The nuclear origin of the non-phosphorylating NADH dehydrogenases of plant mitochondria

R.J. Cook-Johnson[a,*], Q. Zhang[b], J.T. Wiskich[b], K.L. Soole[a]

[a] *School of Biological Sciences, The Flinders University of South Australia, 5042 Melbourne, Australia*
[b] *Department of Environmental Biology, University of Adelaide, South Australia, 5000 Adelaide, Australia*

Received 22 April 1999

Abstract The oxidation of matrix and cytosolic NADH by isolated beetroot and wheat leaf mitochondria was investigated to determine whether the rotenone-insensitive NADH dehydrogenases of plant mitochondria were the products of nuclear or mitochondrial genes. After aging beetroot tissue (slicing and incubating in a $CaSO_4$ solution), the induction of the level of matrix NADH oxidation in the presence of rotenone was greatly reduced in mitochondria isolated from tissue treated with cycloheximide, a nuclear protein synthesis inhibitor. This was also true for the oxidation of cytosolic NADH. Mitochondria isolated from chloramphenicol-treated tissue exhibited greatly increased levels of both matrix and external rotenone-insensitive NADH oxidation when compared to the increase due to the aging process alone. This increase was not accompanied by an increase in matrix NAD-linked substrate dehydrogenases such as malic enzyme nor intra-mitochondrial NAD levels. Possible explanations for this increase in rotenone-insensitive NADH oxidation are discussed. Based on these results we have concluded that the matrix facing rotenone-insensitive NADH dehydrogenase of plant mitochondria is encoded by a nuclear gene and synthesis of the protein occurs in the cytosol.

Key words: Rotenone-insensitive; NADH oxidation; Protein synthesis inhibition; Mitochondrion

1. Introduction

Unlike mammalian mitochondria, plant mitochondria contain additional non-phosphorylating NADH dehydrogenases that are linked to the mitochondrial inner-membrane electron transport chain [1]. It is thought that these dehydrogenases oxidise cytosolic and matrix NADH. The dehydrogenases are insensitive to the Complex I inhibitor rotenone [2]. Little is known about the physiological role of the inner-membrane rotenone-insensitive NAD(P)H dehydrogenases. They do not contribute to the production of ATP at site one and so the free energy released during electron transfer is lost as heat. Consequently, these enzymes represent a potentially energy wasteful system. Thermogenic plants appear to be the exception, where this release of heat is used for reproductive purposes [3]. It has been suggested that the non-phosphorylating enzymes may have a role in stress response. Tissue wounding appears to increase the non-phosphorylating NAD(P)H pathways [4], and other studies have shown cold [5], and nutrient limitations [6] also effect the level of rotenone-insensitive NADH oxidation.

Mitochondria, isolated from fresh beetroot grown in the Adelaide region of South Australia, have the unique distinction of having no detectable external or cytosolic NAD(P)H oxidation rate. However, a significant rate can be induced if the tissue is aged, which refers to wounding by slicing and incubation in an aerated $CaSO_4$ solution over 42 h [4,7]. It has been shown that external NAD(P)H oxidation increases in beetroot mitochondria when the tissue is left in cold storage, for up to 15 weeks at 10°C mimicking the wound or stress response [8]. Nutrient limitation has also been shown to have an effect upon rotenone-insensitive NADH oxidation. Using bean plants grown with or without inorganic phosphate (Pi), matrix rotenone-insensitive NADH oxidation increased in Pi deficient plants [6]. The reason for the response of these proteins to the various treatments is not clear, however it may be that the increase in rotenone-insensitive NADH oxidation acts as a compensatory mechanism for an ETC that has been compromised.

Recently, it has been suggested that in beetroot mitochondria external rotenone-insensitive NAD(P)H oxidation is catalysed by either a 58 kDa and/or 32 kDa protein whilst matrix rotenone-insensitive NADH oxidation occurs via a 43 kDa dimer [8–10]. Sonication studies have shown that both the matrix and external enzymes are peripherally bound to the inner-membrane of plant mitochondria. The external NADH activity has been shown to be released from the membrane by sonication and this activity can therefore be recovered in the soluble fraction, avoiding the complication of contamination by the NADH oxidation activity of Complex I. Using the increase in external NADH oxidation in beetroot mitochondria during aging, Menz and Day [9] have shown that external NADH oxidation is co-incident with the increase of a novel 58 kDa protein. From antibody cross-linking studies this protein was shown to be present on the outerface of the inner-membrane of plant mitochondria.

