Manipulating cytoplasmic pH under anoxia: A critical test of the role of pH in the switch from aerobic to anaerobic metabolism

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Abstract. Ethanol production by maize (Zea mays L.) root tips, measured by an enzymic assay of the suspending medium, was correlated with changes in the cytoplasmic pH, determined by in-vivo $^{31}$P nuclear magnetic resonance (NMR) spectroscopy, following the onset of anoxia. Strong evidence for the role of the cytoplasmic pH in triggering the switch to ethanol production under anoxia was obtained by: (i) varying the pH of the suspending medium between pH 4 and pH 10; and (ii) using the permeant weak base methylamine to combat the acidification of the cytoplasm induced by the anoxic conditions. Experimentally, it proved to be much easier to manipulate the cytoplasmic pH under anoxia after the pH had stabilised, rather than during the initial rapid acidification that occurred following the onset of anoxia, and in the presence of methylamine, it was possible to impose a normal aerobic cytoplasmic pH value on tissue that was metabolising anaerobically. By this means it was possible to demonstrate the reversibility of the pH effect on ethanol production under anoxia and thus to provide good evidence in support of the biochemical pH-stat model of the anoxic response. The NMR measurement of the cytoplasmic pH in the presence of methylamine was achieved by using a manganese pretreatment technique to eliminate interference between the cytoplasmic and vacuolar P$_i$ signals, and it seems likely that the experimental approach used here will have further applications in studies of the metabolic response to anoxia.

Key words: Anoxia – Biochemical pH-stat – Cytoplasmic pH – Ethanol production – Pyruvate decarboxylase – Zea mays

Introduction

Plants vary enormously in their susceptibility to oxygen deprivation (Drew 1990; Crawford 1992) and the poorly aerated conditions that arise through waterlogging, ice formation and compaction of the soil can be a major influence on the distribution of plant species and on the growth and survival of crop plants (Jackson et al. 1991). Retaining control over the cytoplasmic pH appears to be an important factor in determining the survival of a plant tissue under anoxia and it is thought that the initial fall in pH that occurs with the onset of anoxia simultaneously inhibits lactate dehydrogenase (EC 1.1.1.27) and activates pyruvate decarboxylase (EC 4.1.1.1) allowing a switch from the production of lactate to ethanol as the fermentation end product (Davies et al. 1974). The response of pyruvate decarboxylase to pH is consistent with this model (Morrell et al. 1990) and direct experimental evidence in favour of the proposed sequence of events has been obtained using in-vivo nuclear magnetic resonance (NMR) spectroscopy (Roberts et al. 1984a,b, 1985). Thus working with excised maize root tips, and using $^{31}$P NMR to monitor cytoplasmic pH non-invasively, it was concluded: (i) that a fall in cytoplasmic pH under hypoxia triggered the switch from lactate to ethanol production (Roberts et al. 1984a); and (ii) that cytoplasmic acidosis caused cell death and could be used as an indicator of flooding intolerance (Roberts et al. 1984b, 1985). However, despite the experimental evidence in favour of the biochemical pH-stat model, there are at least two major shortcomings in the description of the origin and significance of the cytoplasmic pH changes that occur under anoxia.

Firstly, it is unclear how far the pH must fall to trigger the ethanolic fermentation. Thus published NMR data for maize root tips (Roberts et al. 1984a) and maize root segments (Fan et al. 1988) show falls in pH to 6.8 and 7.15 respectively and it is not clear whether this is a reflection of the underlying metabolic activity of the tissues or whether the pH threshold differs in the two tissues. Similarly ethanol production in rice shoots (Menegus et al.
1991) appears to be associated with a considerably smaller decrease in cytoplasmic pH than that observed in maize root tips. Moreover, in several tissues the cytoplasmic pH apparently passes through a minimum following the onset of hypoxia (Menegus et al. 1991; Saint-Ges et al. 1991; Roberts et al. 1992) and the implications of the partial recovery in pH for the continuation of the ethanolic fermentation have not been addressed.

Secondly, the close correlation between the fall in cytoplasmic pH, the production of lactate and the switch to ethanol observed in the early NMR experiments (Roberts et al. 1984a) has been weakened by subsequent work. For example, experiments on maize root tips showed that the fall in cytoplasmic pH must be partly offset by the metabolic consumption of protons and that it was therefore incorrect to consider the observed pH change in terms of lactate production alone (Reid et al. 1985). More recent work on the same tissue (Saint-Ges et al. 1991) has shown that lactate continues to accumulate after the cytoplasmic pH has fallen, in contrast to the original data (Roberts et al. 1984a), and in rice shoots the fall in pH during the onset of anoxia occurred despite negligible (Menegus et al. 1991; Rivoal et al. 1991) or at best, limited (Menegus et al. 1989) production of lactate.

