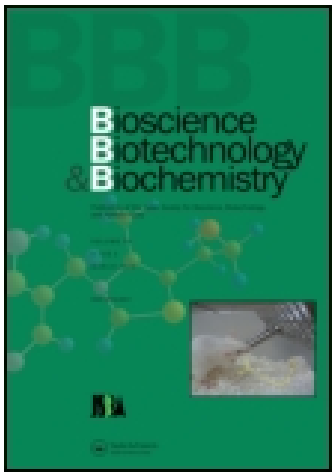

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Specific Interaction of Cytokinins and Their Analogs with Rotenone-sensitive Internal NADH Dehydrogenase in Potato Tuber Mitochondria

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Effects of cytokinins were studied on rotenone-sensitive NADH dehydrogenase in mitochondria from fresh potato tubers (*Solanum tuberosum*), in consideration of the operation of external and rotenone-insensitive internal NADH dehydrogenases that has not been fully accounted for in previous studies. In submitochondrial particles (smp), zeatin was only weakly active, and zeatin riboside (ZR) was inactive. Inhibition rates at 400 μM of isopentenyladenine (iP) and isopentenyladenosine (iPA) were 45% and 30%, respectively, and that of BA (BA) was 64%. In intact mitochondria, the inhibition by iP and BA significantly increased, I_{50} being 50 and 250 μM, respectively, but that by zeatin and iPA decreased. A structure–activity study showed that hydrophobic and steric factors are important for the activity. Cytokinins inhibited the electron flow *via* natural quinone more strongly than that *via* synthetic quinone. These results suggest that among the cytokinins the species that can regulate the electron transport is iP rather than its riboside or zeatin.

Key words: cytokinins; plant mitochondria; NADH dehydrogenase; respiration; potato tuber

Some cytokinins are known to modify plant respiration, and this has been considered to be due to the inhibition of electron flow from internally generated NADH to oxygen *via* a rotenone-sensitive dehydrogenase and the cytochrome pathway.[1–4] Whether this modification of respiration is relevant to any particular action of cytokinins on growth and development of plants has been, however, controversial. The uncertainty arises mainly from two reasons; one is that the effective inhibitory concentration is higher than that usually required for cytokinin assays, especially for the promotion of callus growth, and the other is that the inhibition by naturally occurring zeatin is obscure, although that by synthetic BA is explicit.

After the experiments of Miller[2] on the inhibition of respiratory electron flow by cytokinins, it has been found that plant mitochondria have NADH dehydrogenase on the outer surface of the inner membrane (external NADH oxidase),[5,6] and a rotenone-insensitive NAD(P)H dehydrogenase on the inner surface facing the matrix, in addition to the rotenone-sensitive dehydrogenase (complex I).[7,8] Since the operation of these dehydrogenases has not been fully considered in the previous experiments,[2,4] examination in detail of the cytokinin action on mitochondrial electron flow is needed on the basis of present knowledge. The structure–activity relationship is also to be thoroughly investigated to extend our understanding of the properties of the novel class of respiration inhibitors. Although it has also been reported that BA, but not zeatin, inhibits non-phosphorylating, cyanide-resistant electron flow when it is operating,[2,9–11] in this report we present our results on mitochondria devoid of the alternative pathway.