From the soluble protein profile prepared from fresh beetroot mitochondria, a single polypeptide with a molecular mass of 43 kDa and NADH activity was resolved via column chromatography techniques and after gel filtration chromatography showed a native molecular mass of 86 kDa. Antisera prepared against this protein inhibited rotenone-insensitive NADH oxidation and use of membrane impermeable cross-linkers revealed its location to be on the matrix side of the inner-membrane. It was therefore concluded that the protein responsible for matrix rotenone-insensitive NADH oxidation was a 43 kDa dimer [11]. Despite this information it is not clear whether the mitochondrial or nuclear genome encodes

*Corresponding author. Fax: +61 (8201) 3015.
E-mail: rebecca.cook-johnson@flinders.edu.au

Abbreviations: CHI, cycloheximide; CAP, chloramphenicol; SMPs, sub-mitochondrial particles; MDH, malate dehydrogenase; ME, malic enzyme; ETC, electron transport chain; CI, Complex I

PII: S0014-5793(99)00753-X

the protein responsible for matrix rotenone-insensitive NADH oxidation.

In this study we have shown that during aging of beetroot tissue, both external and matrix rotenone-insensitive NADH oxidation increases, in agreement with previous studies [4,7] and that the matrix rotenone-insensitive NADH dehydrogenase is nuclear encoded. Further, when beetroot and wheat mitochondrial protein synthesis is compromised, both the matrix and external NADH oxidation rates increase, perhaps as a compensatory mechanism.

2. Materials and methods

All biochemicals were purchased from the Sigma Chemical Company, except Percoll, which was supplied by Pharmacia Biotech. Beetroot, *Beta vulgaris* L. were obtained from a market garden at the local town of Virginia, Adelaide, South Australia.

Beetroot mitochondria were isolated using previous methods [4]. Beetroot were aged by thickly grating beetroot and incubating the tissue in a 10^{-4} M $CaSO_4$ solution over 42 h with constant aeration and the solution changed at a minimum of 12 h intervals to ensure the tissue did not go anoxic [4]. The protein synthesis inhibitors, CHI 1.5 mM or CAP 1.5 mM, were added after tissue had been aged for 8 h and then the solution changed at a minimum of 12 h intervals up to a total of 42 h. Sub-mitochondrial particles (SMPs) were prepared using standard methods and the orientation of the SMPs were determined by the latency of cytochrome c oxidase [11,12].

Unless stated otherwise oxygen consumption rates were determined polarographically in standard reaction media, pH 7.2, 0.25 M sucrose, 0.01 M KH_2PO_4, 0.01 M HEPES and 5 mM $MgCl_2$ [4]. The concentrations of the various substrates and inhibitors were 1.5 mM NADH, 10 mM ethylene glycol-bis-β-aminoethylether-*N*,*N*,*N*′,*N*′-tetra acetic acid (EGTA) and 3 mM $CaCl_2$.

The matrix NAD content was estimated in 0.15 M Pyrobuffer (0.25 M tetra sodium pyrophosphate, 68 mM semicarbaside hydrochloride adjusting pH to 8.9 with KOH) [13]. The beetroot mitochondria were incubated with 200 μM oxaloacetic acid (OAA), 3.5 μM carbonyl cyanide *p*-trifluoromethoxy phenyl hydrazone (FCCP) and 2 mM ATP to ensure the total conversion of NADH to NAD. The NADH concentration was measured on a dual wavelength spectrophotometer with reference and assay wavelengths at 374 and 340 nm respectively. The NADH molar extinction coefficient of 4.53×10^3 cm^{-1} was used.

Wheat (*Triticum aestivum*, Machete) seedlings were grown in sand at 25°C for 9–11 days in pots (32×28×8 cm). CAP (200 mg) was dissolved in 200 ml of H_2O and applied to each pot by watering. Leaves were harvested 10–12 days after CAP treatment for mitochondrial preparation and the leaves were then cut into CAP-treated sections (2–3 cm from soil surface, base) and control sections (2–3 cm from tip of leaf, top). Mitochondria and SMPs were isolated following standard methods [14]. For wheat mitochondria oxygen consumption rates were determined in reaction media, pH 7.2, 0.3 M sucrose, 10 mM TES, 10 mM KH_2PO_4, 2 mM $MgCl_2$.

