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Effects of Kaempferol on the Oxidative Properties of Intact Plant Mitochondria

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ABSTRACT

The effects of kaempferol on the oxidative and phosphorylative properties of plant mitochondria from potato tubers and etiolated mung bean (*Phaseolus aureus* Roxb.) hypocotyls were investigated. Kaempferol inhibited the state 3 oxidation rate of malate, NADH, and succinate, but was without effect on the ascorbate-tetramethyl *p*-phenylenediamine oxidation rate. The inhibition was almost the same whether the mitochondria were in state 3 or in an uncoupled state 3. When 180 micromolar kaempferol was added during state 4, the tight coupling of succinate or NADH oxidation was not released. The results obtained indicate that kaempferol inhibits the mitochondrial electron flow at, or just after, the flavoprotein site.

Flavonoids are ubiquitous in higher plants, paralleling the distribution of lignin. Despite this wide distribution, the biological function and physiological properties of flavonoid compounds remain to be discovered (for review, see Ref. 7). However, several of these compounds (*e.g.* phloridzin, rotenone, coumestrol) are physiologically active. For example, the isoflavonoid rotenone, isolated from *Lonchocarpus*, is a potent inhibitor of mitochondrial respiration, acting at one of the several nonhaem iron centers associated with the internal NADH dehydrogenase complex (3, 15). Stenlid (11, 12) found that many flavonoids strongly inhibited ATP formation in isolated plant mitochondria; Koeppe and Miller (4), working on corn mitochondria, provided evidence that kaempferol was acting specifically on the phosphorylation mechanism. On the other hand, Tissut *et al.* (13) demonstrated that kaempferol and several other flavonoids behave as inhibitors of the mitochondrial electron carrier chain. All of these results prompted us to examine the effects of kaempferol (3,5,7,4′ tetrahydroxyflavone) on the oxidative and phosphorylative properties of plant mitochondria isolated from potato tubers and etiolated mung bean hypocotyls.

MATERIALS AND METHODS

Preparation of Mitochondria. Mitochondria from potato (*Solanum tuberosum* L.) tubers and etiolated mung bean (*Phaseolus aureus* Roxb.) hypocotyls cut from bean seedlings grown for 5 days in the dark at 26 C and 60% RH were prepared and purified by methods previously described (2). All operations were carried out at 0 to 4 C. Mitochondria prepared in this manner, in addition to being highly purified, were also tightly coupled; average ADP:O ratio for succinate was 1.8, and respiratory control for the same substrate was 3.1. Integrity assays of the inner and outer membranes were evaluated as indicated by Douce *et al.* (2).

O_2 Uptake measurements. O_2 uptake was followed polarographically at 25 C using a Clark-type electrode system purchased from Hansatech Ltd. (Hardwick Industrial Estate, Kings Lynn, Norfolk, England). The reaction medium contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer, 0.1% defatted BSA, and known amounts of mitochondrial proteins. Unless otherwise stated, all incubations were carried out at pH 7.2.

Protein Determination. Protein was determined by the method of Lowry (6), using BSA (Sigma; fraction V) as the standard.

Mitochondrial Swelling. Potato mitochondria (1 mg protein) were suspended in solutions of 120 mM ammonium phosphate or 100 mM ammonium malate in a final volume of 1 ml. *A* at 520 nm was recorded with a Beckman recording spectrophotometer (model 25). Antimycin A (0.15 μg) was added to the incubation medium in order to inhibit malate oxidation. Pi (2.5 mM) was added to activate swelling in ammonium malate (9).

Split Beam Spectrophotometry. This was performed with the Aminco DW-2 spectrophotometer. The concentrations of the different cytochromes were measured at room or liquid N_2 temperature (77 K) from reduced minus oxidized difference spectra. The wavelengths selected for measurements were those given by Lance and Bonner (5).

Chemical. Kaempferol was purchased from Karl Roth (Karlsruhe, Germany) and dissolved in ethanol. At concentrations greater than 300 μM in the reaction medium, kaempferol can precipitate. Consequently, after each assay, the incubation medium was centrifuged rapidly (10,000*g* for 3 min with a Beckman Microfuge B) and the concentration of kaempferol determined in the supernatant by spectrophotometry.

