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Reduced Cytosolic Fructose-1,6-Bisphosphatase Activity Leads to Loss of O_2 Sensitivity in a *Flaveria linearis* Mutant[1]

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ABSTRACT

The mutant plant of *Flaveria linearis* characterized by Brown *et al.* (Plant Physiol. 81: 212–215) was studied to determine the cause of the reduced sensitivity to O_2. Analysis of CO_2 assimilation metabolites of freeze clamped leaves revealed that both 3-phosphoglycerate and ribulose 1,5-bisphosphate were high in the mutant plant relative to *F. linearis* with normal O_2 sensitivity. The k_{cat} of ribulose-1,5-bisphosphate carboxylase (RuBPCase) was equal in all plant material tested (range 18–22 s^{-1}) indicating that no tight binding inhibitor was present. The degree of RuBPCase carbamylation was reduced in the mutant plant relative to the wild-type plant. Since 3-phosphoglycerate was high in the mutant plant and photosynthesis did not exhibit properties associated with RuBPCase limitations, we believe that the decarbamylation of RuBPCase was a consequence of another lesion in photosynthesis. Fructose 1,6-bisphosphate and its precursors, such as the triose phosphates, were in high concentration in the mutant plant relative to the wild type. The concentrations of the product of the fructose 1,6-bisphosphatase reaction, fructose 6-phosphate, and its isomer, glucose 6-phosphate, were the same in both plants. We found that the mutant plant had up to 75% less cytosolic fructose 1,6-bisphosphatase activity than the wild type but comparable levels of stromal fructose 1,6-bisphosphatase. We conclude that the reduced fructose-1,6-bisphosphatase activity restricts the mutant plant's capacity for sucrose synthesis and this leads to reduced or reversed O_2 sensitivity.

The rate of the photosynthetic reactions occurring in chloroplasts can be limited by the capacity of the rest of the plant to use the products of photosynthesis (19). This feedback limitation on photosynthesis has been the subject of much debate (5, 8, 11, 16). It has been established that O_2 insensitive photosynthesis results from one type of feedback effect on photosynthesis, that of limited starch and sucrose synthesizing capacity (19). However, the biochemical mechanism(s) which underlie this feedback regulation of photosynthesis remain to be determined (8, 16). Plants with altered ratios of CO_2 assimilation capacity to sucrose synthesis capacity may be extremely useful in elucidating the mechanism which causes O_2 insensitive photosynthesis.

While screening plants of the C_3-C_4 intermediate *Flaveria linearis* for variations in O_2 sensitivity, Brown *et al.* (2) discovered a mutant plant that appeared to have reduced sensitivity to O_2 because of feedback limitations within photosynthesis. There was no indication that the reduced O_2 sensitivity resulted from more or less C_4 character in this plant's photosynthetic characteristics. This aberrant plant and its selfed progeny lost O_2 sensitivity at air levels of CO_2 when the leaf temperature was 30°C. The apparent feedback limitation in this plant is more severe than any other plant tested to date.

We freeze-clamped actively photosynthesizing leaves from the mutant plant, a wild-type plant, and from *Phaseolus vulgaris*. Metabolite analyses indicated that the photosynthetic characteristics of the mutant plant were caused by reduced FBPase activity. Enzyme analysis confirmed that cytosolic FBPase activity in the mutant relative to the wild type was reduced.

MATERIALS AND METHODS

Plant Material. Cuttings of *F. linearis* were express mailed from Athens, Georgia to Reno, Nevada. Cuttings of the plant identified as 84-8 (normal O_2 response) and 84-9 (reduced or reversed O_2 response) were grown in Reno in a mixture of peat:sand:perlite, 2:1:1. Plants were grown in an unshaded greenhouse with day/night temperatures of 30°C/20°C. RH was controlled at 60%. Plants were fertilized 3 times weekly with half-strength Hoagland's solution.

