Plant Physiol. (1983) 73, 945-948
0032-0889/83/73/0945/04/$00.50/0

Interaction of Benzylaminopurine with Electron Transport in Plant Mitochondria during Malate Oxidation

Received for publication March 7, 1983 and in revised form June 14, 1983

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ABSTRACT

The effect of 6-benzylaminopurine (BA) was assayed on malate oxidation in mitochondria isolated from fresh and aged potato (*Solanum tuberosum* L.) slices. Depending on the experimental pH, two pathways for malate oxidation were selected. A pH of 7.7 favored the activity of malate dehydrogenase, which is connected with a rotenone-sensitive NADH dehydrogenase, whereas at pH 6.5 malic enzyme, linked to a rotenone-resistant NADH dehydrogenase, was more active.

Experimental results indicate the existence of two sites of inhibition for BA. The first site is common with the site of inhibition of rotenone. The second site is on the classical cyanide-resistant alternative pathway, but is different from the site of salicylhydroxamic acid (SHAM) inhibition, as in succinate oxidation.

Moreover, a distinct cyanide-resistant pathway, sensitive to SHAM but resistant to BA, is found to coexist with the well-known alternative pathway which is sensitive to SHAM and BA. This outlet of electrons can accommodate 10% of the total electron flow in mitochondria from fresh slices, and up to 30% in mitochondria from aged slices.

In plant mitochondria, malate is oxidized by two different enzymes, MDH[1] and NAD^+-dependent ME (1, 4, 13, 14, 19). The location of these two enzymes in the matrix space seems to be widely accepted now (3, 19, 22). Endogenous NADH produced by malate oxidation is also thought to be reoxidized by two distinct NADH dehydrogenases located on the inner face of the inner membrane (15, 18, 19, 22, 25). This occurs through a rotenone-sensitive NADH dehydrogenase connected to MDH while a rotenone-resistant NADH dehydrogenase would be linked to ME (22). However, there is no general agreement on this point at present (26).

The contributions of the two enzyme systems to malate oxidation strongly depend on the mitochondrial environment: pH (2, 13, 23), added NAD^+ (4, 20, 22, 23), concentration of malate (24), and level of NADH in the matrix space (18). The effects of so many factors can easily account for the variability observed in the responses of malate oxidation to rotenone (22, 25, 26).

On the other hand, it is a common observation that malate oxidation is particularly resistant to cyanide inhibition (10, 11) and, thus, is partly mediated by the alternative cyanide-resistant pathway. Moreover, the functioning of ME would be linked to the alternative pathway as shown by Rustin *et al.* (22).

Recently, BA, a synthetic cytokinin, was shown to strongly inhibit the part of succinate oxidation that is resistant to cyanide (7, 16). In contrast, depending on the plant materials used, BA acts in an erratic way on the part of malate oxidation that is similarly resistant to cyanide, particularly in the case of potato tissue (7). On mitochondria from aged potato slices which display cyanide resistance, preliminary results have shown that BA and SHAM act differently on the various pathways of malate oxidation (8). This observation led us to undertake a detailed study of the effect of BA on malate oxidation mediated by specifically defined electron transport pathways in both cyanide-sensitive and cyanide-resistant mitochondria from fresh and aged potato slices respectively.

[1] Abbreviations: MDH, malate dehydrogenase; ME, malic enzyme; BA, 6-benzylaminopurine; SHAM, salicylhydroxamic acid.

MATERIALS AND METHODS

Potato tubers (*Solanum tuberosum* L., var Bintje) were purchased locally. Slices, 1 mm thick and 1 cm in diameter, were cut out of the central part of the tubers and aged during 24 h in an aerated liquid medium as previously described (9). Mitochondria from fresh and aged slices were isolated and purified on sucrose gradient according to conventional procedures (9). Mitochondrial proteins were measured by acid digestion and nesslerization.

O_2 uptake was measured at 25°C in a 2-ml Plexiglas cell fitted with a Clark O_2 electrode. The medium was 300 mM mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer, and 1 mg/ml BSA. Unless otherwise stated, the pH of the medium was 7.2. Depending on the substrate used (10 mM succinate or 30 mM malate), 0.5 to 1 mg mitochondrial protein was used. KCN in water solution, SHAM and rotenone in ethanol, and BA in dimethylsulfoxide were used as inhibitors of electron transfer.