RESULTS

Figure 1 illustrates the effects of kaempferol upon the respiratory rates of potato mitochondria with succinate, NADH, and malate as substrates. When kaempferol is added during state 3, the respiratory rates decrease rapidly with increasing concentrations of kaempferol. The concentration of kaempferol which brings about half-maximum inhibition of respiratory rates in state 3 is not identical for all the substrates used. Thus, the concentration of kaempferol which causes 50% inhibition of malate-driven state 3 respiration is 35 μM, whereas, a greater concentration of inhibitor is required to inhibit NADH and succinate oxidation. The concentration of kaempferol which causes 50% inhibition of malate- or succinate-driven state 3 respiration is lower in a medium free of serum albumin.

In addition, we have observed that the inhibition, by kaempferol, of malate oxidation in the presence of 1 mM NAD^+ is strongly dependent on the pH of the reaction medium (Fig. 2). Thus, at pH 7.5, addition of 100 μM kaempferol to potato mitochondria oxidizing 15 mM malate in the presence of ADP induces a total inhibition of O_2 uptake which shows no tendency to recover, whereas, at pH 6.5, the inhibition is weaker and shows a

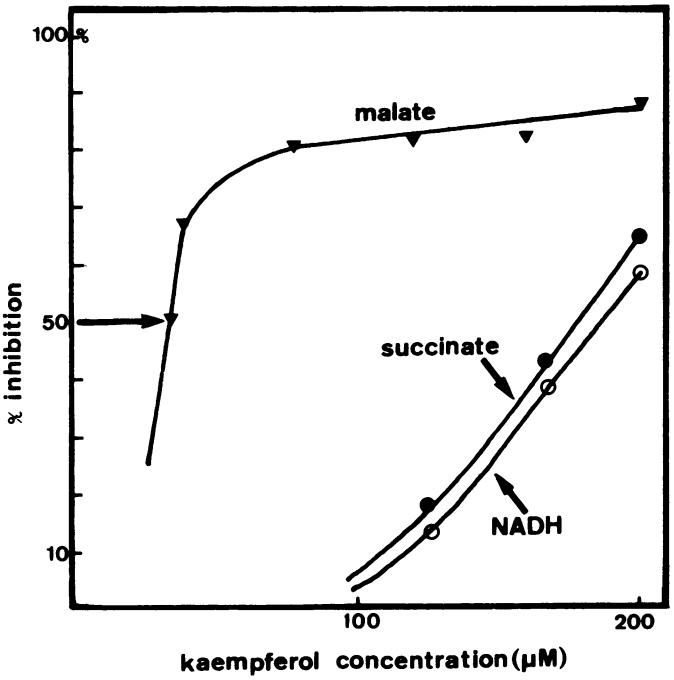

FIG. 1. Effect of kaempferol on state 3 rate of malate, succinate, and NADH oxidation by potato tuber mitochondria (reaction medium, pH 7.2). Substrate concentrations were: 15 mM malate; 10 mM succinate; mM NADH2; and 1 mM ADP.

slow tendency to recover. In contrast, the inhibition by kaempferol of succinate or NADH oxidation is not dependent on the pH in the reaction medium.

In agreement with Koeppe and Miller (4), state 4 respiration is less sensitive to kaempferol (results not shown). The uncouplers, 30 μM DNP[1] and 1 μM FCCP, are unable to reverse the kaempferol inhibition of state 3 respiration. We have observed that the inhibition by kaempferol is almost the same, whether the mitochondria are in state 3 or in an uncoupled state 3 induced by the addition of 30 μM DNP. Moreover, the inhibition of the ADP-stimulated respiration of potato mitochondria by carboxyatractyloside is not relieved by kaempferol (Fig. 3). Similar experiments performed with mung bean mitochondria lead to the same results. These results demonstrate that kaempferol is clearly acting on electron transfer and, perhaps, on the phosphorylation mechanisms as postulated for corn mitochondria (4).

Figure 4 demonstrates that kaempferol is without effect upon the respiratory rate with ascorbate-TMPD as substrate. As this substrate injects electrons in the respiratory chains at the level of the Cyt *c* facing the outer surface of the inner membrane, it is clear that the kaempferol inhibition site is before Cyt *c* in the electron pathway. Difference spectra of antimycin A-treated plant mitochondria (malate + antimycin A oxidized minus oxidized) at liquid N_2 temperature show that classical three α-peaks in the b-region at 553, 557, and 562 nm. In contrast, difference spectra of kaempferol-treated mitochondria (malate + kaempferol oxidized minus oxidized) reveal the absence of reduced *b*-type Cyt (Fig. 5). Consequently, these results demonstrate that kaempferol inhibits the electron flow before the well known antimycin A block. Furthermore, in mung bean mitochondria oxidizing malate, kaempferol strongly inhibits the respiratory pathway through the alternative cyanide-insensitive terminal oxidase. This suggests that the kaempferol inhibition site is before the branching point between the Cyt oxidase pathway and the alternative oxidase pathway (1).

