Biochimica et Biophysica Acta 851 (1986) 93–98
Elsevier

BBA 42088

Quantitative structure-activity relationship of carbonylcyanide phenylhydrazones as uncouplers of mitochondrial oxidative phosphorylation

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(Received January 13th, 1986)

Key words: Uncoupler; Oxidative phosphorylation; Carbonylcyanide phenylhydrazone; (Rat liver mitochondria)

The dependence of the uncoupling activity in the series of 16 carbonylcyanide phenylhydrazones on their physico-chemical properties (partition coefficient, dissociation constant and rate constant for reaction with thiols) is investigated using two physiologically based models, one for protopophoric mechanism of uncoupling and the other assuming the covalent modification of a membrane constituent to be the key step in this process. As indicated by uptake experiments, at the given conditions a lipophilic-hydrophilic equilibrium is attained without any loss of the compounds via chemical reactions. Using this fact to reduce the number of adjustable parameters, a better fit to the data on stimulation of respiration is obtained with the former (protonophoric) model.

Introduction

Reagents dissipating the link between ATP formation and respiration in mitochondria belong to different chemical classes [1–5], molecular mechanism of this action being obviously structure dependent. In this connection three ideas are most frequently stressed. In the case of lipophilic weak acids uncoupling activity is assigned to their involvement in artificial membrane transport of protons leading to decay of the pH gradient as proton-motive force for ATP synthesis [6,7]. Hydrophobic ions also act as uncouplers, presumably because they dissipate the membrane potential necessary for coupling of oxidation and phosphorylation [8]. For reactive electrophilic compounds a covalent binding to functional membrane proteins, preferably to ATPase, is considered as the key step in uncoupling of oxidative phosphorylation [1,9–11].

CCP is classified by a majority of authors [3,4,7,12] as a protonophoric uncoupler. The question of protein modification mechanism of its uncoupling effect, however, remains still open as: (1) certain SH-reagents uncouple oxidative and photosynthetic phosphorylation [11,13] and CCP also belongs to this class of compounds [14–16]; (2) uncoupling effect of CCP can be reversed with thiols [16–18]; (3) CCP modifies membrane proteins [9,10,19,20]. This study represents an attempt to solve this problem using quantitative structure activity relationships methodology [21]. Contrary to previous works dealing with empirical quantitative structure activity relationships of other uncouplers (e.g., dicoumarols [22,23], α-acyl-α-cyanocarbonylphenylhydrazones [24], salicylanilides, 2-trifluoromethylbenzimidazoles and phenols [25]), physiologically based models are used in this case.

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Abbreviations: CCP, carbonylcyanide phenylhydrazones; K_a, dissociation constant; P, apparent partition coefficient; k^*, rate constant for reaction of CCP with protein; k, rate constant for reaction with mercaptoacetic acid; n, number of points; r, correlation coefficients; s, standard deviation; F, F-test.

Materials and Methods

CCP were synthesized by diazotization of the corresponding anilines and subsequent azo coupling reaction with malonodinitrile [26,27].

Aqueous dissociation constants K_a of CCP were determined spectrophotometrically (Specord UV VIS) as reported previously [28].

Reactivity of CCP towards mercaptoacetic acid simulating SH groups of membrane constituents was characterised by rate constants k. The conditions of kinetic measurements and determination of rate constants are described elsewhere [28].

Apparent partition coefficients of CCP in the system 1-octanol/buffer (McIlvaine, pH 7.2, Ref. 29) were determined via spectrophotometric assay of CCP concentration in the aqueous phase at lipophilic-hydrophilic equilibrium [30].

Uptake of CCP by rat liver mitochondria (2.15 mg of protein per ml) in Tris buffer of pH 7.2 [29] after 10 min incubation and separation of mitochondria by centrifugation (20 min, $10000 \times g$) was assessed spectrophotometrically [30].

Uncoupling activity of CCP in rat liver mitochondria (State 4, 7.5 mM glutamate and malate as substrates, 1 mg of proteins per ml) was characterised as the concentration eliciting 50% stimulation of control respiration in medium containing 200 mM sucrose, 10 mM KCl, 5 mM $MgSO_4$, 0.2 mM EDTA, 10 mM potassium phosphate buffer of pH 7.4. Oxygen uptake was recorded polarographically with Clark electrode. Mitochondria were isolated according to Ref. 31.

