Biochimica et Biophysica Acta, 333 (1974) 180–186

BBA 46692

EFFECTS OF QUERCETIN AND F_1 INHIBITOR ON MITOCHONDRIAL ATPase AND ENERGY-LINKED REACTIONS IN SUBMITOCHONDRIAL PARTICLES

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(Received September 10th, 1973)

SUMMARY

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) shares certain properties with the mitochondrial ATPase inhibitor protein. At low concentrations it inhibits both soluble and particulate mitochondrial ATPase and has no effect on oxidative phosphorylation in submitochondrial particles. Unlike the mitochondrial inhibitor protein quercetin inhibits the ATP-dependent reduction of NAD^+ by succinate in fully reconstituted submitochondrial particles. A comparison of various flavones indicates that the hydroxyl groups at the 3′ and perhaps 3 position are important for the inhibition of ATPase activity.

INTRODUCTION

Flavones and flavanones are phenolic compounds found in many green plants. Quercetin and some other bioflavonoids (see Fig. 1) have been shown to have toxic effects on various enzymes [1]. A stimulatory effect on photophosphorylation in chloroplasts has been obsserved [2]. It was also reported [3] that quercetin inhibits oxidative phosphorylation as well as mitochondrial and transport (Na^+-K^+)-ATPase. It was recently observed [4] that quercetin inhibited glycolysis in tumor cells when the mitochondrial ATPase supplied P_i and ADP. The experimental findings suggested that quercetin might inhibit ATPase activity without interfering with oxidative phosphorylation. In the present communication we report that at low concentrations quercetin and other structurally related flavones have an action similar to the mitochondrial ATPase inhibitor protein described by Pullman and Monroy [5].

Abbreviations: F_1, coupling factor 1 (ATPase); A particles (EDTA particles) submitochondrial particles prepared by sonication of beef heart mitochondria in the presence of ammonia and EDTA.

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MATERIALS AND METHODS

Quercetin was obtained from Nutritional Biochemicals Corp. Myricetin, apigenin, and fisetin were from Aldrich Chemical Co. Morin and rutin were obtained from J. T. Baker Chemical Co. Quercetrin was obtained from Fluka Chemical Co., pyruvate kinase from Böhringer. Unresolved submitochondrial particles yielding high P : O ratios; A particles (EDTA particles) (pH 8.9), submitochondrial particles prepared by sonication of beef heart mitochondria in the presence of ammonia and EDTA; submitochondrial particles prepared by sonication of beef heart mitochondria in the presence of pyrophosphate; F_1, coupling factor 1 (ATPase); and oligomycin sensitivity conferring protein were prepared as described [6–10]. F_1 inhibitor was prepared [11] through the $(NH_4)_2SO_4$ precipitation step.

ATPase and P_i–ATP exchange [12], ATP-driven reduction of NAD^+ by succinate [13], oxidative phosphorylation [14], protein [15] and P_i [16] were assayed as described.

RESULTS

Fig. 1 shows the basic ring structure of the plant flavones together with the

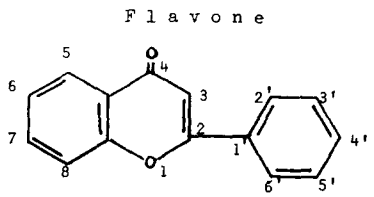

Fig. 1. Basic ring structure of the flavones showing the location of substituent groups of the compounds used in this study as well as the relative amounts of each compound required for 50 % inhibition of the ATPase activity of F_1. Mitochondrial ATPase (2 μg) was incubated for 10 min at 22 °C in a final volume of 0.8 ml of a solution containing 25 μmoles Tris–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 6.7), 10 μg of pyruvate kinase and quercetin. The tubes were then placed at 30 °C and the reaction initiated by the addition of 0.1 ml of reaction mixture containing 2 μmoles sodium ATP (pH 7.4), 3 μmoles phosphoenol pyruvate, 2 μmoles $MgSO_4$, and 25 μmoles Tris–sulfate buffer (pH 7.4). After 10 min 0.1 ml of 50 % trichloroacetic acid was added, the mixture was centrifuged and P_i determined in the supernatant. Quercetin was added as an alcoholic solution and corrections were made for minor inhibition caused by ethanol.

