Oxygen consumption by purified plasmalemma vesicles from wheat roots

Stimulation by NADH and salicylhydroxamic acid (SHAM)

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Received 4 September 1985; revised version received 14 October 1985

Plasmalemma vesicles from wheat (*Triticum aestivum* L.) roots consumed O_2 and the addition of 1 mM NADH increased the rate ~ 3-fold (to 15–30 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$). The NADH-dependent O_2 uptake was abolished by catalase. In the presence of salicylhydroxamic acid (SHAM), an inhibitor of the alternative oxidase pathway in plant mitochondria, NADH-dependent O_2 consumption was stimulated 10–20-fold (to 200–400 nmol $\cdot mg^{-1} \cdot min^{-1}$). Catalase also abolished this stimulation, which was KCN-sensitive but antimycin A-insensitive, and the production of H_2O_2 during SHAM-stimulated NADH-dependent O_2 uptake was demonstrated. Irrespective of the mechanism, SHAM-stimulated respiration by root plasmalemma makes it difficult to interpret results on root respiration obtained using KCN and SHAM.

NADH *Plasmalemma* *Respiration* *(Wheat root)* *Salicylhydroxamic acid*

1. INTRODUCTION

The plasmalemma of higher plant cells appears to contain a redox system. Exogenous NADH (and in some cases NADPH) is oxidized by intact roots [1–4], whole cells [5] and protoplasts [1,6] with a concomitant increase in O_2 uptake. In the absence of added NAD(P)H, external ferricyanide is reduced by protoplasts and intact roots presumably with cytoplasmic NADPH as the electron donor [4,7]. The latter reaction may provide dicotyledonous plants with a mechanism for iron uptake (review [7]). Since both exogenous and endogenous NAD(P)H can act as electron donors, it has been suggested that the plasmalemma contains 2 separate NAD(P)H dehydrogenases [1,2].

The purpose of this study was to see if NADH-dependent O_2 consumption could be detected in a purified preparation of plasmalemma vesicles isolated from wheat roots and to characterize its properties.

Abbreviation: SHAM, salicylhydroxamic acid

2. MATERIALS AND METHODS

Wheat (*Triticum aestivum* L. cv. Drabant) was grown as in [8] and purified plasmalemma vesicles were isolated from 7-day-old roots as described in [9] modified as in [10].

Mg^{2+}-dependent ATPase activity was assayed ± 0.05% Triton X-100 and sidedness of vesicles calculated assuming that the ATPase active site is on the cytoplasmic side of the plasmalemma only [11]. The vesicles were 81% right-side-out according to this criterion.

Cytochrome *c* oxidase activity was measured as the rate of oxidation of reduced cytochrome *c* in the presence of 0.04% (w/v) Triton X-100 [12]. It was 45 ± 17 nmol cytochrome $c \cdot (mg\ protein)^{-1} \cdot min^{-1}$ for 6 independent preparations of

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

plasmalemma. Since the activity of cytochrome *c* oxidase is normally in excess of the activity of the rest of the mitochondrial respiratory chain (e.g. [13]) this indicates that the maximum rate of NADH oxidation due to mitochondrial contamination would be considerably less than 10 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$.

O_2 consumption by the plasmalemma vesicles was measured at room temperature (21–23°C) in a Rank Brothers (Cambridge, England) electrode with a total volume of 1.0 ml. The rate of O_2 consumption was calculated assuming an O_2 concentration of 270 μM [14]. The medium contained 0.25 M sucrose, 10 mM K^+-Mes, pH 6.0, and the appropriate amount of sample. Where indicated, NADH was added to a final concentration of ~1 mM. The concentration of NADH in the stock solution was determined with lactate dehydrogenase (EC 1.1.1.27) and pyruvate.

Lactate dehydrogenase, Triton X-100, catalase, SHAM and antimycin A were from Sigma and NADH was from Boehringer.

3. RESULTS

In the absence of added substrate, O_2 consumption was observed in the presence of plasmalemma vesicles. The addition of NADH increased the rate of O_2 consumption about 3-fold (fig.1a) and this rate was only ~20% inhibited by antimycin A (not shown). Catalase inhibited NADH-dependent O_2 uptake strongly (fig.1b) and catalase added before NADH prevented the stimulation in O_2 uptake caused by NADH (fig.1c). This indicates that H_2O_2 is somehow involved in the reaction. It should be noted that there was little or no release of O_2 when catalase was added (fig.1b,c) and that H_2O_2 was therefore not present at appreciable concentrations.