Non-linear regression analysis was performed with logarithmised data by the method described in Ref. 32.

Results and Discussion

In order to use the relation between physicochemical properties and uncoupling activities in the CCP series for identification of processes involved in this effect, two mathematical models have been constructed: model I, starting from protonophoric action and model II, assuming covalent modification of a membrane constituent. In both cases information about CCP distribution in the suspension of mitochondria is of primary importance.

Uptake of CCP by mitochondria

The dependence of CCP concentration (c_A, Table I) remaining in the medium after 10 min incubation and separation of mitochondria by centrifugation (20 min, $10000 \times g$) on the apparent partition coefficient P (Table I) is given in Fig. 1. The relation can be described adequately by Eqn. 1 resulting from the definition of the partition coefficient $P = n_L V_A / n_A V_L$ (n is the molar amount of CCP in the lipid (L) and aqueous (A) phase, V_L and V_A are the respective volumes) and from the Collander equation [33] relating the partition coefficients determined in systems 1 and 2 as $P_1 = b\, P_2^A$ [34–36]:

$$\frac{c_A}{c_0} = \frac{\text{constant}}{\frac{bV_L}{V_A} P^A + 1} \qquad (1)$$

where c_0 is the initial concentration. Optimization of the constants by non-linear regression analysis provides the following values: constant = 0.662, $bV_L/V_A = 1.063 \cdot 10^{-3}$, $A = 1.329$ [30]. As the values of the statistical parameters ($n = 11$, $r = 0.986$, $s = 0.073$, $F = 33.5$) indicate, the fit is satisfactory. Therefrom it can be concluded that at the given conditions all derivatives investigated attained lipophilic-hydrophilic equilibrium in the mitochondrial suspension without any detectable loss of the compounds via chemical reactions. Furthermore, the concentration of CCP in lipid

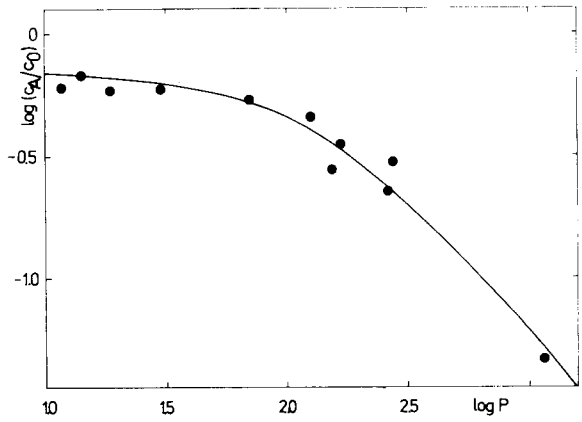

Fig. 1. Ratio of CCP concentration remaining in the medium after 10 min incubation with mitochondrial (c_A) and initial concentration of CCP (c_0) vs. the apparent partition coefficient P. Experimental points in Table I, line corresponds to Eqn. 1 with the optimalized values of the constants.

phases of mitochondria (c_L) can be estimated from Eqn. 2 derived analogically as Eqn. 1

$$\frac{c_L}{c_0} = \frac{\text{constant} \cdot P^A}{\frac{bV_L}{V_A} P^A + 1} \quad (2)$$

To model stimulation of respiration the values of bV_L/V_A and A obtained from uptake experiments can be used with recalculation of the former constant taking into account that V_L depends on concentration of mitochondrial suspension, the changes in V_A being negligible.

Model I

This model assumes protonophoric mechanism of uncoupling whereby the basic idea is that a defined stimulation of respiration elicited by individual derivatives is caused by a constant steady-state transmembrane flux of protons [7]. For the steady-state current I produced by CCP upon application of a constant potential, Eqn. 3 is valid. This expression results from the conventional carrier model [12] sketched in Fig. 2, with acceptable simplifying assumptions [7].

$$I = -\frac{Fr(k'_A - k''_A)k_R[H^+]k_{HA}}{(k'_A + k''_A)(k_D + 2k_{HA}) + 2k_R[H^+]k_{HA}} \quad (3)$$

where F is the Faraday constant, r is molar amount of ionized CCP in the membrane, square brackets denote activity and the other symbols are