Name	Substituents	50 % inhibition of ATPase (μg/ml)
Myricetin	3,3′,4′,5,5′,7 hexa-OH	6
Quercetin	3,3′,4′,5,7 penta-OH	8*
Fisetin	3,3′,4′,7 tetra-OH	11
Morin	2′,3,4′,5,7 penta-OH	40
Apigenin	4′,5,7 tri-OH	not inhibitory
Rutin	Quercetin-3-rutinoside-(rhamnose-glucose)	not inhibitory
Quercetrin	Quercetin-3-rhamnoside	not inhibitory

* About 10 to 12 μg of quercetin/ml inhibited the ATPase activity of A particles.

location of the substituent groups and the approximate concentration of each compound required for 50% inhibition of the ATPase activity of soluble F_1.

From a comparison of the structures and inhibitory activity of the different flavones, it appears that the 3'- and perhaps the 3-hydroxyl group are important for the inhibitory properties of the flavone. Morin (2'-hydroxyl) is a much less effective inhibitor than quercetin (3'-hydroxyl). Apigenin (lacking both 3- and 3'-hydroxyls) as well as rutin and quercetrin (with sugar residues at Position 3), were inactive.

A comparison of the inhibitory activity of quercetin on the ATPase activity of soluble F_1, A particles and unresolved submitochondrial particles revealed similar sensitivity. This is in contrast to the observation [17] that the ATPase activity of submitochondrial particles was considerably more sensitive than that of soluble F_1 to the mitochondrial inhibitor.

It was shown [5] that under comparable conditions F_1 inhibitor protein inhibited mitochondrial ATPase activity but not oxidative phosphorylation. The $^{32}P_i$–ATP exchange was inhibited slightly at high inhibitor concentrations. It can be seen from Table I that quercetin likewise had no effect on oxidative phosphorylation at concentrations that inhibited over 50% of the ATPase activity. Comparative results are shown for the mitochondrial inhibitor. Similar results were obtained with NADH as substrate. As shown in Table II the inhibition of exchange activity by both quercetin and inhibitor were markedly less than the corresponding inhibition of ATPase activity under identical conditions. However, the inhibition of the exchange reaction was higher with quercetin than with an equivalent amount of inhibitor protein.

In 1970 it was reported [13] that the F_1 inhibitor protein inhibited ATP-driven reactions in A particles. These experiments were performed in the presence of a small amount of oligomycin (0.1 μg/ml protein), since in its absence A particles were rather inactive. However, A particles reconstituted with coupling factors [7] did not re-

TABLE I

THE EFFECT OF QUERCETIN ON ATPase ACTIVITY AND OXIDATIVE PHOSPHORYLATION OF UNRESOLVED SUBMITOCHONDRIAL PARTICLES

The effect of quercetin and mitochondrial inhibitor on ATPase activity of 100 μg unresolved submitochondrial particles was assayed as described in the legend of Fig. 1. Oxidative phosphorylation was assayed with 10 mM succinate as substrate. Unresolved submitochondrial particles were incubated for 2 min at 30 °C in the presence of F_1 inhibitor or quercetin prior to starting the reaction with succinate. The low amount of ethanol in the 0.4% solution of quercetin had no effect on oxidative phosphorylation.

Additions	Amount added (μg)	ATPase (μmole ATP hydrolyzed/ 10 min)	% inhibition	Oxygen (natoms)	Glucose 6-phosphate (nmoles)	P:O ratio	% inhibition
None		0.83	0	216	208	0.96	0
Quercetin	4	0.72	13	–	–	–	–
Quercetin	12	0.31	63	222	231	1.04	0
Quercetin	20	0.28	66	210	214	1.02	0
F_1 inhibitor	0.86	0.54	35	216	225	1.04	0
F_1 inhibitor	2.15	0.38	54	216	245	1.13	0

TABLE II

EFFECT OF QUERCETIN AND F_1 INHIBITOR ON ATPase AND $^{32}P_i$–ATP EXCHANGE

Incubation of 100 μg of SMP with the inhibitors was as described in the legend of Fig. 1. Quercetin (0.4 %) was dissolved in dimethylsulfoxide. The samples were then placed at 30 °C and assayed for ATPase or $^{32}P_i$–ATP exchange activity. The ATPase reaction was stopped after 10 min and the exchange reaction after 40 min by the addition of 0.1 ml of 50 % trichloroacetic acid. Results for quercetin are corrected for a small inhibition caused by dimethylsulfoxide.

