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Estimation of flux control coefficients from inhibitor titrations by non-linear regression

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A mathematical model was developed to estimate flux control coefficients (C_o) from titration studies with specific non-competitive inhibitors. In contrast to the normally used graphical determination the model pays regard to the dissociation equilibrium (K_D) that exists between inhibitor and its binding sites (E_o) as well as to an objective estimation of the initial slope. The model was used for the analysis of titration experiments where the respiration of rat liver mitochondria was inhibited with carboxyatractyloside and antimycin A. It is shown that the graphical estimation of E_o and C_o lead to significant overestimation if the ratio K_D/E_o is larger than 10^{-4} which can be avoided by using our model.

Flux control coefficient; Adenine nucleotide translocator; bc_1-Complex; Non-competitive inhibitor

1. INTRODUCTION

After the metabolic control theory had been developed by Heinrich and Rapoport [1] and Kacser and Burns [2], it was successfully used for investigations into the mitochondrial oxidative phosphorylation [3-9]. In most of these studies, specific inhibitors were used to obtain titration curves for graphical determination of the concentration of inhibitor binding sites (E_o) and the initial slope which are required for calculation of flux control coefficients (C_o). Both values are, however, not easily to determine since (A) the dissociation of the inhibitor-enzyme complex has to be taken into account and (B) the titration curves are more or less sigmoidal complicating the calculation of initial slope. For these reasons, we propose a new method for objectively calculating flux control coefficients from experiments in which mitochondrial respiration is titrated by means of irreversible inhibitors. This method employed non-linear regression regarding the dissociation equilibrium between inhibitor and its binding sites. Additionally the method allows to estimate the specific activity of the titrated enzymes. The work in hand intends to show how effective this method is for titrations of rat liver mitochondria with antimycin A and carboxyatractyloside.

1.1. *Theory*

The method of non-linear regression requires a mathematical model that relates the metabolic flux J to the concentration I of the added inhibitor. It will be considered that the inhibitor binds specifically to an enzyme which is in a branch of the total flux J (Fig. 1). Assuming non-competitive inhibition, the binding is independent of the concentration of the intermediates X so that the dissociation equilibrium is

$$K_D = \frac{E \times I_{free}}{EI} \quad (1)$$

With $E_o = E + EI$ and $I = I_{free} + EI$ for the total concentration of enzyme and inhibitor, the square equation follows

$$E^2 + (K_D + I - E_o) \times E - K_D \times E_o = 0 \quad (2)$$

It can be shown that the flux J depends on the enzyme concentration E as

$$J = \frac{\alpha \times E}{\beta + E} + \gamma \quad (3)$$

if (i) the reaction between X_2 and X_3 is proportional to E and (ii) all reactions in Fig. 1 are linear functions of the intermediate concentration X_1, X_2 and X_3. Since linearity is a very restrictive condition a more general equation may be obtained by introducing an empiric exponent n so that

$$J = \frac{\alpha \times E^n}{\beta + E^n} + \gamma \quad (4)$$

The constants α, β and γ are not of interest and can be substituted by the flux rates J_o and J_i in the non-inhibited ($E = E_o$) and completely inhibited ($E = 0$)

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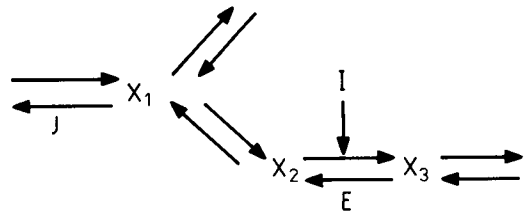


Fig. 1. General scheme relating the inhibition of an enzyme in a metabolic branch to the flux J. X_1, intermediate at the branching point; X_2 and X_3, intermediates converted by the inhibited enzyme.

system and by the flux control coefficient $C_o = (\mathrm{dln}J/\mathrm{dln}E)_{E=Eo}$ in the absence of the inhibitor. Then the final equation results as

$$J = \frac{n\,(J_o - J_i)^2 \times E^n}{C_o \times J_o \times E_o^n + [(n - C_o) \times J_o - n \times J_i)] \times E^n} + J_i \quad (5)$$

2. METHODS

2.1. *Incubation of mitochondria*

Rat liver mitochondria were isolated as previously described [10]. The incubation medium contained 110 mM sucrose, 60 mM Tris, 60 mM KCl, 15 mM glucose, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 μM rotenone, 10 mM succinate, 5 mM ATP, pH 7.4. Active states were adjusted by addition of yeast hexokinase. Inhibitors were added stepwise. Stationary rates of respiration and the first derivatives were measured with a Clark-type electrode at 25°C using a custom-built ratemeter and a two-channel recorder. All chemicals were of the highest purity available.

