondria.

References

[1] D.B.Roodyn, J.W.Suttie and T.S.Work, Biochem. J. 83 (1962) 29.

[2] D.E.S.Truman, Biochem. J. 91 (1964) 59.

[3] B.Kadenbach, Biochim. Biophys. Acta 134 (1966) 430.

[4] E.Wintersberger, in: Regulation of metabolic processes in mitochondria, BBA Library, Vol. 7, eds. J.M.Tager, S.Papa, E.Quagliariello and E.C.Slater (Elsevier Company, Amsterdam-London-New York, 1966) p. 439.

[5] T.W.O'Brien and G.F.Kalf, J. Biol. Chem. 242 (1967) 2172.

[6] D.Haldar, K.B.Freeman and T.S.Work, Biochem. J. 102 (1967) 684.

[7] N.F.Gonzalez-Cadavid and P.N.Campbell, Biochem. J. 105 (1967) 443.

[8] G.E.Kalf, Arch. Biochem. Biophys. 101 (1963) 350.

[9] E.Wintersberger, Biochem. Z. 341 (1965) 409.

[10] J.Mager, Biochim. Biophys. Acta 38 (1960) 150.

[11] R.Rendi, Exptl. Cell. Res. 18 (1957) 187.

[12] O.Z.Sellinger, K.-L.Lee and K.W.Fesler, Biochim. Biophys. Acta 124 (1966) 289.

[13] A.L.Tarentino, D.A.Richert and W.W.Westerfeld, Biochim. Biophys. Acta 124 (1966) 295.

[14] B.Kadenbach, Biochem. Z. 344 (1966) 49.

[15] B.Kleitke, E.-G.Krause and A.Wollenberger, Acta Biol. Med. Germ. 11 (1963) 660.

[16] H.A.Ells, Arch. Biochem. Biophys. 85 (1959) 561.

[17] R.J.Mans and G.D.Novelli, Arch. Biochem. Biophys. 94 (1961) 48.

[18] S.H.Richardson, H.O.Hultin and S.Fleischer, Arch. Biochem. Biophys. 105 (1964) 254.

[19] D.S.Masterson, J. Pharm. Sci. 57 (1968) 305.
[20] A.J.Glazko, L.N.Wolf and W.A.Dill, Arch. Biochem. 23 (1949) 411.
[21] H.M.Worth, C.Kachmann and R.C.Anderson, Toxicol. Appl. Pharmacol. 5 (1963) 719.
[22] O.H.Lowry, N.J.Rosenbrough, A.L.Farr and R.J.Randall, J. Biol. Chem. 193 (1951) 265.
[23] A.M.Kroon, Biochim. Biophys. Acta 108 (1965) 275.
[24] L.W.Wheeldon and A.L.Lehninger, Biochemistry 5 (1966) 3533.