

Reduction of 2,3,5-triphenyltetrazolium chloride by the KCN-insensitive, salicylhydroxamic acid-sensitive alternative respiratory pathway of mitochondria from cultured grapevine cells

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ABSTRACT

The reduction of 2,3,5-triphenyltetrazolium chloride (TTC) by grapevine cells cultured in suspension was studied in order to assess the reliability of using TTC reduction as a measure of cell viability. Similar to the reduction observed in animal cells, TTC can be reduced in grapevine cells by the cytochrome respiratory path of the mitochondria, although it is mostly reduced (about 72 %) by the alternative respiratory path sensitive to salicylhydroxamic acid. Engagement of the alternative path in TTC reduction was calculated through the pV_{11} plot, and was established to be 89 %.

Abbreviations: SHAM, salicylhydroxamic acid; TTC, 2,3,5-triphenyltetrazolium chloride

INTRODUCTION

Cyanide resistant respiration is a characteristic of mitochondria isolated from a wide range of plant tissues (Laties 1982). It results from the transfer of electrons to O_2 via an alternative pathway branching from the cytochrome path at the ubiquinone level (Laties 1982). As the phosphorylation sites of the cytochrome path are bypassed, operation of the alternative path is energetically inefficient and a knowledge of the activities of the alternative and cytochrome paths in vivo is necessary to evaluate the energy available for metabolic processes (Lambers 1982).

In plant respiratory studies, it is generally assumed that the cytochrome path is preferentially used and that the alternative pathway only participates in respiration when the cytochrome path is saturated or inhibited (Lambers 1982). On the basis of this assumption, the activity of the cytochrome and of the alternative path are often measured (Day et al. 1980) by the addition to respiring mitochondria of salicylhydroxamic acid (SHAM), a specific inhibitor of the alternative

pathway (Schonbaum et al. 1971). Thus, the capacity of the alternative pathway disappears after the addition of hydroxamic acid, and it is measured as the hydroxamic acid sensitive part of total KCN non-inhibited respiration (Day et al. 1980).

Tetrazolium salts (i.e. 2,3,5-triphenyltetrazolium chloride, TTC) are well-known compounds with high redox potentials which can be inserted into the mitochondrial respiratory chain and are often reduced to deeply coloured formazans (Möllering et al. 1974). Due to this property, they have been frequently used to determine the viability of cultured cells (see Yokohama et al. 1990). They have the advantage over other methods used to determine cell viability that the amount of the end product (the formazan) can be determined spectrophotometrically. We found that this method is less subjective than counting individual cells, which are frequently difficult to distinguish by microscopic observation, after vital staining (Huang et al. 1986).

In animal cells, most tetrazolium salts are reduced in the cytochrome respiratory chain before the antimycin block, although TTC appears to be reduced after it (Möllering et al. 1974). However, to the best of our knowledge, there are no reports for plant cells on the site of TTC reduction in the two respiratory chains of mitochondria.

In this report we examine the effects of various respiratory chain inhibitors on the capability to reduce TTC by grapevine mitochondria in order to assess the reliability of using TTC reduction as a measure of cell viability. Evidence is presented which suggests that TTC is reduced by the KCN-insensitive SHAM-sensitive alternative pathway of the respiratory chain of grapevine mitochondria.

MATERIAL AND METHODS

Cell cultures

Callus was obtained from immature pericarp tissue of

Monastrell grapes (*Vitis vinifera* L. cv. Monastrell) of approx. 5 mm diameter, and cultured in Gamborg's B5 medium (Gamborg et al. 1968), supplemented with casein hydrolysate (250 mg/L), kinetin (1.0 μ M), α -naphthalene acetic acid (0.5 μ M) and 2 % (w/v) sucrose.

Cultures were maintained in the dark and subcultured at approximately three-week intervals. Starting from friable callus, grape cells were cultured in suspension in the above described culture medium in 100 mL flasks with orbital shaking (125/min). Suspension cultured cells were grown 25°C in darkness.