Materials and Methods

Chemicals. Zeatin, ZR, iP, iPA, BA, kinetin, N^6-*n*-hexyladenine, rotenone and nicotinamide hypoxanthine dinucleotide (deamino NADH) were purchased either from Sigma or from Wako Chemical Co. Synthesis of the following compounds has been reported previously; N^6-*n*- butyladenine, *p*-methoxy- and *p*-nitrobenzyladenines,[12] N^6-phenethyladenine,[13] N^6-*n*-dodecyladenine, *p*-chloro-, *m*-chloro-, *o*-chloro-, *m*-hydroxy-, and *p*-methylbenzyladenines,[14] *m*- and *o*-trifluoromethylbenzyladenines,[15] N^6-geranyladenine,[13] and 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (DB).[16] N^6-Substituted adenines other than those described above were prepared by reacting 6-chloroadenine and an appropriate amine in dimethylsulfoxide at a refluxing temperature.[17] These were purified by recrystalization from ethanol, elemental analyses for C, H, and N of each compound being within the error of $\pm 0.3\%$. *o,p*-Dichlorobenzyladenine, Found: C, 48.74; H, 2.99; N; 23.87. Calcd. for $C_{12}H_9N_5Cl_2$: C, 49.00; H, 3.08; N, 23.81%. *m,p*-Dichlorobenzyladenine, Found: C, 48.89; H, 3.16; N, 23.53. Calcd. for $C_{12}H_9N_5Cl_2$: C, 49.00; H, 3.08; N, 23.81%. *p*-Trifluoromethylbenzyladenine, Found: C, 53.36; H, 3.53; N, 24.10. Calcd. for $C_{13}H_{10}N_5F_3$: C, 53.25; H, 3.44; N, 23.88%. *p*-Fluorobenzyladenine, Found: C, 58.99; H, 4.32; N, 28.93. Calcd. for $C_{12}H_{10}N_5F$: C, 59.25; H, 4.14; N, 28.79%. *p*-Bromobenzyladenine, Found: C, 47.57; H, 3.53; N, 22.98. Calcd. for $C_{12}H_{10}N_5Br$: C, 47.39; H, 3.31; N, 23.03%. N^6-iso-Butyladenine, Found: C, 56.39; H, 6.86; N, 36.73. Calcd. for $C_9H_{13}N_5$: C, 56.53; H, 6.85; N, 36.62%. N^6-*n*-Octyladenine, Found: C, 62.94; H, 8.35; N, 28.29. Calcd. for $C_{13}H_{21}N_5$: C, 63.13; H, 8.56; N, 28.31%. N^6-*n*-Decyladenine, Found: C, 65.71; H, 9.22; N, 25.45. Calcd. for $C_{15}H_{25}N_5$: C, 65.42; H, 9.15; N, 25.43%. N^6-*n*-Tetradecyladenine, Found: C, 68.81; H, 9.86; N, 20.84. Calcd. for $C_{19}H_{33}N_5$: C, 68.84; H, 10.03; N, 20.84%. N^6-*n*-Hexadecyladenine, Found: C, 70.25; H, 10.36; N, 19.52. Calcd. for $C_{21}H_{37}N_5$: C, 70.15; H, 10.37; N, 19.48%.

Isolation of mitochondria. Potato tubers (*Solanum tuberosum* L.) were purchased from local markets. Mitochondria were isolated by the method of Latis.[18] Approximately 600 g of potato tuber was homogenized in 900 ml of a medium containing 0.35 M mannitol, 0.25 M sucrose, 25 mM TES, 1 mM EDTA, 0.05% BSA, 1 mM 2-mercaptoethanol, and 3% (v/v) polyclar SB-100 (pH 7.8) at 4°C. The homogenate was centrifuged at $4000 \times g$ for 5 min and the supernatant was centrifuged at $14{,}000 \times g$ for 20 min. The pellet was resuspended in 30 ml of a medium containing

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Abbreviations: BA, BA; DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; deamino NADH, nicotinamide hypoxanthine dinucleotide; iP, isopentenyladenine; iPA, isopentenyladenosine; smp, submitochondrial particles; ZR, zeatin riboside.

0.35 M mannitol, 0.25 M sucrose, 25 mM TES, and 0.05% BSA (pH 7.8) and centrifuged at $800 \times g$ for 10 min. The mitochondria was pelleted from the supernatant at $14{,}000 \times g$ for 20 min and resuspended in 10 ml of a medium containing 0.35 M mannitol, 0.25 M sucrose, and 25 mM TES (pH 7.8), and again centrifuged at $14{,}000 \times g$ for 20 min. The pellet was resuspended in 0.5 ml of the same medium.

Preparation of smp. Smp were prepared as described in the literature.[19] Mitochondria were diluted with a medium containing 0.3 M sucrose, 10 mM Tris acetate, and 5 mM $MgCl_2$ (pH 7.2) to yield about 20 mg protein/ml. The suspension was sonicated for 6×5 s (maximum output), and the sonicated mitochondria were centrifuged at $24{,}000 \times g$ for 15 min. The supernatant was centrifuged at $105{,}000 \times g$ for 60 min, and the pellet (smp) was suspended in a medium containing 0.3 M sucrose and 10 mM Tris acetate (pH 7.2). These smp were stored at -80°C.

Protein estimation. Protein was estimated by the method of Bradford[20] using BSA as a standard.

Assay procedures. NADH and deamino NADH oxidations by mitochondria and smp were measured spectrophotometrically at 340 nm in a medium containing 0.25 M sucrose, 1 mM $MgCl_2$, and 50 mM potassium phosphate (pH 7.4) at 30°C. The reaction was started by adding 80 μM NADH or deamino NADH. NADH oxidation through rotenone-insensitive NADH dehydrogenase was measured in the medium containing 10 μM rotenone. Succinate-cyt *c* oxidoreductase activity was measured at 30°C in the same medium containing 0.1 μM of an uncoupler SF6847 (2,6-di-*t*-butyl-4-(2,2-dicyanovinyl)phenol), and 2 mM KCN as an inhibitor of complex IV, and 40 μM cyt *c* and 20 mM succinate as substrates were added to start the reaction. The reduction of cyt *c* at 550–540 nm was followed. The final concentration of protein was 30 μg/ml.