Products of malate oxidation were determined by incubating mitochondria in the medium used for O_2 uptake measurements, supplemented with 3.5 mM sodium arsenite to inhibit pyruvate oxidation. The reaction was initiated by addition of 30 mM malate, and stopped by addition of an excess of $HClO_4$. After centrifugation at 3,000*g* for 5 min, the supernatant was neutralized with K_2CO_3, centrifuged at 3,000*g* for 5 min, and used for determinations of oxaloacetate and pyruvate. Oxaloacetate and pyruvate were measured with purified malate and lactate dehydrogenases, respectively, by following the oxidation of NADH at 340 nm in a medium consisting of 100 mM phosphate buffer (ph 7.5), 120 μM NADH, and aliquots of supernatant. The reaction was started by addition of the enzyme.

RESULTS

Effect of pH on Products of Malate Oxidation. Under usual experimental conditions (pH 7.0–7.2), malate oxidation generally proceeds through both ME and MDH. Since the external pH can influence the operation of the malate oxidizing systems (2, 13, 23), two pH values were selected: 6.5 and 7.7.

The participation of the two pathways was determined by analyzing the products of malate oxidation by mitochondria from fresh and aged potato slices (Table I). At pH 6.5, under

Table I. *Products of Malate Oxidation in Mitochondria from Fresh and Aged Potato Slices*

The values were obtained in the presence of ADP (state 3) after a period of 3 min, during which the rates of oxaloacetate and pyruvate formation were approximately linear.

	pH 6.5		pH 7.7	
	Fresh	Aged	Fresh	Aged
	nmol/min·mg protein			
Oxaloacetate	7	9	76	95
Pyruvate	70	101	8	10
O_2 uptake	46	60	41	48

state 3 condition, the formation of pyruvate, and thus the operation of ME was largely prevailing. However, a small amount of oxaloacetate could be measured, indicating a weak participation of MDH. Conversely, at pH 7.7, the formation of oxaloacetate, linked to the operation of MDH, was predominant whereas small amounts of pyruvate were also found to be present.

The oxidation rates appeared to be slightly higher at pH 6.5 than at pH 7.7 (Table I). At the two pH values, they were also higher in mitochondria from aged potato slices than in mitochondria from fresh potato slices. In addition, the O_2 uptakes were found to be rather well correlated with the amounts of oxaloacetate + pyruvate produced.

These results, similar to those of Tobin *et al.* (23) on mitochondria from fresh potato slices, indicate that selecting the pH of the medium permits a rather good discrimination between the two malate oxidizing systems. Moreover, in further experiments conducted at pH 7.7, 2 mM glutamate was added in order to maintain a maximum activity of MDH by preventing its inhibition by oxaloacetate (12).

Effect of BA on Malate Oxidation by MDH. At pH 7.7, the oxidation of malate + glutamate by mitochondria isolated from fresh or aged potato slices appeared to be rather well coupled to phosphorylation (Fig. 1, A and B).

When assayed on mitochondria from fresh slices in which the cyanide-sensitive pathway is the major electron transport pathway, BA inhibited the oxidation rate almost totally (88%) and KCN blocked the slight residual O_2 uptake (Fig. 1A). Since, with succinate as substrate (7), it has recently been demonstrated that BA does not inhibit the Cyt chain *per se*, this effect of BA ought to be located between MDH and ubiquinone, as is rotenone inhibition.

In mitochondria from aged potato slices, the cyanide-resistant electron transfer was 36% of the total state 3 rate (Fig. 1B). The cyanide-resistant oxidation was totally inhibited by BA. SHAM, the usual inhibitor of the cyanide-resistant pathway, had no further effect (not shown). This strong effect of BA suggests that BA could also act on the cyanide-resistant pathway, in the same way as SHAM, as is the case with succinate oxidation (7).

Effect of BA on Malate Oxidation by ME. At pH 6.5, the respiratory control and ADP/O ratios were rather poor even with mitochondria from fresh potato slices, the electrons from ME by-passing the site I of phosphorylation (22).

By contrast with the strong effect observed at pH 7.7, BA slightly inhibited the state 3 rate (7%) of cyanide-sensitive mitochondria from fresh potato slices (Fig. 1C). KCN enhanced the inhibition to an extent of 90%, and SHAM was necessary to achieve complete inhibition. This indicates a weak participation of a BA- and cyanide-resistant electron transport to malate oxidation at this pH.

In mitochondria from aged potato slices (Fig. 1D), the cyanide-resistant electron transfer was greater at pH 6.5 than at pH 7.7 (73% of state 3 rate compared to 36%). BA inhibited the cyanide-resistant malate oxidation to only 60% and SHAM was necessary to block the O_2 uptake totally.