Gas Exchange Measurements and Freeze Clamping. Two to four leaves (5 mm wide) were laid across a circular chamber. Air was mixed from N_2, O_2, and CO_2 and circulated over the leaf material by means of a small pump (Spectrex, Redwood, CA). Upon leaving the circuit, the humidity and CO_2 content of the air was measured with a Binos IRGA (Leybold, Hereaus, Koln, W. Germany). The temperature of the aluminum chamber was controlled to maintain 25°C leaf temperature. All measurements were made with 300 μbar CO_2 and 1000 μmol photons m^{-2} s^{-1}. Measurements were made with 180 mbar O_2 first, then 30 mbar. After the gas exchange characteristics of the leaves had been determined, the leaves were freeze-clamped in the chamber with two liquid N_2 cooled copper blocks. The freezing time was less than 0.25 s. The freeze-clamped leaf material was stored in liquid N_2 until analysis.

Metabolite Analysis. The leaf material which had been freeze-clamped was ground in a mortar and pestle prechilled with liquid N_2. To the ground powder was added 600 μl 3.5% $HClO_4$. After the plant material-perchloric acid homogenate thawed, it was transferred to a microfuge tube and spun for 2 min in an Eppendorf 5414 microfuge. Of the supernatant, 500 μl were transferred to another tube and 135 μl 0.13 M HEPES and 2 N KOH were added. The pH was adjusted to between 5 and 7 if necessary. After centrifugation (as above) to remove the $KClO_4$ precipitate, the supernatant was assayed for metabolite concentrations using NADH and NADP linked enzyme assays (15, 22).

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FBP[2] was measured by adding FBPase (Sigma) and converting the fru 6-P to glc 6-P followed by the glc 6-P dehydrogenase reaction for quantitation. This assay may not detect sedoheptulose 1,7-bisP as does the aldolase reaction commonly used to measure FBP. All assays were carried out with a Sigma ZFP 22 dual wavelength filter photometer (Varex Corporation, Rockville, MD). Metabolite levels are expressed relative to Chl since the leaf area recovered each time varied and RuBPCase catalytic site concentration was not usually measured. Chl was estimated from the pheophytin recovered from the pellet of the acid extract (21).

Enzyme Analysis. Leaf material which had been freeze-clamped was ground in a Ten Brock glass homogenizer for RuBPCase measurements. Carbamylation and k_{cat} measurements were carried out as described in (15). Leaves collected in the light were taken to the lab for FBPase analysis. About 250 mg fresh weight of leaf tissue (about one leaf) was ground in a mortar and pestle with 1.5 ml of extraction buffer (100 mM K-phosphate [pH 7.5], 2 mM EDTA, 10 mM DTT). The homogenate was clarified by centrifugation at 15,000*g* for 2 min in an Eppendorf 5414 microfuge. The pellet was discarded and $(NH_4)_2SO_4$ was added (12.9 g/100 ml). The precipitated protein was removed by centrifugation as above, the pellet was discarded and $(NH_4)_2SO_4$ was again added (an additional 30.7 g/100 ml). The precipitated protein was collected by centrifugation as above and the supernatant was discarded. The pellet was resuspended in a minimum volume of the elution buffer (20 mM phosphate buffer [pH 7.5], 0.4 mM EDTA, 50 mM NaCl, and 10 mM DTT). The two FBPase forms (cytosolic and stromal) were separated by batch ion exchange chromatography using DEAE-Sephadex A-50 (Sigma) as follows. An aliquot (0.1 ml) of the enzyme extract was added to 1 ml of DEAE-Sephadex A-50 preequilibrated with the elution buffer. The enzyme extract was allowed to equilibrate with the DEAE-Sephadex for 2 min at room temperature and then the DEAE-Sephadex was sedimented by a 30 s centrifugation at 200*g*. The solution above the DEAE-Sephadex was removed and retained. The DEAE-Sephadex was washed with one times the bed volume of elution buffer (50 mM NaCl) twice more. The cytosolic FBPase was found to be located in the initial fraction and first wash only. The ionic strength of the elution buffer was then increased to 1 M NaCl. The DEAE-Sephadex was washed twice with one times the bed volume of the high ionic strength buffer. The activity of chloroplastic FBPase was found exclusively in these two fractions. Further washing of the DEAE-Sephadex with high ionic strength buffer did not release more FBPase activity from the DEAE-Sephadex. The chloroplastic and cytosolic FBPase were assayed according to Kelly et al. (6).

