A COMPUTER ANALYSIS OF CYANIDE STIMULATED OXYGEN UPTAKE IN *CHLORELLA PROTOTHECOIDES*

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1. Introduction

Chlorella protothecoides is a unicellular alga which when grown under heterotrophic conditions using a high glucose and low organic nitrogen source has no chlorophyll, little carotenoid and a reduced chloroplast structure [1]. These properties make it very suitable for respiration studies. Interest in respiration has centred around the presence, characteristics and role of inhibitor stimulated oxygen uptake a property which is widespread among the algae. Despite numerous studies [2–8] the mechanism of inhibitor stimulated respiration is unknown.

Grant and Hommersand [9] have investigated the inhibitor responses of whole cells of *Chlorella protothecoides* quantitatively, and the data thus obtained has been used as the basis of the present study.

The present work concerns the elucidation of the mechanism, mathematical symbolism and kinetics of cyanide stimulated oxygen uptake in *Chlorella protothecoides* by means of non-linear optimization procedures to enable one to test the ability of a given mechanism to reproduce the experimental observations.

2. Materials and methods

2.1. *Biochemical methods*

The organism, its culture and the experimental techniques are described in ref. [9]. The experimental data for the present study was obtained from a series of titrations of oxygen uptake rates against cyanide concentration. The final cell density was 12.2 mg dry wt/ml, the initial oxygen concentration was 2.36×10^{-4} M, and the control oxygen uptake rate was 1.74×10^{-7} M O_2/s.

2.2. *Numerical techniques*

The numerical results of non-linear optimizations were obtained using a computer program, FACSIMILE [10]. The differential equations are solved using Gear's method [11] for the numerical integration of large systems of stiff differential equations, and optimization is carried out using a method developed by Powell [12]. Optimized mathematical models are compared to the experimental data by determining the sum squares of deviation (SQD) given by

$$\mathrm{SQD} = \sum_{i=1 \to n} \left(\frac{v_i - u_i/s}{\sigma_i} \right)^2 \qquad (1)$$

where v_i is the observed value, u_i the calculated value, s a scale factor, σ_i the standard error of v_i, and n the number of experimental points. Because SQD depends on n, in order to compare a model to different experiments in which the number of data points varies, the standard deviation (SD) is calculated:

$$\mathrm{SD} = \sigma_i \sqrt{\frac{\mathrm{SQD}}{n - p}} \qquad (2)$$

where p is the number of unknown parameters.

A quantitative measure of how accurately an unknown parameter has been determined by optimization is given by the standard deviation of the natural logarithm of the unknown parameter ($\mathrm{SD}_{\ln}$).

Since rate constants and other parameters need to be varied over a large range of values, the natural logarithm of the unknown parameter is varied and, consequently, SD_{ln} is calculated from the non-linear covariance. Because of the linearity of logarithms less than 0.1, a parameter whose SD_{ln} lies below this value has a relative standard deviation of $\pm SD_{ln}$ and is considered to have a well determined minimum in multidimensional space. For larger values of SD_{ln}, up to 1 in magnitude, the parameter value is determined to within a factor of order $e \simeq 2.72$, and so its order of magnitude is known. Significantly larger values of SD_{ln} show that the observations are inadequate to determine the parameter.

3. Results and discussion

Our model to account for the increased rate of oxygen uptake in *Chlorella protothecoides* in the presence of cyanide is summarized in fig.1. It provides for a hydrogen peroxide generating leak at the branch point of the linear respiratory chain plus catalase to decompose the hydrogen peroxide back to oxygen. The branch point ends in a cyanide insensitive, high oxygen affinity alternate terminal oxidase [13]. The combination of the branch point yielding hydrogen peroxide and the cyanide sensitivity of both cytochrome oxidase and catalase leads to the situation where an increase in cyanide concentration will inhibit cytochrome oxidase and catalase, and increase hydrogen peroxide production. Thus the return of oxygen to the system by the decomposition of hydrogen peroxide is diminished so that the net disappearance of oxygen from the solution, as measured by the oxygen electrode, will increase until cytochrome oxidase and catalase are completely inhibited. At this stage, all the oxygen will be used in hydrogen peroxide production, and the oxygen uptake rate will essentially be doubled for the particular condition that the electron flow rate from substrate to oxygen is approximately constant. However, because of the peculiar conditions of partition of electron flow between the full chain and the branched chain, the doubling will not be exact.