[1] Abbreviations: TMPD, tetramethyl *p*-phenylene diamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DNP, 2-4 dinitrophenol.

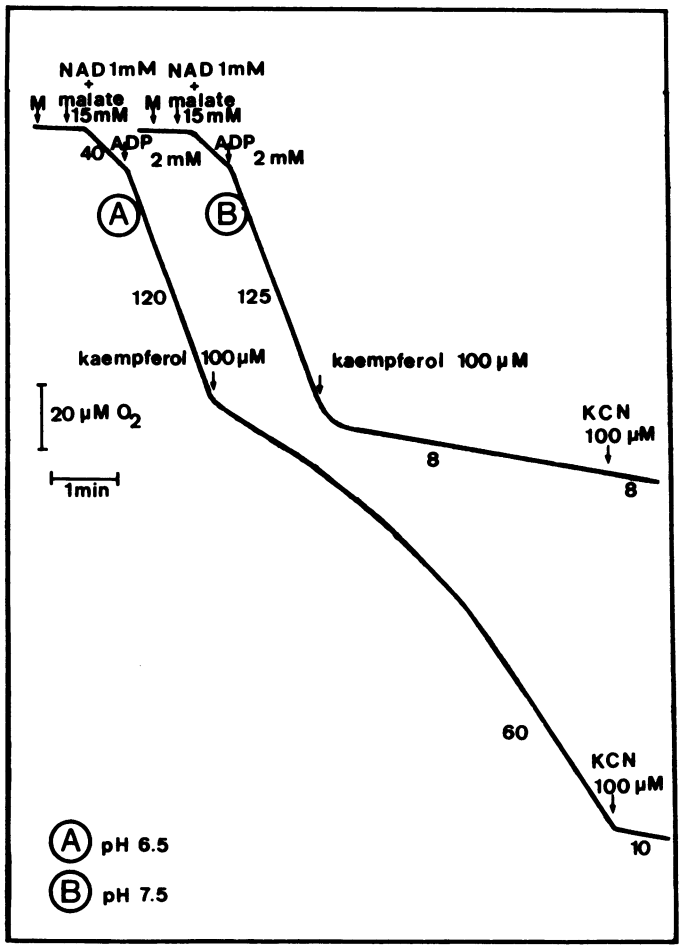

FIG. 2. Effect of kaempferol on malate oxidation by potato tuber mitochondria at pH 6.5 (A) and at pH 7.5 (B). Concentrations given are final concentrations in reaction medium; numbers on traces refer to nmol O_2 consumed/min·mg mitochondrial protein. Because the rate of malate oxidation in potato tuber mitochondria is limited by a lack of endogenous NAD^+ and inasmuch as NAD^+ can penetrate the inner mitochondrial membrane, the incubation medium contained 1 mM NAD (for explanation, see Ref. 14).

DISCUSSION

Our results strongly suggest that the kaempferol inhibition site is either in the flavoprotein region containing FMN or FAD and iron-sulfur protein (internal NADH dehydrogenase, external NADH dehydrogenase, and succinate dehydrogenase) or in the quinone pool. However, the second hypothesis is most unlikely because, for a known amount of mitochondrial protein, the concentration of kaempferol necessary to stop the electron flow is strongly dependent on the substrate used (malate, NADH, or succinate). Except for the NADH, which is oxidized on the external face of the inner membrane, it is also possible that kaempferol could affect the carriers involved in the passage of succinate or malate through the inner mitochondrial membrane. This hypothesis, however, is also unlikely, because kaempferol also inhibits succinate oxidation in submitochondrial particles as it does in intact mitochondria. In addition, and in agreement with Phillips and Williams (9), we have shown that potato mitochondria swell spontaneously when suspended in an ammonium phosphate

