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Inhibition of ATPase activity of *Escherichia coli* ATP synthase by polyphenols

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ABSTRACT

We have studied the inhibitory effect of five polyphenols namely, resveratrol, piceatannol, quercetin, quercetrin, and quercetin-3-β-D glucoside on *Escherichia coli* ATP synthase. Recently published X-ray crystal structures of bovine mitochondrial ATP synthase inhibited by resveratrol, piceatannol, and quercetin, suggest that these compounds bind in a hydrophobic pocket between the γ-subunit C-terminal tip and the hydrophobic inside of the surrounding annulus in a region critical for rotation of the γ-subunit. Herein, we show that resveratrol, piceatannol, quercetin, quercetrin, or quercetin-3-β-D glucoside all inhibit *E. coli* ATP synthase but to different degrees. Whereas piceatannol inhibited ATPase essentially completely (~0 residual activity), inhibition by other compounds was partial with ~20% residual activity by quercetin, ~50% residual activity by quercetin-3-β-D glucoside, and ~60% residual activity by quercetrin or resveratrol. Piceatannol was the most potent inhibitor (IC_{50} ~14 μM) followed by quercetin (IC_{50} ~33 μM), quercetin-3-β-D glucoside (IC_{50} ~71 μM), resveratrol (IC_{50} ~94 μM), quercitrin (IC_{50} ~120 μM). Inhibition was identical in both F_1F_o membrane preparations as well as in isolated purified F_1. In all cases inhibition was reversible. Interestingly, resveratrol and piceatannol inhibited both ATPase and ATP synthesis whereas quercetin, quercetrin or quercetin-3-β-D glucoside inhibited only ATPase activity and not ATP synthesis.

1. Introduction

ATP synthase is the fundamental means of cellular energy production in animals, plants, and almost all microorganisms by oxidative or photophosphorylation. Structurally F_1F_o-ATP synthase is similar whatever the source. In its simplest form in *Escherichia coli* it contains eight different subunits, namely $\alpha_3ß_3\gamma\delta\varepsilon ab_2c_{10}$. The total molecular size is ~530 kDa. F_1 corresponds to $\alpha_3ß_3\gamma\delta\varepsilon$ and F_o to ab_2c_{10}. The ability to separate the water soluble F_1 sector from the F_o sector with the retention of ATP hydrolysis function in the former and proton conduction in the latter have greatly facilitated research in this system. ATP hydrolysis and synthesis occur on three catalytic sites in the F_1 sector, whereas proton transport occurs through the membrane embedded F_o [1,2]. The γ-subunit is part of the "rotor" which is composed of γ, ε, and a ring of C-subunits. γ-Subunit is comprised of three α-helices. Two of these helices form a coiled coil and go right up into the central space of the $\alpha_3ß_3$ hexagon. The "stator" is composed of $b_2\delta$. The function of the stator is to prevent co-rotation of catalytic sites as well as the a subunit with the rotor [3,4]. Proton gradient-driven clockwise rotation of γ (as viewed from the membrane) leads to ATP synthesis and anti-clockwise rotation of γ results from ATP hydrolysis. It works like a motor and in fact it is the smallest known biological nanomotor. Detailed reviews of ATP synthase structure and function may be found in Refs. [5–11].

The enzyme ATP synthase has been implicated in many diseases and is also likely to be targeted therapeutically in the treatment of diseases such as, cancer, heart disease, mitochondrial diseases, immune deficiency, cystic fibrosis, diabetes, ulcers, and tuberculosis [5,12]. The role of mycobacterial ATP synthase is of particular interest as tuberculosis (TB) still claims approximately 2 million lives worldwide. Interestingly, it was found that two mutations in the mycobacterium ATP synthase F_o sector C-subunit, namely D32A and A63P, confer resistance to diarylquinoline drugs [13]. Thus, inhibitory studies of ATP synthase by natural or synthetic compounds may allow the development of lead compounds for therapeutic treatments. A wide range of natural and synthetic products are known to bind and inhibit ATP synthase. A detailed list of inhibitors and their action on ATP synthase in relation to human heath and disease is discussed in Ref. [14].

Polyphenols are naturally occurring plant based phytochemicals. They bind to multiple targets in the body including ATP synthase and inhibition of the mitochondrial enzyme has been reported [14, and references therein]. Polyphenols are antioxidants, possessing chemotherapeutic properties [15–18]. They protect cells and macromolecules against damage caused by free radicals. Some

Abbreviations: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole; DCCD, dicyclohexylcarbodiimide; Bz-423, 1,4-benzodiazepine; Q3G, quercetin-3-β-D glucoside; Mbr, membrane containing ATP synthase; IC_{50}, corresponds to the concentration of inhibitor where 50% of maximal inhibition was observed.

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polyphenols are also known to block the action of enzymes and other substances that promote the growth of cancer cells [19–22]. Polyphenols are also known to have antimicrobial activity. *Streptococcus mutans* is one example where antimicrobial action has been demonstrated. *S. mutans* is a primary microbial agent in the pathogenesis of dental caries. It was shown that polyphenols can inhibit biofilm formation and acid production by *S. mutans*. One of the pathways through which polyphenols are active against *S. mutans* is by the inhibition of proton-translocating F_1-ATPase activity [23,24].

