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Kinetic Study of the Effect of Uncouplers on Substrate Uptake by Rat-liver Mitochondria

G. Prezioso, F. Palmieri and E. Quagliariello

Department of Biochemistry and
Centro di Studio sui Mitocondri e Metabolismo Energetico del CNR
University of Bari, Bari, Italy

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Abstract

The initial rate of the uptake of Pi, succinate, citrate and ketoglutarate, even at low concentrations, is not affected by uncouplers, when added simultaneously with the substrate at concentrations which uncouple oxidative phosphorylation and decrease substrate uptake at equilibrium. The first-order constant of succinate uptake is significantly increased by uncouplers. If, however, mitochondria are preincubated with uncouplers in order to collapse the ΔpH, the rate of substrate uptake is lowered. At high concentrations, of the same order of those used for the substrates, uncouplers have an inhibitory effect also when added together with the substrate. It is concluded that (a) the uncoupling effect is not related to the interaction of uncouplers with substrate carriers or to their entry on these transport systems, (b) the inhibition of substrate uptake at equilibrium can be accounted for only by the collapse of the ΔpH, (c) the interaction of high uncoupler concentrations with the substrate carriers is a secondary and unspecific effect.

Introduction

It is known that uncouplers of oxidative phosphorylation decrease the equilibrium distribution ratio of anionic substrates between the intra- and the extramitochondrial space [1-3]. This and other related observations have been explained on the basis of the hypothesis that uncouplers inhibit the entry of anionic substrates, acting on each of their transport systems [4, 5]. Although considerable evidence supports the alternative interpretation that the inhibition of substrate accumulation by uncouplers is due to a decrease in ΔpH [6-9], recent kinetic measurements of substrate uptake in the presence of DNP* and

* Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazole; DNP, 2,4-dinitrophenol; PCP, pentachlorophenol; CCP, carbonyl cyanide *m*-Cl-phenylhydrazone; ΔpH, the difference between the intra- and the extramitochondrial pH.

dicoumarol have been interpreted to demonstrate the proposed interaction between uncouplers and carriers [10, 11].

In this paper the effect of uncouplers on the rate of substrate uptake by mitochondria has been reinvestigated using a substituted carbonyl cyanide phenylhydrazone (FCCP) and a substituted benzimidazole (TTFB) besides phenols. The results show that uncoupler concentrations, which affect oxidative phosphorylation and decrease substrate uptake at equilibrium, do not inhibit the initial rate of substrate uptake. Some of these results have been presented at the 8th International Congress of Biochemistry [12].

Material and Methods

Rat-liver mitochondria were isolated as previously described [13]. The third wash and resuspension were carried out in 0.25 M sucrose. The mitochondrial protein was determined by a modified biuret method [14].

The kinetics of substrate uptake were studied by using the "inhibitor stop method" [15-19]. In this procedure, mitochondria are incubated under the conditions specified in the legends for 1 min in "Eppendorf" cups, at the desired temperature (1-10°C). The conditions, such as pH, addition of respiratory inhibitors and oligomycin, were chosen on the basis of previous experiments [7, 20] in order that the anionic substrates are accumulated several-fold above the external concentration. The reaction is initiated by addition of labelled substrate, and terminated by rapid addition of an inhibitor. As inhibitors, 2-butylmalonate and 2-phenylsuccinate were used to stop the uptake of dicarboxylic acids and ketoglutarate, 0.5 mM mersalyl to stop the uptake of Pi [21] and 1,2,3-benzenetricarboxylate to stop the uptake of citrate. The permeated substrate is trapped in the mitochondria, since the inhibitor blocks the substrate carrier, but does not penetrate into the mitochondria.

After spinning down the mitochondria in a microcentrifuge (Misco) and carefully removing the supernatant, the radioactivity was measured in the acid soluble fraction of the pellet. Controls were incubated with the inhibitor present before the labelled substrate was added and subtracted from the experimental samples in order to arrive at the amount of substrate taken up into the matrix space [17-20]. Tritiated water (^3H_2O) was added to all samples to correct for variations in the total water space. The rates of uptake were evaluated from the initial and linear part of substrate uptake, limited to the range of a few seconds [17-19].

