THE MINIMUM EFFECTIVE AMOUNT OF UNCOUPLERS FOR RAT LIVER MITOCHONDRIA

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1. Introduction

In a previous report [1], SF 6847 (3,5-di-tert-butyl-4-hydroxy-benzylidenemalononitrile) was shown to be one of the most effective uncouplers of respiratory-chain phosphorylation ever reported, less than 1.2 nmol/g mitochondrial protein being sufficient for half-maximal uncoupling activity. However, the concentration of SF 6847 causing 50% or 100% uncoupling was found to vary according to the experimental methods and conditions used.

Further studies showed that this variation in the effective concentration of uncoupler was mainly due to variation in the concentration of mitochondria in the reaction mixture and that the effective amount of SF 6847 decreased on increasing the concentration of mitochondrial protein. As pointed out by Kraayenhof [2], the uncoupling effects of some weak uncouplers, such as dinitrophenol, are mainly determined by the uncoupler concentration and are slightly dependent on the protein concentration, while the uncoupling effects of potent uncouplers, such as salicylanilide, benzimidazole and carbonylcyanide derivatives are dependent on the protein concentration.

Since relatively little is known about the quantitative relationship between uncoupling effect and protein concentration (but see [3] and [4]), we determined the effects of some uncouplers, such as SF 6847, 2,4-dinitrophenol, pentachlorophenol and flufenamic acid [5–7] at various concentrations of mitochondrial protein. The effective amounts of uncouplers were determined as the amounts per g of protein causing 50% inhibition of the ATP–$^{32}P_i$ exchange reaction and some hypotheses on the uncoupling mechanism were evaluated on the basis of the results.

2. Materials and methods

Rat liver mitochondria were isolated by the method of Hogeboom [8], as described by Myers and Slater [9]. Protein was determined by the biuret method, as described by Cleland and Slater [10]. The ATP–$^{32}P_i$ exchange reaction was measured as described by Hagihara and Lardy [11] in medium containing 50 mM Tris–HCl buffer (pH 7.4), 50 mM sucrose, 1 mM EDTA, 12 mM $MgCl_2$, 75 mM KCl, 4 mM ATP and 10 mM K_2HPO_4 (with 40 000–60 000 cpm of ^{32}P in a total volume of 1.7 ml for 10 min at 25°C. The rates of this reaction were 20–40 nmol per min per mg protein. AT^{32}P synthesis was also measured by the same method in medium containing 20 mM succinate, 20 mM glucose, 100 μg hexokinase, 1 μg rotenone, 530 μM ADP, 2 mM Tris–HCl buffer (ph 7.4), 15 mM KCl and 15 mM K_2HPO_4 (with approx. 11 000 cpm of ^{32}P).

SF 6847 was kindly supplied by Dr Y. Nishizawa, Sumitomo Chemical Industry, Osaka and was used from a stock solution in ethanol.

3. Results and discussion

A number of energy-linked functions of mitochondria can be used to test the sensitivity of mitochondria to uncouplers. Of these we chose the ATP–$^{32}P_i$ exchange reaction and AT^{32}P synthesis to measure the exact concentration of uncouplers giving 50% inhibition at different mitochondrial concentrations, since using ^{32}P the reactions can readily be measured at low and high concentrations of mitochondria. The effects of dinitrophenol on the two reactions were compared. It was shown that the dose-response curve for the effect of

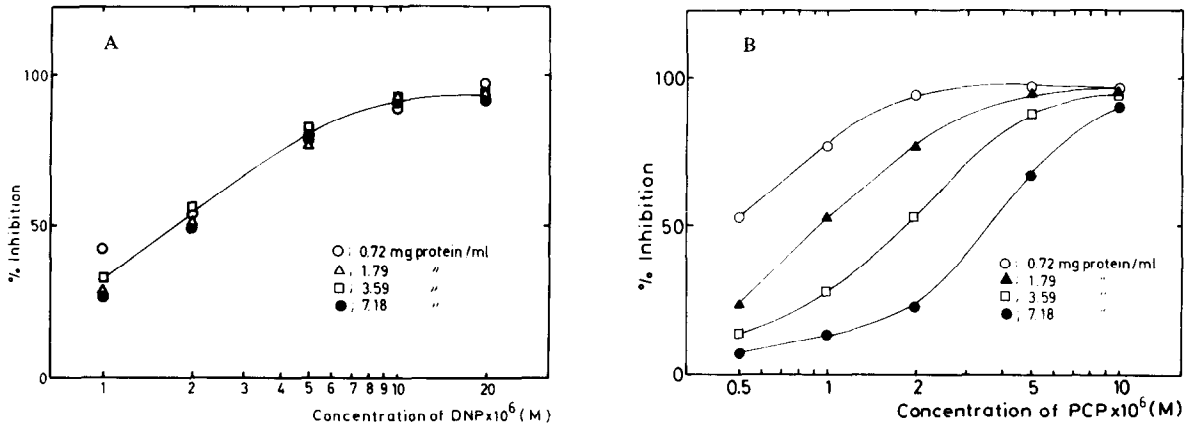

Fig.1. Effect of dinitrophenol (DNP) and pentachlorophenol (PCP) on the ATP–$^{32}P_i$ exchange reaction with various concentrations of rat liver mitochondria. Conditions are as described in the text.