Recently, the polyphenols resveratrol, piceatannol, and quercetin were shown to prevent synthetic and hydrolytic activities of bovine mitochondrial ATP synthase by blocking clockwise or anti-clockwise rotation of the γ-subunit. Thus, the beneficial effect of dietary polyphenols may be in part linked to the blocking of ATP synthesis in tumor cells, thereby leading to apoptosis [15]. The position of hydroxyl groups along with two or more phenolic structures appear to be critical in exerting the inhibitory effect on the ATP synthase [25].

In this paper we present the inhibitory effect of polyphenol compounds, namely resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside on *E. coli* ATP synthase using both purified F_1-ATPase and membrane bound F_1F_o ATP synthase preparations.

2. Materials and methods

2.1. Measurement of growth yield in limiting glucose medium; preparation of E. coli membranes; purification of E. coli F_1; assay of ATPase activity of membranes or purified F_1; measurement of proton pumping in membrane vesicles

The *E. coli* strain was pBWU13.4/DK8 [26]. Growth yield in limiting glucose was measured as in [27]. *E. coli* membranes were prepared as in [28]. It should be noted that this procedure involves three washes of the initial membrane pellets. The first wash is performed in buffer containing 50 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine. The following two washes are performed in buffer containing 5 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine, 0.5 mM DTT, 0.5 mM EDTA. Prior to the experiments, membranes were washed twice more by resuspension and ultracentrifugation in 50 mM $TrisSO_4$ pH 8.0, 2.5 mM $MgSO_4$. F_1 was purified as described in Ref. [29]. Prior to the experiments, F_1 samples (100 μl) were passed twice through 1-ml centrifuge columns (Sephadex G-50) equilibrated in 50 mM $TrisSO_4$ pH 8.0, to remove catalytic site bound-nucleotide. ATPase activity was measured in 1 ml assay buffer containing 10 mM NaATP, 4 mM $MgCl_2$, 50 mM $TrisSO_4$, and pH 8.5 at 37 °C. Reactions were started by addition of 1 ml assay buffer to the purified F_1 or membranes and stopped by addition of SDS to 3.3% final concentration. Pi released was assayed as in [30]. For membranes (30–50 μg protein), reaction times were 20–30 min. For purified F_1 (20 μg protein), reaction times were 2–5 min. All reactions were shown to be linear with time and protein concentration. Membranes were also tested for ATP-driven proton pumping activity which is measured using acridine orange and expressed as percent quench of acridine orange fluorescence in membrane vesicles upon addition of 1 mM MgATP. Measurements were performed as described in [31]. SDS-gel electrophoresis on 10% acrylamide gels was as in [32]. Immunoblotting with rabbit polyclonal anti-F_1-α and anti-F_1-β antibodies was as in [33].

2.2. Source of polyphenols and other chemicals

Resveratrol (R5110-50MG), piceatannol (P0453-25MG), quercetin (Q125-100G), quercetrin hydrate (Q3001-50MG), and quercetin-3-β-D glucoside (17793) were purchased from Sigma Chemical Company. Polyphenols were resuspended in DMSO simply by weighing out. All other chemicals used in this study were ultra pure analytical grade purchased either from Sigma–Aldrich Chemical Company or Fisher Scientific Company.

2.3. Inhibition of ATPase activity by resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside

Membranes or purified F_1 (0.2–1.0 mg/ml) were preincubated with varied concentrations of resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside for 60 min at room temperature, in 50 mM $TrisSO_4$ pH 8.0. Then 1 ml ATPase assay buffer was added to measure the activity.

2.4. Reversal of purified F_1 or membrane bound enzyme ATPase activity from resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside inhibition

Reversibility of inhibition was assayed by dilution of the membrane enzyme and by passing the inhibited purified F_1 through centrifuge columns. Membranes were first reacted with 470 μM resveratrol, 50 μM piceatannol or 94 μM quercetin, 400 μM quercetrin or 400 μM quercetin-3-β-D glucoside for 1 h at room temperature. These concentrations were used based on the maximal inhibition of the ATP synthase (see Figs. 3 and 4). Then 50 mM $TrisSO_4$ pH 8.0 buffer was added to decrease the concentrations to 47, 5, 9.4, 40, or 40 μM for resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside, respectively and incubation continued for 1 additional hour at room temperature before ATPase assay. Reversibility was also tested by passing the resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside inhibited purified F_1 enzyme twice through 1 ml centrifuge columns before measuring the ATPase activity. Control samples without resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside were also incubated for the same time periods as the samples with resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside. Two consecutive passages through centrifuge columns were previously found to decrease the concentration of small molecules bound to ATP synthase and other proteins to non-detectable levels (unpublished results). Thus, after passage through centrifuge columns, reactivation is likely a first-order kinetic process that is a function of release of bound inhibitor.

