CLOSE CORRELATION BETWEEN ANTIMYCIN TITER AND CYTOCHROME b_T CONTENT IN MITOCHONDRIA OF CHLORAMPHENICOL TREATED *NEUROSPORA CRASSA*

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1. Introduction

The variation of the contents of respiratory components can serve as an important tool in attributing particular roles to individual components. Recently, the discrimination between cytochrome b_K and b_T has become important, since cytochrome b_T has been directly associated with energy transfer. The two *b*-type cytochromes usually occur in equimolar amounts. Correlation of a particular function to one of the cytochromes becomes possible when the 1:1 ratio is changed.

Stepwise inhibition of the mitochondrial protein synthesis of *Neurospora crassa* wild type by chloramphenicol leads to mitochondria with a phenotype which becomes more and more similar to that of the mi-1 mutant. The cytochrome b_T content is finally lowered to 1/16, whereas that of cytochrome b_K only to about 1/2. In this study the change of the antimycin-titer of respiratory activity was investigated. Within a definite concentration range of chloramphenicol, a close correlation exists between the antimycin-titer and the content of the cytochrome b_T only. This finding supports the assumption that binding of antimycin is closely related to cytochrome b_T.

Abbreviations:
CAP: chloramphenicol;
UQ : ubiquinone;
SAM: salicylhydroxamate.
Ant : antimycin.

2. Experimental

Cultivation of hyphae (wild type 74A) and preparation of mitochondria as described in [1]. Chloramphenicol (ad us. vet.) purchased from Bayer, Leverkusen, was dissolved in 50% ethanol and added to the hyphae medium. Oxygen consumption was measured amperometrically in a reaction medium according to [1]. The difference spectra of the cytochromes were performed with a special split beam spectrophotometer as described in [2]. The cytochrome contents were calculated with a correction method considering mutual interference of the cytochromes at low temperature as described elsewhere [2]. The extinction coefficients (reduced minus oxidized) used were: for cytochrome aa_3 $\Delta\epsilon_{605-630\,nm}$ 24.0 $mM^{-1}\,cm^{-1}$ [3], for cytochrome *b* $\Delta\epsilon_{560-575}$ = 23.4 $mM^{-1}\,cm^{-1}$ [4], and for cytochrome *c* $\Delta\epsilon_{550-542}$ = 18.7 $mM^{-1}\,cm^{-1}$ [5]. The ubiquinone contents were determined by the extraction method according to [6]. The antimycin titrations were performed in the oxygen electrode vessel. The concentration of the antimycin solution was controlled spectrophotometrically, using an extinction coefficient of ϵ_{320} = 4.78 $mM^{-1}\,cm^{-1}$, according to [7].

3. Results

Cultivation of *Neurospora crassa* wild type in a growth medium containing increasing concentrations of CAP, culture No. 1 (0 g CAP/liter) to No. 5 (5 g CAP/liter), leads to a drastic change in the content

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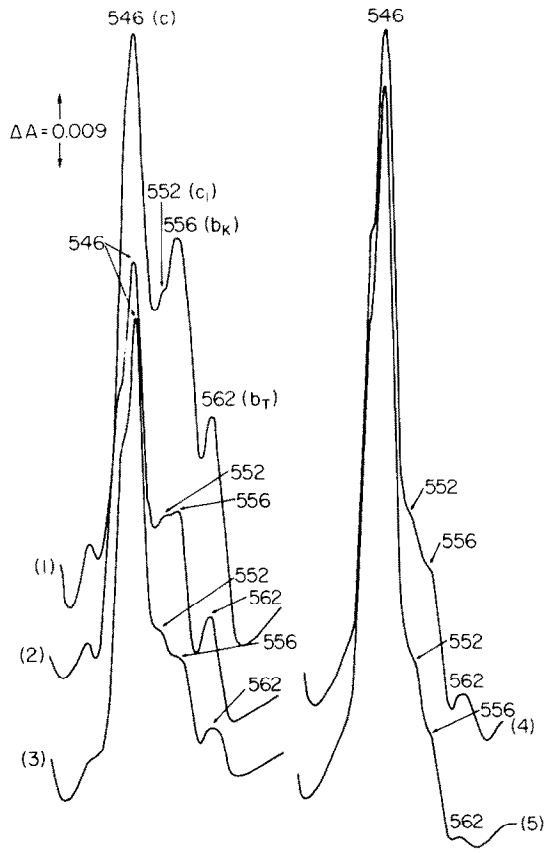