Oxygen consumption by mitochondria was measured using a Clark type oxygen electrode (Rank Brothers Co.) in a medium containing 0.4 M mannitol, 25 mM TES, 5 mM $MgCl_2$, 5 mM potassium phosphate, and 0.05% BSA (pH 7.7) at 25°C. The concentration of protein was 0.3 mg/ml. The reaction was started by adding 0.5 mM NAD^+, 5 mM glutamate, 5 mM malate and 0.15 mM ADP. Mitochondria were activated by passing through a single state 3/state 4 transition.

Results

Effects of representative cytokinins

Intact mitochondria and smp prepared form fresh potato slices were totally cyanide-sensitive. When deamino NADH, a selective substrate for rotenone-sensitive dehydrogenase,[21] was used as an electron donor, BA inhibited 64% of its oxidation in smp at 400 μM, I_{50} being 90 μM (Fig. 1 and Table). The inhibition rate by naturally occurring zeatin was 12% at 400 μM and 26% at 800 μM, but ZR was essentially inactive. iP was considerably stronger than zeatin, retarding 45% of the respiration at 400 μM ($I_{50} = 570$ μM), and the inhibition rate of iPA was 30% ($I_{50} = 800$ μM). The inhibition rate of kinetin was as much as that of iP ($I_{50} = 580$ μM). When ferricyanide was used as electron acceptor and deamino NADH as substrate, little inhibition was observed (data not shown).

These cytokinins did not inhibit rotenone-insensitive NADH dehydrogenase in smp, and external NADH dehydrogenase and succinate-cyt *c* oxidoreductase in intact mitochondria, in the concentration range tested (0–800 μM) (data not shown). The NADH oxidation experiments with smp and intact mitochondria include the electron flow *via* complexes III and IV, and succinate oxidation in intact mitochondria that *via* complex III. Thus the results show that the cytokinins are specific inhibitors of rotenone-sensitive dehydrogenase in plant mitochondria.

When a synthetic quinone, DB,[16] was used, the inhibitory potency of the cytokinins was greatly reduced; that at 800 μM of BA diminished to 27% from 76% inhibition in the assay *via* endogenous quinone.

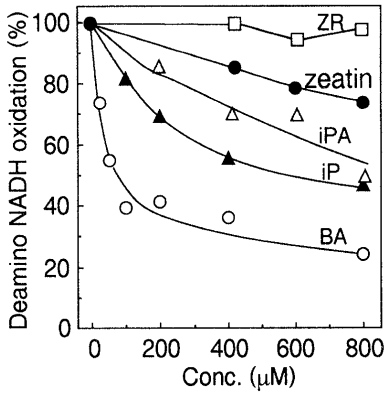

Fig. 1. Effects of Representative Cytokinins on the Oxidation of Deamino NADH in Smp.

The reaction without an inhibitor was taken as a control (100%).

Table Effects of N^6-Adenylate Cytokinins on the Oxidation of Deamino NADH in Smp

Compound	I_{50} (μM)	Compound	I_{50} (μM)
Zeatin	26%[a]	*m*-OH-BA	134
Zeatin riboside	2%[a]	*p*-Me-BA	43
Isopentenyladenine	567	*p*-F-BA	55
Isopentenyladenosine	800	*p*-Br-BA	8
Benzyladenine	90	*p*-MeO-benzyladenine	74
Kinetin	580	*p*-NO_2-benzyladenine	64
N^6-Phenyladenine	550	N^6-*n*-Butyladenine	542
N^6-Phenethyladenine	156	N^6-iso-Butyladenine	474
p-Cl-benzyladenine	13	N^6-*n*-Hexyladenine	17
m-Cl-benzyladenine	97	N^6-*n*-Octyladenine	8
o-Cl-benzyladenine	100	N^6-*n*-Decyladenine	1.1
o,*p*-Cl-benzyladenine	11	N^6-Geranyladenine	7.5
m,*p*-Cl-benzyladenine	13	N^6-*n*-Dodecyladenine	0.6
p-CF_3-benzyladenine	19	N^6-*n*-Tetradecyladenine	0.44
m-CF_3-benzyladenine	87	N^6-*n*-Hexadecyladenine	10
o-CF_3-benzyladenine	89	Rotenone	0.25

[a] Inhibitory percentage at 800 μM.