As a first step towards a critical reappraisal of the biochemical pH-stat model, we have developed a novel experimental procedure that allows the cytoplasmic pH to be manipulated independently of the oxygen supply to the tissue. A key observation in the early NMR work on plant anoxia was that the switch to the production of ethanol could be brought forward by lowering the cytoplasmic pH with acetic acid (Roberts et al. 1984a). The converse of this experiment, which has apparently not been attempted before, would be to observe the development of the hypoxic response under conditions in which the cytoplasmic pH is maintained at a high level, either by exposure to a permeant weak base or by exposure to a high external pH. This approach could provide a critical test of the pH model by providing further evidence in-vivo for the physiological significance of the pH sensitivity of lactate dehydrogenase and pyruvate decarboxylase. Thus, if ethanol production were prevented at high cytoplasmic pH under anoxia, the pH model would be supported; whereas if the switch to ethanol production still occurred, then the idea that pH acts as a signal for the switch from aerobic to anaerobic metabolism would be seriously weakened. Accordingly, this paper describes experiments in which the aim was either to prevent or at least retard the usual fall in pH under anoxic conditions and then to correlate the production of ethanol with the resulting time dependence of the cytoplasmic pH. The results provide strong support for the critical role of pH in the switch from lactate to ethanol by showing that ethanol production ceases as the cytoplasmic pH returns to a normal aerobic value under anoxia.

### Materials and methods

**Plant material.** Maize seeds (Zea mays L., cv. LG11; Force Lima-grain, Cambridge, U.K.) were washed with tap water for 30 min and germinated in the dark between sheets of absorbent paper soaked in 0.1 mM CaSO 4 at 21°C. After 3 d germination, 150 5-mm root tips were cut into approximately 50 ml of a continuously aerated medium containing either 10 mM 2-(N-morpholino)-ethane sulphonic acid (Mes), 0.1 mM CaSO 4, at either pH 4.0 or 6.0, or 10 mM 3-(cyclohexylamino)-1-propane sulphonic acid (Caps), 0.1 mM CaSO 4 at pH 10.0.

For some experiments it was necessary to pretreat the tissue with manganese to reduce the intensity of the vascular inorganic phosphate (Pi) signal, and this was done using a procedure similar to that described by Quiquampoix et al. (1993). The seedlings were aerated in 5-l tanks containing 10 mM Mes, 0.1 mM CaSO 4, 1 mM MnSO 4·4H 2O at pH 6.0 for 1 h and then transferred to tanks containing aerated 10 mM Mes, 0.1 mM CaSO 4 at pH 6.0 for 3 h; 150 5-mm root tips were then cut into the desired medium.

After cutting, the tissue was transferred into 6 ml of medium in a 10-mm-diameter NMR tube where it was oxygenated using an air-lift system operating with an oxygen gas flow rate of 30 ml·min -1 (Fox et al. 1989). In addition, oxygenated medium was circulated through the tube at 1 ml·min -1 to maintain a fixed external pH. The tissue was allowed to stabilise in the NMR tube for 30 min at 21°C before starting the NMR experiment. Spectra were acquired for 1 h before anoxia was induced by switching the gas supply from oxygen to nitrogen.

**In-vivo 31P nuclear magnetic resonance spectroscopy.** The 31P NMR spectra were recorded at 121.49 MHz on a Bruker, Rheinstetten, Germany) CXP300 spectrometer with an Oxford Instruments (Oxford, UK) 7.05 T superconducting magnet using a double-tuned 10 mm-diameter probehead. 1H-decoupled 31P NMR spectra were acquired with a 45° pulse angle, a recycle time of 0.5 s and a total acquisition time of either 5 or 30 min. Chemical shifts were measured relative to the signal from a capillary containing a 2% (v/v) aqueous solution of the tetraethyl ester of methylene diphosphonic acid and are quoted relative to the resonance at 0 ppm from 85% orthophosphoric acid (Kime et al. 1982a). Cytoplasmic pH values were determined as described previously (Fox and Ratcliffe 1990). As discussed elsewhere (Roberts et al. 1981), changes in pH can be measured more accurately than absolute pH values and changes of 0.05 pH units can be measured reliably by the method used here. In contrast, the absolute values of pH are probably only accurate to within 0.1-0.2 pH units.

**Ethanol measurements.** Ethanol in the effluent from the NMR tube was measured by the enzymic method described elsewhere (Bernt and Gutmann 1974). The effluent was collected in 1-ml fractions, and each quoted measurement is the mean value for three consecutive fractions collected over a period of 3 min. The time resolution in the ethanol-production curves was further reduced by the dilution effect caused by the release of the ethanol into the 6-ml volume of the suspending medium within the NMR tube. Accordingly, there was an intrinsic lag of several minutes between the pH measurements and the corresponding ethanol determinations.

**Respiration rate and oxygen content measurements.** Thirty root tips or root segments were cut and placed in 50 ml of continuously aerated medium. After 1.5 h the tissue was placed in 5 ml of the same medium in the chamber of a Rank oxygen electrode. Oxygen consumption was measured three times over 10-min periods.

To determine the amount of oxygen available to the roots in the NMR tube, the whole air-lifting and circulating system was reconstructed and fixed into the chamber of the oxygen electrode. When the air-lift system was used without any net flow of the suspending medium through the NMR tube, the oxygen content fell from 1.36 to less than 0.01 µmol·ml -1 on switching from oxygen to nitrogen. However, when nitrogenated medium was circulated through the NMR tube at 4 ml·min -1, in the absence of air-lifting, the oxygen content was as high as 0.22 µmol·ml -1, indicating that oxygen was entering the medium either through the tubing or the static air pumps. When the airlift was combined with circulation, a satisfactorily low level of oxygen (less than 0.02 µmol·ml -1) could only be