Pyrophosphate-dependent phosphofructokinase activity was measured spectrophotometrically in a reaction medium containing 100 mM Tricine, 5 mM $MgCl_2$, 0.15 mM NADH, 2 U/ml aldolase, 3 U/ml triose-P isomerase, 3 U/ml α-glycerol-P dehydrogenase, 1 μM fru 2,6-bisP and 1 mM pyrophosphate. The reaction was initiated by the addition of 2 mM fru 6-P.

RESULTS

Gas Exchange Analysis. The sensitivity of photosynthetic CO_2 assimilation to O_2 level was tested. The rate of assimilation in normal air was 91% of the rate in low O_2 in the mutant *F. linearis* line 84-9 (Table I). The wild type, line 84-8, had only 69% of the rate of assimilation in normal air compared to low O_2, a typical value for C_3 plants. Results from *P. vulgaris* are also presented. In this experiment the loss of O_2 sensitivity in the mutant plant was not as extreme as reported by Brown *et al.* (2) but in a qualitative way the results are the same as described previously for these two lines of *F. linearis*.

Table I. *Ratio of Assimilation Rate in 21% O_2 to Assimilation Rate in 3% O_2*

Standard errors are presented to indicate variability of the ratios.

Plant Material	Ratio
Flaveria linearis	
84-9 (n = 5)	0.91 ± 0.07
84-8 (n = 4)	0.69 ± 0.03
Phaseolus vulgaris (n = 4)	0.79 ± 0.02

Table II. *Effect of Feeding 5m KH_2PO_4 to Twig of Flaveria linearis Line 84-9 on Photosynthesis at Low O_2*

Leaf temperature was 23.5°C and light was 1000 μmol $m^{-2}s^{-1}$.

Condition	O_2	CO_2	Photosynthesis
	mbar	*μbar*	*μmol CO_2 $m^{-2}s^{-1}$*
	181	348	25.3
	24	425	21.8
After Pi feeding			
For 10 min	24	422	22.9
For 120 min	24	445	21.8

Phosphate was fed to a twig of the mutant plant to see if this treatment would reverse the loss of O_2 sensitivity. This experiment was done at a slightly higher CO_2 partial pressure and so upon switching to low O_2, an inhibition of photosynthesis was observed. Feeding 5 mM phosphate for over two h did not stimulate the rate of photosynthesis (Table II).

Metabolite and RuBPCase Analysis. Leaves were freeze-clamped at air levels of CO_2 and either normal (180 mbar) or low (30 mbar) O_2. In addition to leaves from the normal line of *F. linearis* (84-8) and the reduced O_2 sensitivity line (84-9), data from *P. vulgaris* is presented. The pool of RuBP was not affected by O_2 in these experiments (Fig. 1). The 84-8 leaf material had less RuBP than the 84-9. Using conversion factors determined from other leaves we calculate that the RuBP pool size was 2.6 mol RuBP mol^{-1} binding sites for 84-8 and 4.2 mol mol^{-1} for the 84-9 leaves.

RuBPCase activity was assessed by measuring k_{cat} and the degree of carbamylation. The k_{cat}, mol CO_2 mol^{-1} enzyme s^{-1}, was between 18 and 22 s^{-1} and did not vary between treatments (data not shown). Carbamylation was reduced by low O_2 in the 84-8 plant but the effect of O_2 was opposite in the 84-9 plant. In *P. vulgaris*, carbamylation was not affected by O_2 (Fig. 1).

The amount of PGA was low in *F. linearis* relative to *P. vulgaris* and little effect of O_2 was observed (Fig. 1). The 84-9 plant had more PGA than the 84-8 plant. The level of triose phosphates in the 84-9 plant was similar to that in *P. vulgaris* and about double that in the 84-8 *F. linearis* (Fig. 2).

The most dramatic difference between 84-8 and 84-9 *F. linearis* plants was in the pool size of FBP. Almost no FBP was found in the 84-8 plant but in the 84-9 plant the FBP pool was very high, nearly twice the level in *P. vulgaris*. Despite the large pool size of FBP, the pools of fru 6-P and glc 6-P were nearly identical in the 84-8 and 84-9 plants. The pools in *P. vulgaris* were much higher than in *F. linearis*.

