View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

ANTIMYCIN- AND CYANIDE-RESISTANT RESPIRATION AND SUPEROXIDE ANION PRODUCTION IN FRESH AND AGED POTATO TUBER MITOCHONDRIA

Alberto BOVERIS*, Rodolfo A. SÁNCHEZ** and Martha T. BECONI***

*Instituto de Química, Biológica, Facultad de Medicina; **Departamento de Ecología and ***Departamento de Química, Facultad de Agronomía; Universidad de Buenos Aires, Buenos Aires, Argentina*

Received 20 June 1978

1. Introduction

Higher plant mitochondria possess a special pathway of electron transport to molecular oxygen, which is resistant to cytochrome chain inhibitors, frequently referred to as the alternate oxidase [1,2]. The fraction of total respiration accounted for the alternate oxidase varies widely between plant species and with the physiological condition of the tissues. For instance, the alternate pathway accounts for less than 10% of the oxygen taken up by mitochondria isolated from potato tubers, whereas it represents more than 70% of the respiration of skunk cabbage spadix at the moment of heat production [2,3].

Although the role of the alternate oxidase in plants is poorly understood, it has been found to be associated with thermogenesis [3], and suggested to be important in plant productivity [4], seed germination [5] and fruit ripening [6], which underlines the need for more basic knowledge of this respiratory activity. Identification of the primary product of oxygen reduction is of major importance when considering the physiological role of the alternate oxidase. It has been shown that H_2O_2 is the observable product, under appropiate conditions [7]. Hydrogen peroxide can be produced by either a two-electron or a one-electron transfer process; in the latter case O_2^- being the stoichiometric precursor. In recent reviews it has been suggested that the alternate oxidase may produce H_2O_2 via O_2^- in a superoxide dismutase-catalyzed reaction [2,6], although direct evidence was not available.

Incubation of potato tuber slices in a moist atmosphere (ageing) develops considerable alternate oxidase activity [8], offering an useful system to study the relationship between O_2^- production and the oxygen uptake resistant to cytochrome chain inhibitors.

The data presented here show that the increase in antimycin-resistant mitochondrial respiration taking place in ageing potato tuber slices is not accompanied by a simultaneous enhancement in O_2^- production; this latter activity being sensitive to cyanide and salicylhydroxamic acid (SHAM).

2. Materials and methods

2.1. *Isolation of mitochondria*

Recently harvested potato tubers were sliced into discs (2 mm thick and 45 mm diameter) which were used either immediately or incubated in a humid chamber on filter paper moistened with 1 mM $CaCl_2$, for 24 h or 48 h at 28°C. Mitochondria were isolated according to the general procedure described by Bonner [9], after homogenization of the potato discs in 0.3 M mannitol, 1 mM EDTA, 4 mM cysteine, 0.5% polivinylpirrolidone and 5 mM Tris–HCl, (pH 7.3), in a blendor during 7 s at low speed. The homogenate was immediately centrifuged at 14 000 × *g* for 10 min to separate mitochondria from cytosolic components. The sediment was resuspended in the homogenization medium and, after centrifugation at 600 × *g* for 10 min, mitochondria were

Address reprint requests to: Dr A. Boveris, Instituto de Química Biológica, Facultad de Medicina, Paraguay 2155, 1121 Buenos Aires, Argentina

isolated by centrifugation at 8000 × *g* for 10 min and finally suspended in 0.3 M mannitol, 1 mM EDTA (pH 7.3). Protein was measured using the Folin-Ciocalteau's reagent [10]. The amount of mitochondria in the tissue was calculated from the cytochrome oxidase activities of isolated mitochondria and homogenate.

2.2. *Measurements of respiration*

Oxygen uptake was determined in a KIC-Oxygraph (Gilson Medical Elect. Middleton, WI, USA) at 30°C. The reaction medium contained 0.3 mannitol, 5 mM NaH_2PO_4, 2 mM $MgCl_2$, 20 mM Tris–HCl buffer (pH 7.3), 10 mM succinate, 6 mM glutamate and 0.8–1.5 mg protein/ml. ADP, when used, was 0.1 mM.

2.3. *Superoxide anion production*

Production of O_2^- was determined as the SOD-sensitive rate of adrenochrome formation, which was measured at 485–575 nm (ΔE = 2.97 $mM^{-1} \cdot cm^{-1}$; [11–12]) in an Aminco-Chance dual wavelength spectrophotometer (American Inst. Co., Silver Springs, MD, USA). The reaction medium was the same as that used for oxygen uptake measurements but supplemented with 1 mM epinephrine. Temperature was 30°C.

