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Regulation of Climacteric Respiration in Ripening Avocado Fruit[1]

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ABSTRACT

Ripening of avocado fruit is associated with a dramatic increase in respiration. *In vivo* ^{31}P nuclear magnetic resonance spectroscopy revealed large increases in ATP levels accompanying the increase in respiration. Both glycolytic enzymes, phosphofructokinase, and pyrophosphate: fructose-6-phosphate phosphotransferase were present in avocado fruit with the latter activity being highly stimulated by fructose 2,6-bisphosphate. Fructose 2,6-bisphosphate levels increased approximately 90% at the onset of ripening, suggesting that the respiratory increase in ripening avocado fruit may be regulated by the activation of pyrophosphate:fructose-6-phosphate phosphotransferase by an increase in fructose 2,6-bisphosphate.

Ripening of a number of fruits, including avocado, is accompanied by a dramatic increase in respiration (6-8). Ripening and the associated increase in respiration is triggered by endogenous production of or by application of ethylene (8). Based on analysis of glycolytic metabolite levels before and after the respiratory climacteric it has been demonstrated that the rate of carbon flux through glycolysis is enhanced during the respiratory increase in ripening bananas, avocados, and tomatoes (2, 4, 12). It has further been suggested that the increase in glycolysis is brought about by activation of PFK[3] (2, 4, 12). However, most studies of PFK activity and of PFK effector levels have not supported a role for PFK in contributing to the respiratory increase during ripening (11).

Two recent advances prompted us to reexamine the possible role of glycolytic regulation in contributing to the increase in respiration during ripening. The first was the availability of ^{31}P-NMR spectroscopy using an external surface coil as a means to monitor phosphorylated nucleotide levels *in vivo* during fruit ripening. Previous measurements of phosphorylated nucleotide levels in tissue extracts of ripening avocado fruit were complicated by changes in tissue extractability that accompany textural changes of the fruit and by hydrolysis of ATP during extraction, as indicated by exceptionally high ADP levels in the tissue extracts (16). The use of conventional high resolution NMR spectroscopy to monitor *in vivo* levels of phosphorylated nucleotides in plant tissues has found a variety of applications (9). This approach has been restricted to tissues that can be excised and confined in 10 to 20 mm spectrometer tubes with appropriate precautions to prevent tissue anaerobiosis. In the present study involving bulky storage organs we found that tissue excision induced changes in the tissue during acquisition of the spectrum. The use of a surface NMR coil overcame these difficulties and allowed completely noninvasive monitoring of phosphorylated nucleotide levels in the same fruit over the entire period of ripening.

A second advance prompting these studies was the identification in plant tissues of PFP (3). This is an alternative enzyme catalyzing the same glycolytic reaction as PFK, with the exception that PFP utilizes PPi rather than ATP as the phosphate donor and PFP is reversible. It seemed possible that regulation of PFP rather than of PFK may contribute to the enhancement of glycolysis and respiration during avocado fruit ripening. Using ^{31}P-NMR spectroscopy and analysis of PFP and an activator of PFP, $F2,6P_2$, we have reexamined the involvement of glycolytic regulation of climacteric respiration in ripening avocado fruit.

MATERIALS AND METHODS

Plant Material. Avocado fruits (*Persea americana* Mill. cv Hass) were obtained within 24 h after harvest from the University of California South Coast Field Station. Individual fruit were ripened at 20°C in sealed 1 L chambers with continuous flow (6 L/h) of ethylene-free air.

Respiration. Respiration was measured by monitoring CO_2 production. Gas samples (1 ml) were withdrawn from the outlet port of each ripening chamber and CO_2 analyzed by IR gas analysis as described (5).

NMR Spectroscopy. Avocado fruits were removed from the ripening chambers at the indicated times and ^{31}P-NMR spectra recorded. The spectrum in Figure 1 was recorded from an excised 10 mm diameter avocado tissue plug. The spectrum was recorded using a General Electric NM-200 spectrometer at 81 MHz; 8000 transients were acquired using a 60° pulse, a 0.45 s pulse repetition time, 4000 time domain points, and an 8 KHz spectral width. Line broadening of 25 Hz was applied.

The spectra in Figures 2A and 3A were recorded from intact, whole avocado fruit set on a 3.5 cm diameter surface NMR coil. Fruit were marked and placed in the same orientation with respect to the surface coil each day. Spectra were recorded using a ORS TMR 32/310 spectrometer at 32.5 MHz. One thousand transients were acquired using a 30 μs pulse, 2000 time domain points, a 2 KHz spectral width, and a 1.5 s pulse repetition time. Line-broadening of 10 Hz was applied.