Table 1
Relationship between uncoupling activity and protein concentration

Uncoupler	Concn. of protein (mg/ml)	Concn. of uncoupler causing 50% inhibition (M)	Amount of uncoupler causing 50% inhibition	
			nmol/g protein	mol/mol of cytochrome *a**
Dinitrophenol	0.72	1.63×10^{-6}	2,260	10.8
	1.79	1.98×10^{-6}	1,110	5.29
	3.59	1.76×10^{-6}	490	2.33
	7.18	2.09×10^{-6}	291	1.39
			M.E.A.** 60	0.29
Flufenamic acid	0.46	1.82×10^{-6}	3,960	18.9
	0.72	2.18×10^{-6}	3,030	14.4
	1.15	2.80×10^{-6}	2,430	11.6
	2.30	3.80×10^{-6}	1,650	7.86
	4.60	5.60×10^{-6}	1,220	5.81
			M.E.A. 1,090	5.19
Pentachlorophenol	0.72	4.61×10^{-7}	640	3.05
	1.79	1.08×10^{-6}	600	2.86
	3.59	1.89×10^{-6}	526	2.50
	7.18	3.90×10^{-6}	543	2.59
			M.E.A. 516	2.46
SF 6847	0.75	1.30×10^{-9}	1.73	8.23×10^{-3}
	1.88	2.42×10^{-9}	1.29	6.14×10^{-3}
	3.76	3.60×10^{-9}	0.96	4.57×10^{-3}
			M.E.A. 0.84	4.00×10^{-3}

* Value of 210 nmol/g protein from [13].
** Minimum effective amount of uncoupler.

dinitrophenol on AT^{32}P synthesis was sigmoidal, especially during a short incubation period such as 10 min, whereas the relation for ATP–$^{32}P_i$ exchange reaction was linear. It was also shown that the concentration of dinitrophenol causing 50% inhibition of the exchange reaction was 1/5–1/10 of that for 50% inhibition of ATP–$^{32}P_i$ synthesis. This may be related to the fact that the effective concentration of uncoupler becomes lower when the respiration rate is low [12]. Thus, the ATP–$^{32}P_i$ exchange reaction was measured in the presence of different uncouplers over a wide range of protein concentrations of 0.72 mg to 7.2 mg/ml.

Dinitrophenol was found to be a typical example of an uncoupler, which was not affected by protein concentration, while pentachlorophenol was. The concentration of dinitrophenol required for 50% inhibition was constant over a wide range of protein concentrations, as shown in fig.1A, whereas that of pentachlorophenol was dependent on protein concentration, as shown in fig.1B. Therefore, to obtain a constant level of inhibition, the concentration of pentachlorophenol must be increased on increasing the concentration of protein. The activities of SF 6847 and flufenamic acid were less dependent on protein.

Table 1 shows that amount of dinitrophenol (nmol/g protein) required for 50% inhibition decreased on increasing the concentration of protein, whereas that of pentachlorophenol hardly changed. A plot of the reciprocal of the mitochondrial concentration against the amount of uncoupler causing 50% inhibition per g protein was linear, and a value for the amount of uncoupler per g protein could be obtained from the intercept on the ordinate (fig.2). This value is denoted as the minimum effective amount of uncoupler (M.E.A.). It is noteworthy that dinitrophenol itself is essentially a very potent uncoupler, although the apparent uncoupling activity of dinitrophenol is not so high. This would be related to the low binding affinity of dinitrophenol to mitochondria as reported by Bakker et al. [4]. They found that when uncouplers were added at pH 7.2 to 0.7 mg mitochondrial protein/ml the bound percentage was less than 4% for dinitrophenol and 84% for pentachlorophenol. It is suggested that the amount of dinitrophenol bound to mitochondria increases with increase in the concentration of mitochondrial protein in the reaction system, but that the amount of bound pentachlorophenol hardly changes due to the strong

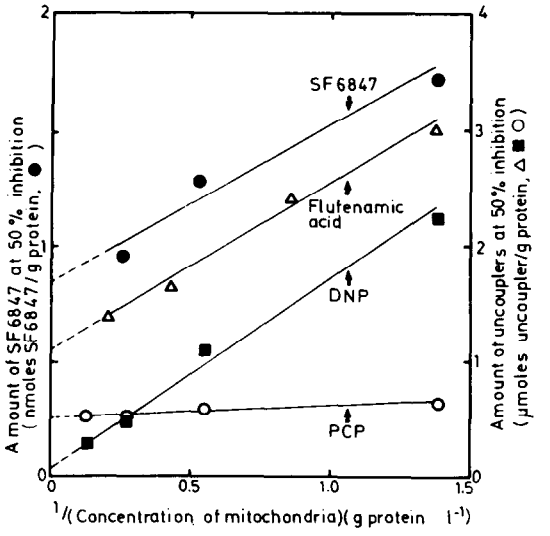

Fig.2. Relationship between reciprocal of the mitochondrial concentration and amount of uncoupler per g protein causing 50% inhibition of the ATP–$^{32}P_i$ exchange reaction. Results are taken from table 1.
DNP, dinitrophenol; PCP, pentachlorophenol.

binding to mitochondria even at low protein concentration.