Fig. 1. Low temperature absorption spectra of the mitochondrial *b*-type and *c*-type cytochromes from different hyphae cultures containing increasing concentrations of chloramphenicol. The CAP concentrations of the different cultures (no. 1–5) are given in the second column of table 1. The protein concentration in the cuvette (mg/ml) of the preparations 1–5 was, 4.0, 2.7, 3.0, 3.5 and 2.7, respectively. The spectra were obtained with a light path of 0.5 cm as described in Experimental; reduction of the measuring sample by dithionite; the reference sample was kept aerobic by oxygen.

of the respiratory chain components. This is illustrated in fig. 1 with the low temperature difference spectra (dithionite-reduced minus oxidized) of the *b*-type and *c*-type cytochromes in the α-region. The four α-bands at 562, 556, 552 and 546 nm, representing cytochrome b_{562}, b_{556}, c_1 and c, respectively, are visible in all preparations. The corresponding absorption maxima for all cytochromes show no wavelength shift, but varying heights, indicating that addition of CAP to the growth medium causes no alteration of the cytochromes, but of their contents. In this study cytochrome b_{562} of *Neurospora crassa* is defined as cytochrome b_T, since its characteristics are, by analogy to mammalian mitochondria: energy-dependent reduction in the anaerobic state [8, 9], complete reduction only in the antimycin-inhibited state and reoxidation when cytochrome c_1 is reduced under this condition [10–12]; cytochrome b_{556}, which is almost completely reduced in uncoupled anaerobic state, corresponds to cytochrome b_K [13].

The contents of the cytochromes, as calculated from the low temperature spectra (fig. 1), are shown in table 1; furthermore, table 1 shows the change of UQ content and of KCN-sensitivity of respiration in dependence on the CAP concentration. With increasing inhibition of mitochondrial protein synthesis, the content of UQ and the *c*-type cytochromes increases, whereas that of the *b*-type cytochromes and cytochrome aa_3 decreases. In a culture containing 5 g CAP/liter, the UQ content amounts to about 3 μmoles/g protein, coming close to the value obtained from mammalian mitochondria. Moreover, under these conditions, the contents of cytochrome c_1 and c reach the high values of 0.51 and 2.07 μmoles/g protein, respectively. With regard to the *b*-type cytochromes, it is observed that the content of cytochrome b_K is diminished to about 1/2, whereas that of cytochrome b_T is lowered to about 1/15 of the original value. The synthesis of cytochrome aa_3 is affected in a similar manner as that of cytochrome b_T. Fig. 2 gives the ratios of actual cytochrome content over original cytochrome content as a function of the CAP concentration in the growth medium. The effect of CAP on the synthesis of all cytochromes except cytochrome *c* reaches its maximum at 5 g CAP/liter hyphae growth medium (culture No. 5).

Another parameter in the simulation of the mi-1 mutation [14, 2] is the KCN-insensitive respiration. Besides the NADH respiration (see last column of table 1), oxidation of all other substrates linked either to endogenous NAD or to UQ becomes insensitive to KCN as well as to antimycin (not shown). Thus, parallel to increasing deficiency of the normal pathway, a capacity for electron transport from the substrates to oxygen via a new pathway becomes available. The redox changes of UQ in the course of

Table 1
Alteration of the contents of respiratory chain components and of sensitivity of NADH-respiration to KCN as a function of chloramphenicol concentration in growth medium.