3. Results

3.1. Inhibition of ATPase activity of purified F_1 and F_1F_o ATP synthase in membranes by resveratrol, piceatannol, or quercetin

Fig. 1 shows the recently solved F_1-reseveratrol, F_1-piceatannol and F_1-quercetin complex structures from bovine ATP synthase by molecular replacement using data to 2.3, 2.4, and 2.7 Å, respectively. The distinct binding pocket for resveratrol, piceatannol, and quercetin lies between the β_{TP}-subunit and the C-terminal region of γ-subunit [15]. Polyphenols resveratrol, piceatannol, and quercetin were also shown to bind in a slightly distorted planar conformation through H-bonds and hydrophobic interactions. The hydrophobic interactions occur between the inhibitors and γLys-260, γIle-263, β_{TP}Val-279, and β_{TP}Ala-278. Polyphenol inhibited mitochondrial ATP synthase[1] X-ray structures also show that residues γAla-256, γThr-259, γGlu-264, α_{TP}Glu-292, α_{TP}Gly-290 and α_{DP}Glu-292 are within 4 Å of the bound compounds thus providing additional non-polar interaction [15]. We studied the inhibitory effect of the

[1] *E. coli* residue numbers used throughout.

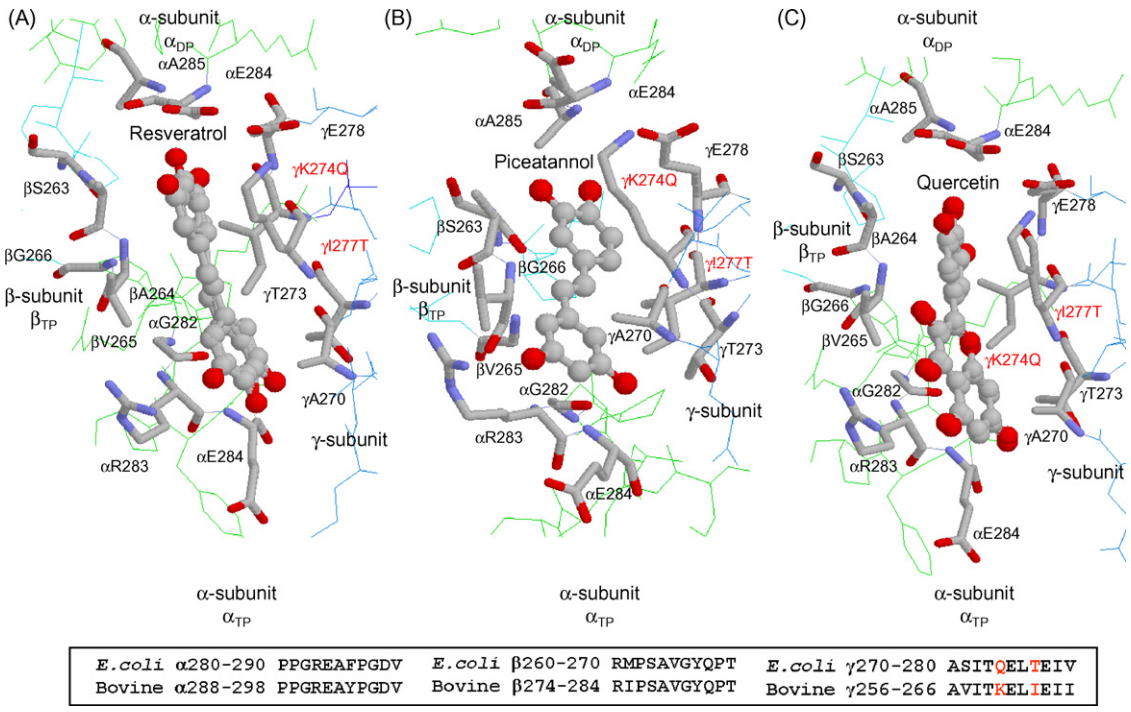

Fig. 1. X-ray structures of mitochondrial ATP synthase showing resveratrol, piceatannol or quercetin. Rasmol software was used to generate these figures. PDB files used were 2jj1, 2jj2, and 2jjZ [31]. (A) Reacted resveratrol in contact with α-, β-, and γ-subunits. (B) Reacted piceatannol in contact with α-, β-, and γ-subunits. (C) Reacted quercetin in contact with α-, β-, and γ-subunits. Green color represents α-subunit, cyan color is for β-subunit and blue color is for γ-subunits. Residues involved in the interaction with compounds are identified. γQ274K, γT277I in red is showing the difference between bovine and *E. coli* ATP synthase. In place of Q and T bovine has K and I residues. *E. coli* residue numbering is shown. At the bottom *E. coli* and bovine α-, β-, and γ-subunit binding pocket residue are aligned. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

above three polyphenols, and two more polyphenol compounds quercetrin and quercetin-3-β-Dglucoside (Fig. 2) on the purified F_1 and membranes of *E. coli* synthase to understand the inhibitory effect of polyphenol compounds on prokaryotic enzyme *E. coli* ATP synthase. Conservation of *E. coli* ATP synthase residues suggests that it possess an identical binding pocket except for two changes, namely γK260Q and γI263T. Corresponding *E. coli* residue positions are γGln-274 and γThr-277.