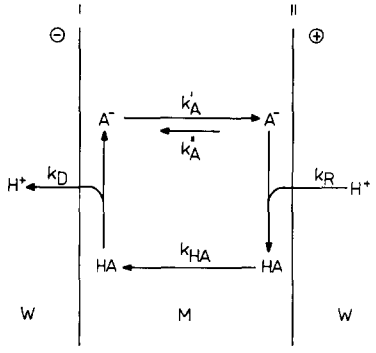

Fig. 2. Schemical outline of the membrane proton transport mediated by CCP [7]. The circled positive and negative signs indicate a potential difference across membrane. A and HA stand for the ionised and unionised form of CCP. The rate constants k refer to the processes: recombination of H^+ with A (R), dissociation of HA (D), diffusion of the respective molecules (HA, A). The back steps omitted for clarity. M, membrane, W, aqueous phases.

as in Fig. 2. The quantity r can be calculated from the total amount of CCP in membrane (n) described by Eqn. 2 using the dissociation constant $K_a = k_D/k_R$ as:

$$r = \frac{nK_aBP^AV_L}{V_A(K_a + [H^+])\left[\frac{bV_L}{V_A}P^A + 1\right]} \quad (4)$$

Combination of Eqns. 3 and 4 for I = constant and $k_D \gg k_{HA}$ (experimentally verified in Ref. 7) yields final equation for correlation of uncoupling activity with physicochemical properties:

$$\frac{1}{c} = \frac{\text{constant } P^AK_a}{(BP^A + 1)(CK_a + 1)(DK_a + 1)} \quad (5)$$

where c is the concentration of CCP-stimulating mitochondrial respiration to a certain degree (50% of the original rate in our case), $B = b\ V_L/V_A$, $C = 1/[H^+]$ and $D = (k'_A + k''_A)/2\ [H^+]k_{HA}$. Providing that the last term can be considered as a constant in the series tested, Eqn. 5 should describe the data. The values of adjustable coefficients are to be determined by non-linear regression analysis of the uptake data (A and B, the latter being recalculated for the concentration of mitochondrial suspension in respiration experiments) and of the uncoupling activities (C, D and constant).

Model II

For stimulation of respiration caused by covalent modification of a membrane protein (R) two possible types of interaction are considered: reaction 6, i.e., direct formation of the covalent bond and reaction 7 representing noncovalent interaction followed by covalent bond formation:

$$CCP + R \xrightarrow{k^*} CCP - R \quad (6)$$

$$CCP + R \overset{K}{\rightleftarrows} CCP - R^+ \xrightarrow{k^*} CCP - R \quad (7)$$

where K is the equilibrium constant of noncovalent complex ($CCP - R^+$) formation, k^* is the rate constant for formation of covalent bond. If, for simplicity's sake, it is assumed that (a) the loss of CCP caused by its interaction with the proteins is negligible; (b) non-covalent interactions are practically instantaneous [37]; (c) formation of

covalent bonds is irreversible; (d) stimulation of respiration is the instantaneous consequence of the protein modification and is linearly proportional to the concentration of the covalent complex CCP-R; (e) the concentration of CCP in the membrane is given by Eqn. 2; (f) the rate constant k^* is related to the same parameter for reaction with mercaptoacetic acid according to the Hammett equation $k^* = Dk^E$ [38], then the expressions relating the isoeffective concentration c to the relevant physico-chemical properties for reactions 6 and 7 read [39]:

$$\frac{1}{c} = \frac{\text{constant t } P^A k^E}{BP^A + 1} \tag{8}$$

$$\frac{1}{c} = \frac{\text{constant } KP^A\left(1 - e^{-Dk^E t} - C\right)}{BP^A + 1} \tag{9}$$

Providing that the value of K is independent of structure in the series tested, uncoupling activity should be determined by the partition coefficient P, the rate constant k and time t in this case. In principle, this treatment is also applicable to the case where the decisive step is that of a non-covalent binding of uncouplers to membrane proteins (e.g., H-bond or charge-transfer complex formation), as the ability to form such bonds is probably linearly dependent on the rate constant k. The adjustable coefficients A and E and the constant are optimalized analogically as in model I.

