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Direct Inhibition of Plant Mitochondrial Respiration by Elevated CO_2[1]

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Doubling the concentration of atmospheric CO_2 often inhibits plant respiration, but the mechanistic basis of this effect is unknown. We investigated the direct effects of increasing the concentration of CO_2 by 360 μL L^{-1} above ambient on O_2 uptake in isolated mitochondria from soybean (*Glycine max* L. cv Ransom) cotyledons. Increasing the CO_2 concentration inhibited the oxidation of succinate, external NADH, and succinate and external NADH combined. The inhibition was greater when mitochondria were preincubated for 10 min in the presence of the elevated CO_2 concentration prior to the measurement of O_2 uptake. Elevated CO_2 concentration inhibited the salicylhydroxamic acid-resistant cytochrome pathway, but had no direct effect on the cyanide-resistant alternative pathway. We also investigated the direct effects of elevated CO_2 concentration on the activities of cytochrome *c* oxidase and succinate dehydrogenase (SDH) and found that the activity of both enzymes was inhibited. The kinetics of inhibition of cytochrome *c* oxidase were time-dependent. The level of SDH inhibition depended on the concentration of succinate in the reaction mixture. Direct inhibition of respiration by elevated CO_2 in plants and intact tissues may be due at least in part to the inhibition of cytochrome *c* oxidase and SDH.

Respiration rates are often lower when plants are grown at elevated C_a than when they are grown at ambient CO_2 levels (Wullschleger et al., 1994; Amthor, 1996). Two effects of elevated C_a on apparent dark respiration in intact plants or tissues have been reported (Amthor, 1991): (a) a direct, immediate effect in which respiration is reversibly reduced by exposure to elevated C_a; and (b) an acclimation effect in which respiration of plants grown in elevated C_a differs from respiration of plants grown in ambient C_a (when measured at a common value of C_a). The acclimation effect generally results in reduced respiration in plants and tissues grown at elevated C_a (Bunce and Caulfield, 1991; Azcón-Bieto et al., 1994), although in some plants acclimation leads to increased respiration (Thomas et al., 1993). Acclimation of respiration in photosynthetic tissues of plants grown at elevated C_a can be related to a reduction in the maximum activity of Cyt *c* oxidase (Azcón-Bieto et al., 1994; Aranda et al., 1995).

The direct effect of CO_2 on dark respiration is reversible and is observed in most plants as a reduction in respiration within minutes of a step change in C_a (Amthor, 1996). A reversible inhibition of CO_2 evolution by *Rumex crispus* leaves was observed when C_a was increased stepwise through the range of 0 to 1000 μL L^{-1} (Amthor et al., 1992). Inhibition of respiration by increasing C_a has also been reported in whole plants (Bunce, 1990; Ryle et al., 1992), leaves (Reuveni and Gale, 1985; Bunce, 1990; El Kohen et al., 1991; Amthor et al., 1992; Byrd et al., 1992; Thomas and Griffin, 1994; Ziska and Bunce, 1994), roots (Reuveni and Gale, 1985; Palta and Nobel, 1989; Qi et al., 1994), microorganisms (Koizumi et al., 1991), and animal tissues (Palet et al., 1991). Respiration can also be unaffected or even increased when C_a increases (Palet et al., 1991; Ryle et al., 1992). Although direct effects of C_a on apparent respiration in plant tissues have long been reported (e.g. Kidd, 1916), these effects have recently been reevaluated in the context of the rising C_a, which is expected to reach a value of twice the preindustrial concentration during the second half of the next century. A reduction of dark respiration in aerial plant tissues by elevated C_a would have important consequences for the carbon balance in terrestrial ecosystems.

The site of action of the direct, short-term inhibition of respiration by CO_2 is unknown. Levels of C_a 5% or higher may inhibit some enzymes of the glycolytic pathway (Kerbel et al., 1988, 1990), as well as mitochondrial O_2 uptake (Shipway and Bramlage, 1973; Palet et al., 1992).