Fig. 3 shows the inhibition of ATPase activity of purified F_1 or membrane bound enzyme in presence of varied concentrations of resveratrol, piceatannol or quercetin. While complete, potent inhibition (~100% inhibited; IC_{50} ~14 μM) occurs in presence of piceatannol, the maximum amount of inhibition in presence of quercetin was slightly less (~80%; IC_{50} ~33 μM), and resveratrol appear to be the least potent (~40% inhibited; with a midpoint of ~94 μM). We consistently found that the F_1 data and the membrane data were the same for these inhibitors. Previously we established that inhibition of ATPase activity can be assayed using either membrane preparations or purified F_1 with equivalent results [34,35].

3.2. Inhibitory effect of quercetrin or quercetin-3-β-D glucoside on the purified F_1 or membrane bound enzyme

Fig. 4 shows the inhibitory effect of quercetrin or quercetin-3-β-D glucoside. Both quercetrin (IC_{50} ~120 μM) and quercetin-3-β-D-glucoside (IC_{50} ~71 μM) beget partial inhibition of about 40 and 50%, respectively. Again the F_1 data and the membrane data were the same for both the inhibitors. Partial inhibition of ATP synthase is not uncommon. In previous studies [34–40], we have noted several instances where mutant ATP synthase were incompletely inhibited

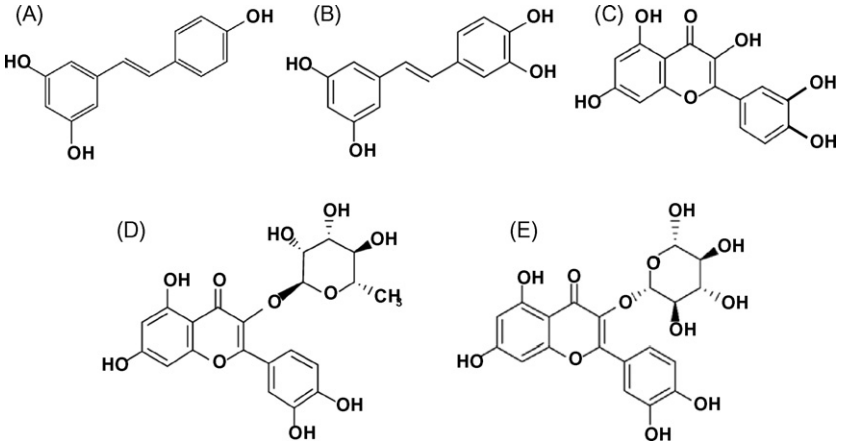

Fig. 2. Structures of polyphenol inhibitors of *E. coli* ATP synthase (A) resveratrol, (B) piceatannol, (C) quercetin, (D) quercitrin, and (E) quercetin-3-β-D glucoside.