Chemical shifts in all spectra are reported relative to 85% H_3PO_4. Peak assignments are as follows: I, cytoplasmic Pi; II, vacuolar P; III, γ-P of ATP and β-P of ADP; IV, α-P of ATP and of ADP (may also contain resonances of UDP-glucose or

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[3] Abbreviations: PFK, phosphofructokinase; PFP, pyrophosphate:fructose-6-phosphate phosphotransferase; $F2,6P_2$, fructose 2,6-bisphosphate.

other pyrophosphodiesters); V, β-P of ATP. The unlabeled peak near 5 ppm arises from phosphomonoesters (*i.e.* sugar phosphates).

Enzyme and $F2,6P_2$ Assays. Individual avocado fruits were allowed to ripen in a ripening chamber and CO_2 production was monitored. One g tissue samples were removed with a cork borer and immediately frozen in liquid N_2 and the avocado returned to the ripening chamber. Previous studies have indicated that removal of tissue plugs does not alter avocado ripening (1). For PFK and PFP determinations, frozen tissue samples were powdered in liquid N_2 and extracted and assayed as described by Smyth et al. (10). Activity of PFP was assayed in the absence and presence of 1 μM $F2,6P_2$. For determination of $F2,6P_2$ levels, frozen tissue samples were powdered in liquid N_2 and extracted as described by Stitt *et al.* (14). Determination of $F2,6P_2$ in tissue extracts was carried out using the potato PFP bioassay described by Van Schaftingen *et al.* (15).

RESULTS

The feasibility of obtaining *in vivo* ^{31}P-NMR spectra from avocado fruit tissue was demonstrated using 10 mm tissue cylinders excised from avocado fruit and recording ^{31}P-NMR spectra in a conventional high resolution NMR spectrometer (Fig. 1). Spectra were obtained over a 1 h time period and showed clearly resolved peaks. Repeated scanning of excised tissue, however, revealed time dependent decreases in the cytoplasmic Pi and possibly other peaks (data not shown). This most likely resulted from restricted O_2 supply to the tissue (9) or from a physiological wound response.

The use of an external, surface NMR coil in a large bore (20 cm) NMR spectrometer magnet allowed noninvasive acquisition of ^{31}P-NMR spectra from intact avocado fruit (Figs. 2A, 3A). The two series of spectra are representative of seven fruits that were monitored in the early (Fig. 2, March 1985) or late season of fruit maturity (Fig. 3, October 1985). Avocado fruit were obtained within 24 h after harvest and allowed to ripen at 20°C in sealed chambers with continuous flow of air. CO_2 production was monitored (Figs. 2B and 3B) and the fruit removed at the indicated times for acquisition of ^{31}P-NMR spectra. The lower field strength of the large bore magnet resulted in decreased resolution of ^{31}P peaks as compared to the spectrum in Figure 1.

Relative changes in ATP levels during ripening were determined by measuring the area of the β-ATP peak (V) in each spectrum (Figs. 2B, 3B). In seven fruits examined, the ATP levels increased between 86 and 94% following the increase in respiration. The level of the cytoplasmic Pi peak (I) appeared to decrease as the β-ATP peak (V) increased (compare Fig. 3A, 49 h with 202 h). Although the level of cytoplasmic Pi is difficult to quantify because of low resolution in this region of the spectrum, the apparent decrease in cytoplasmic Pi is consistent with cytoplasmic Pi serving as the pool of Pi for increasing levels of phosphorylated nucleotides. No notable changes were detected in the chemical shift of cytoplasmic or vacuolar Pi suggesting that large changes in the pH of these compartments do not accompany avocado ripening.

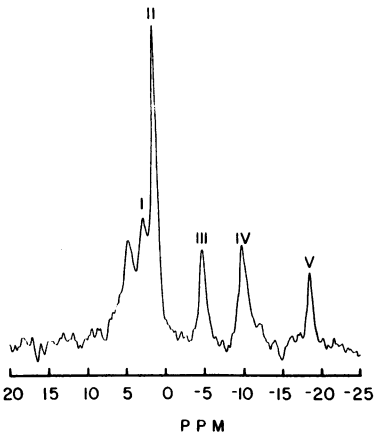

FIG. 1. ^{31}P-NMR spectrum of excised avocado fruit tissue at 81 MHz. Peak assignments are as follows: I, cytoplasmic Pi; II, vacuolar Pi; III, γ-P of ATP + β-P of ADP; IV, α-P of ATP and of ADP (may also contain resonances of UDPG or other pyrophosphodiesters); V, β-P of ATP.