Comparison of the models with the experimental data

As the dependence of uncoupling activity on the partition coefficient is deduced from the uptake studies only the coefficients related to K_a or k are to be determined. For this purpose the isoeffective concentrations (see Fig. 3 for illustration of determination) were processed by substracting, in bilogarithmic plot, the function of the partition coefficient $f(P)$. This new variable is plotted against the respective physico-chemical properties (Table I) in Fig. 4, together with best fit lines. As the dependence on log k is clearly nonlinear, Eqn. 9 resulting from the two-step modification of the protein (reaction 7) was chosen to describe the experimental data. The optimalised values of coefficients are: $A = 1.329$, $B = 4.944 \cdot 10^{-4}$, both from the uptake experiments, $C = 4.535 \cdot 10^5$ M and 0.500, $D = 1.609 \cdot 10^4$ M and 0.029, constant $= 3.311 \cdot 10^{10}$ and 0.662 (for model I (Eqn. 5) and model II (Eqn. 9), resp.), $E = 0.320$. The statistical parameters ($n = 16$, $r = 0.962$ and 0.721, $s = 0.113$ and 0.259, $F = 18.62$ and 1.626) indicate that Model I gives obviously better fit.

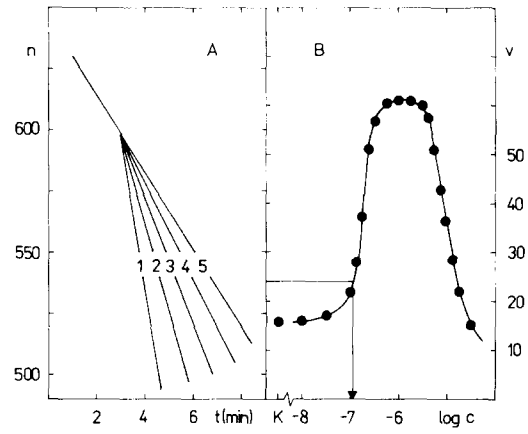

Fig. 3. (A) Kinetics of respiration under influence of the derivative 7 able I), (B) graphical determination of concentration c causing 50% stimulation of control respiration in mitochondria (1 mg protein per ml). Concentration of CCP (in μM): 1 (1), 10 (2), 14 (3), 0.1 (4), 0.01 (5).

Under our experimental conditions the requirements for maximal uncoupling potency can be formulated as: lipophilicity (1-octanol/buffer partition coefficient P) above $2 \cdot 10^3$ and acidobasicity (pK_a in dilute aqueous solution) between 4.5

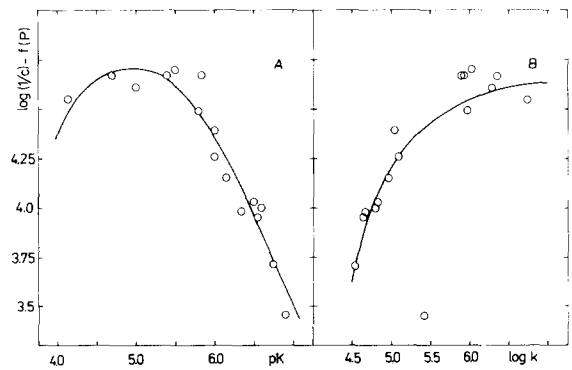

Fig. 4. Dependence of the corresponding parts of the Eqns. 5 and 9 on the dissociation constant K_a (A) and the rate constant k (B). The experimental points in Table I, c as in Fig. 3, $f(P)$ determined from the uptake experiments (Fig. 1). Lines correspond to Eqns. 5 (A) and 9 (B) with the optimalized values of the constants.

and 5.5. The shifts in the parameters elicited by changes in experimental conditions can be estimated on the basis of the following considerations. The lipophilicity limit reads approximately as $P = 1/B = V_A/bV_L$ and is, therefore, inversely proportional to the concentration of mitochondrial suspension. The boundaries for optimal acidobasicity are given as $K_a = 1/C$ and $= 1/D$ and ought to be proportional to concentration of protons in the medium. Structural unspecificity of model I warrants extrapolation of the conclusions made for CCP derivatives also to other protonophoric uncouplers.