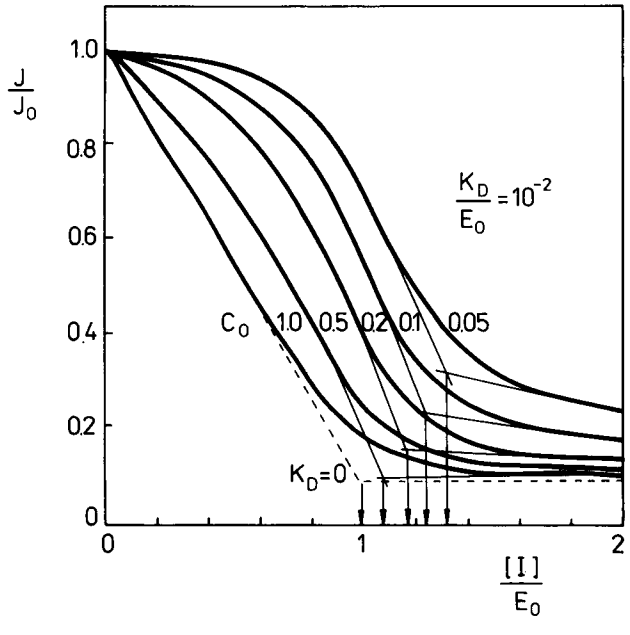
3. RESULTS AND DISCUSSION

3.1. *Properties of the model*

Essential properties of the model are illustrated in Fig. 2. The influence of the flux control coefficient C_o on the shape of the titration curves is shown in Fig. 2A. Only for large flux control coefficients ($C_o = 1$) the simulated titration curve shows a sharp fold at an inhibitor enzyme ratio of one. In this case the graphical determination of the E_o value yields the theoretical value. With decreasing flux control coefficients, however, the curves become more and more sigmoidal reaching the minimum fluxes at higher inhibitor concentrations only. Also with increasing dissociation constants (Fig. 2B) the steepness of the curves diminishes. It is easily to see, that with decreasing C_o- and with increasing K_D/E_o-values the graphical method proposed by Groen et al. [8] for an estimation of the concentration of binding sites leads to an increasing overestimation of this value.

3.2. *Estimation of model parameters from antimycin A titrations*

Application of our method to the estimation of model parameters from experimental data is shown in Figs 3 and 4. The active rate of mitochondrial respiration was titrated with antimycin A which binds to the bc_1 complex with a high affinity [11]. Due to a slow dismutation of the semiquinone in the center 'o' of the Q-cycle leading to production of superoxide and H_2O_2 [12] the oxygen consumption can not be inhibited completely even in excess of antimycin A. The relative



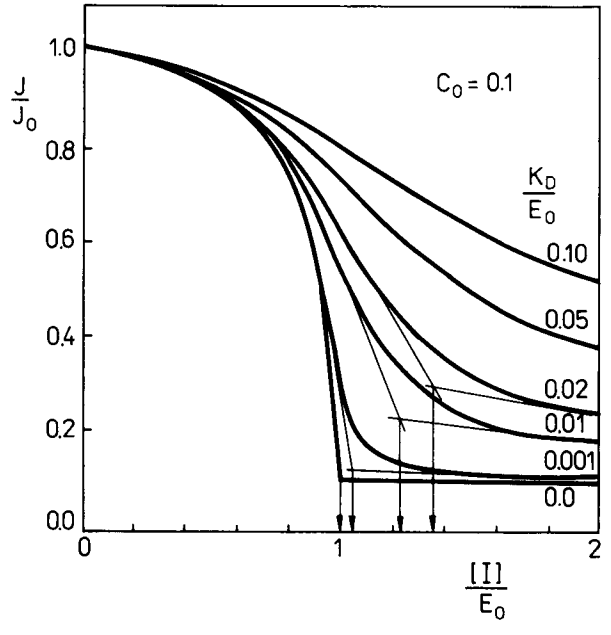


Fig. 2. Properties of the model: relation of the normalized flux to the inhibitor-enzyme ratio. The simulated curves were calculated from Eqn. (5) with the following parameter values: $J_i/J_o = 0.1$, $n = 1$, K_D/E_o and C_o as indicated. The lines and the arrows mark the graphical determination of E_o-values.