TTC reduction assay

To study the ability of cells to reduce TTC, about 0.4 g of fresh cells were suspended in 10 mL of 50 mM K-phosphate buffer (pH 7.0) containing 15 mM TTC. After incubation overnight in the dark at 25°C, about 0.1 g of cells were recovered by filtration on GF/A fibre glass filters. The formazan was extracted by heating in 6 mL of 95 % ethanol at 60°C for 15 min. The formazan was quantified by measurement of absorbance at 485 nm. Conversion of the absorbance values to μ moles of TTC reduced was carried out by calibration with an external standard obtained by reduction of TTC in water with an excess of sodium dithionite.

The effect of the following inhibitors on the TTC reduction assay was monitored: i) KCN and antimycin A (from a 200 μ M stock in methanol) as well known inhibitors of the cytochrome pathway of respiration, and ii) salicylhydroxamic acid (from a 1.75 M stock in 2-methoxyethanol) as inhibitor of the cyanide-resistant alternative respiratory pathway (Schonbaum et al. 1971). Solvents, at the concentration used, had no effect on the TTC reduction assay. Inhibitors were added 45 min before the addition of TTC.

RESULTS AND DISCUSSION

Optimum pH and localization of the TTC reduction product in grapevine cells

Reduction of TTC by grapevine cells shows a strong dependence on external pH, having an optimal value at pH 7.0 (Fig. 1). At this pH, reduction of TTC was almost totally enzymatic since it was lowered by 95 % after heating (100°C for 5 min). Linearity with respect to the cell fresh weight was reached under our assay conditions up to values of 35 μ mol of TTC reduced after 16 h of incubation at 25°C.

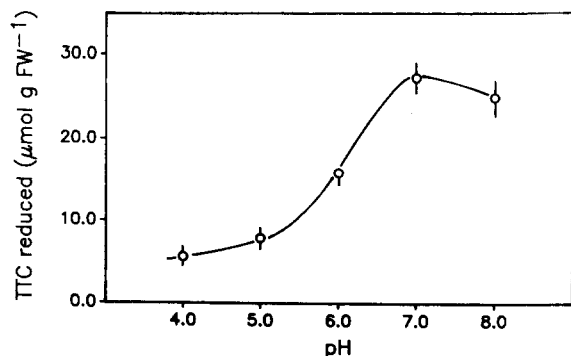


Figure 1. Effect of the pH of the 50 mM K-phosphate incubation buffer on the capability of grapevine cells to reduce TTC. Bars show SE (n=3).

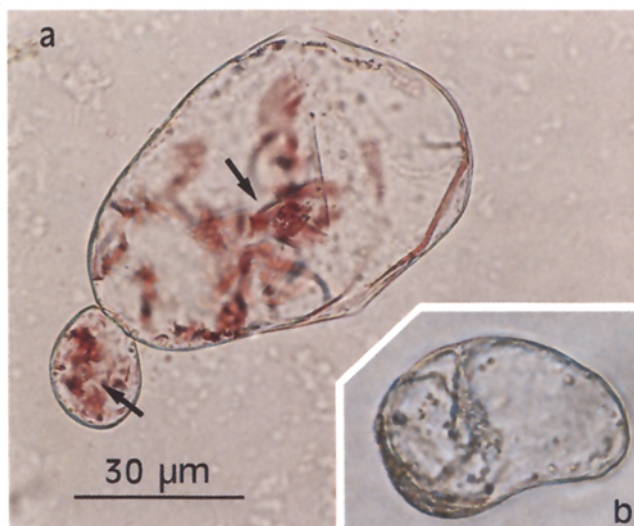


Figure 2. Light micrograph of grapevine cells stained with TTC for 16 h at 25°C (a) showing the presence of deeply coloured red crystal-like structures inside the cells (arrows) and b) view of a non-stained cell.