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Abbreviations: C_a, concentration of CO_2 in the air; DIC, dissolved inorganic carbon; SDH, succinate dehydrogenase; SHAM, salicylhydroxamic acid; $V_{cyt\text{-}KCN}$, SHAM-resistant O_2 uptake, the activity of the Cyt pathway in the presence of SHAM; $V_{alt\text{-}SHAM}$, cyanide-resistant O_2 uptake, the activity of the alternative pathway in the presence of KCN.

This includes the activities of mitochondrial enzymes such as SDH (Zeylamaker et al., 1970) and Cyt *c* oxidase (Miller and Evans, 1956; Palet et al., 1991, 1992). Although the dissolved CO_2 concentration can reach up to 1.7 mM (equivalent to 0.5% C_a) in nongreen tissues (Raven and Newman, 1994), there are no reports showing that elevated C_a inhibits enzyme activity associated with respiration of photosynthetic tissues at more physiological levels of C_a (up to 1000 μL L^{-1}). It has also been suggested that extramitochondrial factors such as dark CO_2 fixation or measurement artifacts (Reuveni et al., 1993; Wullschleger et al., 1994; Amthor, 1996) contribute to the apparent inhibition of respiration by elevated C_a.

The goal of this work was to determine whether the direct effects of CO_2 reported in tissues can be seen in isolated plant mitochondria and, if so, to study the sites of action of any such inhibition. To accomplish this we isolated mitochondria from soybean (*Glycine max* L.) cotyledons and exposed them to an increase in DIC equivalent to 360 μL L^{-1} above the current ambient level of C_a.

MATERIALS AND METHODS

Seeds of soybean (*Glycine max* L. cv Ransom) were planted in a 1:1 mixture of sand and perlite. Plants were grown in growth chambers in the Duke University Phytotron at 25°C under a 13-h/11-h (light/dark) photoperiod at 1000 μmol photons m^{-2} s^{-1}. In late spring of 1994 seeds were also planted and grown in the greenhouse with no control of incident light or temperature. In both cases plants were watered at least once a day and cotyledons were collected between 7 and 10 d after sowing.

Mitochondrial Isolation and Assay

Cotyledon mitochondria were isolated using a Percoll gradient as described by Day et al. (1985) with minor modifications (Umbach and Siedow, 1993).

Isolated mitochondria were assayed at 25°C in 10 mM Tes-buffered medium, pH 7.2, containing 0.3 M Suc, 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$, and 0.1% BSA. Oxygen uptake was measured polarographically using a Clark-type O_2 electrode (Rank Brothers, Cambridge, UK) and initiated by the addition of different substrates. When the reaction of mitochondrial oxidases with oxygen was initiated with 5 mM succinate, mitochondria were preincubated with 0.15 mM ATP, and SDH was further activated by a single state 3/state 4 transition. Oxidation of 2 mM NADH (30 μM Ca^{2+}) was carried out in the presence and absence of pyruvate (5 mM). All experiments described here were carried out under state 3 conditions, in which ADP was present in excess, to avoid ATP control of mitochondrial O_2 uptake.

$V_{cyt\text{-}SHAM}$ was assessed in the presence of 2 mM SHAM, an inhibitor of the alternative oxidase. $V_{alt\text{-}KCN}$ was assessed in the presence of 1 mM KCN, an inhibitor of the Cyt pathway.

Mitochondria were preincubated in a closed cuvette for 10 min in the presence or absence of elevated C_a concentration (see "CO_2 Treatments") because preliminary trials showed that this was necessary to obtain stable rates of O_2 uptake after the step change in C_a. Respiratory control (state 3-to-state 4 ratio) and ADP-to-O ratios of the mitochondria in the cuvette remained constant for at least 30 min at room temperature, after which they started to decline.