It should be possible to evaluate the various hypotheses proposed for the mechanism of uncoupling on the basis of the minimum effective amounts of potent uncouplers. According to some hypotheses [14–16], uncouplers act as proton conductors across the energy-conserving membrane. These hypotheses propose that the uncoupler molecule moves across the mitochondrial membrane in the charged and uncharged form, respectively, the net result being the transport of one proton per cycle of movement. Assuming a stoichiometry of $2H^+$ per high-energy bond [14], a rate of 40 nmoles/min·mg protein of ATP–P_i exchange (see Materials and methods) would be 50% uncoupled at 40 neq H^+/min·mg protein. Since the minimum effective amount of SF 6847 is 0.84 nmol/g protein, the uncoupler has to cycle almost 800 times per sec across the membrane, which is difficult to imagine.

A stoichiometric interaction of uncouplers with the energy-conserving site [17–22] also seems unlikely for SF 6847, since rat liver mitochondria contain much larger amounts of components of the respiratory chain and ATP synthesis system than the minimum effective

amount of SF 6847 (cf. also [23]). Wilson [21] proposed that one mole of S-13 (5-chloro-3-tert-butyl-2'-chloro-4'-nitro-salicylanilide) interacts stoichiometrically with one mole of cytochrome *a* at the third site of respiratory chain phosphorylation. However, the M.E.A. of SF 6847 corresponds to 4.0×10^{-3} mole per mole of cytochrome *a* as shown in table 1. Therefore, a new concept is required to explain how one mole of uncoupler can affect several hundred sites of energy conservation in the mitochondria.

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References

[1] Muraoka, S. and Terada, H. (1972) Biochim. Biophys. Acta 275, 271–275.
[2] Kraayenhof, R. (1971) in: Uncoupling of Energy Conservation in Chloroplast and Mitochondria, Mondeel-Offsetdrukkerij, Amsterdam.
[3] Nicholls, P. and Wenner, C.E. (1972) Arch. Biochem. Biophys. 151, 206–215.
[4] Bakker, E. P., van den Heuvel, E. J. and van Dam, K. (1974) Biochim. Biophys. Acta 333, 12–21.
[5] Saeki, K., Muraoka, S. and Yamasaki, H. (1972) Jap. J. Pharmacol. 22, 187–199.
[6] Terada, H. and Muraoka, S. (1972) Molecular Pharmacol. 8, 95–103.
[7] Terada, H., Muraoka, S. and Fujita, T. (1974) J. Med. Chem., 17, 330–334.
[8] Hogeboom, G. H. (1955) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N.O., eds.), Vol. 1, pp. 16–18, Academic Press, New York.
[9] Myers, D. K. and Slater, E. C. (1957) Biochem. J. 67, 558–572.
[10] Cleland, K. W. and Slater, E. C. (1953) Biochem. J. 53, 547–556.
[11] Hagihara, B. and Lardy, H. A. (1960) J. Biol. Chem. 235, 889–894.
[12] Tsou, C. S. and van Dam, K. (1969) Biochim. Biophys. Acta, 172, 174–176.
[13] Muraoka, S., Takahashi, K. and Okada, M. (1972) Biochim. Biophys. Acta 267, 291–299.
[14] Mitchell, P. (1966) in: Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glyn Res. Ltd., Bodmin.
[15] van Dam, K. and Slater, E. C. (1967) Proc. Natl. Acad. Sci. U.S. 58, 2015–2019.
[16] Liberman, E. A., Topaly, V. P., Tsofina, L. M., Jasaitis, A. A. and Skulachev, V. P. (1969) Nature, 222, 1076–1078.
[17] Margolis, S. A., Lenaz, G. and Baum, H. (1967) Arch. Biochem. Biophys. 118, 224–230.
[18] Kurup, C. K. R. and Sanadi, D. R. (1968) Arch. Biochem. Biophys. 126, 722–724.
[19] Sanadi, D. R. (1968) Arch. Biochem. Biophys. 128, 280.
[20] Wilson, D. F. and Azzi, A. (1968) Arch. Biochem. Biophys. 126, 724–726.
[21] Wilson, D. F. (1969) Biochemistry 8, 2475–2481.
[22] Wilson, D. F. and Brooks, E. (1970) Biochemistry, 9, 1090–1094.
[23] Terada, H. and van Dam, K. (1975) Biochim. Biophys. Acta, in press.