Mitochondrial protein was determined by standard methods (Lowry) using BSA as standards.

3. Results

3.1. Effect of mitochondrial and nuclear protein synthesis inhibitors on the external NADH dehydrogenase

Mitochondria isolated from tissue treated with the various protein synthesis inhibitors were presented with NADH as a substrate. As this molecule can not traverse the inner-membrane oxidation rates are due to an external facing NADH dehydrogenase. External NADH oxidation is virtually absent in fresh beetroot mitochondria (Fig. 1). As previously reported [4] when beetroot was aged in $CaSO_4$, external NADH oxidation was induced. This induction was sensitive to CHI as the addition of CHI to the aging beetroot tissue inhibited the induction of external NADH oxidation in isolated mitochondria. This indicates that the protein responsible for this oxidation is a nuclear gene product. Interestingly, there was an additional increase in external NADH oxidation over and above the increase caused by aging in tissue treated with the mitochondrial protein synthesis inhibitor CAP (Fig. 1).

3.2. Effect of aging on external and matrix rotenone-insensitive NADH oxidation

To establish the activity of the matrix rotenone-insensitive NADH dehydrogenase, the oxidation rate of an NAD-linked substrate such as malate was determined in the presence of rotenone. The activity in the absence of rotenone (total NADH oxidation) represents both Complex I and rotenone-insensitive dehydrogenase activity. Although total malate oxidation was not significantly affected by aging the activity of the rotenone-insensitive malate oxidation increased three-fold, at both pH 6.8 and pH 7.2 (Table 1). At pH 7.2, the predominant substrate dehydrogenase is malate dehydrogenase. It has been reported that in some isolated mitochondria a low level of malate dehydrogenase can be found in association with the outer-membrane [15]. Therefore an increase in malate-dependent oxygen uptake at this pH may reflect the participation of the external malate dehydrogenase and NADH oxidation via the external facing NADH dehydrogenase. However, the similar response at pH 6.8 where malic enzyme is the predominant malate oxidising enzyme and is exclusively found in the matrix, suggests that the response was due to the matrix facing rotenone-insensitive dehydrogenase. The specific activity of malic enzyme was not different between fresh and aged beetroot mitochondria (data not shown) suggesting that the increase in the rotenone-insensitive rate of malate oxidation was not due to an increase in the substrate dehydrogenase, but due to an increase in the activity of the matrix rotenone-insensitive NADH dehydrogenase (Table 1). The increase in absolute rotenone-insensitive malate oxidation rate was also reflected in the level of rotenone resistance that increased with aging (Fig. 2).

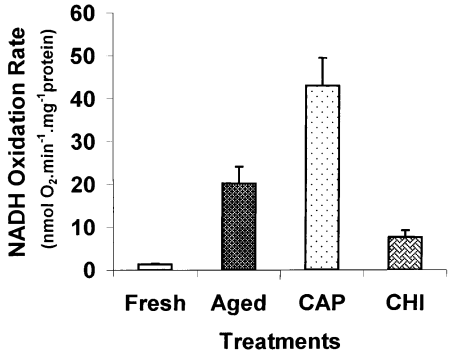

Fig. 1. Effect of aging and protein synthesis inhibitors on external NADH oxidation by beetroot mitochondria. Mitochondria were isolated from fresh tissue or from tissue incubated in a 10^{-4} M $CaSO_4$ (aged) solution over 42 h. Additional treatments included a final concentration of either 1.5 mM CHI or 1.5 mM CAP after 8 h aging. The NADH oxidation rate was determined by the addition of 1.5 mM NADH in the presence of 26 μM rotenone to ensure no possible contribution by CI. The oxidation rate was sensitive to inhibition by the addition of 10 mM EGTA and restored by the addition of 3 mM $CaCl_2$. Numbers in brackets are standard errors, $n=3$.