Cyt *c* Oxidase Assay

Plant Cyt *c* oxidase activity was measured in mitochondria broken by osmotic shock. Commercial beef-heart Cyt *c* oxidase was from Sigma. Both enzymes were assayed polarographically at 25°C as the rate of azide (2.5 mM)-sensitive O_2 uptake in the reaction medium used for mitochondria (pH 7.2), with the addition of 1 mM lauryl maltoside, 8 mM ascorbate, 1 mM *N,N,N'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride, and 30 μM Cyt *c*.

SDH Assay

SDH activity was determined by measuring SDH-phenazine methosulfate reductase activity (Burke et al., 1985). Mitochondria were placed in a medium containing 30 mM Tricine-NaOH (pH 7.5), 0.25 to 8 mM succinate, and 1 mM phenazine methosulfate. Malonate (5 mM)-sensitive O_2 uptake was then measured at 25°C.

CO_2 Treatments

The free CO_2 concentration dissolved in aqueous solution is linearly proportional to the C_a in the gas phase in equilibrium at constant temperature. In ambient atmospheric conditions, the free CO_2 dissolved in water is near 12 μM at 25°C. However, the quantity of DIC is a function of the pH, determined by the Henderson-Hasselbalch equation. A stock solution of DIC was prepared fresh daily in the mitochondrial reaction medium using either potassium or sodium bicarbonate and was kept in containers with no gas phase at 25°C (pH 7.2), at which level equilibrium between the dissolved chemical species was established. For the elevated C_a treatment, 0.1 mM DIC (12.2 μM CO_2 and 88 μM HCO_3^-, pK_1 = 6.35) was added to the closed cuvette to emulate increasing ambient C_a by 363 μL L^{-1} in the liquid phase. In the case of the SDH-phenazine methosulfate reductase activity the reaction was carried out at pH 7.5. Therefore, 0.17 mM DIC (12.2 μM CO_2 and 158 μM HCO_3^-) was used for the elevated C_a treatment. The reaction medium placed in the reaction cuvette was previously equilibrated with ambient air that contained a CO_2 concentration less than 420 μL L^{-1} (measured with a gas analyzer [6262 IR, Li-Cor, Lincoln, NE).

For the incubation experiments 0 or 0.1 mM DIC (10 μL from the stock solution) was added to the closed cuvette containing the reaction medium and mitochondria 10 min before the substrates (succinate or NADH) were added. Depending on the substrate used, either ATP (succinate) or Ca^{2+} and pyruvate (NADH) were also added to the closed cuvette during the 10-min incubation.

RESULTS

The Direct Effect of Atmospheric CO_2 on Plant Mitochondria

Elevated C_a inhibited mitochondrial O_2 uptake (Fig. 1). The rates of O_2 uptake depended on the substrate used, but elevated C_a inhibited O_2 uptake in all cases. When oxidizing succinate, elevated C_a inhibited mitochondrial O_2 uptake 16% ($P < 0.001$) (Fig. 1, SUCC). Elevated C_a inhibited the oxidation of NADH by soybean mitochondria (9%; $P = 0.056$) (Fig. 1, NADH) significantly in the presence of pyruvate (15%; $P = 0.029$, rank summary test) (Fig. 1, NADH+PYR). Elevated C_a inhibited the oxidation of succinate and NADH in the absence of pyruvate by 15% ($P < 0.001$) (Fig. 1, SUCC+NADH).