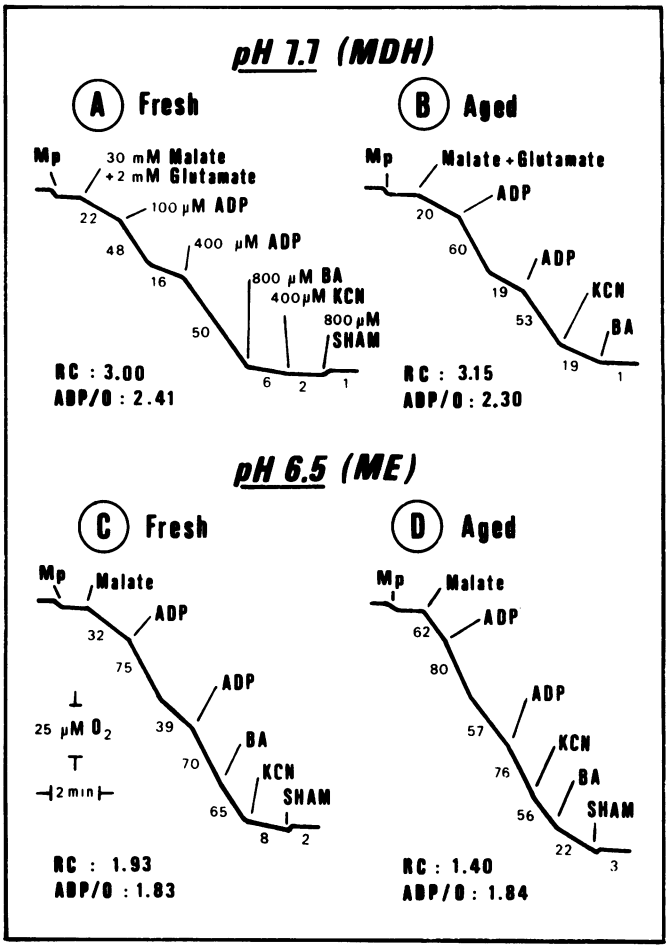

FIG. 1. Effect of BA on malate oxidation at pH 7.7 (A, B) and pH 6.5 (C, D). The assays were performed on mitochondria isolated from fresh (A, C) and aged (B, D) potato slices. Numbers along the traces are nmol O_2/min·mg protein.

The rather weak action of BA observed at pH 6.5 could not be explained by a direct effect of pH on BA since with succinate as substrate no difference was observed in BA inhibition at pH 7.2 and 6.5 (see Fig. 3).

Sites of BA Inhibition. The above results have shown that BA could act at two places in malate oxidation: on the MDH-ubiquinone segment and on the cyanide-resistant alternative pathway.

Since rotenone is the most classical inhibitor of the MDH-ubiquinone segment (NADH dehydrogenase or complex I), it was interesting to compare the effects of BA and rotenone on fresh and aged potato mitochondria (Table II). These experiments were carried out in the presence of 800 µM SHAM. As could be expected (6), SHAM decreased slightly (15%) the rate of malate oxidation in aged potato mitochondria and had no effect on fresh potato mitochondria (compare the values of oxidation rates in Table I and II). Under these conditions, the alternative pathway, and thus any side-effect of BA on this pathway, was eliminated.

In both types of mitochondria, BA inhibited malate oxidation about 10% at pH 6.5 and up to 85% at pH 7.7. The levels of rotenone inhibition closely paralleled those observed with BA, showing that the two compounds exert a strong inhibition only when MDH is active (pH 7.7). Only a very slight inhibition was observed when ME was the prevailing enzyme (pH 6.5). However, inhibition by BA or rotenone was not complete at pH 7.7.

Table II. *Effect of BA and Rotenone on Malate Oxidation Linked to Cyanide-Sensitive Electron Transport*

All values correspond to state 3 rates. The inhibitors were assayed on cyanide-sensitive electron pathway, in the presence of 800 μM SHAM. Two mM glutamate was added to malate at pH 7.7.

	pH 6.5		pH 7.7	
	Fresh	Aged	Fresh	Aged
	nmol O_2/min·mg protein			
Cyanide-sensitive rate	44	51	40	44
	%			
Inhibition				
BA (800 μM)	9	11	85	81
Rotenone (10 μM)	7	5	87	80
BA + KCN (400 μM)	96	95	97	93
Rotenone + KCN	99	96	97	96