1,4-^{14}C-succinic acid, U-^{14}C-L-malic acid, 1-^{14}C-malonic acid (sodium salt), ^{32}P-phosphoric acid, 5-^{14}C-2-ketoglutaric acid (sodium salt), U-^{14}C-sucrose and ^3H-H_2O were obtained from the Radiochemical

Centre (Amersham, England). 1,5-^{14}C-citric acid was bought from the New England Biochemical Corp. Rotenone was obtained from F. P. Penick and Co. (New York). 2-Butylmalonate was kindly supplied by Dr. J. D. MacGivan. 2-Phenylsuccinate was obtained from K and K Laboratories Inc. (Plainview, N.Y.). FCCP was a gift from Dr. P. Heytler (du Pont) and TTFB was provided by Dr. A. Kröger. Other reagents were of the highest purity obtainable commercially.

Results

Figure 1A illustrates the time course of succinate uptake by liver mitochondria in the absence and in the presence of 3 μM FCCP at 10°. The uncoupler was added together with 0.5 mM ^{14}C-succinate at time 0. During the first seconds of incubation the uptake of succinate in the presence of FCCP is virtually the same than in its absence. Only after 6 sec an inhibition of succinate uptake supervenes, which becomes more marked as the equilibrium is reached. The time at which succinate uptake inhibition by FCCP appears, depends on the temperature and on the uncoupler concentration. For example in an experiment carried out under the same conditions as in Fig. 1 but at 5°, the inhibition of

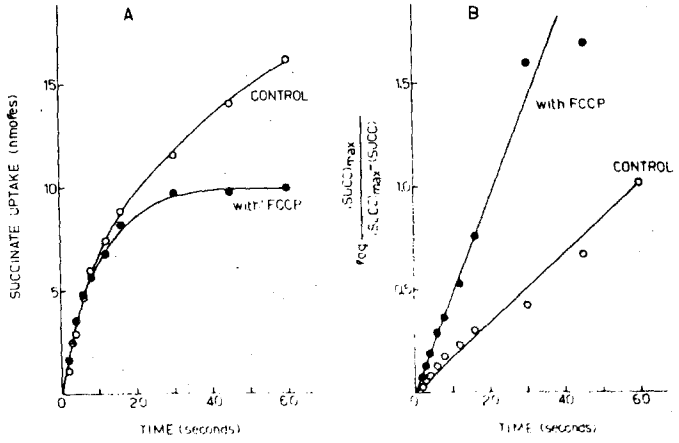

Figure 1. A. Time course of succinate uptake by rat-liver mitochondria in the absence and in the presence of FCCP. The reaction mixture contained 80 mM KCl, 50 mM Tris-HCl, 1 mM EDTA, 1 μg rotenone, 1 μg antimycin, 10 μg oligomycin, 2.14 mg protein and (added at time 0) 0.5 mM ^{14}C-succinate, pH 6.3. Where indicated, 3 μM FCCP was also added together with the labelled substrate. Temperature: 10° C. Other conditions as indicated in Methods. B. Logarithmic plot of succinate uptake by rat-liver mitochondria, demonstrating first order type kinetics:

$$2.3 \log \frac{\text{Succ}_{max}}{\text{Succ}_{max} - \text{Succ}} = K \cdot t$$

substrate uptake appeared not before 12 sec. Similarly the inhibition of succinate uptake supervened later with 0.3 μM than with 3 μM FCCP. The succinate uptake appears to follow a first order type of reaction (Fig. 1B and ref. 17). As shown in Fig. 1B, the presence of FCCP increases the apparent first order constant, K, from 2.3 to 6.5 min^{-1}. It can be derived that the apparent first order constant is given by the following equation: $K = (V_i/V_o)K_1 + K_2$, where K_1 and K_2 are the apparent kinetic constants of the entry and the efflux respectively, and V_i and V_o are the intra- and the extramitochondrial volume. Since $V_i \ll V_o$, K is approximately equal to K_2. Thus, in our experimental conditions, the uncoupler stimulates the rate of succinate efflux, without affecting the rate of substrate entry.

