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Viewpoints

The Cyanide-Resistant Oxidase: To Inhibit or Not to Inhibit, That Is the Question

In recent years, there have been considerable advances in the characterization of the cyanide-resistant alternative pathway associated with plant respiration. The pathway consists of a single enzyme, a cyanide-resistant quinol oxidase called the AO, that shunts electrons off the cyanide-sensitive Cyt pathway at the level of the ubiquinone pool and reduces oxygen to water with no conservation of energy (for recent reviews, see Day et al., 1995; Siedow and Umbach, 1995; Wagner and Krab, 1995). cDNA and gene sequences for the AO from several species have been reported (Day et al., 1995), which has led to some understanding of the structural features of the oxidase (Day et al., 1995; Siedow and Umbach, 1995).

Studying the partitioning of electron flow between the alternative and Cyt pathways using isolated mitochondria, Bahr and Bonner (1973) found that low concentrations of Cyt pathway inhibitors such as cyanide or antimycin diverted electrons onto the alternative pathway. However, specific inhibition of the AO, using compounds such as SHAM, did not divert electrons onto the Cyt pathway. These results suggested that electron flow through the alternative pathway took place only when electron transfer through the Cyt pathway was either at or near saturation. The validity of the Bahr-Bonner, or electron overflow, paradigm has been debated within the plant respiration community (Laties, 1982; Lambers, 1985). Most experimental evidence was consistent with the paradigm, and simultaneous measurement of oxygen uptake and ubiquinone redox poise qualitatively confirmed the Bahr-Bonner model (Dry et al., 1989). During succinate oxidation by isolated mitochondria, AO activity appeared only when the ubiquinone pool was more than 40 to 50% reduced, whereas the activity of the main pathway increased as a linear function of reduced ubiquinone at levels well below those needed for significant AO activity.

The general acceptance of the electron overflow paradigm had several consequences. First, regulation of the alternative pathway through an electron overflow mechanism culminated in the proposal that the AO acted primarily in an "energy overflow" capacity (Lambers, 1985). The alternative pathway was envisioned as being active when the cytoplasm contained excess reducing equivalents and/or had a high adenylate charge (i.e. ATP:ADP ratio). The latter circumstance, in particular, will restrict electron flow through the Cyt pathway. Shunting electrons onto the alternative pathway allows continued operation of glycolysis and the TCA cycle, providing carbon skeletons and oxidation of excess carbohydrate, among other possibilities (Lambers, 1985). Second, acceptance of the electron overflow paradigm led to the use of AO inhibitors to quantify alternative pathway activity during respiration by intact plant tissues (Laties, 1982; Lambers, 1985). Any inhibition of oxygen uptake following the addition of SHAM could be taken as a measure of alternative pathway activity in the uninhibited condition because the diversion of electrons onto the saturated Cyt pathway after inhibitor addition could not occur. Numerous caveats attended the application of this approach (Møller et al., 1988), but its theoretical underpinnings appeared sound. Notable, however, was the high frequency with which tissues showing reasonable rates of alternative pathway activity following inhibition of the Cyt pathway, in turn, showed no inhibition when an alternative pathway inhibitor such as SHAM was added (Laties, 1982). This suggested that no electron flow was taking place through the alternative pathway in the absence of added inhibitor.

Several recent observations have been made that call into question the electron overflow paradigm. First, the Cyt pathway never attains a saturated rate, even at high levels of ubiquinone reduction (Van den Bergen et al., 1994). This means that there will always be some diversion of electrons to the Cyt pathway following inhibition of the AO, resulting in a systematic underestimation of alternative pathway activity in the absence of added inhibitor. Equally important, the AO is subject to potential posttranslational regulation through the operation of two systems. One involves a regulatory sulfhydryl-disulfide redox system on the AO protein in which the reduced state shows 4- to 5-fold greater activity than the oxidized state (Umbach and Siedow, 1993). This sulfhydryl-disulfide system responds rapidly to changes in the redox poise of the mitochondrial matrix in isolated mitochondria (Vanlerberghe et al., 1995). The second potential regulatory feature of the AO involves its allosteric activation by α-keto acids, of which pyruvate is, physiologically, the most relevant (Millar et al., 1993). In the presence of pyruvate, the AO shows significant activity at low levels of reduced ubiquinone (Umbach et al., 1994; Day et al., 1995). If the mitochondrial pyruvate level in planta is high enough to place the AO in the "activated" state, the alternative pathway can compete with the Cyt pathway for electrons when neither pathway is near saturation. Addition of SHAM to such a tissue could show no inhibition of oxygen uptake; the electrons are accommodated by the excess capacity available on the Cyt pathway. Applying the electron overflow paradigm to this example would lead to the erroneous conclusion that there was no alternative pathway activity in the uninhibited condition.