The consumption of O_2 by the plasmalemma vesicles was proportional to the amount of sample up to at least 0.45 $mg \cdot ml^{-1}$ in both the absence and presence of NADH (fig.2). The lines intersect the *y*-axis around 0.5 nmol min^{-1} which is the consumption of O_2 by the electrode itself.

The NADH-dependent O_2 uptake was inhibited by 1 mM KCN (not shown). However, when SHAM, an inhibitor of the alternative oxidase pathway in plant mitochondria [15], was added, a very rapid rate of O_2 consumption followed (fig.3),

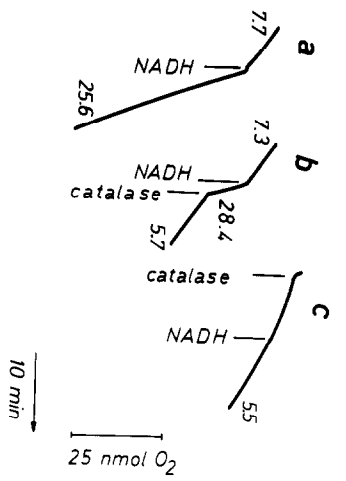

Fig.1. Oxygen consumption by plasmalemma vesicles from wheat roots and the effect of NADH (final concentration 0.88 mM) and catalase (50 $\mu g \cdot ml^{-1}$). Vesicles (0.28 mg protein $\cdot ml^{-1}$) which had been stored in liquid N_2 were used but similar results were observed on freshly prepared vesicles.

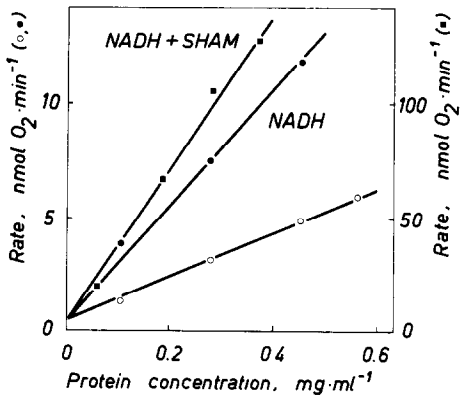

Fig.2. Dependence of rate of O_2 consumption upon amount of plasmalemma vesicles from wheat roots. The rate was measured without NADH (○), after the addition of 0.84 mM NADH (●) and after the addition of 0.84 mM NADH in the presence of 2 mM SHAM (■). The lines were fitted by eye and the numbers indicate the slopes in nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$. Note the 2 different scales on the *y*-axis. Freshly prepared vesicles were used.

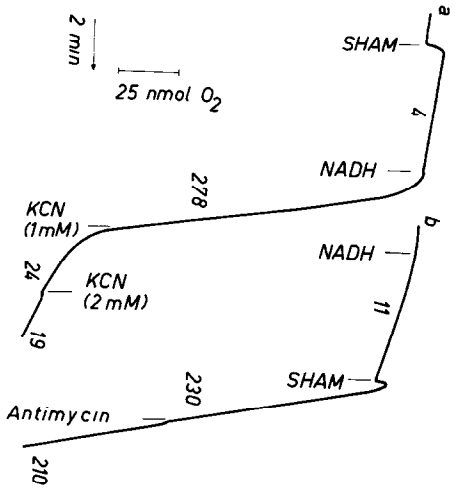

Fig.3. Effect of sequence of addition of NADH and SHAM on rate of O_2 consumption by wheat root plasmalemma vesicles. NADH (0.86 mM), SHAM (2 mM), KCN (1.0 and 3.0 mM) and antimycin A (0.9 μM) were added to 0.30 mg·ml^{-1} vesicles where indicated. Numbers on traces indicate rates in nmol O_2·mg^{-1}·min^{-1}. Freshly prepared vesicles were used.