2.4. *Superoxide dismutase and cytochrome oxidase activities*

Superoxide dismutase (SOD) activity was determined according to the method of Forman and Fridovich [13] using 0.25 mM xanthine and 3 μg/ml xanthine oxidase (specific activity 0.61 Unit/mg protein) as O_2^- scource and 10 μM cytochrome c^{3+} as O_2^- scavenger in 50 mM Tris–HCl buffer (pH 8.1). Cytochrome *c* reduction was monitored at 550 nm in a Gilford 2000 spectrophotometer. SOD activity was determined in the 14 000 × *g* supernatant (cytosol) and in the mitochondrial fraction.
In the cytosol, total SOD and cyanide-insensitive (in the presence of 0.1 mM KCN) SOD (Mn-enzyme) were measured; the cyanide-sensitive SOD (Cu-Zn-enzyme) was calculated by difference [14–15]. Mitochondrial SOD was measured in the presence of 0.1 mM KCN. Cytochrome oxidase was determined by measuring the oxidation rate of 20 μM cytochrome c^{2+} in 50 mM phosphate buffer (pH 7.0). Activities were calculated as first order reaction constants by the integral procedure [16].

2.5. *Chemicals*

Bovine SOD, xanthine oxidase, xanthine, ADP and antimycin A were purchased from Sigma Chemical Co. and salicylhydroxamic acid from Aldrich Chemical Co.

3. Results

Ageing of potato tubers leads to an increase in the mitochondrial mass of the tissue. The newly synthetized mitochondria have a loose coupling and a decreased cytochrome oxidase activity (table 1). After ageing for 24 h and 48 h, total mitochondrial protein increased 3.2 and 3.0 times, the respiratory control ratio decreased by 52% and 98%, and the cytochrome oxidase activity decreased by 49% and 54%, respectively, in agreement with previous reports [8,17]. Antimycin-insensitive respiration accounted for 9%, 56% and 90% of the state 3 oxygen uptake in the mitochondria isolated from the slices aged for 0 h, 24 h and 48 h, respectively (table 1 and fig.1). Mitochondria from aged tissue were more sensitive to cyanide than to antimycin, especially at KCN concentrations higher than 0.2 mM (fig.2). The fact that cyanide inhibition in mitochondria from aged potato tissue is not maximal at 0.1 mM KCN has been already noticed by Hackett et al. [8]. Cyanide-resistant oxygen uptake amounted to 8%, 12% and 27% of the state 3 respiration in the mitochondria from fresh, 24 h and 48 h aged potato tissue, and was similar irrespective of the presence or absence of antimycin. Consequently, cyanide was able to inhibit the antimycin-resistant respiration of the same mitochondria by 15%, 79% and 71%, respectively (table 1). SHAM, the known inhibitor of alternate oxidase activity [18], depressed state 3 respiration by 27%, 49% and 31% in the mitochondria isolated from discs incubated for 0 h, 24 h and 48 h and on a percentage basis was slightly more effective on the antimycin–insensitive respiration of the same mitochondria inhibiting by 29%, 62% and 51%, respectively. It is important to note that the antimycin- and cyanide-insensitive respiration, i.e., the oxygen uptake resistant to the sum of both inhibitors was

Table 1
Mitochondrial properties, oxygen consumption and superoxide anion production in potato tuber mitochondria and mitochondrial and cytosolic superoxide dismutase activity in potato tuber tissue

	Potato tuber discs incubated		
	0 h	24 h	48 h
Mitochondrial properties			
Total mitochondrial protein (mg/g tissue)	0.42	1.31	1.28
Isolated mitochondrial protein (mg/g tissue)	0.17	0.36	0.38
Respiratory control	3.55	2.24	1.05
Cytochrome oxidase (min^{-1}/mg protein)	10.9	5.51	5.05
Oxygen consumption (nmol O_2/min/mg prot)			
State 4	22	45	117
State 3	78	101	123
+ 0.4 μM antimycin	7	57	111
+ 1 mM KCN	6	12	33
+ antimycin + KCN	6	12	37
+ 2 mM SHAM	57	51	85
+ antimycin + SHAM	5	22	55
+ antimycin + SHAM + KCN	5	7	18
Production of O_2^- (nmol/min/mg prot)			
State 1	4.5	9.6	11.2
State 4	14.5	14.2	13.8
State 3	13.2	13.5	13.4
+ 0.2 μM antimycin	15.2	15.6	13.2
+ 1 mM KCN	0.9	0.7	0.9
+ 1 mM SHAM	0	0	0.2
Superoxide dismutase activity (Units/mg prot)			
Mitochondrial Mn-enzyme	9.0	8.6	10.5
Cytosolic Mn-enzyme	17.1	14.2	10.2
Cytosolic Cu-Zn-enzyme	10.6	38.0	76.4