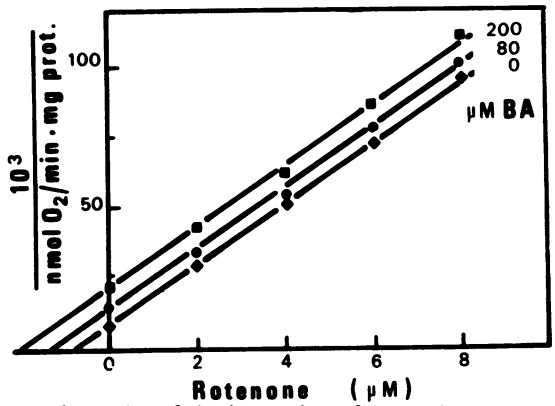

FIG. 2. Dixon plot of the interaction of BA and rotenone on the cyanide-sensitive electron transfer in mitochondria from fresh potato slices. Mitochondria were oxidizing malate in the presence of 2 mM glutamate at pH 7.7 in order to favor electron transfer through MDH. Rotenone was added in the presence of increasing concentrations of BA.

This could be related to a slight participation of ME as mentioned above (Table I). Similarly, a weak participation of MDH at pH 6.5 could explain the slight inhibition observed with the two inhibitors. Finally, a further addition of KCN totally inhibited the oxidation in all conditions. Thus, the two inhibitors, BA and rotenone, act in the same manner on the electron transport linked to the oxidation of malate by MDH.

A detailed study of BA and rotenone interaction was carried out on mitochondria devoid of alternative pathway (fresh potato slices) and at pH 7.7 to favor electron transport through MDH maximally (Fig. 2). The effect of rotenone was measured in the presence of various concentrations of BA. A Dixon plot (5) of the data gave parallel lines. According to Yonetani and Theorell (27), such a result indicates that the two inhibitors act in a competitive manner on the same site.

Considering the alternative pathway itself, it had been previously observed with succinate as substrate that BA and SHAM have identical effects (7). By contrast with this result, the cyanide-resistant part of malate oxidation, *via* ME, was inhibited only to a maximum of 60% by BA (Fig. 1D), whereas it is well known that it is totally inhibited by SHAM (7, 10).

An analysis of the inhibition pattern of the alternative pathway was made at pH 6.5 with mitochondria from aged potato slices (Fig. 3). With succinate, a Dixon plot of the results gave a straight line. At pH 6.5, the rate of succinate oxidation was smaller than at pH 7.2 (the usual pH of succinate oxidation), but no difference was observed between the two pHs in the degree and mode of inhibition. With malate, a biphasic curve was obtained. A break

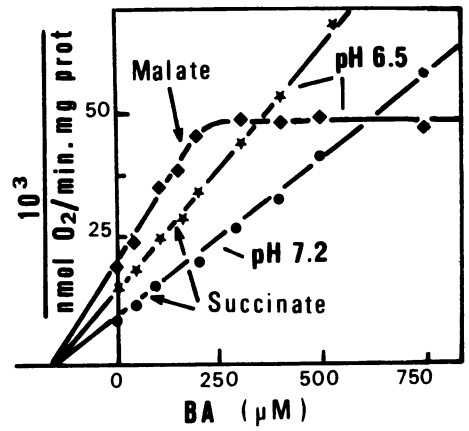

FIG. 3. Dixon plot of BA inhibition of the cyanide-resistant succinate and malate oxidation. The experiments were performed with mitochondria from aged potato slices in the presence of 400 μM KCN. Malate oxidation was studied at pH 6.5 in order to by-pass the rotenone-sensitive site and succinate oxidation was studied at both pH 6.5 and 7.2.

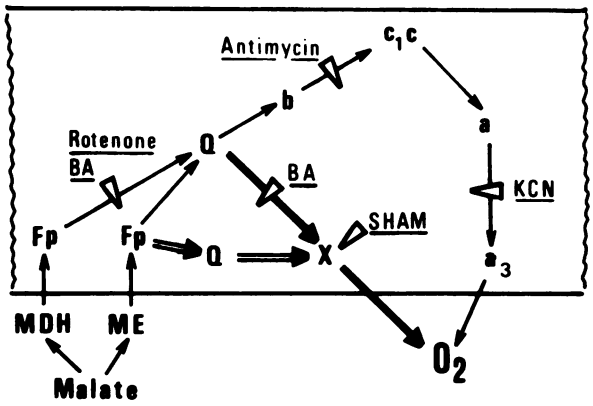

FIG. 4. Different electron transport pathways for malate oxidation. Single heavy arrow, classical alternative cyanide-resistant pathway, sensitive to SHAM and BA. Double arrow, additional cyanide-resistant pathway, SHAM-sensitive but BA-resistant and linked to ME. (a, a_3, b, c, c_1), Cyt; (Fp), NADH dehydrogenases; (Q), ubiquinone pools; (X), 'terminal oxidase' of the alternative pathway.

in the curve occurred at 200 μM BA. The first part of the curve corresponds to the inhibition exerted by BA on the cyanide-resistant pathway. The effects were the same with succinate and malate oxidation, yielding identical apparent K_i values (160 μM), from which one can conclude that BA has only one site of inhibition on the alternative pathway. The second part of the curve corresponds to the cyanide- and BA-resistant electron transfer which is only sensitive to SHAM (Fig. 1D).