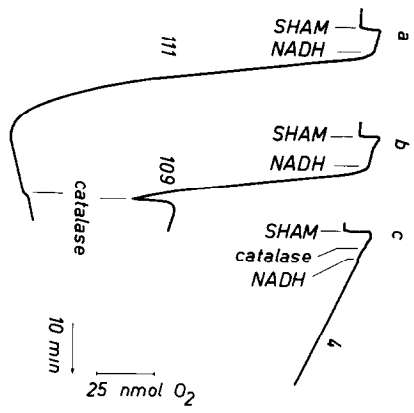

Fig.4. Stoichiometry and product formation during oxidation of NADH by wheat root plasmalemma vesicles in the presence of SHAM. SHAM (2 mM), a limiting amount of NADH (0.30 mM) and catalase (50 μg·ml^{-1}) were added where indicated. Freshly prepared vesicles (0.19 mg·ml^{-1} in a,b and 0.28 mg·ml^{-1} in c) were used.

but only in the presence of NADH. SHAM by itself had no stimulatory effect on O_2 consumption (fig.3a). Similar results (not shown) were obtained with *m*-chlorobenzhydroxamic acid, an analogue of SHAM and also inhibitor of the alternative oxidase [15]. The SHAM-stimulated, NADH-dependent O_2 consumption was proportional to the amount of sample (fig.2) and it was >90% inhibited by 1 mM KCN (fig.3a), but unaffected by 0.9 μM antimycin A (fig.3b).

When a limited amount of NADH (300 nmol) was added to plasmalemma vesicles in the presence of SHAM, the O_2 consumption eventually stopped and the O_2 concentration actually increased slightly. The total O_2 consumption was 146 nmol in fig.4a, corresponding to a 2.1:1 ratio of NADH:O_2. More detailed experiments confirmed that the ratio was 2:1 (not shown). When catalase was added after about 20 min, only a minute release of O_2 was observed (fig.4a). However, catalase added during the rapid phase of O_2 consumption caused a significant release of O_2 (fig.4b). Little or no O_2 consumption was observed after the addition of catalase (fig.4b) although the NADH had not been exhausted (cf. fig.4a and b).

When catalase was added before NADH (in the presence of SHAM) no stimulation of O_2 uptake was observed upon NADH addition (fig.4c). Note that the rate of oxidation at 0.3 mM NADH (fig.4a,b) was less than half that at 0.86 mM NADH (fig.3) indicating a K_m(NADH) above 1 mM.

4. DISCUSSION

The plasmalemma vesicles used here are purified and predominantly right-side-out (see section 2). Since NADH does not cross the plasmalemma of intact protoplasts [1], the oxidation of NADH in our experiments probably took place on the outer surface of the vesicles which would be the side facing the apoplastic space in vivo. Consistent with this conclusion is the observation that 0.025% (w/v) Triton X-100 inhibited O_2 consumption slightly (not shown). A stimulation would have been expected if the activity observed before Triton addition were due only to the 20% inside-out or leaky vesicles present in our plasmalemma preparation (see section 2). A possible source of apoplastic NADH in vivo could be the malate dehydrogenase bound to the cell wall [16].

Mitochondrial contamination was not large enough to account for more than a fraction of the

NADH-dependent O_2 consumption by purified plasmalemma vesicles (see section 2). Furthermore, plasmalemma respiration was only inhibited 20% by antimycin A, which would be expected to inhibit NADH oxidation by wheat root mitochondria completely [17]. Our results confirm that previous observations of NADH-stimulated O_2 consumption by protoplasts [1,6], whole cells [5] and intact roots [1–4] were caused by a redox system present in the plasmalemma.

Oxygen was the terminal acceptor for the electrons from NADH and no H_2O_2 formation was detected in the absence of SHAM (fig.1b). However, addition of excess catalase totally abolished the NADH-stimulated O_2 consumption (fig.1b,c) and H_2O_2 may therefore be involved in the reaction. Its degree and mode of involvement is difficult to assess since the preparations contained some endogenous catalase activity as indicated by the results in fig.4. Judging from the results in [5] water was also the final product of NADH oxidation by carrot cells.

The very strong stimulation of NADH-dependent O_2 consumption by SHAM (fig.3) was a surprise although the results of Pupillo and De Luca [18] indicated that SHAM stimulates duroquinone-dependent NADH oxidation by cucumber plasmalemma. In our system, the addition of duroquinone is not required. H_2O_2 was formed during the SHAM-stimulated, NADH-dependent O_2-consumption and, as in the absence of SHAM, the addition of excess catalase completely inhibited any further O_2 consumption (fig.4) again implying a role for H_2O_2. We need to know more about the stoichiometry of the reaction to be able to draw any firm conclusions about the enzyme system involved and the precise mechanism. However, SHAM may interact with a P420/450 system in the plasmalemma vesicles and enhance its turnover. Such a mechanism would explain the inhibition by SHAM of the photoreduction of a membrane-bound *b*-type cytochrome [19] probably identical to the P420/450 system identified in purified plasmalemma vesicles by Kjellbom et al. [20].