In Fig. 2 the effect of FCCP on the rate of succinate uptake is analysed in the presence of various substrate concentrations as a Lineweaver-Burk plot. The K_m and V_{max} values for the rate of succinate

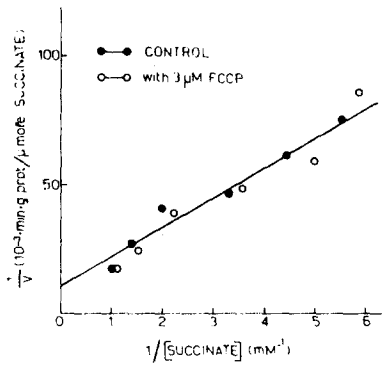

Figure 2. Effect of FCCP on the rate of succinate uptake at various substrate concentrations. Experimental conditions as in Fig. 1 except that ^{14}C-succinate was used at the concentrations indicated. Mitochondrial protein was 1.9 mg.

uptake are 1.1 mM and 92 μmoles succinate/min g protein respectively at 10°C in agreement with previous reports [17, 19, 22]. The uncoupler has no effect on the rate of succinate uptake even with substrate concentrations much lower than the K_m.

The dependence on uncoupler concentration of the rate of malonate uptake is shown in Fig. 3. The rate of malonate uptake is not affected by 0.2-3 μM FCCP, when added simultaneously with the substrate. Under these conditions, even 30 μM FCCP had no effect (see ref. 18). If, however, the uncoupler was present during the preincubation time in order to collapse the ΔpH [9, 22-25], the rate of malonate uptake, calculated from measurements at 3 sec, is markedly inhibited.

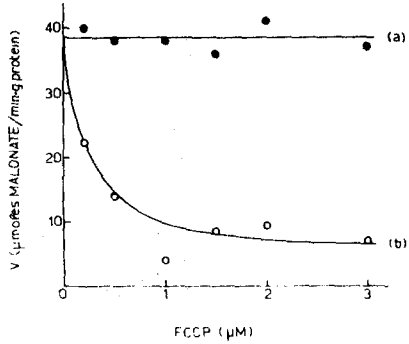

Figure 3. Dependence on FCCP concentration of the rate of malonate uptake. The reaction mixture contained 100 mM KCl, 50 mM Tris-HCl, 1 mM EGTA, 1 μg rotenone, 10 μg oligomycin, 2.3 mg protein and (added at time 0) 0.5 mM ^{14}C-malonate. FCCP at the concentrations indicated was added together with the substrate in (a) and was present during 1.5 min preincubation in (b). Final pH: 6.3. Temperature: 9° C. Other conditions as indicated in Methods.

The effect of increasing concentrations of FCCP was also tested on the rate of Pi uptake on its own carrier. In this case, 5 mM butylmalonate was present in order to inhibit the uptake of Pi on the dicarboxylate carrier. Figure 4 shows that the rate of Pi uptake is decreased when mitochondria are preincubated with FCCP, while it is not affected if the uncoupler is added simultaneously with Pi. In the former case, with both the Pi and the dicarboxylate carrier, the uncoupler was effective in the concentration range used to uncouple oxidative phosphorylation. Fifty per cent inhibition of malonate and Pi uptake was obtained with about 0.25 μM FCCP.

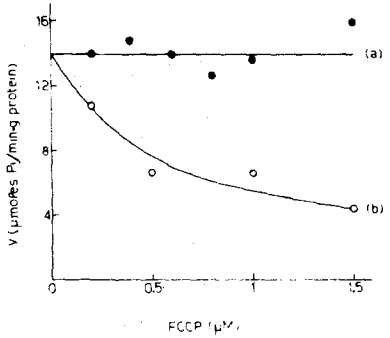

Figure 4. Dependence on FCCP concentration of the rate of Pi uptake. The reaction mixture contained 100 mM KCl, 50 mM Tris-HCl, 1 mM EGTA, 1 μg rotenone, 10 μg oligomycin, 5 mM butylmalonate, 2.1 mg protein and (added at time 0) 0.2 mM ^{32}P-orthophosphate. FCCP at the concentrations indicated was added together with the labelled substrate in (a) and was present during 2.5 min preincubation in (b). Temperature: 1° C. Final pH: 6.7. Other conditions as indicated in Methods.