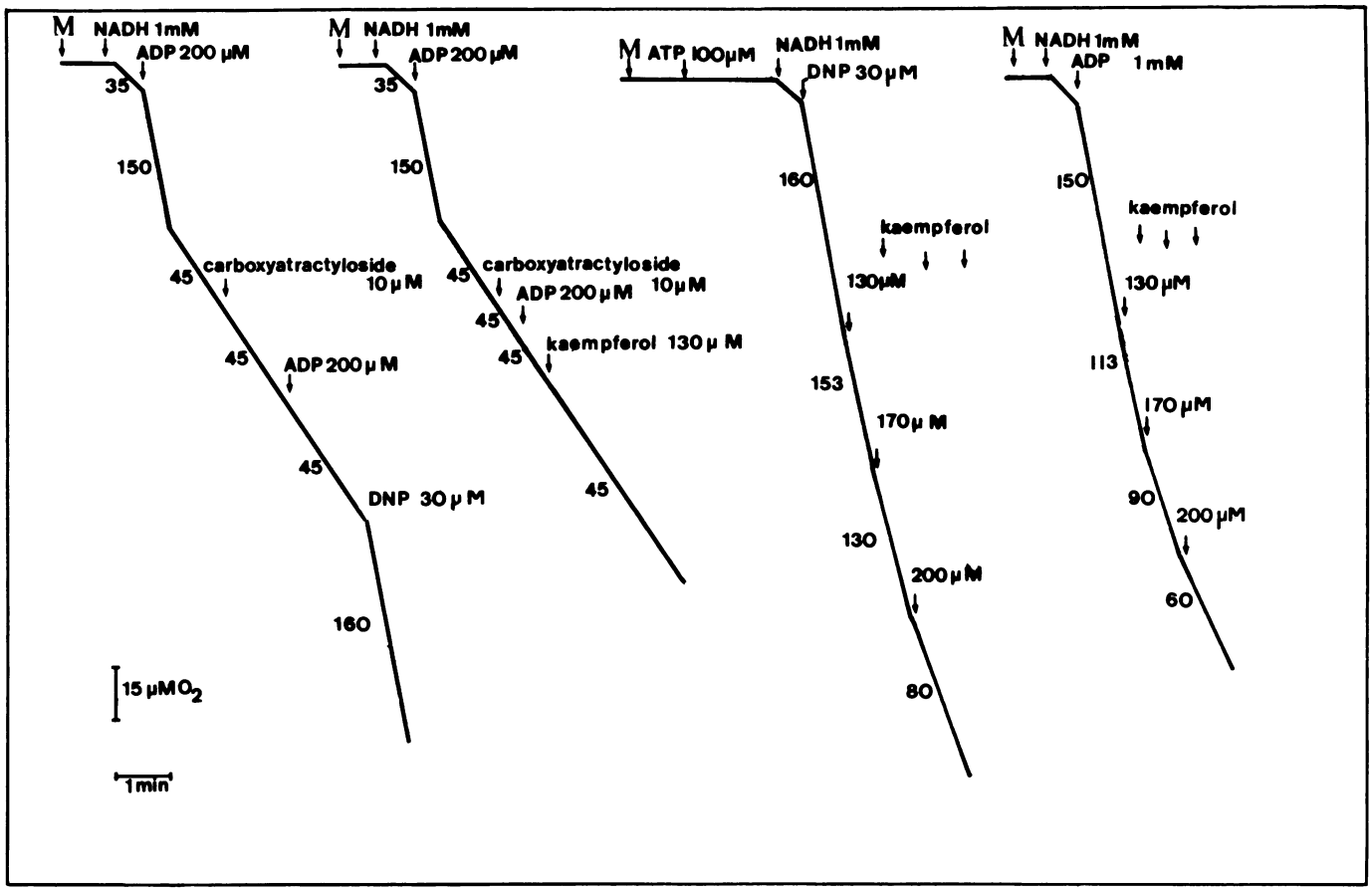

FIG. 3. Effect of carboxyactractyloside and kaempferol on the rate of O_2 consumption by potato mitochondria with NADH as substrate. Concentrations given are final concentrations in reaction medium (pH 7.2). Numbers on traces refer to nmol O_2 consumed/min·mg mitochondrial protein.