FBPase. Our first measurements of FBPase indicated that the mutant plants, which had high levels of FBP, had high levels of FBPase (data not shown). However, when these crude extracts were treated in a way that deactivates the stromal FBPase (7) the mutant had substantially less residual FBPase activity. The residual activity is likely due to the cytosolic form of the enzyme and so we set about to physically separate the two forms to

[2] Abbreviations: FBP, fructose 1,6-bisphosphate; FBPase, fructose-1,6-bisphosphatase; PGA, 3-phosphoglycerate, RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase EC 4.1.1.39; PFP, pyrophosphate-dependent phophofructokinase.

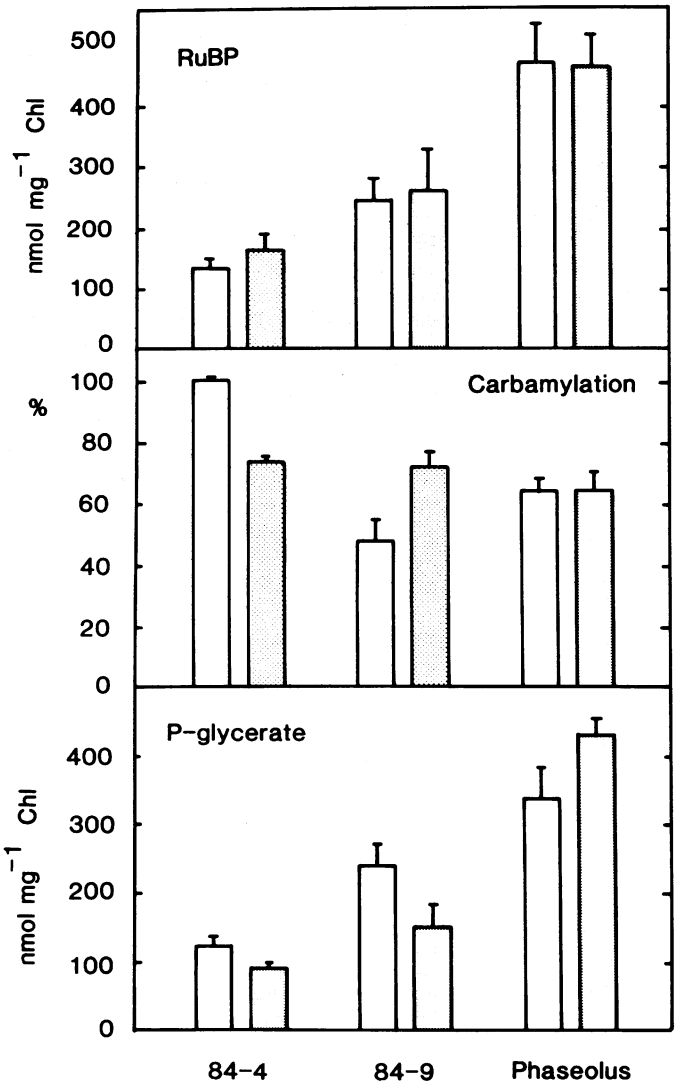

FIG. 1. Substrate pool, product pool and CO_2-Mg^{2+} activation (carbamylation) of RuBP-1,5-bisphosphate carboxylase for wild-type (84-8) and mutant (84-9) *F. linearis* and *P. vulgaris*. The open bars are data obtained in 180 mbar O_2 and the hatched bars are data obtained in 30 mbar O_2.