effectively inhibited by SHAM (42% and 52% in the 24 h and 48 h incubated tissue). Similarly, the oxygen uptake in the presence of both antimycin and SHAM was effectively decreased (68%) by 1 mM cyanide in the mitochondria from aged tissue (table 1). Apparently, three independent oxygen-consuming activities are present in mitochondria from aged potato tissue, and these show different sensitivities to antimycin, cyanide and SHAM.

Superoxide anion production was about 13–15 nmol/min/mg protein in the mitochondria isolated form either fresh or aged tissue. Generation of O_2^- by potato mitochondria was only slightly affected by ADP or antimycin. Conversely, 1 mM KCN and 1 mM SHAM were effective inhibitors (table 1). The determination of O_2^- generation is illustrated in fig.3. Adrenochrome formation, in absence of respiratory substrate amounted to 12 nmol/min/mg of protein. The addition of succinate and glutamate increased this rate by about 100%, whereas antimycin showed a small (9%) enhancing effect. Addition of SOD at a saturating concentration, gives specificity to the assay regarding O_2^- involvement and allows the calculation of O_2^- production rates. Figure 4 shows titrations of the rate of adrenochrome formation by SOD, KCN and SHAM. Comparison of the effect of the three inhibitors shows that potato mitochondria have an adrenochrome-forming capacity that does not involve O_2^- production and therefore is insensitive to SOD, but it is sensitive to cyanide and

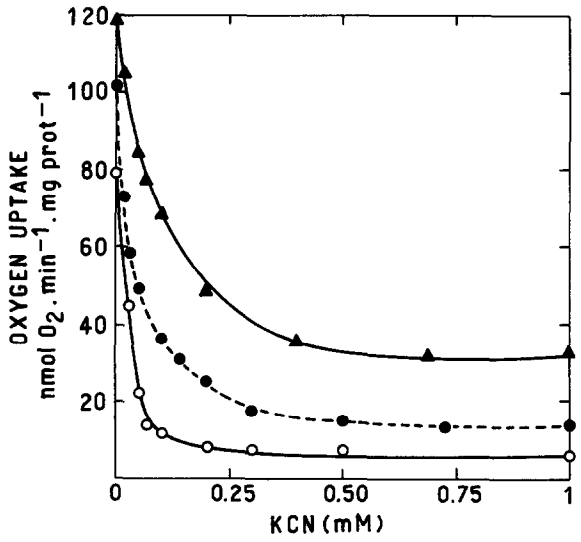

Fig.1. Effect of antimycin on state 3 respiration of potato mitochondria isolated from discs incubated 0 h (○), 24 h (●) and 48 h (▲). Exptl. conditions as described in section 2.

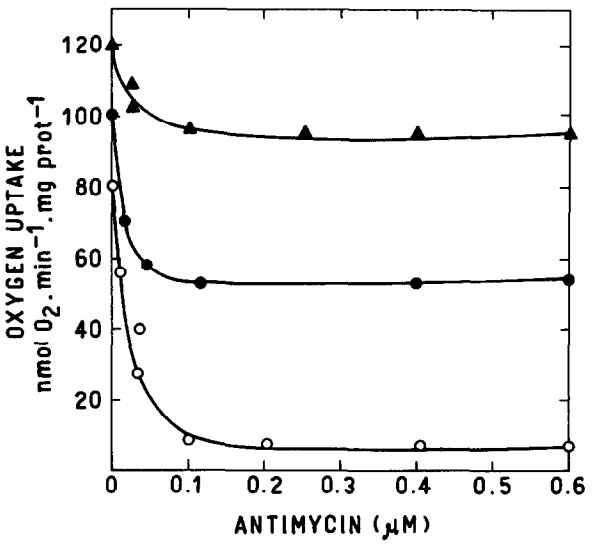

Fig.3. Production of O_2^- by fresh potato tuber mitochondria. Experimental conditions as described in section 2. 0.073 mg protein/ml.