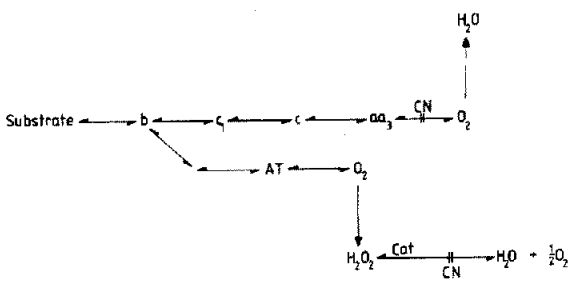

Fig.1. The model of cyanide stimulated respiration in *Chlorella protothecoides*. It consists of the linear (cytochromes *b*, c_1, *c* and aa_3) and branched (the alternate terminal oxidase, AT) portions of the respiratory chain, the latter providing a H_2O_2 generating leak, plus catalase (Cat) to decompose the H_2O_2 back to oxygen.

The steady state rate equations used to describe this system are derived from the following chemical equations:

$$O_2 \xrightarrow{aa_3} 2H_2O \qquad (3(a))$$

$$aa_3 + CN \rightleftharpoons aa_3\cdot CN \qquad (b)$$

$$2H_2O_2 \xrightarrow{Cat} O_2 + 2H_2O \qquad (4(a))$$

$$Cat + CN \rightleftharpoons Cat\cdot CN \qquad (b)$$

$$O_2 \xrightarrow{AT} H_2O_2 \qquad (5(a))$$

$$AT + CN \longrightarrow \text{no reaction} \qquad (b)$$

where aa_3 is cytochrome oxidase, Cat is catalase, AT the alternate terminal oxidase, and aa_3·CN and Cat·CN the cyanide inhibited cytochrome oxidase and catalase complexes, respectively.

In the derivation of the steady state equations, the inhibition of both cytochrome oxidase and catalase by cyanide was considered to be non-competitive [14,15].

The velocity of oxygen consumption by cytochrome oxidase (v_{aa_3}) is given by

$$v_{aa_3} = \frac{V_m^{aa_3} K_i^{aa_3} [O_2]}{(K_i^{aa_3} - [CN])([O_2] - K_m^{aa_3})} \qquad (6)$$

where $V_m^{aa_3}$ is the maximum velocity of oxygen consumption by cytochrome oxidase, $K_m^{aa_3}$ the Michaelis-Menten constant of eq. (3(a)) and $K_i^{aa_3}$ the equilibrium constant of eq. (3(b)).

The velocity of oxygen production by catalase (v_{Cat}) is given by

$$v_{Cat} = \frac{V_m^{Cat} K_i^{Cat} [H_2O_2]}{(K_i^{Cat} - [CN])([H_2O_2] - 2K_m^{Cat})} \quad (7)$$

where V_m^{Cat} is the maximum velocity of oxygen production by catalase, K_m^{Cat} the Michaelis-Menten constant of eq. (4(a)), and K_i^{Cat} the equilibrium constant of eq. (4(b)).

The velocity of oxygen consumption by the alternate terminal oxidase (v_{AT}) is given by

$$v_{AT} = \frac{V_m^{AT} [O_2]}{([O_2] - K_m^{AT})} \quad (8)$$

where V_m^{AT} is the maximum velocity of oxygen consumption by the alternate terminal oxidase and K_m^{AT} the Michaelis-Menten constant of eq. (5(a)).

Thus the net rate of oxygen consumption by the system is given by

$$\frac{d[O_2]}{dt} = v_{aa_3} + v_{AT} - v_{Cat} \quad (9)$$

and the net rate of hydrogen peroxide production is given by

$$\frac{d[H_2O_2]}{dt} = v_{AT} - 2v_{Cat} \quad (10)$$

Four solutions of the optimization problem are presented (table 1). In all cases, the standard error of the data, σ_i, was assumed to be 2%, and subsequent optimizations achieved a SD of this value.