No. of culture	CAP	UQ	Cytochromes					Inhibition of NADH-respiration by KCN
			b_T	b_K	c_1	c	aa_3	
	(g/l)		(μmoles/g protein)					(%)
1	0	0.40	0.32	0.39	0.36	1.30	0.30	100
2	0.25	0.45	0.23	0.37	0.37	1.30	0.26	90
3	0.50	0.62	0.15	0.24	0.44	1.42	0.22	78
4	1.00	1.10	0.07	0.26	0.42	1.47	0.13	32
5	5.00	3.21	0.02	0.23	0.51	2.07	0.01	24

Addition of CAP, measurement of respiratory activity, determination and calculation of ubiquinone and cytochrome contents as described in Experimental.

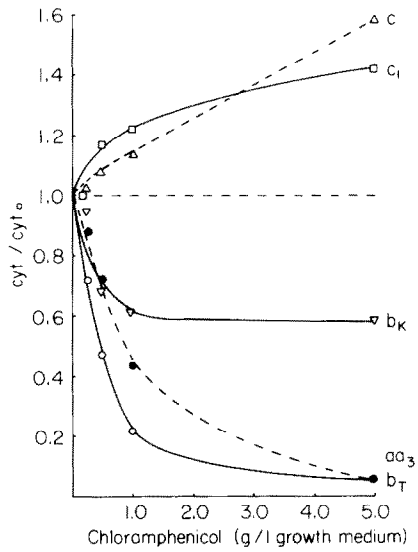

Fig. 2. Change of mitochondrial cytochrome contents as a function of the chloramphenicol concentration in the hyphae growth medium. The actual cytochrome content (cyt) over original cytochrome content (cyt_0) was plotted against the CAP concentration present in the hyphae growth medium.

titrations by various inhibitors show that UQ is functionally included in both paths, acting as the branching system in the respiratory chain of CAP-treated wild type (unpublished), similar as in the mi-1 mutant [2]. Salicylhydroxamate completely inhibits the KCN-insensitive NADH respiration (addition of SAM after KCN) without interfering with the KCN-sensitive oxidation (comparison of the values obtained by addition of KCN alone and of SAM before KCN).

The high specificity of SAM makes possible antimycin titrations in mitochondria equipped with the second oxidase pathway. Control experiments with wild type prove that there is no difference between antimycin titrations performed in the presence and in the absence of SAM. The antimycin titrations shown in fig. 3 were performed in the presence of 2 mM SAM. In graph A, the activity of NADH respiration is plotted against the amount of antimycin referred to mitochondrial protein. The titer for full inhibition of the different preparations continuously decreases from 0.26 to 0.04 μmoles/g protein. As shown in table 1, parallel to the decrease of the antimycin titer, the content of cytochrome b_T is diminished from 0.32 to finally 0.02 μmoles/g protein. The titration curves exhibit nonlinear shape [15]. In graph B, these titration curves are plotted against the amount of antimycin as referred to cytochrome b_T. In this plot, the first three titration curves form one family of curves which intercept the abscissa at about equimolar ratio of antimycin to cytochrome b_T. In titrations of preparations with very low cytochrome b_T content (curve 4 and 5) the equimolar ratio is exceeded, reaching a ratio of antimycin/cytochrome b_T of 1.5 or 2. It might be speculated that small amounts of cytochrome b_T-apoenzyme, with the ability to bind antimycin, could cause this increase. Taking into account that