Localization of the TTC reduction product in cultured cells was carried out by observation of stained cells by light microscopy. Results shown in Fig. 2 illustrate the presence of large deeply coloured red crystal-like structures inside the cells, absent in both the cell wall and the plasmalemma.

Titration of the TTC reduction with KCN and SHAM

Preliminary inhibition studies (Table I) showed that cyanide (0.1 mM) inhibited TTC reduction by 15 %, while antimycin A (2 μ M) and SHAM (1 mM) had little inhibitory effects. However, when cyanide and SHAM were present both, TTC reduction was inhibited by 49 % (Table I). This effect was hardly significant in the presence of antimycin A, in which case an inhibition of 56 % was attained (Table I).

From these results, and in accordance with Day et al. (1980), it can be concluded that TTC reduction in grapevine cells can be mediated by both mitochondrial respiratory pathways, and that the cyanide-resistant pathway remains less active until the cytochrome system is inhibited. Under such circumstances, cyanide and SHAM together exhibit greater inhibitory activity than either inhibitor alone (Table I).

Day et al. (1980) stated that the maximum capacity of the alternative path (V_{alt}) in mitochondria can be calculated by measuring electron transport in the presence of cyanide. In order to calculate V_{alt} , grapevine cells were titrated with KCN in the absence of SHAM. The results shown in Fig. 3 illustrate that, although at lower concentrations (0.05 mM) KCN

stimulated (around 15 %) TTC reduction, a saturable inhibition of the cytochrome path was reached with KCN concentrations in the order of 0.1 mM. For this KCN concentration, the inhibition was established at about 20 %. Thus, 0.1 mM KCN, which totally inhibits the cytochrome path (Bingham and Farrar 1987), causes electrons to be diverted down the uninhibited alternative path, and no further inhibition in the TTC reduction can be observed. Under these circumstances, the maximum capacity of the alternative path (V_{alt}) was estimated to be 80 % of the total capability of grapevine cells to reduce TTC, or 20.2 μ moles TTC reduced/g fresh weight.

Table I. Effect of KCN (0.1 mM), antimycin A (2 μ M) and SHAM (1 mM), applied alone or together, on the capability of grapevine cells to reduce TTC at 25°C.

Inhibitor(s)	TTC reduction (%)		
	Mean	SE	n
---	100	4	8
KCN	85	5	8
Antimycin A	93	7	7
KCN + Antimycin A	92	1	4
SHAM	91	6	8
SHAM + Antimycin A	90	5	4
SHAM + KCN	51	6	4
SHAM + KCN + Antimycin A	44	5	7

Contrary to the alternative path, maximum cytochrome flux (V_{cyt}) can be obtained both in the presence and absence of fully inhibitory levels of hydroxamic acids (Day et al. 1980). For this reason, TTC reduction was also titrated at various concentrations of SHAM in the presence and absence of 0.1 mM KCN. In the absence of KCN, SHAM had no significant influence on TTC reduction at 1 mM (Fig. 4A), but above this level a substantial inhibition was observed. Thus, 25 mM SHAM inhibited TTC reduction by 65 % (Fig. 4A).

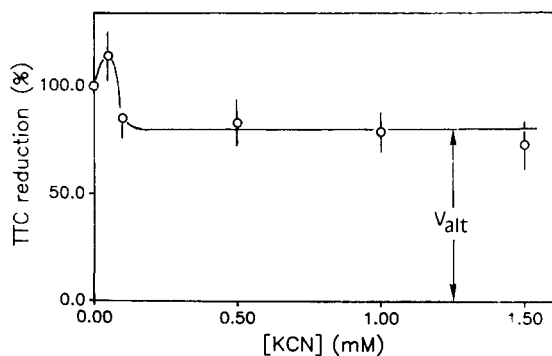


Figure 3. Effect of increasing KCN concentrations on the capability of grapevine cells to reduce TTC at 25°C. Bars show SE (n=4).