In solution 1, the parameters $V_m^{aa_3}$, $K_m^{aa_3}$, $K_i^{aa_3}$, V_m^{Cat}, K_m^{Cat}, K_i^{Cat}, V_m^{AT} and K_m^{Cat} were varied. $K_m^{aa_3}$ and K_m^{AT} were undetermined (SD_{ln} of 6860 and 3510, respectively), as were $K_i^{aa_3}$, $V_m^{aa_3}$ and K_m^{Cat} (SD_{ln} of 170, 15.8 and 6.77, respectively). In solution 2, $K_m^{aa_3}$ and K_m^{AT} were set to zero (i.e., over the time course of the experimental data, both cytochrome oxidase and the alternate terminal oxidase were assumed to be fully saturated with oxygen). Imposing these constraints, all the parameters were well determined to within an order of magnitude with the exception of $K_i^{aa_3}$ which was poorly determined (SD_{ln} of 7.67). In solution 3, a further constraint was imposed: $K_i^{aa_3}$ was set to 1×10^{-6} M and held constant during optimization. This resulted in all the remaining parameters being well determined.

Table 1
Optimized values of the parameters (the SD_{ln} are shown in brackets)

Parameter	Dimensions	Solution 1		2		3		4	
$V_m^{aa_3}$	nM O_2/s	9.76	(15.8)	6.58	(0.884)	11.7	(0.475)	0[a]	
$K_m^{aa_3}$	nM	475	(6860)	0[a]		0[a]		0[a]	
$K_i^{aa_3}$	μM	0.0634	(170)	17.3	(7.67)	1[a]		0[a]	
V_m^{Cat}	nM O_2/s	337	(0.361)	439	(0.756)	916	(1.04)	2870	(4.01)[b]
K_m^{Cat}	nM	52.9	(6.77)	250	(1.28)	151	(1.54)	4620	(0.823)
K_i^{Cat}	μM	3.17	(0.500)	2.32	(1.14)	0.831	(1.20)	0.341	(4.04)[b]
V_m^{AT}	nM O_2/s	337	(0.0591)	334	(0.00816)	332	(0.00606)	332	(0.00600)
K_m^{AT}	nM	2.93	(3510)	0[a]		0[a]		0[a]	

[a] Constrained during optimization
[b] The correlation coefficient between V_m^{Cat} and K_i^{Cat} was −0.999 and the SD_{ln} of the product $V_m^{Cat} \times K_i^{Cat}$ was 0.141

Table 2
A comparison of the calculated and observed rates of oxygen uptake as % control rate, at a series of cyanide concentrations (the control rate was 1.74×10^{-7} M O_2/s)

[Cyanide] (μM)	O_2 Uptake as % control rate				
	Observed	Calculated			
		1	2	3	4
4	105	106	107	102	106
6	126	124	126	125	125
8	140	135	139	139	140
10	150	144	148	148	151
20	170	163	168	167	174
40	182	175	180	176	187
60	187	180	184	180	191
80	189	182	186	182	193
100	190	183	187	183	195
200	191	186	190	185	197
400	191	188	191	186	199
600	190	188	192	186	199
800	189	188	192	186	199
1000	188	189	192	186	199
2000	186	189	192	187	200

The poor determination of $K_i^{aa_3}$ can be accounted for by the observation that the value of $K_i^{aa_3}$ varies from 5×10^{-8} M to 5×10^{-4} M depending on the oxidation state of cytochrome oxidase [14]. Thus, a poorly determined $K_i^{aa_3}$ indicates that our model is independent of the oxidation state of cytochrome oxidase.

As a low value of $V_m^{aa_3}$ was obtained by optimization, a fourth solution was carried out with no cytochrome oxidase (i.e., $V_m^{aa_3}$ was set to zero). This constraint did not increase the value of the SD, and resulted in the determination of V_m^{AT}, K_m^{Cat} and the product $V_m^{Cat} \times K_i^{Cat}$, with K_m^{AT} set to zero. Thus this solution indicates that the oxygen electrode experiments yield information about the net rate of oxygen utilization and oxygen production. These experiments are therefore not able to distinguish between the contribution of cytochrome oxidase and the alternate terminal oxidase to the rate of oxygen uptake.

A comparison of the calculated and observed rates of oxygen uptake at a series of cyanide concentrations is shown in table 2.

We therefore conclude that the increase in respiration by cyanide and other inhibitors, characteristic of *Chlorella protothecoides* and a number of other algal systems, can be attributed to the role of catalase in supplying oxygen from hydrogen peroxide.

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