SHAM. This activity is probably due to the phenol oxidase (*o*-diphenol:O_2 oxidoreductase, EC 1.10.3.1) of potato tissue [19].

Concerning SOD activity, neither the mitochondrial nor the cytosolic Mn-enzyme (probably leaked from broken mitochondria) showed variation upon ageing (table 1). Conversely, the activity of the cytosolic Cu-Zn-containing SOD increased 3.6 and 7.2 times after aerobic incubation of the potato discs for 24 h and 48.

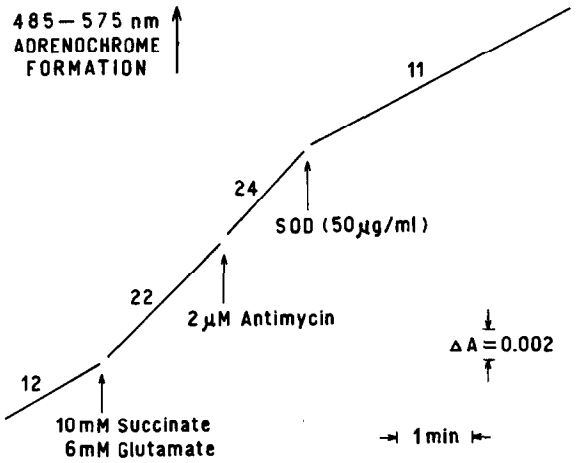

Fig.2. Effect of cyanide on state 3 respiration of potato mitochondria isolated from discs incubated 0 h (○), 24 h (●) and 48 h (▲). Exptl. conditions as described in section 2.

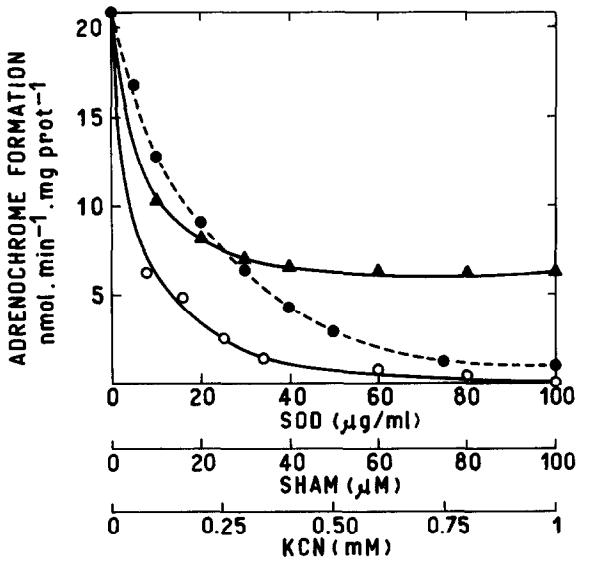

Fig.4. Effect of superoxide dismutase (▲) SHAM (○) and cyanide (●) on the rate of adrenochrome formation by fresh potato mitochondria.

4. Discussion

The antimycin-resistant and the cyanide-resistant repiration of potato tuber mitochondria are markedly increased upon ageing, but no enhancement of O_2^- generation was measured under such conditions, and therefore the theoretical possibility that O_2^- is the primary product of the alternate oxidase [2,6] is not experimentally substantiated.

Moreover, the O_2^- production in the mitochondria isolated from the aged tissue represents only a small fraction of total oxygen uptake. To calculate the ratio O_2^- production/O_2 uptake it is necessary to take into account that in potato mitochondria the following reactions take place:

$$O_2^- + O_2^- + 2\,H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (1)$$

$$H_2O_2 + H_2O_2 \xrightarrow{Catalase} 2\,H_2O + O_2 \quad (2)$$

$$H_2O_2 + AH_2 \xrightarrow{Peroxidase} 2\,H_2O + A \quad (3)$$

A pathway catalyzed by SOD and catalase will result in the production of 4 O_2^- per O_2 taken up, whereas a SOD and peroxidase catalyzed pathway will show 2 O_2^- per O_2 consumed. Consequently, even considering that reaction (3) absolutely prevails over reaction (2), production of O_2^- would only account for 7–12% of the antimycin-resistant respiration and less than that if some of the produced H_2O_2 is decomposed by catalase. Therefore, O_2^- production is not enough to account for the activity of the alternate oxidase.