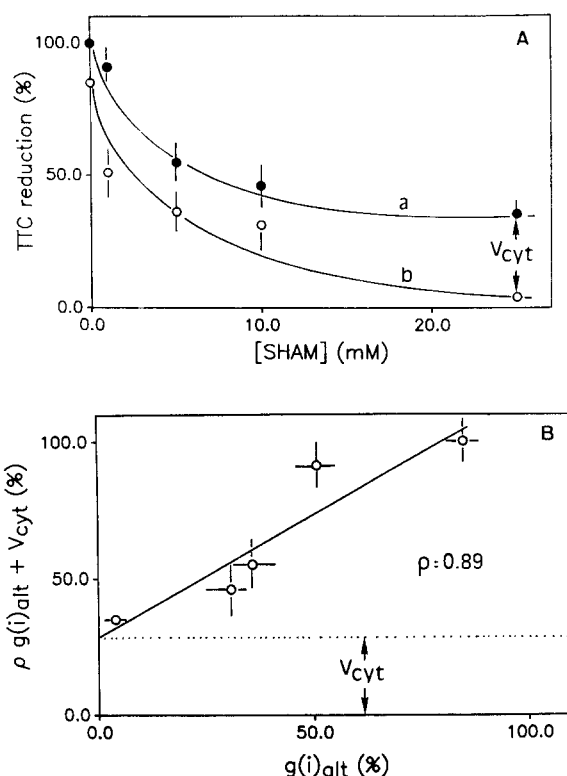


Figure 4. A) Effect of increasing SHAM concentrations on the capability of grapevine cells to reduce TTC in the absence (a) and the presence (b) of 0.1 mM KCN. Bars show SE (n=4-10). B) ρV_{alt} plot of data from A. To obtain the ρV_{alt} plot, the values of curve a [$\rho g(i)_{alt} + V_{cyt}$] in A were plotted against the values of curve b [$g(i)_{alt}$] in A.

Unlike observations in the absence of KCN, concentrations of 1 mM SHAM in the presence of 0.1 mM KCN, inhibited TTC reduction by 49 %, reaching 95 % inhibition at 25 mM SHAM (Fig. 4A).

No residual TTC reduction was observed for saturating concentrations of KCN and SHAM (Fig. 4A).

From these titration curves, the contribution of the cytochrome path to total TTC reduction by grapevine mitochondria was calculated to be 28 % and the maximum cytochrome flux (V_{cyt}) was determined at 7.1 μ moles TTC reduced/g fresh weight.

In the O_2 -consuming respiratory process, involvement of the alternative pathway in planta is often calculated from plots of the respiration rates in the presence of SHAM but absence of KCN (i.e. curve a in Fig. 4A) against the respiration rate in the presence of both SHAM and KCN (i.e. curve b in Fig. 4A). This constitutes the so-called ρV_{alt} plot (Møller et al. 1988), introduced by Bahr and Bonner (1973).

Construction of this plot from data shown in Fig. 4A provides a straight line (Fig. 4B, $r=0.928$, statistically significant at $p<0.05$), where the slope represents the engagement (ρ) of the alternative pathway (Møller et al. 1988).

The value calculated for ρ was 0.89, which suggests that the alternative pathway is almost totally engaged in TTC reduction by grapevine mitochondria. For this value, the predicted value for V_o was $V_o = \rho V_{alt} + V_{vt} = 25.1$, which is in good accordance with the experimental value ($V_o = 25.3$ μ moles TTC reduced/g fresh weight).

From these titration curves it can be concluded that, in the absence of inhibitors, reduction of TTC by the cytochrome path accounts for about 28 % (V_{cyt}) of the total, while this is mostly reduced by 72 % (ρV_{alt}) by the KCN-insensitive SHAM-sensitive alternative path of grapevine mitochondria.

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