Table 1
Effect of aging or protein synthesis inhibitors on the level of malate oxidation in beetroot mitochondria

Tissue treatment	Malate oxidation rate (nmol O_2 min^{-1} mg^{-1} protein)			
	pH 6.8		pH 7.2	
	Total	+Rotenone	Total	+Rotenone
Fresh	41.12 (3.70)	6.51 (1.34)	51.37 (6.64)	9.52 (0.07)
Aged	54.98 (4.67)	21.47 (2.68)	47.90 (5.41)	18.53 (3.61)
CAP	48.93 (3.98)	34.56 (3.62)	51.20 (7.83)	37.95 (4.24)
CHI	47.59 (2.61)	4.81 (1.34)	44.71 (7.10)	2.57 (0.41)

The total malate oxidation rate was determined by addition of 10 mM malate to beetroot mitochondria in the presence of 1 mM ADP and represents the combination of CI and the matrix rotenone-insensitive NADH dehydrogenase. With the addition of 26 μM rotenone, CI activity is inhibited and the resulting rate is attributable to the matrix rotenone-insensitive NADH dehydrogenase. Tissue treatments are as indicated in Fig. 1. Numbers in brackets are standard errors for $n = 3$.

In an attempt to measure the activity of the matrix facing rotenone-insensitive NADH dehydrogenase directly, the rate of NADH oxidation was determined in SMPs prepared from the isolated mitochondria. Although the SMPs were determined to be 70–80% inside out, EGTA was included in the assay to ensure that the oxidation rate of SMPs was due only to the matrix rotenone-insensitive NADH dehydrogenase and not due also to the external NADH dehydrogenase. It has been shown that the external NADH dehydrogenase is inhibited by addition of EGTA [10]. The increase in the rotenone resistance of matrix NADH oxidation was not as marked in SMPs as in intact mitochondria (Fig. 2). However, this may be due to the loss of some enzyme during the sonication step to generate the SMPs as the rotenone-insensitive NADH dehydrogenases are believed to be only peripherally bound to the inner-membrane [9–11].

3.3. *Effect of mitochondrial and nuclear protein synthesis inhibitors on the matrix rotenone-insensitive NADH dehydrogenase*

It has been known for some time that Complex I is a multimeric protein with its peptides being encoded for by both the mitochondrial and nuclear genome. There is however no information concerning which genome encodes the matrix rotenone-insensitive NADH oxidising protein of plant mitochondria. To address this, beetroot slices were treated with either the nuclear protein synthesis inhibitor CHI or the mitochondrial protein synthesis inhibitor CAP during aging. Mitochondria were isolated from inhibitor-treated tissues and the malate oxidation rates determined either in the absence or presence of rotenone. The incubation of beetroot tissue with CHI prevented the increase in rotenone-insensitive malate oxidation and rotenone resistance that was observed in the absence of this inhibitor (Table 1, Fig. 2). This response was observed in both intact mitochondria and EGTA-resistant NADH oxidation with beetroot SMPs. This suggests that the increase in matrix rotenone-insensitive NADH dehydrogenase was due to protein synthesis and that this synthesis was associated with the nuclear genome.

Conversely, when the beetroot tissue was incubated with CAP the matrix rotenone-insensitive malate oxidation rate increased 1.5-fold compared to the increase observed with aging alone (Table 1, Fig. 2). Again as with aging treatment alone, this rise was due to an increase in matrix rotenone-insensitive NADH dehydrogenase activity as there was no increase in state 3 rates (in the presence of excess ADP) (Table 1) or malic enzyme activity (data not shown). The increase in rotenone resistance of matrix NADH oxidation after CAP treatment was not as marked in SMPs (Fig. 2) as observed for malate oxidation in intact mitochondria which again may reflect the loss of this peripherally bound enzyme during the sonication step of SMP preparation.

This response of matrix rotenone-insensitive NADH oxidation to CAP incubation was not restricted to beetroot as an increase was also observed with wheat seedlings watered with CAP (Table 2). After exposure to CAP, wheat leaves continue to grow though somewhat etiolated [14]. In a previous study, it was shown that in tissue that emerges after CAP treatment, which is defined as base (Table 2), the properties of the mitochondria are altered. It was shown that the alternative oxidase and external NADH oxidation increases in mitochondria from CAP-treated tissue [14]. In this report it can be seen that CAP treatment dramatically increased the level of rotenone resistance of EGTA-resistant NADH oxidation in wheat SMPs (Table 2). This increase was not observed in top leaf tissue that developed prior to CAP addition.