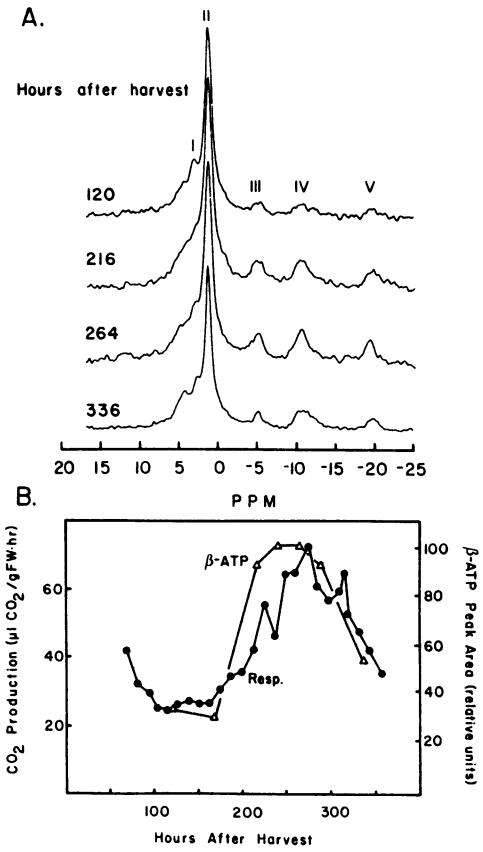

FIG. 2. A, ^{31}P-NMR spectra of intact avocado fruit during ripening. Spectra were obtained at 32.5 MHz by placing the same fruit in the same orientation on a 3.5 cm diameter surface coil at the time indicated. Peak assignments and chemical shifts are as in Figure 1. The spectra were normalized according to the amplitude of the ^{1}H resonance of water in the fruit taken with the same probe tuning. Normalization according to the area of the vacuolar Pi resonance gave identical results. B, Respiration rate and relative ATP levels during avocado fruit ripening. β-P-ATP peak areas (Δ) were determined from the area of the β-P-ATP peak of a series ^{31}P-NMR spectra, some of which are shown in Figure 2A, taken with the same ripening fruit. The rate of CO_2 production (●) was monitored as described in "Materials and Methods."

Because avocado PFK is inhibited by ATP (Solomos [11] and references therein), the increased steady state ATP level determined by *in vivo* NMR spectroscopy was inconsistent with a role of PFK activation in enhancing glycolytic activity during ripening. Increases in cytoplasmic Pi have been suggested to play a key role in activating PFK during tomato fruit ripening (4). However, our results indicate that in avocado, cytoplasmic Pi levels decrease at the onset of ripening which is again inconsistent with activation of PFK during avocado fruit ripening.

To determine whether PFP may provide an alternative pathway for carbon flux through glycolysis during ripening the activity of PFK and PFP was assayed in extracts of avocado tissue sampled throughout ripening (Fig. 4A). At all stages of fruit

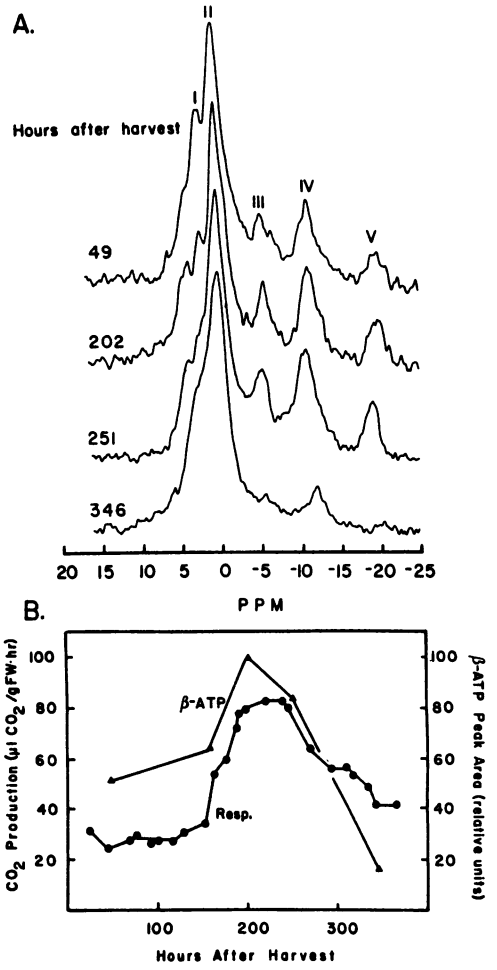

FIG. 3. A, ^{31}P-NMR spectra of intact avocado fruit during ripening. Spectra were normalized according to the area of the vacuolar Pi resonance. Parameters were the same as for Figure 2A except that a 3 s pulse repetition time was used. B, Same as Figure 2B.