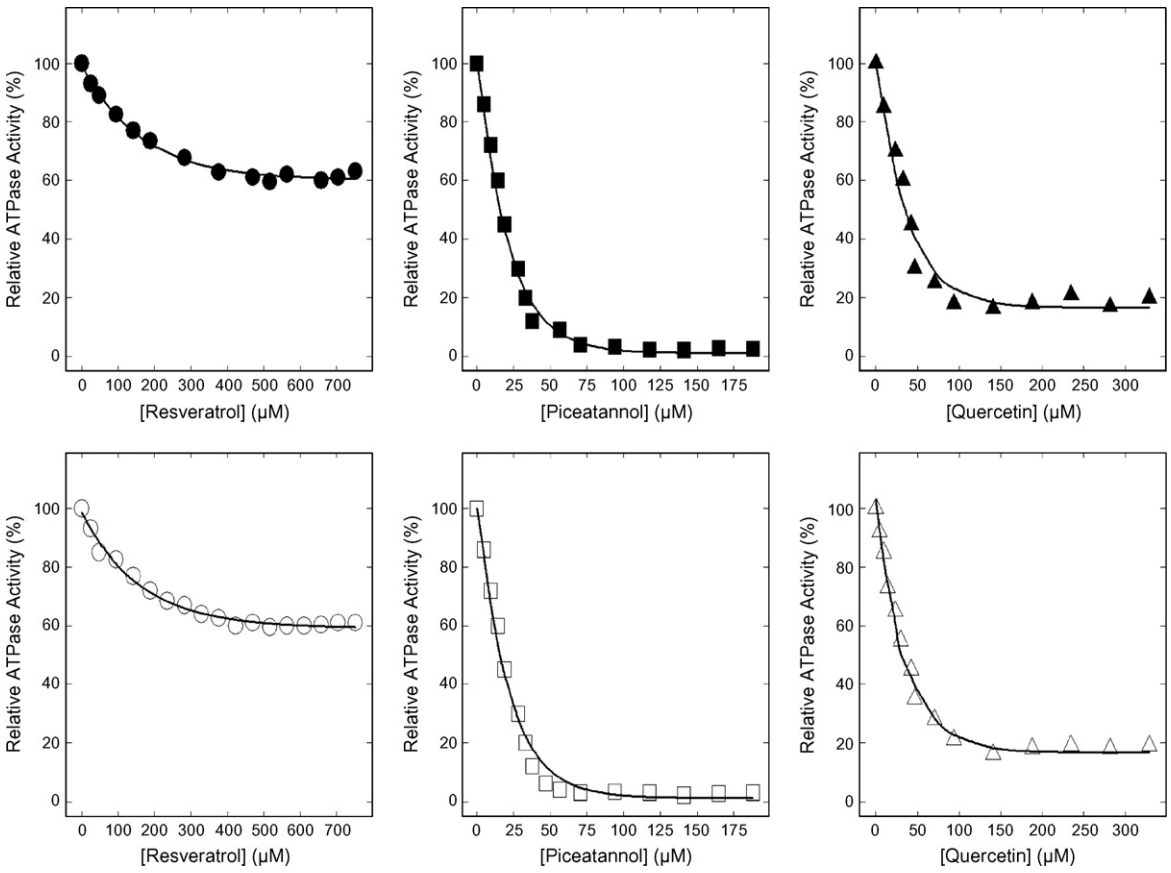

Fig. 3. Inhibition of ATPase activity in purified F_1 or membrane-bound ATP synthase by resveratrol, piceatannol or quercetin. Membranes or purified F_1 were preincubated for 60 min at 23 °C with varied concentration of resveratrol, piceatannol or quercetin, and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Details are given in Section 2. Symbols used are: circles (● and ○), resveratrol; squares (■ and □), piceatannol; triangles (▲ and △), quercetin. Filled symbols are for membranes while open are for purified F_1. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within ±10%.

by potent inhibitors like fluoroaluminate, fluoroscandium, sodium azide, or NBD-Cl. For example, detailed studies on NBD-Cl inhibition showed that fully reacted purified F_1 or membranes retained residual activity. To be sure that the maximal inhibition with resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside had been reached, we incubated each membrane preparation or purified F_1 with 376 μM resveratrol, 50 μM piceatannol, 100 μM quercetin, 400 μM quercetrin or 376 μM quercetin-3-β-D glucoside for 1 h as in Figs. 3 and 4 followed by supplementary pulses of the compounds and continued the incubation for an additional hour before assaying ATPase activity. As shown in Fig. 5A and B very little or no additional inhibition occurred consistent with Figs. 3 and 4 data. This shows that the inhibition by resveratrol, quercetin, quercitrin or quercetin-3-β-D glucoside was maximal and fully inhibited F_1 or membranes retained residual activity. Although, we used 1 h incubation time it was observed that the maximal inhibition of purified F_1 or membrane bound enzyme was achieved within 15 min.

3.3. *Reversal of ATPase activity of purified F_1 or membrane enzyme from the resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside inhibition*

This experiment was carried out in two ways (i) the purified F_1 or membrane bound enzyme was inhibited with the higher concentration of resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside. Then the samples were diluted to a non-inhibitory concentration. It was found that the inhibition was totally reversible (Fig. 5C and D). (ii) 20 μg purified F_1 samples were inhibited with 470 μM resveratrol, 50 μM piceatannol, 94 μM quercetin, 400 μM quercetrin or 400 μM quercetin-3-β-D glucoside for 1 h. Again these inhibitory concentrations were determined based on data from Figs. 3 and 4. Then they were passed twice through 1 ml centrifuge columns and ATPase activity was measured. It was found that in all cases activity was restored back to the near normal level as in absence of the compounds similar to Fig. 5C and D (data not shown). These data indicate that the observed inhibition is not the result of protein denaturation and that the enzyme retains the ability to reactivate upon release of the compound after dilution.

3.4. *Inhibition of growth on LB, limiting glucose, and succinate medium in presence of resveratrol, piceatannol, quercetin, quercitrin, or quercetin-3-β-D glucoside*

Inhibitory effects on ATP synthesis were studied by growing the *E. coli* strain pBWU13.4 on succinate plates, limiting glucose, or LB media in presence or absence of resveratrol, piceatannol, quercetin quercitrin, or quercetin-3-β-D glucoside. We found that pBWU13.4 growth was inhibited in presence of resveratrol or piceatannol but was not affected in presence of quercetin, quercitrin, or quercetin-3-β-D glucoside. Loss of growth on succinate plates and limiting glucose suggests the loss of oxidative phosphorylation (see Table 1).

