A NOVEL INHIBITOR OF NADH DEHYDROGENASE IN *PARACOCCUS DENITRIFICANS*

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1. Introduction

In [1] low concentrations of Tinopal AN, (1,1-bis-(3,*N*-5-dimethylbenzoxazol-2-yl)-methine *p*-toluene sulphonate), which is present in the optical brightener Uvitex AN (Ciba-Geigy Ltd) [2], inhibited the aerobic respiration of *Paracoccus denitrificans*. Using inverted membrane vesicles of *P. denitrificans* [1], this inhibition was shown to occur in the NADH dehydrogenase (EC 1.66.99.3) region of the respiratory chain because, although respiration through segments II and III of the respiratory chain was unaffected by relatively high concentrations of the compound, it was markedly inhibited when NADH was used as the electron donor.

The sensitivity of growing cells of *P. denitrificans* to rotenone, a well-known segment I inhibitor, has been ascribed [3] to the possession of iron–sulphur centre N-2 in the NADH dehydrogenase complex of the respiratory chain. This iron–sulphur centre was absent when rotenone-insensitive cells of *P. denitrificans* were examined by electron paramagnetic resonance spectroscopy [3]. When amytal and piericidin A (two other classical inhibitors of the NADH dehydrogenase region of the respiratory chain) were compared to rotenone in beef-heart submitochondrial particles, it was concluded [4] that both amytal and piericidin A bound to the same site as rotenone, since they competed with [^{14}C]rotenone for binding at the rotenone-binding site. It was thus deduced that all 3 inhibitors share the same binding site.

We report here, by comparison between rotenone-sensitive and rotenone-insensitive cells of *P. denitrificans*, that Tinopal AN did not share the same specific site as rotenone (and also, presumably, amytal and piericidin A), and therefore provides the first example of an inhibitor of the NADH dehydrogenase region of the respiratory chain whose binding site is not associated with the iron–sulphur centre N-2.

2. Materials and methods

Paracoccus denitrificans NCIB 8944 was grown and maintained on a defined succinate–nitrate medium [5]. Respiration by washed cell suspensions was monitored in a Clark-type oxygen electrode [6].

Tinopal AN was kindly donated by Dr A. M. Paton, Division of Agricultural Bacteriology, School of Agriculture, University of Aberdeen. Stock ethanolic solutions of Tinopal and rotenone were prepared at 20 mM and 5 mM, respectively.

3. Results

Fig.1 shows the growth kinetics of *P. denitrificans* as determined by optical density measurements. Addition of 50 μM rotenone (212 nmol/mg dry wt) to early exponential-phase cells caused an immediate cessation of growth. Growth resumed after ~1.75 h at ~40% of the rate obtaining before rotenone addition, indicative of a loss of the rotenone-sensitive iron–sulphur centre N-2 [3]. This growth rate then increased, presumably due to the elevated cell concentration as growth proceeded. Similar data were obtained in [3]. Addition of 25 μM Tinopal AN, when rotenone was already present in the culture medium (fig.1a), resulted in a delay in the onset of growth inhibition; growth continued normally for ~30 min before being affected. However, no resumption of growth occurred after the addition of Tinopal AN whether rotenone was present (fig.1a) or absent (fig.1b).