DISCUSSION

Cytokinins, such as BA, have been found to inhibit the cyanide-resistant alternative pathway of plant mitochondria (7, 16). This fact was clearly established with succinate, but in the case of malate variable results were obtained due to the complexity of the oxidation of this substrate in plant mitochondira (7, 16). In this work, by using pH conditions favoring MDH (pH 7.7) or ME (pH 6.5), it has been possible to gain more information on the mode of action of BA (Fig. 4).

Two sites can be postulated for BA inhibition. On the one hand, the results clearly show that BA acts on the same site as rotenone. A similarity in the actions of these two inhibitors has also been suggested recently (8, 17, 21). Moreover, this common

site of inhibition appears to be confined exclusively to malate oxidation by MDH in agreement with the findings of Rustin *et al.* (22) for rotenone. On the other hand, BA also acts on the cyanide-resistant electron transfer during malate oxidation by ME, and this site of action was found to be the same whether malate or succinate is oxidized. However, it has been recently demonstrated that BA and SHAM do not inhibit the alternative pathway at the same site (7).

When malate is oxidized at pH 7.2, it has been previously observed that the extent of BA inhibition on the cyanide-resistant pathway was more pronounced with succinate oxidation than with malate oxidation (7). Therefore, at this pH, MDH and ME are both operative. The use of discriminating pH values has shown that the cyanide-resistant electron transfer mediated by MDH is totally BA-sensitive (Fig. 1B). In contrast, BA partly inhibits the cyanide-resistant electron transfer mediated by ME (Fig. 1D). Thus, the site of BA action on the alternative pathway could be located between ubiquinone and the site of SHAM inhibition. In the case of malate oxidation *via* MDH„ the electrons could be transferred through both BA- and SHAM-sensitive sites. In malate oxidation *via* ME, the electrons could be transferred partly (60%) in this way and partly (40%) directly to the SHAM-sensitive site. A specific pool of ubiquinone could eventually be implicated as an intermediary electron carrier in this pathway.

In cyanide-sensitive mitochondria from fresh potato slices, when MDH operates at pH 7.7, the electrons are channeled through the first site of phosphorylation to ubiquinone *via* the rotenone- and BA-sensitive site. Then, the Cyt pathway carries all the electron transfer (6). By contrast, at pH 6.5, a slight cyanide-resistant electron transport is observed, comparable to that mentioned by Rustin *et al.* (22). However, this particular cyanide-resistant transfer which is SHAM-sensitive is also BA-resistant (Fig. 1C).

In mitochondria from aged potato slices, the problem is more complex, due to cyanide resistance (6, 9). The higher level of cyanide inhibition at pH 7.7 (64%) compared to that at pH 6.5 (26%) and higher respiratory control and ADP/O ratios clearly show that the alternative pathway is preferentially linked to ME than to MDH (Fig. 1, B and D), even if this could be due in part to an inhibition of aspartate aminotransferase, as recently suggested (26). At pH 6.5, ME being mainly operative, part of the electrons are transferred by the Cyt chain. Another part goes through the alternative pathway either *via* the BA- and SHAM-sensitive sites or directly *via* the SHAM-sensitive site only. This latter pathway is the same in cyanide-sensitive or in cyanide-resistant potato mitochondria in which it can accommodate about 10% and 30%, respectively, of the total electron flow.

Concerning the discrepancies between the level of BA inhibition observed during malate oxidation in the absence and in the presence of KCN (7, 16), it is quite clear now that the intramitochondrial and external pH could influence the flow of electrons in the different pathways. Under usual conditions, at pH around 7.2, the two malate-oxidizing systems are functional and intermediary results are generally observed. The methods for mitochondrial isolation, the degree of purification of mitochondria and slight variations of pH could be responsible for the extent of engagement of the different routes of malate oxidation. The level of intracellular NAD^+, whose importance has often been emphasized (4, 20, 22, 23), could also play an essential role.

Acknowledgments—We wish to thank Ms. E. Moreau and M. Pouget for their helpful technical assistance.

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