4. Discussion

The goal of this study was to examine the inhibitory role of polyphenols resveratrol, piceatannol, quercetin, quercitrin, or

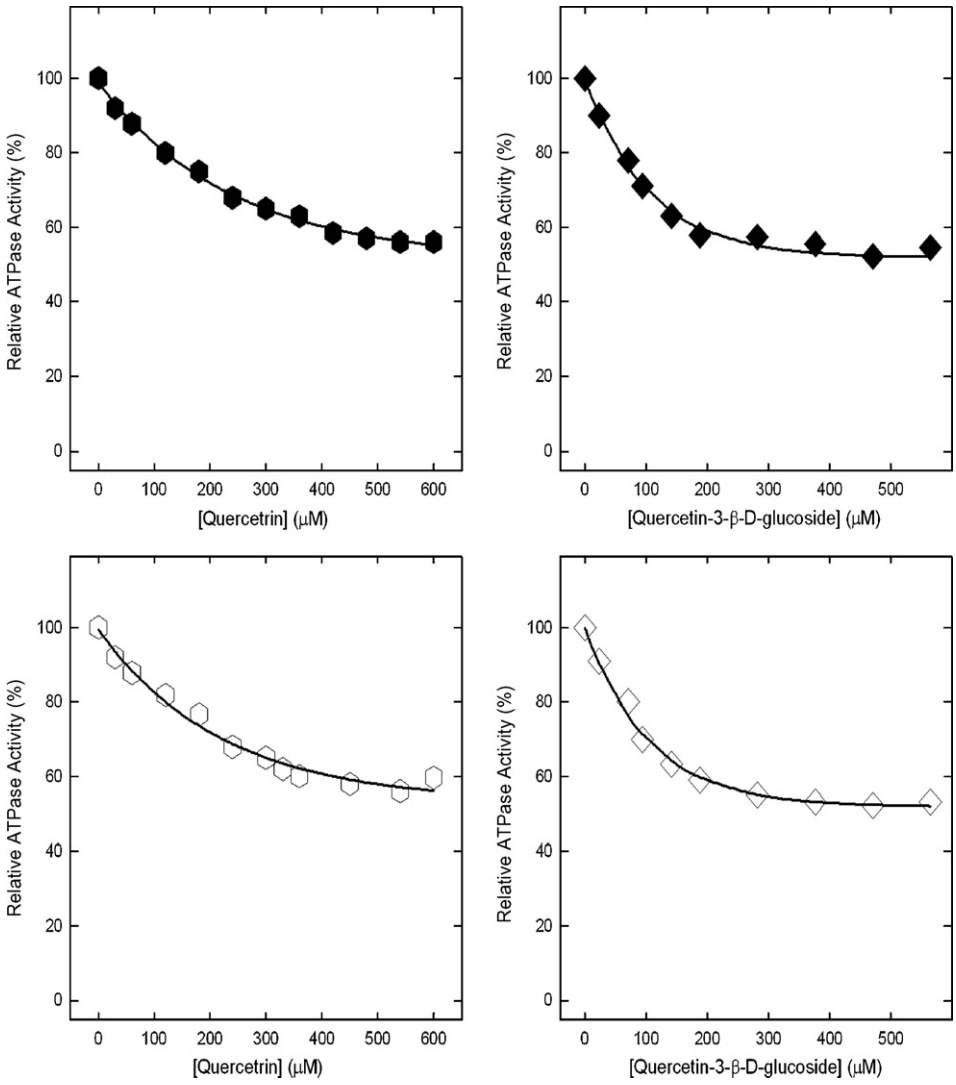

Fig. 4. Inhibition of ATPase activity in purified F_1 or membrane-bound ATP synthase by quercitrin or quercetin-3-β-D glucoside. Purified F_1 or membranes were preincubated for 60 min at 23 °C with varied concentration of quercitrin or quercetin-3-β-D glucoside and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Details are given in Section 2. Symbols used are: (♦ and ◊), quercitrin; (⬢ and ⬡), quercetin-3-β-D glucoside. Filled symbols represent membrane data while open symbols represent data for purified F_1. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within ±10%.

quercetin-3-β-D glucoside on the *E. coli* ATP synthase. X-ray crystal structures of F_1-resveratrol, F_1-piceatannol and F_1-quercetin show that they bind at the C-terminal tip of γ-subunit and interact with the binding pocket between β_{TP}-subunit and the C-terminal tip of γ-subunit (Fig. 1). The hydrophobic interaction between the inhibitor compounds and mitochondrial enzyme was shown to involve γK260, γI263, β_{TP}V279, and β_{TP}A278. Other residues, which are within 4 Å of the bound compounds and contribute to non-polar interactions, are γAla-256, γThr-259, γGlu-264, α_{TP}Glu-292, α_{TP}Gly-290, and α_{DP}Glu-292. Two H-bonds are also formed between β_{TP}V279 and α_{TP}Glu-292 and the bound polyphenol compounds [15]. The equivalent residues in the *E. coli* enzyme are γQ274 (γK260), γT277 (γI263), β_{TP}V265 (β_{TP}V279) and β_{TP}264 (β_{TP}A278). Parentheses show the bovine numbers.

Table 1
Effects of polyphenols on the *E. coli* cell growth.