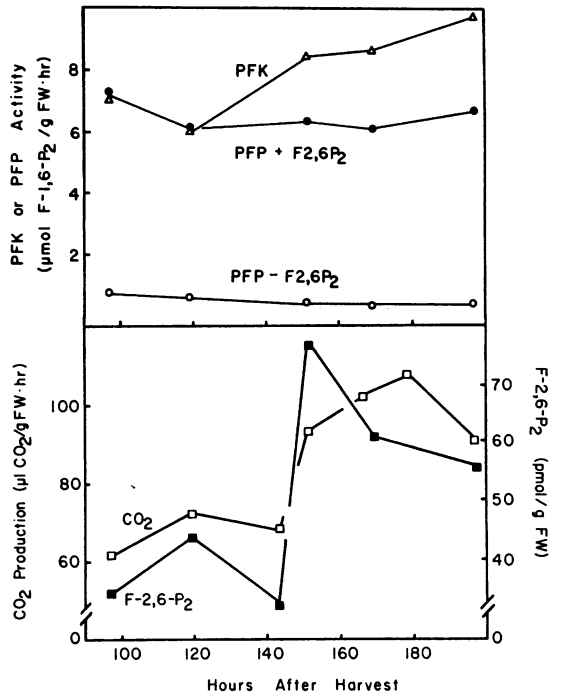

FIG. 4. Phosphofructokinase (PFK), phosphofructophosphotransferase (PFP) activities, fructose-2,6-bisphosphate ($F2,6P_2$) levels and respiration rate (CO_2 production) in a ripening avocado fruit. An avocado fruit was allowed to ripen in a respiration jar and CO_2 production (□) monitored. One g tissue samples were removed with a cork borer at the indicated time points and immediately frozen in liquid N_2. PFK (△) and PFP (○, ●) activities were extracted and assayed as described in "Materials and Methods." PFP activity was determined in the absence (○) or presence of 1 μM $F2,6P_2$ (●). $F2,6P_2$ (■) was extracted and assayed as described in "Materials and Methods."

ripening both enzymes were present. Under the assay conditions employed, PFP activity was stimulated approximately 10-fold by $F2,6P_2$ (Fig. 4A) with the concentration of $F2,6P_2$ giving half-maximal activation of PFP of 21 nM (data not shown). When PFP was assayed in the presence of $F2,6P_2$ the activity of this enzyme in tissue extracts was similar to that of PFK, suggesting that, if fully activated, both enzymes could contribute significantly to glycolytic activity.

The level of $F2,6P_2$ in the tissue samples from ripening avocado fruit was found to increase by 80 to 90%, coincident with the increase in respiration (Fig. 4B). The timing of the increase in $F2,6P_2$ suggests that the respiratory increase during avocado fruit ripening may result from the activation of PFP by increases in $F2,6P_2$. This proposal reconciles a great deal of data that identifies the glycolytic reaction catalyzed by PFK as the point of activation of glycolysis, but which fails to account for this activation by changes in the most important modulators of PFK (11).

DISCUSSION

We have reported here *in vivo* ^{31}P-NMR measurements indicating large increases in ATP during avocado ripening. The use of an external surface NMR coil avoided metabolite alterations associated with wounding and allowed monitoring of individual fruit over the entire period of ripening. Our results confirm a previous report of increases in ATP during avocado ripening (16). Attempts to measure turnover rates of ATP by saturation transfer NMR were unsuccessful due to the relatively low sensitivity of the instrument used in this study. Successful application of this technique will be required to determine whether ATP turnover is stimulated in ripening fruit. This information will be useful in evaluating to what extent, if any, climacteric respiration is required to fulfill increased energy demand associated with ripening.

Activation of the glycolytic reaction catalyzed by PFK and PFP has long been recognized to contribute to the respiratory increase associated with ripening (2, 4, 12). A recent report has demonstrated that ethylene treatment of carrots and potatoes (nonclimacteric storage tissues) resulted in a large increase in $F2,6P_2$ (13). We observed here an increase in $F2,6P_2$ of similar magnitude in a naturally ripening avocado. This increase in $F2,6P_2$ is temporally correlated with the increase in respiration, suggesting that activation of PFP may play a role in climacteric respiration. Because the levels of a number of other potential modulators of PFK and PFP change at the onset of ripening, a more complete analysis will be required to assign a definite role for PFP in the regulation of climacteric respiration.

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