The Direct Effect of Atmospheric CO_2 on the Activity of Cyt *c* Oxidase and SDH

Elevated C_a inhibited the activity of Cyt *c* oxidase obtained from several different sources (Fig. 2A). Inhibition of Cyt *c* oxidase activity was similar for soybean cotyledons (19%; $P = 0.003$) and roots (20%; $P = 0.018$). Slightly greater inhibition was observed for purified Cyt *c* oxidase from beef heart (28%; $P = 0.019$). The average inhibition of plant

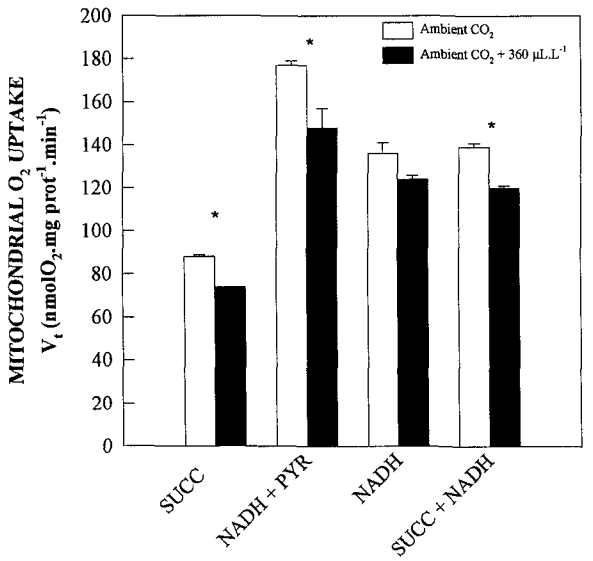

Figure 1. The direct effect of elevated C_a on soybean cotyledon mitochondrial respiration. Oxidation of either succinate (SUCC), NADH and pyruvate (NADH+PYR), NADH alone (NADH), or succinate and NADH (SUCC+NADH) was measured in the presence of ADP (state 3 conditions). Values shown are for mitochondria from plants grown in the greenhouse, except SUCC and NADH+SUCC plants, which were grown in growth chambers. Mitochondria were incubated for 10 min with 0 (□) or 0.1 (■) mM DIC at 25°C. Respiratory controls and ADP-to-O ratios were 1.34 ± 0.05 and 1.23 ± 0.06, 1.49 ± 0.05 and 1.54 ± 0.03, and 1.42 ± 0.07 and 1.66 ± 0.08 for succinate, NADH, and succinate plus NADH, respectively. Values are means ± SE of three to nine replicates. *, Significant difference in the mean ($P < 0.05$) using a Student's *t* test or a rank summary test (see text). V_t, Total velocity of O_2 uptake of intact mitochondria.

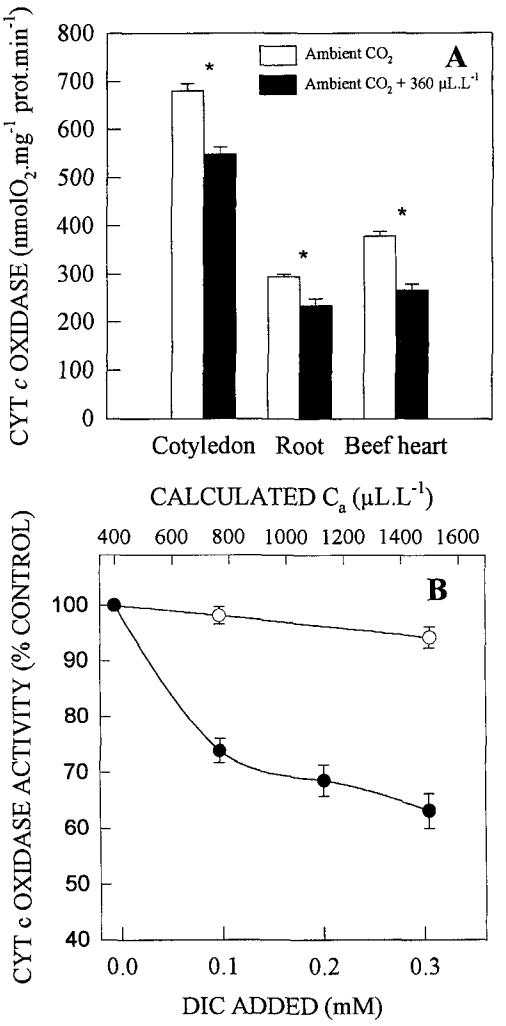

Figure 2. The direct effect of elevated C_a on Cyt *c* oxidase activity. A, Cyt *c* oxidase from soybean cotyledon and root mitochondria or isolated from beef heart was incubated for 10 min with 0 (□) or 0.1 (■) mM DIC at pH 7.2 in a closed cuvette before the measurements were taken. B, The effect of elevated DIC on beef-heart Cyt *c* oxidase activity measured without preincubation time (○) and with a 10-min DIC preincubation (●). Values are means ± SE of three to eight replicates.