To estimate the degree of engagement of the mitochondrial alternative oxidase pathway in whole roots, the respiration (O_2 uptake) of the roots is measured in the presence of a series of SHAM concentrations in either the absence or presence of KCN. In the subsequent calculations it is assumed that only mitochondrial O_2 consumption is sensitive to the inhibitors (reviews [17,21]). The presence of a very rapid SHAM-stimulated, KCN-sensitive O_2 consumption associated with plasmalemma vesicles from roots (fig.3) indicates that the above approach may yield misleading results. The degree of the error depends on the relative size in vivo of the SHAM-stimulated O_2 uptake by the plasmalemma and the reduction in O_2 consumption caused by SHAM inhibition of the alternative oxidase in the mitochondria. At least in one case, SHAM has been observed to stimulate the respiration of intact roots [22] and this effect may have been due to O_2 consumption by the plasmalemma redox system.

ACKNOWLEDGEMENTS

We are grateful to Dr T. Lundborg and Professor A. Kylin for continuous support and to Dr Knud M. Møller for useful suggestions. This study was made possible through a special grant from the Swedish Natural Sciences Research Council to I.M.M.

REFERENCES

[1] Lin, W. (1982) Proc. Natl. Acad. Sci. USA 79, 3773–3776.
[2] Rubinstein, B., Stern, A.I. and Stout, R.G. (1984) Plant Physiol. 76, 386–391.
[3] Kochian, L.V. and Lucas, W.J. (1985) Plant Physiol. 77, 429–436.
[4] Qiu, Z.-S., Rubinstein, B. and Stern, A.I. (1985) Planta, in press.
[5] Misra, P.C., Craig, T.A. and Crane, F.L. (1984) J. Bioenerg. Biomembranes 16, 143–152.
[6] Thom, M. and Maretzki, A. (1985) Plant Physiol. 77, 873–876.
[7] Bienfait, H.F. (1985) J. Bioenerg. Biomembranes 17, 73–83.
[8] Wignarajah, K., Lundborg, T., Björkman, T. and Kylin, A. (1983) Oikos 40, 6–13.
[9] Widell, S., Lundborg, T. and Larsson, C. (1982) Plant Physiol. 70, 1429–1435.
[10] Møller, I.M., Kay, C.J. and Palmer, J.M. (1984) Biochem. J. 223, 761–767.
[11] Larsson, C., Kjellbom, P., Widell, S. and Lundborg, T. (1984) FEBS Lett. 171, 271–276.
[12] Møller, I.M. and Palmer, J.M. (1982) Physiol. Plant. 54, 267–274.

[13] Møller, I.M., Lundborg, T. and Bérczi, A. (1984) FEBS Lett. 167, 181–185.
[14] Wise, R.R. and Naylor, A.W. (1985) Anal. Biochem. 146, 260–264.
[15] Schonbaum, G.R., Bonner, W.D., Storey, B.T. and Bahr, J.T. (1971) Plant Physiol. 47, 124–128.
[16] Gross, G.G. (1977) Phytochemistry 16, 319–321.
[17] Lance, C., Chauveau, M. and Dizengremel, P. (1985) in: Higher Plant Cell Respiration (Douce, R. and Day, D.A. eds) Encyclopedia of Plant Physiology, New Series, vol.18, pp.202–247, Springer, Berlin.
[18] Pupillo, P. and De Luca, L. (1982) Plasmalemma and Tonoplast: Their Functions in the Plant Cell (Marmé, D. et al. eds) pp.321–328, Elsevier, Amsterdam, New York.
[19] Caubergs, R.J., Goldsmith, M.H.M. and Briggs, W.R. (1977) Carnegie Inst. Yearb. 77, 356–357.
[20] Kjellbom, P., Larsson, C., Askerlund, P., Schelin, C. and Widell, S. (1985) Photochem. Photobiol., in press.
[21] Lambers, H. (1985) in: Higher Plant Cell Respiration (Douce, R. and Day, D.A. eds) Encyclopedia of Plant Physiology, New Series, vol.18, pp.418–473, Springer, Berlin.
[22] De Visser, R. and Blacquière, T. (1984) Plant Physiol. 75, 813–817.