To determine the effect of Tinopal AN on the

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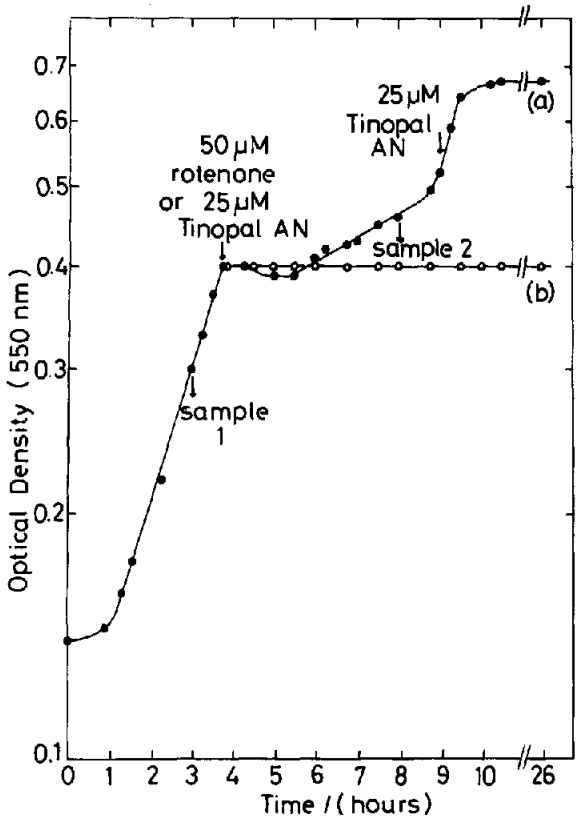

Fig.1. Growth kinetics of *Paracoccus denitrificans*. Cells from an overnight culture grown in succinate–nitrate medium [5], were inoculated into 500 ml fresh medium at 30°C and incubated aerobically. Growth was measured turbidometrically at 550 nm. Samples of 100 ml were taken from the growing culture (•——•) before and after the addition of 50 μM rotenone. Tinopal AN was then added to give 25 μM final conc. In the absence of inhibitors the final absorbance of the cultures would have exceeded 5 absorbance units. The effect of Tinopal AN on the growth kinetics in the absence of rotenone (○——○) is also shown.

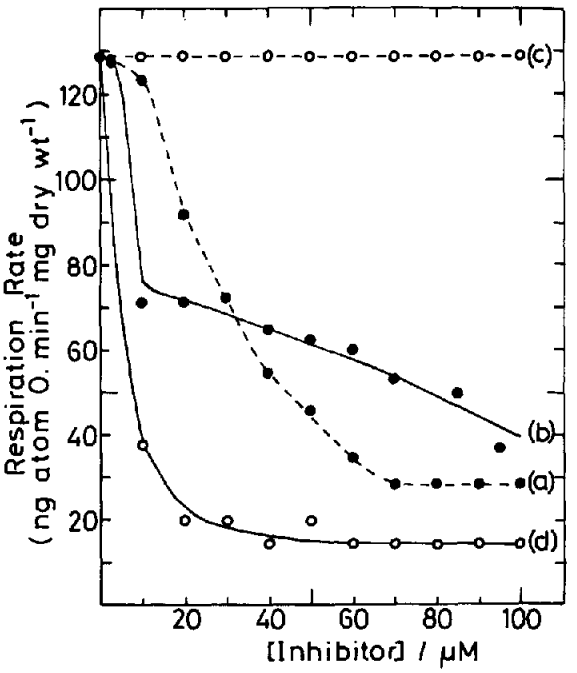

Fig.2. Effect of Tinopal AN (——) and of rotenone (– – –) on the respiration of rotenone-sensitive (•) and rotenone-insensitive (○) cells of *Paracoccus denitrificans*. Cell samples (fig.1) were harvested, washed once and resuspended in 0.1 M sodium phosphate buffer (pH 7.3). Oxygen uptake by cells (3.9 mg dry wt) from this suspension was assayed in 3 ml 0.1 M sodium phosphate buffer (pH 7.3). Tinopal AN or rotenone were added to the final concentrations indicated, and respiration was monitored as in section 2.

respiration of the rotenone-sensitive and rotenone-insensitive cells, samples were taken from the growing culture at the times indicated in fig.1. The effect on the respiration of washed cells of adding increasing concentrations of Tinopal AN or rotenone is shown in fig.2, where it is apparent (if unsurprising) that the oxygen uptake by the cells of sample 1, taken prior to rotenone addition, was rotenone-sensitive, since successive additions of rotenone at 10–70 μM elicited a rapid decrease in respiratory activity. Rotenone at >70 μM had no further effect (fig.2a). Addition of 10 μM Tinopal AN also caused a rapid decrease in the respiration rate; higher concentrations produced a more gentle decrease in respiration (fig.2b, [1]).