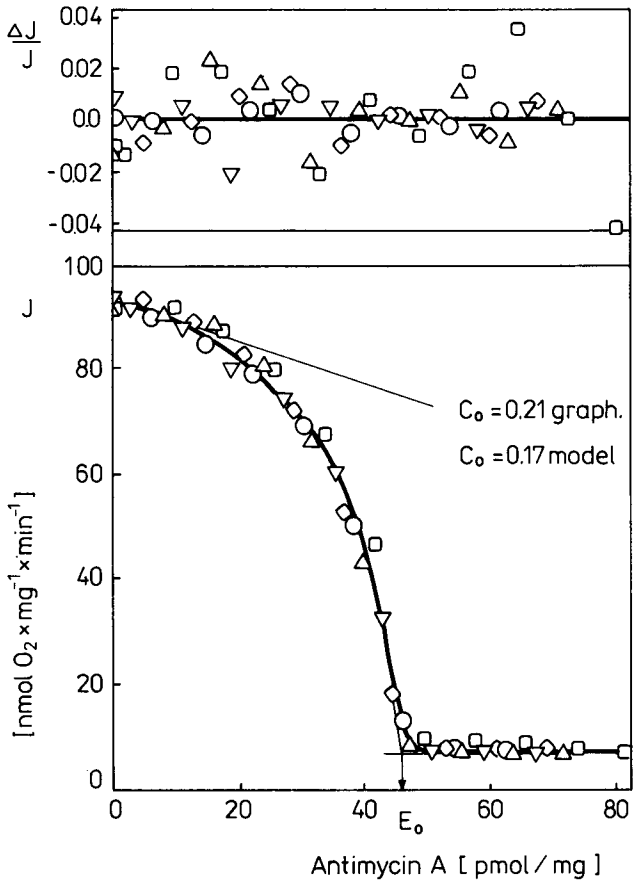


Fig. 3. Titration of rat liver mitochondria with antimycin A. Mitochondria (0.84 mg/ml) were incubated as described in section 2. Antimycin A was added stepwise to 5 separate incubations which were marked by different symbols. The curve of the linear model ($n = 1$) was fitted to all experimental points assuming a constant relative error. The parameters estimated in this fit and other separate fits for the 5 incubations are shown in Table I. The graphically determined value for C_o is 0.21. For this the initial slope was determined from the first 14 points by means of linear regression. The upper panel shows the relative deviations of the experimental points from the curve in the case of the separate fits.

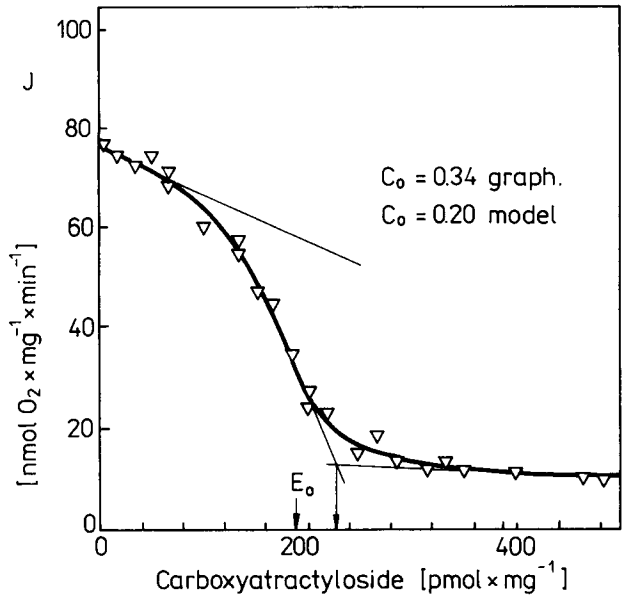


Fig. 4. Titration of rat liver mitochondria with carboxyatractyloside. Mitochondria (0.49 mg/ml) were incubated as described in section 2. Carboxyatractyloside was added stepwise. The 25 experimental points were obtained in 4 separate incubations of one preparation of mitochondria. The curve was fitted to the linear model ($n = 1$) assuming an absolute constant error. Estimated parameters: C_o = 0.21, E_o = 0.20 nmol/mg, K_D = 0.86 nM, J_o = 76.3 nmol O_2/mg/min, J_i = 9.0 nmol O_2/mg/min. The graphically determined value for C_o is C_o = 0.36. The initial slope was calculated from the first 4 experimental points by means of linear regression.