Addition	Amount added (μg)	ATPase (μmoles ATP hydrolyzed/10 min)	% inhibition	$^{32}P_i$–ATP exchange (nmoles [^{32}P]ATP formed/40 min)	% inhibition
None		1.69	0	380.8	0
Quercetin	4	1.33	21	329.6	14
Quercetin	12	0.94	44	326.0	14
Quercetin	20	0.68	61	305.6	20
F_1 inhibitor	0.86	0.75	56	355.2	7
F_1 inhibitor	1.72	0.54	68	358.0	6
F_1 inhibitor	3.44	0.37	78	339.6	11

TABLE III

EFFECT OF QUERCETIN AND F_1 INHIBITOR ON THE ATP-DRIVEN NAD^+ REDUCTION BY SUCCINATE

A particles (200 μg) were assayed for ATP-driven reduction of NAD^+ by succinate. The particles were incubated in the presence of the indicated amounts of quercetin or F_1 inhibitor in a final volume of 1.0 ml of reaction mixture at 30 °C containing 0.1 μg of rutamycin per mg particle protein. The reaction was started by the addition of 0.15 μmole of NAD^+. For reconstitution A particles (3 mg) were incubated with 150 μg of F_1 and 233 μg of oligomycin sensitivity conferring protein at 22 °C for 30 min. The particles were kept in an ice bath and assayed for ATP-driven reduction of NAD^+ as described above except that no rutamycin was added.

Additions	Amount added	A particles (nmoles NADH/min per mg)	% inhibition	A particles (reconstituted) (nmoles NADH/min per mg)	% inhibition
Complete system		19.0	0	74.6	0
Quercetin	4	14.5	24	84.3	0
Quercetin	12	7.7	59	38.5	48
Quercetin	20	2.4	87	16.8	77
F_1 inhibitor	2.15	13.3	30	75.8	0
F_1 inhibitor	4.30	6.5	66	80.6	0
F_1 inhibitor	6.45	5.2	73	74.6	0
Complete system without rutamycin		4.4	–	–	–

quire oligomycin. As shown in Table III we confirmed the findings of Asami et al. [13] on the effect of inhibitor on the ATP-driven reduction of NAD^+ by succinate in A particles. The optimal concentration of rutamycin was 0.1 μg/mg particle protein, the same as with oligomycin. It can be seen that the reaction was strongly inhibited by quercetin.

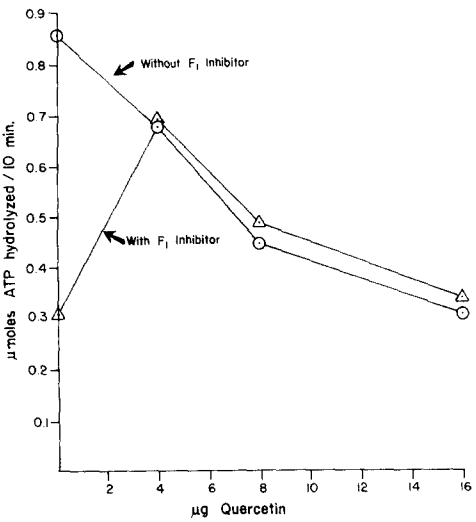

Fig. 2. The displacement of F_1 inhibitor by quercetin from soluble ATPase. F_1 (3.4 μg) was incubated with the indicated amounts of quercetin in the absence (○–○) or in the presence (△- - -△) of 3.44 μg of F_1 inhibitor for 10 min at room temperature. The samples were then placed at 30 °C and assayed for ATPase activity.

TABLE IV

EFFECT OF INHIBITOR AND RUTAMYCIN ON THE ATP-DRIVEN REDUCTION OF NAD^+ BY SUCCINATE IN RECONSTITUTED A PARTICLES

A particles were combined with F_1 and oligomycin sensitivity conferring protein as described in the legend of Table III. The reconstituted particles (134 μg protein) were incubated at 30 °C in the presence of the indicated amounts of F_1 inhibitor and/or rutamycin for 5 min. The reaction was initiated by the addition of NAD^+.