mitochondrial Cyt *c* oxidase, 19 to 20%, was similar to the percentage of inhibition seen with mitochondrial electron transport (Fig. 1). A titration of the beef-heart Cyt *c* oxidase activity showed that the effect of increasing C_a on the activity of the enzyme was largest when C_a was increased from normal ambient to twice ambient levels (Fig. 2B). Much less inhibition of Cyt *c* oxidase was observed when it was not preincubated with the elevated C_a for 10 min prior to taking the measurements (Fig. 2B).

The activity of SDH from mitochondria from soybean cotyledons was also inhibited by increasing C_a in the reaction medium (Table I). The percentage of inhibition of SDH activity by elevated C_a was dependent on the concentration of succinate present: 20% at 0.25 mM succinate ($P = 0.003$), 12% at 2 mM succinate ($P = 0.022$), and 7% at 8 mM

Table I. *The direct effect of elevated C_a on the activity of SDH from soybean cotyledon mitochondria*

SDH was preincubated for 10 min with 0 (ambient C_a), 0.17 (ambient C_a + 360 μL L^{-1}), or 0.51 (ambient C_a + 1080 μL L^{-1}) mM DIC at pH 7.5 before the measurements were taken. Values are means ± SE of three to six replicates. Letters indicate statistical differences in the degree of inhibition of an increase in ambient C_a in 360 μL L^{-1}, $P < 0.05$ (one-way analysis of variance). For other statistical details, see text.

C_a	Succinate Concentration			
	0.125 mM	0.25 mM	2 mM	8 mM
	nmol O_2 mg^{-1} protein min^{-1}			
Ambient	32 ± 0.5	40 ± 0.8	110 ± 2.1	124 ± 3.7
Ambient + 360 μL L^{-1}	24 ± 0.7	32 ± 0.9	97 ± 2.0	115 ± 4.0
Ambient + 1080 μL L^{-1}	17 ± 0.6	22 ± 0.7	–[a]	–
	0.75a	0.80b	0.88c	0.93c

[a] –, Not determined.

succinate ($P = 0.143$). The degree of inhibition of SDH activity by elevated C_a was lessened as the concentration of succinate was increased ($P < 0.05$) (Table I).

The Direct Effect of Atmospheric CO_2 on the Cyt and Alternative Pathways

Elevated C_a inhibited the $V_{cyt\text{-}SHAM}$ with a variety of substrates (Fig. 3). Elevated C_a caused a greater inhibition of the Cyt pathway than O_2 uptake by mitochondria in the absence of any inhibitor (Fig. 1), except for the oxidation of NADH alone. The percentage of inhibition of the Cyt pathway by elevated C_a during oxidation of succinate was 16% ($P < 0.001$) (Fig. 3, SUCC); 22% when external NADH and pyruvate were supplied ($P = 0.030$) (Fig. 3, NADH+PYR); and 17% with both NADH and succinate ($P = 0.009$; plants grown in growth cabinets) (Fig. 3, SUCC+NADH). Oxidation of NADH alone gave the lowest inhibition (9%, $P = 0.056$) (Fig. 3, NADH).

Increased C_a had little or no effect on $V_{alt\text{-}KCN}$ (Fig. 4). Elevated C_a inhibited the alternative pathway only when the mitochondria were oxidizing succinate (17%; $P = 0.145$, rank summary test) and not when NADH, either alone or in the presence of pyruvate, was the substrate.