Polyphenols	Growth on succinate plates[a]	Growth yield in limiting glucose[b] (%)	Growth on LB media[b] (%)
Control[c]	++++	100	100
Null[c]	–	43	45
Resveratrol	–	42	43
Piceatannol	–	43	44
Quercetin	++++	96	98
Quercitrin	++++	96	97
Quercetin-3-β-D glucoside	++++	97	98

[a] Growth on succinate plates after 3 days was determined by visual inspection. (++++) Heavy growth; (–) no growth.
[b] Growth yield on limiting glucose and LB was measured as OD_{595} after ~20 h growth at 37 °C.
[c] Control, pBWU13.4/DK8; null, pUC118/DK8. Growth of positive and negative controls in absence of polyphenol compounds. Data are means of four to six experiments each at 37 °C. Each individual experimental point is itself the mean of duplicate assays.

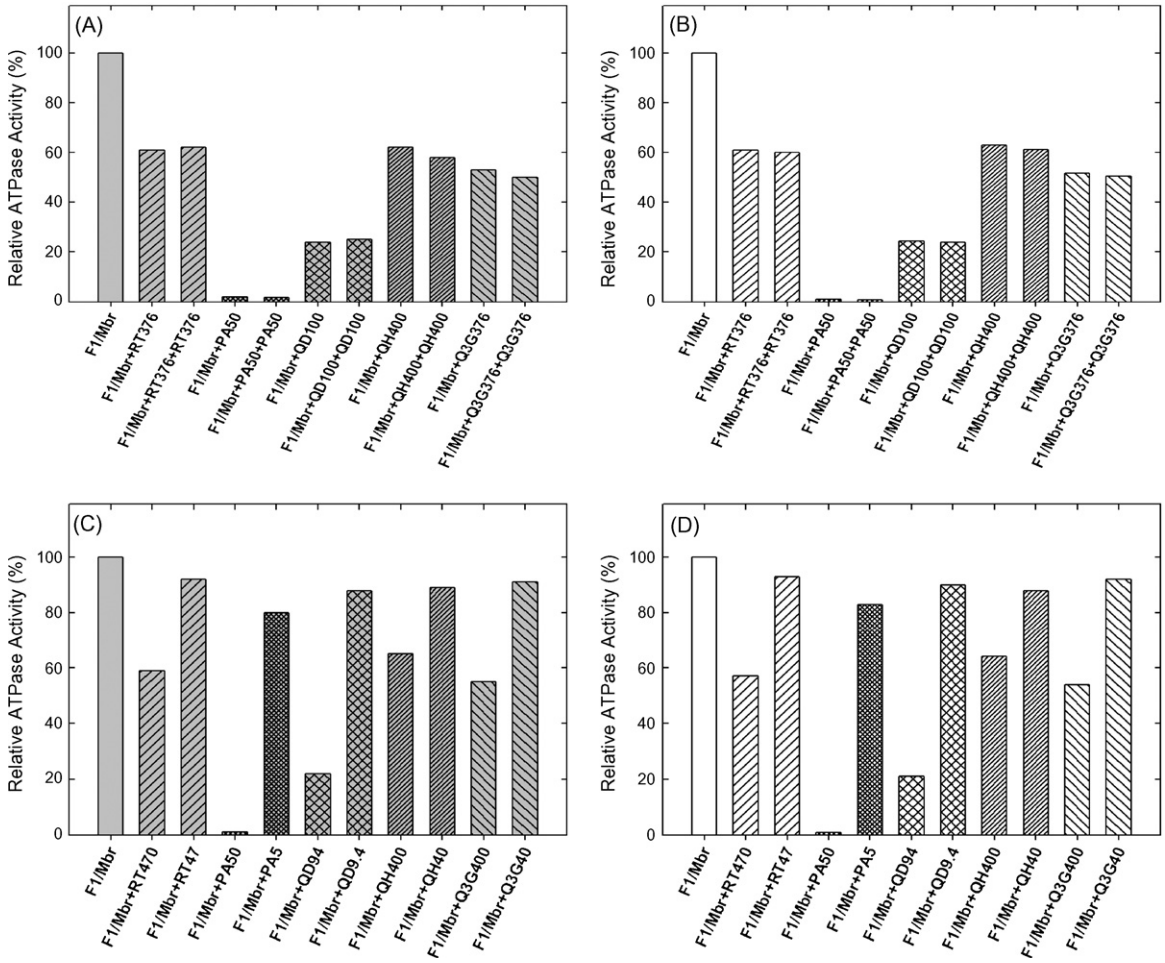

Fig. 5. Results of extra pulse of resveratrol, piceatannol, quercetin, quercitrin, or quercetin-3-β-D glucoside on purified F_1 or membrane bound ATP synthase and reversal of inhibition by dilution. (A and B), Membrane bound ATP synthase (Mbr, gray background) or purified F_1 (F_1, white background) was inhibited with 376 μM resveratrol, 50 μM piceatannol or 100 μM quercetin, 400 μM quercitrin or 376 μM quercetin-3-β-D glucoside for 60 min under conditions as described in Figs. 2 and 3. Then a further pulse of 376, 50, 100, 400, and 376 μM for resveratrol, piceatannol, quercetin, quercitrin or quercetin-3-β-D glucoside, respectively, was added and incubation continued for 1 h before assay. (C and D) Membrane bound ATP synthase (Mbr, gray background) or purified F_1 (F_1, white background) was incubated with 470 μM resveratrol, 50 μM piceatannol, 94 μM quercetin, 400 μM quercitrin or 400 μM quercetin-3-β-D glucoside for 60 min under conditions as described in Figs. 2 and 3. Concentrations were lowered to 10-fold by adding $TrisSO_4$ buffer and incubation continued for 1 h before assay. The first bars are purified F_1 or membrane enzyme with no compound (F1/Mbr), followed by resveratrol (RT), piceatannol (PA), quercetin (QD), quercitrin (QH) or quercetin-3-β-D glucoside (Q3G) from left to right. The last digits represent the compound concentrations.