As expected, oxygen uptake by the cells of sample 2 (see fig.1a) was rotenone-insensitive, and rotenone at up to 100 μM (=75 nmol/mg dry wt) produced no significant inhibition of respiration (fig.2c). However, these cells still remained very sensitive to additions of Tinopal AN. On addition of 20 μM Tinopal AN respiration was almost completely inhibited. Further additions had little further effect (fig.2d). The respiration of rotenone-insensitive cells is in fact even more sensitive to Tinopal AN than that of rotenone-sensitive cells (fig.2b,d).

4. Discussion

The growth inhibition of *P. denitrificans* by rotenone in [3] was confirmed here using 50 μM rotenone

(212 nmol/mg dry wt). When growth resumed after 1.75 h it initially reached ~40% of the rate before rotenone addition (fig.1a, [3]).

To determine the sensitivity of respiration of the rotenone-sensitive and rotenone-insensitive cells to Tinopal AN or to rotenone, the respiration of washed cells was assayed in the presence of increasing concentrations of the inhibitors. The respiration of rotenone-sensitive cells could be inhibited to a maximum of ~80%, using 70 μM rotenone (54 nmol/mg dry wt) (fig.2a), indicating that there is a supply of substrates whose oxidation is unaffected by rotenone. In [3] 30 μM rotenone also produced a maximal inhibition of ~70%. The effect of Tinopal on the rotenone-sensitive cells agrees with [1].

Rotenone-insensitive cells, whose respiration rate was unaffected by the addition of up to 100 μM rotenone (75 nmol/mg dry wt) (fig.2) due to a loss of the sensitive iron–sulphur centre N-2 [3], were still extremely sensitive to Tinopal AN (fig.2d). The sensitivity of these cells to Tinopal AN clearly indicates that the 2 inhibitors do not share the same binding site. Indeed, Tinopal AN was a significantly more potent respiratory inhibitor in these cells than in rotenone-sensitive cells (fig.2b,d). The increased sensitivity towards Tinopal AN of the rotenone-insensitive cells is most simply ascribed to an easier accessibility of the compound to its site of respiratory inhibition in the absence of iron–sulphur centre N-2 in the NADH dehydrogenase complex.

The delayed inhibition of growth of rotenone-insensitive *P. denitrificans* upon addition of Tinopal AN (fig.1a) may be ascribed to an interaction (observed by UV spectroscopy) between the 2 inhibitors in the aqueous medium (unpublished).

We conclude that during respiratory inhibition, Tinopal AN does not act at the same site as rotenone (or, by implication, piericidin A and amytal), and that removal of the rotenone-sensitive binding site may render the Tinopal AN binding site more accessible to Tinopal AN. Tinopal AN could be a useful tool in the study of the molecular constitution of the NADH dehydrogenase complex.

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References

[1] Phillips, M. K. and Kell, D. B. (1981) FEMS Microbiol. Lett. 11, 111–113.
[2] Paton, A. M. and Jones, S. M. (1971) in: Methods in Microbiology (Norris, J. R. and Ribbons, D. W. eds) vol. 5A, pp. 135–144, Academic Press, London.
[3] Meijer, E. M., Schuitenmaker, M. G., Boogerd, F. C., Wever, R. and Stouthamer, A. H. (1978) Arch. Microbiol. 119, 119–127.
[4] Horgan, D. J. and Singer, T. P. (1968) J. Biol. Chem. 243, 834–843.
[5] McCarthy, J. E. G., Ferguson, S. J. and Kell, D. B. (1981) Biochem. J. 196, 311–321.
[6] Kell, D. B., John, P. and Ferguson, S. J. (1978) Biochem. J. 174, 257–266.