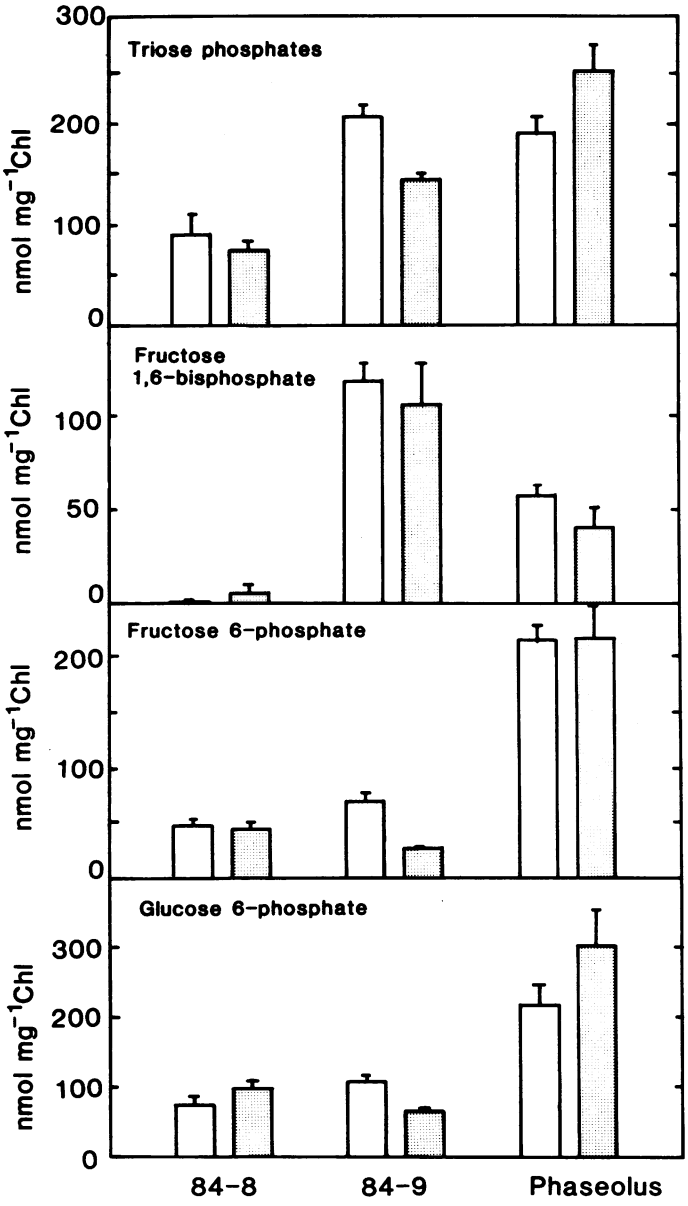

FIG. 2. Metabolite pool sizes in *F. linearis* and *P. vulgaris*. Symbols as in Figure 1.

determine if the mutant had less cytosolic FBPase than the wild type. When the cytosolic and stromal FBPases were separated, we found substantially less cytosolic FBPase in the mutant than the wild-type plant. The activity of the stromal enzyme was higher in the mutant, accounting for the slightly greater total activity (Table III).

We tested for the presence of PFP. In the fractions containing the cytosolic FBPase we found no PFP and in the stromal fraction we found PFP equal to 4% of the activity of FBPase. This is consistent with the finding that PFP elutes from DEAE-cellulose at 0.24 M NaCl (23). These data indicate that some of the FBPase activity we measure as stromal FBPase could be the result of PFP but none of the cytosolic FBPase activity is the result of PFP.

Table III. *Fructose-1,6-Bisphosphatase Activity in Flaveria linearis Plants 84-8 and 84-9*

Plant	Fructose-1,6-bisphosphatase Activity		
	Cytosolic	Stromal	Total
	μmol min^{-1}g^{-1} fr wt		
84-8	0.555	0.58	0.63
84-9	0.012	0.67	0.68
Ratio (%)	22	121	

DISCUSSION

Reduced carbamylation of RuBPCase is commonly seen during feedback limited photosynthesis (13, 18). There is good evidence that the decarbamylation is a consequence of feedback limited photosynthesis, not a cause (12, 14). The high levels of PGA and the gas exchange behavior of the mutant both indicate that the lack of carbamylated RuBPCase was not the cause of the aberrant behavior of the mutant. FBP and precursor metabolite pools were high while fru 6-P and glc 6-P pools were the same in the mutant and wild-type plants. These results indicated that FBPase activity was lower in the mutant.

The relative amounts of FBPase in the two lines of *F. linearis*

were determined after $(NH_4)_2SO_4$ fractionation and subsequent batch separation by DEAE-Sephadex. This procedure should eliminate complications resulting from small effector molecules such as AMP and fru 2,6-bisP. It is usually preferable to assay enzyme activities with as little purification as possible to prevent loss of activity during purification. However, in this case it was necessary to physically separate the two FBPases since the activity of the stromal enzyme overshadows the activity of the cytosolic enzyme. Relating enzyme activity in extracts to *in vivo* behavior is fraught with difficulties but in this case a clear signal from metabolite measurements is confirmed by measurements of enzyme activity *in vitro*.

Brown *et al.* (2) reported that the mutant plant lost O_2 sensitivity, an observation confirmed here (Tables I and II). This requires a change in activity of an enzyme which is required for CO_2 assimilation but not for photorespiration. The stromal FBPase is required for photorespiration but the cytosolic FBPase is not (17).