With the oxidation of succinate, the time needed to attain maximal inhibition of O_2 uptake by increased C_a was greater for the $V_{cyt\text{-}SHAM}$ than for $V_{alt\text{-}KCN}$ (Fig. 5). With the Cyt pathway, maximal inhibition was achieved after 5 to 6 min, whereas only 2 to 3 min were needed to maximally inhibit the alternative pathway.

DISCUSSION

The results of this study show that the reported direct inhibitory effect of increasing C_a on plant respiration (Amthor et al., 1992) is also seen in isolated plant mitochondria. The effect was mediated at least in part by inhibition of Cyt *c* oxidase and SDH. Elevated C_a did not inhibit the alternative oxidase. The levels of inhibition obtained in soybean cotyledon mitochondria matched those reported for soybean leaves and whole plants by doubling ambient levels of C_a (Bunce, 1990; Byrd et al., 1992; Thomas and Griffin, 1994).

Elevated C_a inhibited the oxidation of succinate and NADH by mitochondria (Fig. 1). Inhibition of O_2 uptake

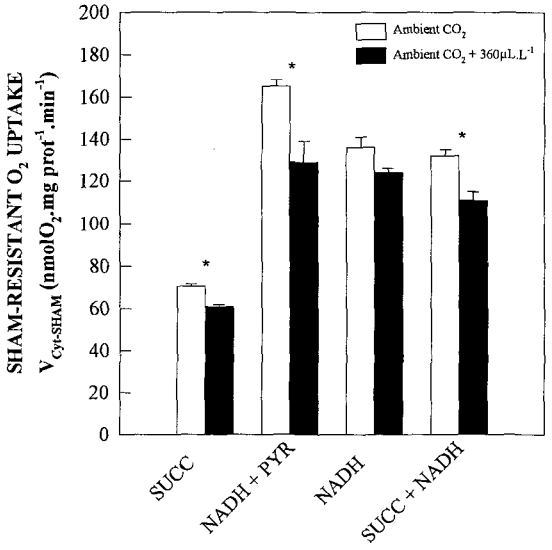

Figure 3. The direct effect of elevated C_a on Cyt pathway activity. $V_{cyt\text{-}KCN}$ was measured in mitochondria preincubated for 10 min with 0 (□) or 0.1 (■) mM DIC in the presence of 2 mM SHAM. For other details see "Materials and Methods" and the legend to Figure 1.

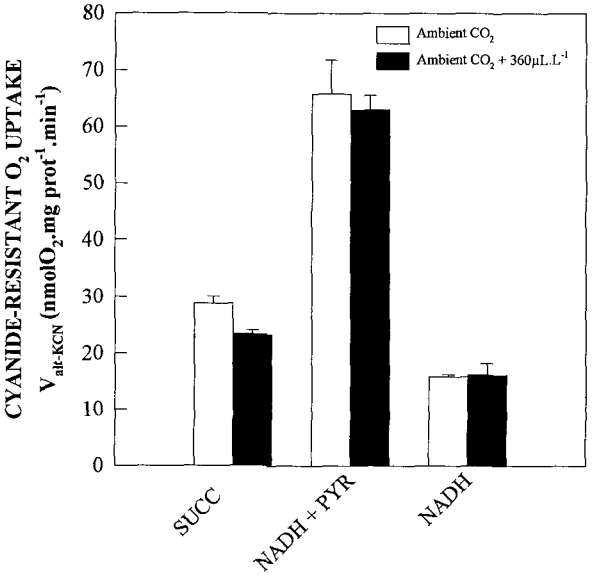

Figure 4. The direct effect of elevated C_a on cyanide-resistant alternative pathway activity. $V_{alt\text{-}KCN}$ was measured in mitochondria preincubated for 10 min with 0 (□) or 0.1 (■) mM DIC in the presence of 1 mM KCN. For other details see "Materials and Methods" and the legend to Figure 1.