3.4. *Effect of mitochondrial and nuclear protein synthesis inhibitors on NAD production*

Matrix rotenone-insensitive NADH oxidation is known to be sensitive to matrix levels of NAD(H) as it has a poor affinity for its substrate NADH [10,11]. Consequently, the increase in matrix rotenone-insensitive NADH oxidation

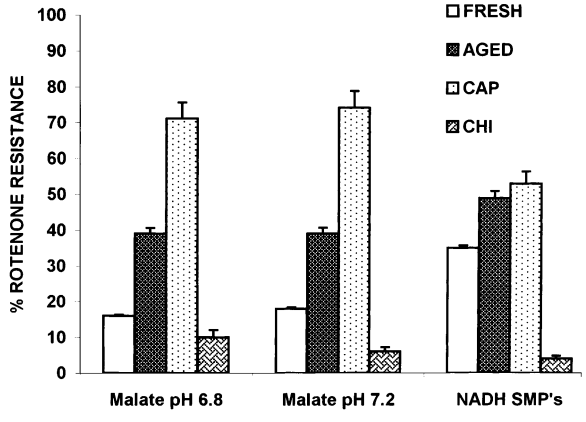

Fig. 2. Effect of aging and protein synthesis inhibition on the level of rotenone resistance of malate oxidation in whole beetroot mitochondria and NADH oxidation in SMPs isolated from beetroot mitochondria. Malate oxidation conditions were as for Table 1 and SMP NADH as for Table 2. Rotenone resistance represents the rate in the presence of rotenone as a percentage of the uninhibited rate for each substrate. Numbers in brackets are standard errors, $n = 3$.

Table 2
Effect of CAP treatment on NADH oxidation in wheat SMPs

Tissue treatment	NADH	+Rotenone	% Rotenone resistance
	(nmol O_2 min^{-1} mg^{-1} protein)		
Control top	11.2 (1.3)	6.7 (0.2)	60
Control basal	1.9 (0.4)	0.7 (0.06)	37
CAP top	22.2 (3.5)	13.2 (1.0)	60
CAP basal	7.3 (0.9)	4.6 (0.7)	63

Wheat SMPs were prepared from mitochondria isolated from either control or CAP-treated wheat leaves. SMP NADH oxidation rates were determined by addition of 160 μM NADH in the presence of 2 mM EGTA to give a total NADH oxidation rate. Rotenone (10 μM) was included to determine the contribution to the NADH oxidation rate by the matrix rotenone-insensitive NADH dehydrogenase. Rotenone resistance was then determined as a percentage of total NADH oxidation. Numbers in brackets are standard errors for $n = 4$.

may lie either in the increase in the level of the enzyme itself or may be linked to an increase in the level of matrix NAD. An increase in matrix NAD would increase the rotenone-insensitive NADH oxidation rate due to its high $K_{m\,NADH}$. Therefore the levels of matrix NAD in mitochondria isolated from the variously treated beetroot tissue were determined. There was no notable increase in matrix NAD levels of mitochondria isolated from fresh beetroot, aged or tissue treated with either protein synthesis inhibitor (Table 3).

4. Discussion

In this study incubation with the protein synthesis inhibitors CHI and CAP have been used to determine which genome is responsible for the increase in matrix rotenone-insensitive NADH oxidation and external rotenone-insensitive NADH oxidation. As CAP affects mitochondrial protein synthesis it would be assumed that any proteins within the mitochondrion or those that contain mitochondrial encoded subunits would be affected if protein turnover occurred within the experimental period. The same argument applies to CHI but in relation to nuclear protein synthesis.

From the results presented it can be concluded that the external NADH dehydrogenase (at least the 58 kDa species) and the matrix rotenone-insensitive NADH dehydrogenase are nuclear encoded as the increase in their activity observed during aging is dramatically decreased by the nuclear protein synthesis inhibitor CHI. As it has been clearly demonstrated that the increase in external NADH oxidation in beetroot mitochondria occurs concomitantly with an increase in a 58 kDa polypeptide that has NADH dehydrogenase activity, it can be concluded that this 58 kDa polypeptide is nuclear encoded. In response to CAP treatment an increase in both the external and matrix NADH rotenone-insensitive oxidation rates was observed in mitochondria isolated from beetroot and wheat tissue. Presumably this is a response to decreased activity within the major route of NADH oxidation by CI. However, from malate oxidation rates it appears that the ETC can still cope with the input of NADH from both MDH and ME. The increase in internal NADH oxidation may reflect a response to a requirement for the continual turnover of glycolytic NADH. CI may not be limited when supplied with individual substrates (as in the oxygen electrode experiments) but may be potentially limiting when the complete ETC is turning over in vivo. The NAD assay indicated no significant increase in matrix NAD levels or substrate dehydrogenases, therefore it can be assumed that the increase in matrix rotenone-insensitive NADH oxidation must be due to an increase in the protein itself.