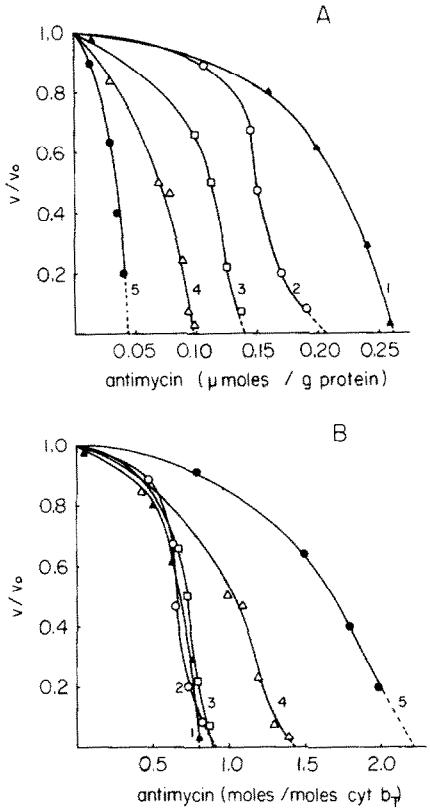

Fig. 3. Activity of NADH respiration plotted as a function of antimycin concentration, A referred to mitochondrial protein, B referred to cytochrome b_T content. The NADH respiration of the different preparations (no. 1–5 of table 1) was titrated by antimycin in the presence of 2 mM SAM at 25° as described in Experimental. Each point of the curve was obtained by a separate incubation, since preincubation with the inhibitors was necessary for full effect.

the assembling process of cytochrome b_T is strongly affected by the high CAP concentration [16, 17], the occurrence of such subunits is not improbable.

4. Discussion

In this study chloramphenicol treated *Neurospora crassa* is analyzed with respect to the alteration of the respiratory chain. It is observed that a simulation of the mi-mutation is obtained by addition of high concentration of CAP to hyphae growth-medium. The advantage of inhibition of the mitochondrial protein synthesis by increasing concentrations of CAP over mutant preparations is that the phenotype may be altered gradually. The final stage comes close to the mi-1 mutant, which, in contrast to the mi-3 mutant, has a very low content not only of cytochrome aa_3 but also of cytochrome b_T [2]. In both the mi-mutant and CAP-treated hyphae the decreased respiration via cytochrome oxidase is compensated by electron flow via a KCN-insensitive oxidase. This is illustrated in fig. 4 in a simplified scheme of the bifurcated chain.

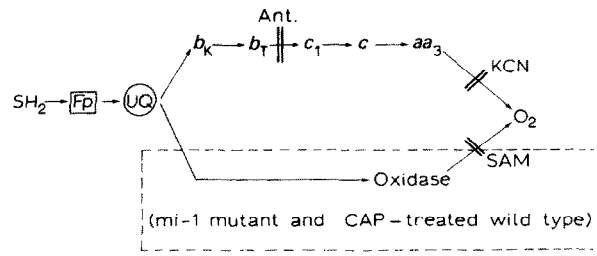

Fig. 4. Scheme of pathways to oxygen in wild type, chloramphenicol-treated wild type and mi-1 mutant mitochondria.

Table 2
Change of antimycin/cytochrome ratios in dependence on increasing inhibition of mitochondrial protein synthesis by chloramphenicol.

CAP	Antimycin titer	ant / b_T	ant / b_K	ant / c_1
(g/l)	(nmoles / mg)	(moles/moles)		
0	0.26	0.81	0.67	0.72
0.25	0.20	0.87	0.54	0.54
0.50	0.13	0.87	0.54	0.29
1.00	0.10	1.40	0.38	0.24
5.00	0.04	2.00	0.17	0.08

In mitochondria from hyphae grown in increasing CAP concentrations the titer for full inhibition of NADH oxidation by antimycin decreases continuously. For a detailed analysis, table 2 gives the ratios of the titers referred to cytochrome b_T, b_K and c_1. With regard to cytochrome b_K and c_1, the results given in table 2 show clearly that neither of these carriers is affected by antimycin in such a way as to give an equimolar ratio. This stoichiometry of inhibition, one antimycin per cytochrome b, holds

for cytochrome b_T in pigeon heart [18] and beef heart [19, 20]. In *Neurospora* the antimycin/cytochrome b_T ratio is between 0.8 and 0.9, provided that CAP-concentration does not exceed 0.5 g/liter. The deviation from equimolarity at low CAP concentrations is simply the result of the use of different ϵ-values for calculation of cytochrome *b* (cf. [18]). It can be speculated that the increase of the ratio up to 2 at higher CAP concentrations results from the binding of antimycin to cytochrome b_t-apoenzyme (cf. Results) but the presence of such subunits must yet be proved.