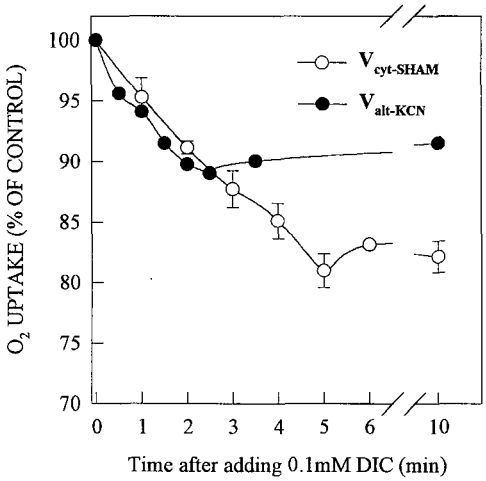

Figure 5. The time course of inhibition of the Cyt ($V_{cyt\text{-}SHAM}$) and alternative ($V_{alt\text{-}KCN}$) pathways by elevated C_a in soybean cotyledon mitochondria. Concentrations used were: 5 mM succinate, 1 mM KCN, 2 mM SHAM, and 0.1 mM DIC. Values are the means of two different experiments for each parameter.

should reflect the inhibition of those components of the mitochondrial electron transport chain that control the overall rate of respiration (Padovan et al., 1989; Moore, 1992). The inhibition of Cyt *c* oxidase activity from different sources by elevated C_a (Fig. 2A) and SDH activity (Table I) supports the results obtained with the entire mitochondrial electron transport chain, and suggests that the direct effect of elevated C_a on intact mitochondrial respiration mainly affects the activity of the Cyt pathway. In our study, inhibition was greater under conditions in which Padovan et al. (1989) reported that substantial control of respiration resided at the level of Cyt *c* oxidase (Fig. 1, SUCC).

The inhibition of Cyt *c* oxidase was greater when the enzyme was preincubated with elevated C_a prior to taking the measurement. Very high concentrations of DIC (10–20 mM, equivalent to 5–10% C_a) inhibited the activity of purified beef-heart Cyt *c* oxidase 40 to 50% (Palet et al., 1991, 1992). However, Palet et al. (1991, 1992) did not preincubate the enzyme with elevated C_a prior to measurement. Without preincubation, titration of the activity of the purified beef-heart Cyt *c* oxidase versus C_a showed no significant inhibition of activity at physiological levels of C_a (Fig. 2B), but showed 50% inhibition when 10 mM DIC (5% C_a; data not shown) was added without preincubation (González-Meler, 1995). These data show that the reaction between Cyt *c* oxidase and any form of DIC is time-dependent.

The time-dependent lag in the effect of elevated C_a on the activity of Cyt *c* oxidase (Fig. 2B) is also observed in intact mitochondrial respiration (Fig. 5). The slow equilibrium reaction among soluble species of inorganic carbon in the mitochondrial matrix (Forster et al., 1969; Balboni and Lehninger, 1986) may explain this time lag, at least for the alternative pathway (Fig. 5). However, the time needed to reach maximal inhibition for the Cyt pathway was considerably longer than the time expected for equilibrium of DIC chemical species to be attained (Fig. 5). Other proteins (e.g. Rubisco or hemoglobin) also react slowly with CO_2 (Mitz, 1979; Lorimer, 1983). The fact that CO_2 can readily cross biological membranes (Balboni and Lehninger, 1986) and the facility with which it carbamylates proteins (Mitz, 1979) suggest that a reversible protein carbamylation may be involved in the inhibition of Cyt *c* oxidase. Although from this study we cannot conclude which chemical species of inorganic carbon inhibited Cyt *c* oxidase, Palet et al. (1991, 1992) showed that inhibition of Cyt *c* oxidase from carnation callus and pea leaf mitochondria depended on the concentration of free CO_2 dissolved in the reaction medium. Bicarbonate can also inhibit plant Cyt *c* oxidase competitively, but only at very high concentrations (Miller and Evans, 1956).