Based on these results, the inhibition by cytokinins of rotenone-sensitive dehydrogenase was examined in intact mitochondria using malate as a substrate at pH 7.7. At this pH, malate dehydrogenase has been reported to supply NADH preferentially to the rotenone-sensitive dehydrogenase.[3] In our experiments, about 20–30% of the respiration was not inhibited by rotenone. As Fig. 2 shows, inhibition by BA of the rotenone-sensitive respiration was raised to 82% at 400 μM, I_{50} being 50 μM. The activity of iP was also progressive in intact mitochondria, the inhibitory percentage being 63% at 400 μM ($I_{50} = 250$ μM), while that of zeatin was 17%. On the contrary, the inhibitory percentage of iPA decreased from 30% to 25% in smp at 400 μM. ZR was substantially inactive in intact mitochondria as well. The results indicate that, for the inhibitory action, hydrophobicity of the molecules is important in intact mitochondria more than in smp.

Effects of other N^6-substituted adenine derivatives

N^6-Adenylate cytokinin analogs in which the structure was systematically varied were tested for the rotenone-sensitive NADH dehydrogenase using smp. They are N^6-(substituted benzylamino)- and N^6-alkylaminopurines. The

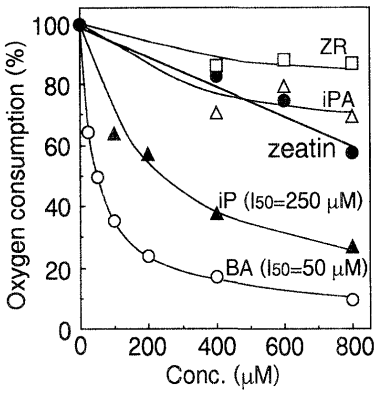

Fig. 2. Effects of Representative Cytokinins and Their Analogs on Malate Oxidation at pH 7.7 in Mitochondria.

The oxidation was measured in terms of oxygen consumption, and that without an inhibitor was taken as a control (100%).

structure and the assay data are summarized in the Table.

Among monochlorobenzyladenines, the *p*-substituted analog had the highest activity, and the potency of the *o*-compound was the lowest. A similar structure–activity profile was also seen for trifluoromethyl derivatives. An introduction of a second substituent into the *meta* or *ortho* position of the *p*-chloro derivative had little effect on the activity. Among the *p*-substituted benzyladenines, the *p*-bromo derivative was the most active compound, the I_{50} being 8 μM. The activity of *p*-methoxy and *p*-nitro derivatives was far lower. I_{50} values of phenyl- and phenethyladenines were 550 and 156 μM, respectively. An introduction of a substituent (bromo) into the benzene moiety made these compounds almost insoluble into the medium and thus their activity was not measured.

In an alkylaminopurine series, the activity in terms of I_{50} increased from 542 μM of the *n*-butyladenine to 0.44 μM of *n*-tetradecyl derivative with the number of carbon atoms of the N^6-side chain, and then decreased to 10 μM of *n*-hexadecyladenine. Introduction of a branch, an unsaturation or both somewhat modified the activity, as exemplified by N^6-isobutyl- and N^6-geranyladenines. The activities of *n*-tetradecyladenine was comparable to that of rotenone.[22] The compound did not inhibit other respiratory enzymes.

Discussion

Adenylate cytokinins were found to be specific inhibitors of rotenone-sensitive internal NADH dehydrogenase. The activity of iP was significantly stronger than that of zeatin. In intact mitochondria, 50% inhibition was observed for iP at 250 μM, but it was not observed for zeatin even at 800 μM. The inhibition of zeatin has been reported to be 9% and that of iP to be 36% in mung bean hypocotyl mitochondria at 500 μM.[2] By extrapolation, the inhibitions in this study are calculated to be 20% for zeatin and 65% for iP at 500 μM. Although the sensitivity to inhibitors may fluctuate somewhat between mitochondria from different species of plants, the figures reported previously[1,2] are significantly smaller than those estimated here, and are thought to be calculated based on the respiration that obviously contains the contributions from external NAD(P)H and rotenone-insensitive internal NADH dehydrogenases. The actual potency seems stronger in these mitochondria as well.

iP had significantly higher potency in intact mitochondria than in smp, but the activity of iPA was lower in intact mitochondria than in smp. The activity of zeatin was nearly the same in intact mitochondria and smp. Since iP is more hydrophobic than zeatin and iPA, iP appears to permeate into and accumulate at the site of action in mitochondrial membranes more effectively than zeatin and iPA, and this difference is more prominent with intact mitochondria than with smp. These results suggest that among the endogenously occurring cytokinins the species that can regulate electron transport from NADH to the cytochrome pathway is iP rather than its riboside or zeatin.