Exploiting the effects of pyruvate, the sharing of electrons between the two pathways has been clearly demonstrated in

Abbreviations: AO, alternative oxidase; SHAM, salicylhydroxamic acid.

isolated mitochondria (Hoefnagel et al., 1995; Ribas-Carbo et al., 1995). Therefore, under appropriate conditions the AO can compete with the Cyt pathway for electrons and the diversion of electrons from the alternative pathway to the Cyt pathway can take place following the addition of SHAM. These studies have been limited to isolated mitochondria, but high concentrations of pyruvate can be generated in planta under physiological conditions, for example during the deacidification phase in CAM plants or during photosynthesis in NAD^+-malic enzyme-type C_4 plants (Day et al., 1995). Therefore, any inhibition of respiration following the addition of an alternative pathway inhibitor to intact tissue or isolated mitochondria only allows the conclusion that some alternative pathway activity was present prior to inhibition. No quantitative estimate of how much activity can be derived from this measurement. Particularly problematic is the often-reported lack of inhibition of respiration following addition of SHAM (Laties, 1982; Lambers, 1985). In light of the new understanding of AO regulation, no conclusions can be drawn about the functioning of the alternative pathway from such a result.

No less confounding are measurements of "maximum" alternative pathway activity made following the addition of inhibitors of the Cyt pathway, because such inhibition can lead to perturbation of the reduction state of the pyridine nucleotide pool in the mitochondrial matrix. As inferred from the data of Vanlerberghe et al. (1995), increases in the NAD(P)H/$NAD(P)^+$ poise in the mitochondrial matrix can lead to rapid activation of the AO through reduction of the regulatory sulfhydryl-disulfide system. This would result in the observation of more alternative pathway activity than existed prior to inhibition. Similar problems could attend the addition of uncouplers, which will lower the cell adenylate charge, and could affect both the level of cytosolic pyruvate and the redox poise of the mitochondrial pyridine nucleotide pool.

Eliminating the concept of electron overflow as a primary regulatory feature of the alternative pathway does not rule out the possibility that a role for the alternative pathway in metabolism is one of energy overflow. The feed-forward nature of the allosteric regulation of the AO by pyruvate and the response of the AO's regulatory sulfhydryl-disulfide system to an increase in the reduction state of the mitochondrial pyridine nucleotide pool are complementary mechanisms. Both enhance AO activity under the conditions envisioned previously as leading to alternative pathway activity during "energy overflow" (e.g. excess cellular levels of reducing substrate and/or a high cellular adenylate charge) (Lambers, 1985).

The only method that exists at present for making quantitative measurements of alternative pathway activity, and even this technique is still in the developmental stage, is the use of oxygen isotope fractionation (Robinson et al., 1995). Cyt *c* oxidase and AO differentially fractionate ^{18}O when reducing oxygen to water, and this fractionation can be accurately measured with a mass spectrometer. This allows calculation of the partitioning of electron flow between the two pathways in the absence of added inhibitors. Comparative results of oxygen electrode and mass spectrometer studies using isolated mitochondria support the validity of such measurements (Ribas-Carbo et al., 1995). Alternative approaches have been considered, including saturation transfer NMR and calorimetric measurements of plant respiration (Wagner and Krab, 1995), but both of these procedures are dogged by theoretical considerations that preclude their applicability.

In summary, measurements using the oxygen electrode in conjunction with metabolic inhibitors should not be used in the future for purposes of deriving alternative pathway activity. Recalling the title of this essay, the answer is not to inhibit. However, it is important to remember, "There are more things in heaven and earth, Horatio, than are dreamt of in your philosophy" (Shakespeare, 1695). No doubt this will apply to the alternative oxidase as we come to understand further this fascinating and important feature of plant metabolism.

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