SDH activity of soybean cotyledons was also inhibited by elevated C_a (Table I). However, the relative inhibition of SDH was greater at lower concentrations of succinate. Dicarboxylic (malonate, acetoacetate, and oxaloacetate) or monocarboxylic acids (formate, glycolate, and glyoxylate) all inhibit competitively the activity of SDH (DerVartanian and Veeger, 1964). Bicarbonate is also a monocarboxylic acid and has been reported to be a competitive inhibitor of SDH (Zeylamaker et al., 1970). The effect of succinate concentration on the inhibition of SDH activity by elevated C_a is consistent with the competitive nature of the inhibition by bicarbonate reported by Zeylemaker et al. (1970). Inhibition of the activity of SDH by 1200 μL L^{-1} C_a has also been observed in root mitochondria (Reuveni et al., 1995).

Oxygen uptake through the $V_{Cyt\text{-}SHAM}$ was always inhibited by C_a independent of the substrate used, although in the case of NADH alone the inhibition was minimal (9%) (Fig. 3). During the oxidation of succinate, significantly more control of respiration resides at Cyt *c* oxidase than during the oxidation of NADH (Padovan et al., 1989). This means that inhibition of the Cyt *c* oxidase by C_a (Fig. 2) will not be able to reduce the rate of oxidation of NADH alone, as is observed in oxidation of succinate (Fig. 3), in which C_a also inhibited SDH activity (Table I). Inhibition of the Cyt pathway by increased C_a was considerably greater during oxidation of NADH and pyruvate than with NADH alone. It should be noted that the rate of Cyt pathway activity during the oxidation of NADH alone is lower than that for the oxidation of NADH and pyruvate (Fig. 4) (see also Ribas-Carbó et al., 1995), suggesting that the sites of metabolic control during the oxidation of NADH alone versus NADH and pyruvate are not comparable.

The alternative oxidase is apparently not inhibited by elevated C_a, because its pathway ($V_{alt\text{-}KCN}$) was not inhibited when mitochondria were oxidizing NADH, when significant metabolic control at the level of the alternative oxidase would be expected (with or without pyruvate) (Fig. 4). Thus, the inhibition of the Cyt pathway shown in Figure 4 for the oxidation of NADH can be attributed to the inhibition of Cyt *c* oxidase. However, the alternative pathway was inhibited when mitochondria oxidized succinate. Such an inhibition is a consequence of inhibition of SDH by elevated C_a (Figs. 4 and 5).

Our results suggest that at least part of the so-called direct effect of elevated C_a on respiration reported in tissues (e.g. Amthor et al., 1992) may be located at the level of the mitochondria. An increase in the concentration of CO_2 equivalent to 360 μL L^{-1} inhibited the rate of mitochondrial O_2 uptake by 10 to 15%, depending on the substrate utilized. Greater inhibition was found during the oxidation of succinate. This can be explained by the direct inhibition of SDH and Cyt *c* oxidase by elevated C_a. There was no direct effect of increased C_a on the alternative oxidase. Because all of the decarboxylations in the Krebs cycle form CO_2 (Balboni and Lehninger, 1986), and because bicarbonate concentration in the mitochondrial matrix may fluctuate between 0.05 and 0.4 mM (calculated from Raven and Newman, 1994, and refs. therein), identifying which chemical species (free CO_2 or bicarbonate) inhibits mitochondrial activity will be useful in establishing the physiological consequences of this effect. It is also noteworthy that the acclimation effect of C_a on plants or tissues grown in elevated C_a affects the activity and amount of Cyt *c* oxidase (Azcón-Bieto et al., 1994) and SDH (Frenkel and Patterson, 1973), suggesting that these enzymes are important for the control of respiration in a high-CO_2 world.

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