In Table I the results obtained with different uncouplers on the rate of uptake of substrates linked to the dicarboxylate, tricarboxylate and ketoglutarate transport systems are summarized. It can be seen that FCCP and TTFB, representatives of two classes of uncouplers, do not affect the rate of uptake of all the substrates tested when used at optimal concentrations for full uncoupling. Also the substituted phenols, at the concentrations indicated in Table I, which fall in the range effective to uncouple oxidative phosphorylation and to inhibit substrate uptake at equilibrium, have generally no significant effect on the dicarboxylate, tricarboxylate and ketoglutarate carriers.

TABLE I. Effect of various uncouplers on the rate of malate, citrate and ketoglutarate uptake

Additions	Rate of uptake (μmoles/min . g protein)		
	Malate	Citrate	Ketoglutarate
–	30.5 (27.8-35.0)	24.4 (23.0-26.4)	32.2 (31.2-33.5)
FCCP 1 μM	30.1 (26.0-34.5)	25.6 (21.7-29.6)	34.7 (32.0-37.4)
TTFB 3 μM	30.4 (27.6-35.2)	23.9 (23.6-24.2)	32.9 (29.8-37.2)
Dicoumarol 15 μM	31.5 (29.1-32.9)	27.8 (23.9-29.0)	24.0 (21.1-29.5)
PCP 6.6 μM	–	25.8 (22.4-28.5)	36.6 (29.4-44.2)
DNP 50 μM[a]	–	20.8 (18.2-23.2)	22.1 (16.4-31.6)
Citrate 5 mM	–	6.0 (5.3- 8.6)	–
Malate 5 mM	1.9 (0-4.2)	–	1.1 (0-2.8)

[a] 100 μM DNP was used with citrate.

The reaction mixture contained 100 mM KCl, 50 mM Tris-HCl, 1 mM EGTA, 1 μg rotenone, 10 μg oligomycin, pH 6.4. In the experiments with citrate and ketoglutarate, the mitochondria were first preincubated 1 min in the presence of 0.5 and 0.2 mM malate respectively and then cooled at 8° C. After 1 min incubation at 8° C, 0.5 mM ^{14}C-malate, 0.5 mM ^{14}C-citrate or 0.5 mM ^{14}C-ketoglutarate were added. Where indicated, the uncouplers listed in the table or unlabelled malate or citrate were added simultaneously with the labelled substrates. Mitochondrial protein was 2.4 mg in the experiments with malate and ketoglutarate and 2.1 mg in the experiment with citrate. Each value is mean of three measurements, with the ranges in brackets. –, not determined.

At higher concentrations, but still in the uncoupling range, the phenols inhibit the rate of substrate uptake, in agreement with previous observations [10, 11]. This dual behaviour of the substituted phenols is illustrated in Fig. 5 in the case of DNP. The initial rate of succinate uptake is not affected by 20 μM DNP (Fig. 5A), although the amount of substrate taken up at equilibrium is decreased. In this experiment, the uptake of succinate in the presence of 20 μM DNP is virtually the same than in its absence during the first 10 sec. The inhibition of substrate uptake appears earlier with increasing DNP concentrations and, in the presence of 200 μM DNP (Fig. 5B), is already present at 2 sec.

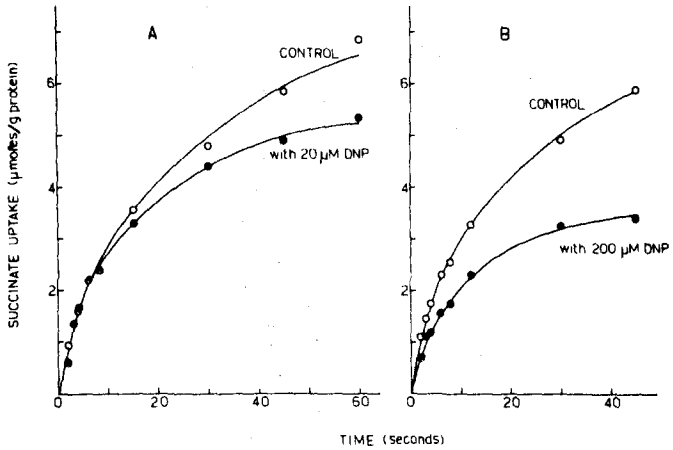

Figure 5. Time course of succinate uptake in the absence and in the presence of 20 μM (A) or 200 μM (B) DNP. Experimental conditions as in Fig. 1. Where indicated, DNP was also added together with the labelled substrate. Mitochondrial protein was 1.68 mg in A and 2.66 mg in B.