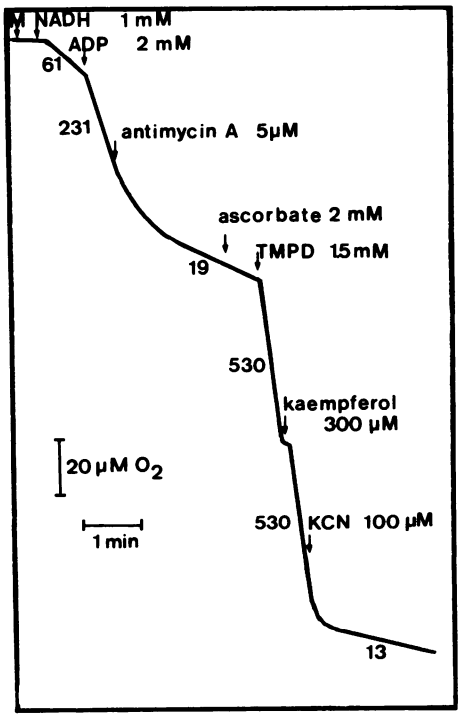

FIG. 4. Effect of kaempferol on ascorbate-TMPD oxidation by potato tuber mitochondria. Concentrations given are final concentrations in reaction medium (pH 7.2). Numbers on traces refer to nmol O_2 consumed/min·mg mitochondrial protein.

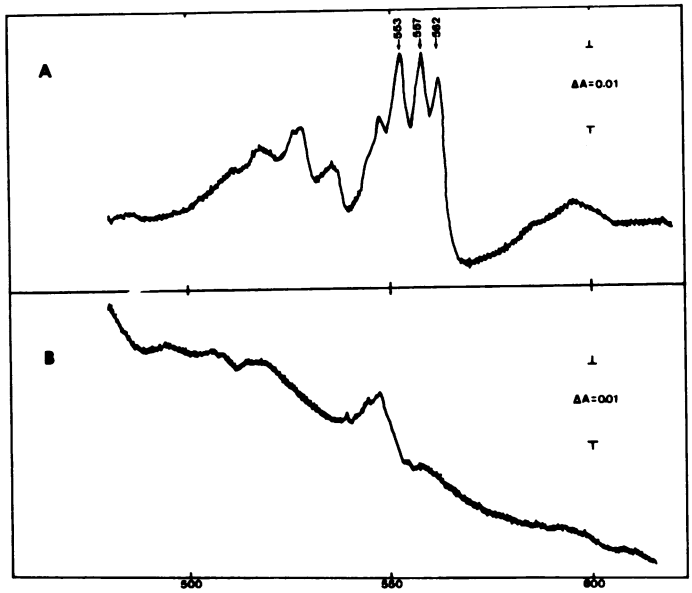

FIG. 5. Difference spectra of potato mitochondria at liquid N_2 temperature (77 K). A, 20 mM malate + 10 µM antimycin A, aerobic minus aerobic; B, 20 mM malate + 300 µM kaempferol, aerobic minus aerobic. Mitochondria were suspended in the reaction medium. Optical path, 2 mm (Plexiglas cuvettes). Mitochondrial protein, 3 mg/ml.

solution or in an ammonium malate solution in the presence of Pi. Addition of 200 μM kaempferol to the incubation medium is without effect on the rate of swelling in ammonium phosphate or ammonium malate. These results strongly suggest that kaempferol does not inhibit entry of malate or phosphate in potato mitochondria.

The possibility that kaempferol could also inhibit the phosphorylation mechanism in mitochondria cannot be ruled out. As a matter of fact, the mitochondrial coupling factor is facing the matrix space and, thus, could be nonaccessible to kaempferol molecules. This is in contrast with broken chloroplasts, where the coupling factor is facing the external medium and where we have shown that kaempferol uncouples phosphorylations (13).

The fact that the inhibition by kaempferol of malate oxidation is strongly dependent on the pH in the reaction medium is comprehensible, inasmuch as, at pH 7.5, only the malate dehydrogenase is operating, whereas, at pH 6.5, it is the NAD-linked malic enzyme which is operating (8). Addition of kaempferol to mitochondria supplemented with malate causes immediate reduction of pyridine nucleotide at all the pH values tested, because this inhibitor slows down the rate of electron flow (14). Under these conditions, the equilibrium of the malate dehydrogenase will move towards malate formation, and this could explain the fact that kaempferol inhibition of malate oxidation is more powerful at pH 7.5 than it is at pH 6.5.

Finally, the effects of kaempferol on the oxidative properties of plant mitochondria contrast markedly with those of the flavone (10). As a matter of fact, flavone, in contrast with kaempferol, is a potent inhibitor of the external NADH dehydrogenase.

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