Expt No.	Additions	nmoles NADH/min per mg A particles
I	Complete system	74.6
	+ F_1 inhibitor (6.45 μg)	74.6
	+ rutamycin (0.05 μg/mg)	75.8
	+ rutamycin (0.1 μg/mg)	80.6
	+ rutamycin (0.15 μg/mg)	13.2
	+ inhibitor + rutamycin (0.05 μg/mg)	62.6
	+ inhibitor + rutamycin (0.1 μg/mg)	18.0
	+ inhibitor + rutamycin (0.15 μg/mg)	0.0
II	Complete system	75.8
	+ F_1 inhibitor (6.45 μg)	60.1
	+ rutamycin (0.1 μg/mg)	45.1
	+ rutamycin (0.125 μg/mg)	30.1
	+ inhibitor + rutamycin (0.1 μg/mg)	8.4
	+ inhibitor + rutamycin (0.125 μg/mg)	7.2

However, when A particles were reconstituted with oligomycin sensitivity conferring protein and F_1 and assayed in the absence of rutamycin (Table III), quercetin strongly inhibited the reaction whereas the inhibitor protein gave little or no inhibition. This reveals a pronounced difference between quercetin and the mitochondrial inhibitor. Experiments with unresolved submitochondrial particles, without added F_1, gave similar results. There was little or no inhibition of the ATP-driven reduction of NAD^+ by succinate with amounts of inhibitor that inhibited ATPase activity over 50%.

When F_1 was incubated with a fixed amount of inhibitor protein, approx. 60% inhibition of ATPase was obtained as shown in Fig. 2. If both quercetin and inhibitor protein were incubated with F_1, the inhibition did not correspond to the sum of the effect of the two inhibitors, but rather corresponded to that of quercetin alone. In fact, at low quercetin concentration, there was actually a stimulation of ATPase activity as compared to the value with inhibitor protein alone. If F_1 was first incubated with inhibitor and quercetin added later the results were the same. It therefore appears that quercetin can either displace the inhibitor from the enzyme or render it ineffective as an inhibitor of ATP hydrolysis.

Since A particles activated by coupling factors were much less sensitive to the inhibitor protein than A particles in the presence of rutamycin, the possibility was considered that this apparent discrepancy may be caused by a difference in susceptibility to rutamycin. To test this possibility the effect of various concentrations of rutamycin were tested on particles in the presence and absence of inhibitor protein. It can be seen from Table III that in reconstituted A particles, 0.1 μg rutamycin per mg protein only partially inhibited (0–40%) the rate of ATP-driven reduction of NAD^+ by succinate. However, in the presence of the inhibitor protein this amount of rutamycin caused 80–90% inhibition.

DISCUSSION

It is clear from the data presented that low concentrations of quercetin and related flavones inhibited mitochondrial ATPase without interfering with oxidative phosphorylation. Since these flavones are plant constituents it is of interest to note that at low concentrations quercetin also inhibited the ATPase activity of coupling factor CF_1, the coupling factor of spinach chloroplasts, without a marked effect on photophosphorylation (Deters, D. and Racker, E., unpublished). It is possible that these compounds participate in the regulation of the energy metabolism in plants. Since the glycosides are inactive (Fig. 1) a metabolic control mechanism depending on the ATP level can be visualized.

There are some differences in the response of the mitochondrial particles to quercetin and the protein inhibitor. Probably the most pronounced one is that quercetin inhibits the ATP-driven reduction of NAD^+ by succinate under conditions at which the protein inhibitor is ineffective.

The mechanism of action of quercetin appears to be similar to that of the mitochondrial protein inhibitor. It was proposed [18] that the major function of the inhibitor is to block the undesirable hydrolysis of ATP catalyzed by the coupling factor F_1. A finer control mechanism of ATPase activity by inhibitor, dependent on substrate oxidation and the ATP/ADP ratio, has been proposed by van de Stadt

et al. [19].

A broader type of regulation by the inhibitor was suggested by Asami et al. [13] based on their experimental observations that the inhibitor protein impaired ATP-driven reactions in A particles. While we have confirmed these experimental findings an alternative explanation may be proposed, namely that in the presence of the inhibitor A particles are more sensitive to rutamycin. Nevertheless, the suggestion of control of the ATP-driven reaction by inhibitor is an attractive one, particularly since such a control appears to be operative in chloroplasts [11].

We find [4] that quercetin inhibits glycolysis in tumor cells. The relationship between this effect and the inhibition of mitochondrial ATPase and of other transport systems [3] is now under investigation. We are also studying the relative potency of a number of analogs of quercetin to obtain clues to the relationship of its chemical structure and function as an ATPase inhibitor.

ACKNOWLEDGEMENTS

This work was supported by United States Public Health Service Grant CA-08964 from the National Cancer Institute.

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