Superiority of model I is supported also by the linear time-course of stimulated respiration within 5–8 min of application (Fig. 3), what is consistent with Eqn. 5. According to model II (Eqn. 9) uncoupling activity ought to be either time-dependent or completely independent of reactivity. On the other hand, it should be noted that there is only one significant outlier in model II (Fig. 4B, derivative 14 in Table I). Its exclusion from the correlation results in substantial improvement of the fit: $n = 15$, $r = 0.973$, $s = 0.099$, $F = 23.88$. The good description of the experimental data by both the models can be explained by: (1) a rather high collinearity between acidobasicity and reactivity of the series tested in spite of its significant decrease due to the use of 2-substituted derivatives (Fig. 5) as proposed by one of us (P.S.); (2) a quite similar graphical form of the dependences in Fig. 4.

It can be concluded that protonophoric model I describes the dependence of uncoupling activity on physico-chemical properties in CCP series better than model II which assumes protein modification mechanism of this effect. Its superiority is, in addition to a better description of the time-course of the stimulated respiration, based on a large deviation of only one derivative tested (num-

TABLE I

STRUCTURE, PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERISTICS OF CARBONYL CYANIDE R-PHENYLHYDRAZONES

P, the apparent partition coefficient, k, the rate constant (in $M^{-1} \cdot s^{-1}$) for the reaction with mercaptoacetic acid, K_a, the dissociation constant in M, c_A/c_0, ratio of concentration remaining in the medium after 10 min incubation with mitochondria and initial concentration, c, concentration (in M) eliciting 50% stimulation of mitochondrial respiration

No	R	log P	log k	pK_a	$-\log \frac{c_A}{c_0}$	$\log \frac{1}{c}$
1	H	1.85	4.65	6.55	0.26	6.35
2	2-Br	2.20	4.68	6.35	–	6.75
3	2-NO_2	1.27	5.98	5.80	0.23	6.17
4	2-CF_3	2.00	6.29	5.00	–	7.18
5	2-Cl	1.75	5.94	5.83	–	6.95
6	3-Cl	2.19	5.04	6.00	0.56	7.15
7	4-Cl	2.23	4.97	6.15	0.45	6.95
8	4-OCF_3	2.42	5.10	6.00	0.65	7.22
9	2,6-diCl	2.01	5.90	4.70	–	7.25
10	2,3-diCl	1.80	6.35	5.15	–	7.01
11	2-Cl,4-NO_2	2.00	6.73	4.15	–	7.12
12	2-CH_3,4-NO_2	1.85	6.03	5.50	–	7.10
13	2,6-diCH_3	2.10	4.80	6.60	–	6.68
14	2,5-diCH_3	2.60	5.42	6.90	–	6.52
15	4-CH_3	2.10	4.53	6.75	0.35	6.39
16	4-$(CH_2)_2Cl$	2.44	4.83	6.50	0.53	7.00
17	4-$COCH_3$	1.15	5.40	5.85	0.17	–
18	3-OH	1.48	–	–	0.23	–
19	4-N=N-C_6H_5	3.06	4.20	7.33	1.35	–
20	4-$NHCOCH_3$	1.07	–	–	0.22	–
21	2-CH_3	1.90	5.32	6.90	–	–
22	2-NO_2,4-CH_3	1.30	5.83	6.00	–	–

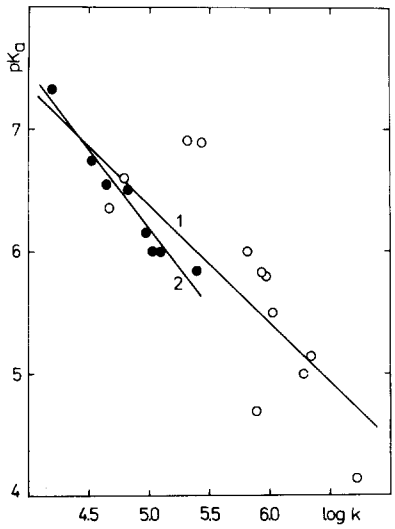

Fig. 5. Collinearity between acidobasicity and reactivity of CCP derivatives. Open points, ortho-derivatives; full points, the other derivatives. The equation $pK_a = A \log k + B$ is valid for all derivatives (1) and for non-orthoderivatives (2) with $A = -0.969$ and -1.283, $B = 11.232$ and 12.603, $n = 20$ and 8, $r = 0.846$ and 0.973, $s = 0.438$ and 0.123, $F = 45.3$ and 106.6.

ber 14, Table I) from the predicted value. Therefore we consider the results of this study only as indication of a protonophoric mechanism of uncoupling effects in CCP series, rather than as an unambiguous evidence for it.

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