Cytokinins inhibited the electron flow *via* endogenous quinone far more strongly than *via* a synthetic quinone, DB. This suggests that binding sites of endogenous and artificial quinones are different, and that cytokinins specifically inhibit natural flow of electrons. This may add a support to that the inhibition of rotenone-sensitive dehydrogenase is a natural role of cytokinins. This phenomenon is thought to be specific to the adenylate structure of cytokinins rather than hydrophobicity, since rotenone inhibited the electron flow to both the natural and synthetic quinones equally strongly.

The effectiveness of cytokinin derivatives on respiration has been suggested to be related to their lipophilicity.[2,4] This, systematic structure–activity relationship study showed that structural characteristics are also important. The dependence on the structural characteristics is evidently seen in BA series. In N^6-*n*-alkyladenine series, the activity increased from $I_{50}=542$ μM of *n*-butyl derivative to $I_{50}=0.44$ μM of *n*-tetradecyl compound with the increasing number of carbon atoms at the side-chain, and then decreased to $I_{50}=10$ μM of *n*-hexadecyladenine. This seems to be typically hydrophobicity dependent, but the effects of branching and unsaturation is thought to be a steric effect. The most active compound in this series was N^6-tetradecyladenine, which was as active as rotenone. The activity in intact plants and tissues of exogenous compounds is decided by a balance between permeability to and intrinsic activity at the site of action. Thus, the intrinsically active but highly hydrophobic compounds like N^6-tetradecyladenine may not seem effective *in vivo* activity, but are considered to be of value as tools for interpreting mitochondrial function *in vitro*. Incidentally, they were also the species that specifically inhibit the electron flow *via* natural quinone.

Zeatin is a weak cytokinin for the inhibition of respiration but it is known as a highly active one for long-term assays like callus tests, and this has prevented the interpretation of the relevance of the effect on respiration to cytokinin action. However, after finding that cytokinin action delayed senescence in *Xanthium* leaves,[23,24] many reports have suggested linking of short-term responses to cytokinins of tissues to the inhibition of respiration.[25-30] If these quick actions of cytokinins in assays have physiological significance in intact plants as well, then the active species could be iP, rather than zeatin. Incidentally, zeatin has been reported to be weakly active in some senescence tests.[1,11,31] So far as we know, there has been no report that rotenone has effects for plants *in vivo*, including cytokinin-like activity, though it is highly toxic for insects and fishes. It may be unable to permeate into plant tissues.

iP has been identified in a number of plant species as a

major cytokinin species or a coexisting one with zeatin.[32] In some cases, measurements of both species have been made. For example, the concentrations of zeatin and iP were nearly the same in the xylem sap of a mistletoe (*Amyema pendulum*) in the mimicking condition, 430 and 434 ng/ml, respectively.[33] A cold treatment (vernalization) of *Cichorium intybus* root to induce flowering caused an increase in amounts of iP and zeatin from a trace or a few μg/100 g dry weight to 16–20 μg.[34] The concentration of iP remained at the 12 μg level thereafter till maximum flowering, but that of zeatin decreased to zero. iP, iPA, and ZR were found in flower buds of *Citrus* (Valencia orange) but zeatin was not, and the levels of iP, iPA, and ZR increased with blossom development, from less than 5 ng/g fresh weight to about 15 ng.[35] Although the localization of cytokinins in the cells or subcellular concentration is not known, these facts suggest that in some cases the concentration of iP is comparable to, or more than, those of other active species like zeatin, and in some other cases iP is the sole active species.

In the past, the examination of effects of naturally occurring cytokinins on respiration has been substantially restricted to zeatin. In reference to these results, it seems to be needed to examine the effects of iP, rather than zeatin, in mitochondria from diverse species of plants, in consideration of the operation of external and internal rotenone-insensitive dehydrogenases. Hydroxylation of iP to zeatin and deribosylation of iPA to iP are known as parts of the cytokinin biosynthetic and metabolic processes,[36] but these may be in certain cases processes to lower or raise the level of iP and thus to regulate respiration. The physiological significance of the inhibition of respiration could be an alteration of biochemical processes connected to the rotenone-sensitive NADH dehydrogenase, and this is the question to be examined.

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