We have, then, evidence from metabolite pool size measurements, from enzyme activity assays and from analytical gas exchange that is all consistent with the statement that the cytosolic FBPase has a much reduced activity in the mutant line of *F. linearis* discovered by Brown *et al.* (2). We conclude that the cytosolic FBPase activity is reduced *in vivo* in the mutant plant and it is specifically this reduced activity that leads to the aberrant gas exchange behavior seen in this plant. A number of possibilities could account for the reduced FBPase activity seen in 84-9. Transcription or translation of the FBPase gene could be reduced, one of several isozymes could be lost, or the gene for cytosolic FBPase could be changed with the same amount of cytosolic FBPase protein produced but with reduced activity. Now that the cause of the aberrant behavior has been established, we can further examine how a plant responds to the specific reduction in the activity of a single enzyme required for photosynthesis.

Transitions between Limitations in Gas Exchange Behavior. Brown *et al.* (2) found that the mutant behaved the same as the wild type at low CO_2. However, the characteristics of photosynthesis of the mutant changed dramatically as the CO_2 level was increased. This behavior indicates that the cytosolic FBPase exerts no influence on photosynthesis at low CO_2 (and low photosynthesis rate). As the rate photosynthesis increases, the influence of cytosolic FBPase changes from negligible to dominant. The transition in intact leaf photosynthesis behavior is abrupt (Fig. 3 in Ref. 2) as had been discussed by Sharkey (17).

Biochemical Mechanism of Loss of O_2 Sensitivity. The loss of O_2 sensitivity in C_3 plants has been explained in a number of ways. Most recent explanations focus on the phosphate status of the leaves. Sharkey (16) hypothesized that starch and sucrose synthesis reactions are at a maximum rate when plants fail to respond to O_2. Leegood and Furbank (8) hypothesized that the phosphate level in the cytosol falls to below the phosphate optimum for chloroplast photosynthesis but that sucrose synthesis is not at its maximum rate. They fed phosphate to leaves at low temperature and observed a dramatic stimulation of the rate of CO_2 fixation indicating that under their conditions, increased phosphate in the cytosol could stimulate the rate of starch and sucrose synthesis. The observations that leaf pieces fed mannose lost O_2 sensitivity (4) also support the position of Leegood and Furbank. In contrast, the loss of O_2 sensitivity observed in this mutant of *F. linearis* is caused by a loss in enzyme activity. Phosphate feeding did not reverse the loss of O_2 sensitivity in this mutant (Table II). In this case it is the enzymic capacity which is limiting sucrose synthesis. Under natural conditions it is likely that both conditions, phosphate level imbalance and limited enzyme capacity, can lead to a loss of O_2 sensitivity. However, we note that spinach plants grown with restricted phosphate nutrition do not show O_2 insensitivity (1) indicating that this phenomenon is not a symptom of poor phosphate nutrition but rather occurs primarily in very robust plants with high rates of photosynthesis.

Reversed O_2 Sensitivity. The sensitivity to O_2 is often reversed during feedback limited photosynthesis and this is correlated with a reversal of sensitivity to CO_2 (2, 16). If starch and sucrose synthesis capacity (enzyme activity) limits photosynthesis during feedback limited photosynthesis, then reversed sensitivity to O_2 must occur by a reduction in either starch or sucrose synthesis capacity as O_2 is reduced or CO_2 is increased. Starch synthesis could be inhibited by excessively high PGA levels (19) inhibiting hexose monophosphate isomerase (3). Sucrose synthesis could be inhibited if the phosphate optimum of chloroplasts changed in low O_2 (20). Low temperature does cause an increase in the phosphate optimum of isolated chloroplasts (9, 10). If the phosphate level in the cytosol was not at the optimum for photosynthesis in the chloroplasts, photosynthesis in the intact leaf could be reduced from what might otherwise be allowed by starch and sucrose synthesis enzyme capacity as has been demonstrated by Leegood and Furbank (8) at low temperature. It is easy to imagine this mechanism leading to a reversal of the sensitivity to O_2 and CO_2 but this mechanism cannot account for the reverse sensitivity to O_2 seen in *F. linearis*.

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