These results demonstrate a communication between the mitochondrial and nuclear genomes as when one is compromised, additional proteins are synthesised in response presumably in an attempt to maintain oxidative capacity perhaps to fuel maintenance respiration. Research using *Nicotiana sylvestris* cytoplasmic male sterile (CMS) mutants I and II that have recently been well characterised on the molecular, developmental and functional levels lends support for our findings [16]. These mutants were deficient in some of their mitochondrially encoded CI subunits and the authors concluded that when CI activity was compromised the activity of the external NADH dehydrogenase increased as a compensatory mechanism [16].

In this study we have observed that under conditions where potentially newly synthesised CI subunits will be defective, both external and matrix rotenone-insensitive NADH oxidation have increased. This suggests a role for the non-phosphorylating NADH dehydrogenases as an adaptive mechanism for growth periods when CI is compromised.

Table 3
Effect of aging and protein synthesis inhibitors on matrix NAD levels of beetroot mitochondria

Tissue treatments	NAD assay (nmol NAD mg^{-1} protein)
Fresh	1.23 (0.15)
Aged	1.15 (0.25)
CAP	1.72 (0.01)
Cyclo	1.37 (0.10)

Matrix NAD levels were determined in mitochondria isolated from fresh and treated beetroot tissue. These treatments are as indicated in Fig. 1. Numbers in brackets are standard errors for $n = 3$.

Acknowledgements: The financial support for this work was from the Australian Research Council to K.L.S. and J.T.W.

References

[1] Siedow, J.N. and Umbach, A.L. (1995) Plant Cell 7, 821–831.
[2] Soole, K.L., Menz, I. and Wiskich, J.T. (1992) in: H. Lambers and L.H.W. van der Plas (Eds.), Molecular, Biochemical and Physiological Aspects of Plant Respiration, SPB Academic Publishing, The Hague, The Netherlands, pp. 65–71.
[3] Palmer, J.M. and Ward, J.A. (1985) in: R. Douce and D.A. Day (Eds.), Encyclopedia of Plant Physiology, Vol. 18: Higher Plant Cell Respiration, Springer-Verlag, Berlin, pp. 173–200.
[4] Rayner, J.R. and Wiskich, J.T. (1983) Aust. J. Plant Physiol. 10, 55–63.
[5] Fredlund, K.M., Rasmusson, A.G. and Moller, I.M. (1991) Plant Physiol. 97, 99–103.

[6] Rychter, A.M., Chauveau, M., Bomsel, J. and Lance, C. (1992) Physiol. Plant. 84, 80–86.
[7] Day, D.A., Rayner, J.R. and Wiskich, J.T. (1976) Plant Physiol. 58, 38–42.
[8] Day, D.A., Neuburger, M. and Douce, R. (1985) Aust. J. Plant Physiol. 12, 219–228.
[9] Knudten, A.F., Thelen, J.J., Luethy, M.H. and Elthon, T.E. (1994) Plant Physiol. 106, 1115–1122.
[10] Menz, R.I. and Day, D.A. (1996) Plant Physiol. 112, 607–613.
[11] Menz, R.I. and Day, D.A. (1996) J. Biol. Chem. 271, 23117–23120.
[12] Möller, I.M. and Palmer, J.M. (1982) Physiol. Plants 54, 267–274.
[13] Klingenberg, M. (1974) in: H.U. Bergmeyer (Ed.), Methods in Enzymatic Analysis, Vol. 4, Academic Press, New York, pp. 2045–2059.
[14] Zhang, Q., Mischis, L. and Wiskich, J.T. (1996) Aust. J. Plant Physiol. 23, 583–592.
[15] Soole, K.L., Dry, I.B., James, A.T. and Wiskich, J.T. (1990) Physiol. Plants 80, 75–82.
[16] Gutierres, S., Sabar, M., Lelandais, C., Cherit, P., Diolez, P., Degand, H., Boutry, M., Vedel, F., de Kouchkovsky, Y. and De Paepe, R. (1997) Proc. Natl. Acad. Sci. USA 94, 3436–3441.