Table II compares the effectiveness on the rate of succinate uptake of various uncouplers, at a concentration as high as 0.1 mM. FCCP and TTFB, at this concentration which is about two orders of magnitude higher than that required for full uncoupling, also inhibit the rate of substrate uptake similarly to the substituted phenols.

TABLE II. Effect of various uncouplers at 0.1 mM concentration on the rate of succinate uptake.

Additions (0.1 mM)	Rate of succinate uptake (μmoles/min . g protein)
–	16.2
FCCP	6.4
TTFB	8.4
PCP	3.0
DNP	4.5
Dicoumarol	6.1

The reaction mixture contained 80 mM KCl, 50 mM Tris-HCl, 1 mM EGTA, 1 μg rotenone, 1 μg antimycin, 10 μg oligomycin, 2.6 mg protein and (added at time 0) 0.5 mM ^{14}C-succinate. Where indicated, the uncouplers listed in the table were added together with the labelled substrate. Final pH: 6.3. Temperature: 10° C.

Discussion

The results reported in this paper show that the initial rate of uptake of several substrates linked to the Pi, dicarboxylate, tricarboxylate and ketoglutarate carriers is not affected by powerful uncouplers, like FCCP and TTFB, at concentrations which give complete uncoupling. DNP and CCP were earlier found not to inhibit the rate of ADP uptake and to stimulate ATP uptake [15]. These findings give direct evidence that uncouplers do not compete with the entry of anionic substrates in the mitochondria at the level of their specific transport systems and therefore do not support the hypothesis that uncouplers are transported through the mitochondrial membrane by the substrate carriers, as postulated in a suggested mechanism of uncoupling [4, 26].

Experiments like that illustrated in Fig. 1A clearly show that the decrease of substrate uptake at equilibrium is not due to an inhibition of the rate of substrate entry. On the other hand, several experimental evidence reported in this paper support the interpretation that the effect on the substrate accumulation by uncouplers can be explained only by the collapse of the ΔpH, on which the distribution of anionic substrates across the mitochondrial membrane depends [6, 7, 20]. Thus, preincubation of mitochondria with FCCP, which causes a decrease of ΔpH, inhibits the flux of substrate uptake long before than the simultaneous addition of FCCP with the substrate. Furthermore the inhibition of the flux of substrate uptake by FCCP appears earlier on increasing the uncoupler concentration or the temperature, conditions which increase the rate of the uncoupler-induced H^+ uptake. It is visualized that, due to the H^+-linked translocation of the anionic substrates [20], the net flux of substrate uptake is a function of the difference between the intra- and extramitochondrial concentration of both protons and substrates. Thus, in the presence of a highly buffered medium, uncouplers inhibit the net flux of substrate uptake by inducing H^+ uptake [7, 22, 27] and therefore increasing the rate of substrate exit at the same substrate internal concentration (Fig. 1).

At concentrations of the same order of those used for the substrates, uncouplers inhibit the rate of substrate uptake, in agreement with the observations obtained by Kraayenhof *et al.* [10] with DNP and by Papa *et al.* [11] with DNP and dicoumarol. In the case of FCCP and TTFB, it should be noticed that an appreciable inhibition of succinate uptake is found only at concentrations about two orders of magnitude higher than those fully uncoupling. This result, together with the lack of inhibition by the substituted phenols at low uncoupling concentrations, clearly shows that the interaction of uncouplers with the substrate carriers is a secondary effect, not related to the uncoupling action and probably due to an unspecific binding of the nucleophilic compounds to proteins. The lipid-solubility of the uncouplers would favour their binding to the translocators within the membrane.

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