Our results then show that there is more than one alternative O_2 consuming system in addition to the classical cytochrome oxidase. One of them produces O_2^-, although is resistant to antimycin and sensitive to cyanide and SHAM. Another one is that usually referred to as the alternate oxidase, resistant to antimycin and cyanide but sensitive to SHAM; this system apparently produces H_2O_2 [7], but without the participation of O_2^- as an intermediate product. There is still evidence in table 1, for another O_2 consuming system, inhibited by cyanide and resistant to antimycin and SHAM.

Ageing brings about a considerable increase in mitochondrial mass and in SOD activity, which implies an enhanced O_2^- [14] and H_2O_2 generation in aged tissue. According to our measurements, production of O_2^- represents a small fraction of the total oxygen uptake of potato mitochondria, however, its potential physiological importance is not to be disregarded since O_2^- is an extremely reactive species [14]. The interaction of O_2^- with H_2O_2 may produce $HO^\cdot$ and $^1O_2$ [20], capable of starting chain reactions with a high amplifying effect.

One might speculate about the physiological impact of increased O_2^- and H_2O_2 production in the aged tissue. Among the possible effects, a faster rate of lipid peroxidation [20,21] could be considered. Increased lipid and organic peroxide formation may lower the NADPH/NADP ratio, which is of far reaching consequences and may help to explain the observed increase in activity of the pentose phosphate shunt [22] in the aged potato tuber tissue.

Acknowledgements

The authors wish to thank Dr P. R. Rich for enlightening discussions and Prof A. O. M. Stoppani for encouragement during this work. This investigation was supported by grants from CAFPTA, CONICET and the Secretary of State of Science and Technology, Argentina. Mrs M. I. D. de Rodríguez gave efficient technical assistance.

References

[1] Ikuma, H. (1972) Ann. Rev. Plant Physiol. 23, 419–436.
[2] Bonner, W. D. jr. (1977) in: Oxygen and Physiological Function (Jobsis, F. F. ed) pp. 26–38, Professional Information Library. Dallas, Texas, USA.
[3] Meeuse, B. J. D. (1975) Ann. Rev. Plant Physiol. 26, 117–126.
[4] Zelitch, I. (1975) Science 18, 626–633.
[5] Yentur, S. and Leopold, A. C. (1976) Plant Physiol. 57, 274–276.
[6] Solomos, T. (1977) Ann. Rev. Plant Physiol. 28. 279–297.
[7] Rich, P. R., Boveris, A., Bonner, W. D. and Moore, A. L. (1976) Biochem. Biophys. Res. Commun. 71, 695–703.
[8] Hackett, D. P., Haas, D. W., Griffiths, S. K. and Niederpruem, D. J. (1960) Plant Physiol. 35, 8–18.

[9] Bonner, W. D. jr. (1967) in: Methods in Enzymology (Estabrook, R. W. and Pullman, M. E. eds) vol. 10, pp. 126–133, Academic Press, New York.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Misra, H. and Fridovich, I. (1972) J. Biol. Chem. 274, 3170–3175.
[12] Cadenas, E., Boveris, A., Ragan, C. I. and Stoppani, A. O. M. (1977) Arch. Biochem. Biophys. 180, 248–257.
[13] Forman, H. J. and Fridovich, I. (1973) Arch. Biochem. Biophys. 158, 396–450.
[14] Fridovich, I. (1974) Adv. Enzymol. 41, 35–97.
[15] Beauchamp, C. O. and Fridovich, I. (1973) Biochim. Biophys. Acta 317, 50–64.
[16] Yonetani, T. and Ray, G. S. (1965) J. Biol. Chem. 240, 3392–3398.
[17] Van der Plas, L. H. W. and Verleur, J. D. (1975) Plant Sci. Lett. 7, 149–154.
[18] Schonbaum, G. R., Bonner, W. D. jr., Storey, B. T. and Bahr, J. R. (1971) Plant Physiol. 47, 124–128.
[19] Kubowitz, F. (1937) Biochem. Z. 292, 221–229.
[20] Kellog, E. W. and Fridovich, I. (1977) J. Biol. Chem. 252, 6721–6728.
[21] Chin, C. K. and Frenkel, C. (1976) Nature 264, 60.
[22] Kahl, G. (1973) Botanical Review 39, 274–299.