It has been considered that a component X, arranged in the chain between cytochromes b_T and c_1, is the carrier which actually binds antimycin; the redox state of this component is supposed to be responsible for the redox changes of the *b*-type cytochromes [21, 22]. The present study does not exclude the existence of this carrier, yet it postulates that by inhibitors of mitochondrial protein synthesis, such as CAP, the biosynthesis of the component X is decreased a) in parallel to that of cytochrome b_T at low inhibitor concentrations, b) to a degree smaller than that of cytochrome b_T at high inhibitor concentrations.

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References

[1] H. Weiss, G. v. Jagow, M. Klingenberg and Th. Bücher, European J. Biochem. 14 (1970) 75.
[2] G. v. Jagow, H. Weiss and M. Klingenberg, European J. Biochem., *submitted for publication.*
[3] B.F. van Gelder, Biochem. Biophys. Acta 118 (1966) 36.
[4] W.S. Zaugg and J.S. Rieske, Biochem. Biophys. Res. Commun. 9 (1962) 213.
[5] E. Margoliash and N. Frohwirt, Biochem. J. 71 (1959) 570.
[6] A. Kröger and M. Klingenberg, Biochem Z. 344 (1966) 317.
[7] F.M. Strong, J.P. Dickie, M.E. Loomans, E.E. van Tamelen and R.S. Dewey, J. Am. Chem. Soc. 82 (1960) 1513.
[8] D.F. Wilson and P.L. Dutton, Biochem. Biophys. Res. Commun. 39 (1970) 59.
[9] B. Chance, D.F. Wilson, P.L. Dutton and M. Erecinska, Proc. Natl. Acad. Sci. U.S. 66 (1970) 1175.
[10] A.M. Pumphrey, J. Biol. Chem. 237 (1962) 2384.
[11] J.S. Rieske, Archives 145 (1971) 179.
[12] D.F. Wilson, M. Koppelmann, M. Erecinska and P.L. Dutton, Biochem. Biophys. Res. Commun. 44 (1971) 759.
[13] P.L. Dutton and B.T. Storey, Plant Physiol. 47 (1971) 282.
[14] A.M. Lambowitz, C.W. Slaymann, C.L. Slaymann and W.D. Bonner, J. Biol. Chem. 247 (1972) 1536.
[15] A. Kröger and M. Klingenberg, Vitamins and Hormones 28 (1970) 533.
[16] J. Mager, Biochim. Biophys. Acta 38 (1960) 150.
[17] G.D. Clark-Walker and A.W. Linnane, Biochem. Biophys. Res. Commun. 25 (1966) 8.
[18] J.R. Brandon, J.R. Brocklehurst and C.P. Lee, Biochemistry 11 (1972) 1150.
[19] A.J. Berden and E.C. Slater, Biochim. Biophys. Acta 216 (1970) 237.
[20] P.L. Dutton, M. Erecinska, N. Sato, Y. Mukai, M. Pring and D.F. Wilson, Biochim. Biophys. Acta 267 (1972) 15.
[21] H. Baum, J.S. Rieske, H.J. Silman and S.H. Lipton, Proc. Natl. Acad. Sci. U.S. 57 (1967) 798.
[22] M.K.F. Wikström, in: International Conference on Mechanism in Bioenergetics, Bari 1972, Acad. Press, in preparation.