residual plot (Fig. 3B) indicates that only small and randomly distributed deviations of the experimental points from the estimated curve occur. As shown in Table I both the linear ($n = 1$) and the non-linear ($n = 1.1$) model yielded practically the same estimated model parameters. From this we conclude that the linear model ($n = 1$) is sufficient to estimate the model parameters. The estimated K_D = 7.8 pM is comparable to those estimated by others (30–300 pM [13,14]). Due to the very low dissociation constant (K_D/E_o = 0.0001) both the computed and the graphically estimated E_o- and C_o-values are similar. The flux control coefficient of the bc_1-complex (C_o = 0.2) is between that reported by others (C_o = 0.03–0.5 [7,9]). The results for single incubations (Columns 2–6) demonstrate that a titration curve with about 10 experimental points is sufficient for the estimations.

3.3. *Estimation of model parameters from carboxyatractyloside titrations*

Carboxyatractyloside binds non-competitively and irreversibly to the mitochondrial adenine nucleotide translocator [15] with an 1:1 stoichiometry [16]. As in the case of antimycin A titration the computer-fitted curve sufficiently covers the experimental points. The estimated dissociation constant K_D = 0.86 nM is somewhat lower than reported earlier (K_D = 5–10 nM [15,16]). Since the binding of carboxyatractyloside is weaker than that of antimycin A a remarkable part of the inhibitor is dissociated from the enzyme in the range of E_o and therefore the enzyme is not completely inhibited and needs higher inhibitor concentrations for complete inhibition. For this reason the graphical method results in overestimated E_o- and C_o-values. Besides the uncertainty of the E_o-determination the problem of initial slope is the reason for that. It seems therefore that the C_o-values of the adenine nucleotide translocator of rat liver mitochondria for the fully active succinate induced respiration reported earlier are overestimated (0.29–0.45 [3,4,9,17]). This work shows, that the graphical analysis of titration curves [8] is limited to inhibitors with very low dissociation constants e.g. antimycin A. With increasing constants ($K_D/E_o > 0.0001$) the dissociation equilibrium has to be taken into account. Another uncertainty of the

Table I

Estimated model parameters from titration of rat liver mitochondria with antimycin A

PARAMETERS	ALL TITRATIONS	SINGLE TITRATIONS				
	(1)	(2)	(3)	(4)	(5)	(6)
number of points	50	10	12	10	9	9
C_o	0.17 (0.16)	0.16	0.15	0.19	0.22	0.18
E_o (pmol/mg)	45.7 (46.0)	44.3	46.7	46.5	45.5	45.8
K_D (pM)	7.8 (8.8)	9.2	5.3	4.2	8.5	13.0
J_o (nmol O_2/min/mg)	93.1 (92.8)	93.5	93.1	92.8	96.1	92.1
J_i (nmol O_2/min/mg)	7.2 (7.2)	6.9	7.8	7.1	7.0	6.8

Column 1 shows the results of all of the 50 experimental points seen in Fig. 3 using the linear and the non-linear model (n in parenthesis). SD = 0.554 ($n = 1$); SD = 0.559 ($n = 1.1$). Columns 2–6 show the results of the fits to the points of single incubations.

graphical method is the true determination of the initial slope which critically depends on the location of the first points of the titration curve. The usefulness of the computer-model to describe the titration curves is shown by (i) the sufficient coincidence of our estimated with the published dissociation constants, (ii) the absence of systematic deviations of the computed curves from the experimental points as shown with the residual plots and (iii) the finding that the introduction of an additional parameter (n) did not improve the fit. Probably our method is also applicable to other non-competitive inhibitors such as mersalyl [18], oligomycin [19] or myxothiazol [20] and permits to be extended to competitive inhibitors, if their inhibition kinetics is included into the model.

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