One of the important aspects of the inhibitory mechanism of resveratrol, quercetin, and piceatannol on bovine ATP synthase was their effect on rotary mechanism of the enzyme. This conclusion was also supported by the conserved nature of the C-terminal of γ-subunit along with some mutagenesis studies [15, and references therein]. However, contrary to the conclusion from bovine mitochondrial enzyme, γ-subunit C-terminal deletion of 3, 6, 9, 12, 15, and 18 amino acid residues from *E. coli* enzyme [41], up to 20 and 21 amino acid deletion from chloroplast and Bacillus PS3 enzyme [42] does not affect the catalytic activity of the enzymes suggesting that the C-terminal of γ-subunit is a dispensable part.[2] The inhibition of *E. coli* enzyme by the five polyphenols used in this study suggest that at least the hydrophobic binding pocket between the γ-subunit C-terminal tip and the hydrophobic inside of the surrounding annulus is required for the binding of these compounds.

Previous studies on resveratrol bound five different proteins [43–47] and quercetin bound seven proteins [48–54] along with bovine mitochondrial enzyme [15] demonstrated that the mode of binding of resveratrol and quercetin to F_1-ATPase is same. In all these proteins the bound resveratrol or quercetin conformation appears to be slightly distorted. Importantly all the residues including γLys-274 and γIle-277 involved in binding of resveratrol or quercetin are highly conserved among bovine, rat and human enzymes [55,56]. In *E. coli* enzyme residues γLys-274 and γIle-277 are replaced by γGln-274 and γThr-277. The changed residues are shown in red in Fig. 1.

A comparative analysis of distance measurements between bovine and *E. coli* enzyme residues and bound polyphenol compounds based upon the crystal structure of the bovine enzyme is shown in Table 2. Theoretical estimates of distance values for the *E. coli* enzyme were obtained using a model where the bovine residues were replaced with *E. coli* residues using the site mutagenesis feature of Deep View Swiss-Pdb Viewer, Version 4.01 (see table legend). These distance measurements suggest that residue γGln-274 and γThr-277 are much closer to the bound polyphenol compounds in *E. coli* than γLys-274 and γIle-27 in mitochondrial enzyme. Gln and Thr side chain are a little shorter than that of Lys and Ile, respectively. The orientation of their side chains in the direction of bound polyphenol inhibitors makes γGln-274 closer by 1.6–0.53 Å and γThr-277 closer by 1.6–0.66 Å than γLys-274 or γIle-277 to the bound polyphenol compounds. The H-bonds network

[2] *E. coli* enzyme mutants namely αR283A/Q, αE284A/Q, βS263A/Q, γT273A/Q, γQ274A/K, γT277A/Q, and γE278A/Q resulted in the loss of inhibition by resveratrol, piceatannol, and quercetin (P.K. Dadi, Z. Ahmad, unpublished results).

Table 2
Distance variation due to change in amino acid at position αK274Q and αI277T.[a].

Residue	Resveratrol	Piceatannol	Quercetin
αLys-274 (αGln-274)	4.88 (3.28)	4.53 (2.92)	4.19 (3.66)
αIle-277 (αThr-277)	4.09 (3.44)	4.04 (3.69)	4.83 (3.23)

[a] The distances shown are between the nearest atom of the residue and the bound polyphenol resveratrol, piceatannol or quercetin, in angstroms (Å) as determined by X-ray crystallography [28] using PDB codes 2jj1, 2jj2, and 2ji2. Theoretical estimates of distance values for the *E. coli* enzyme were obtained using a model where the bovine residues were replaced with *E. coli* residues using the site mutagenesis feature of Deep View Swiss-Pdb Viewer, Version 4.01 (N. Guex, A. Diemand, M.C. Peitsch, T. Schwede (2008) at http://spdbv.vital-it.ch/).

between bound polyphenol inhibitors and binding site residues involves β_{TP}Ser-263, β_{TP}Val-265, β_{TP}Gly-266, γThr-273, α_{TP}Arg-283, and α_{TP}Glu-284 [15]. The changed residue Thr at position γI277T in *E. coli* generates an additional H-bond with the OH group of γSer-281. Earlier using a neural network based predication [57] it was found that Gln or Thr residues has similar propensity for ligand binding as that of positively charged Lys residues [58].

E. coli enzyme was inhibited to different degrees by resveratrol, piceatannol, quercetin, quercetrin, or quercetin-3-β-D glucoside (Figs. 3 and 4). Piceatannol causes maximal inhibition with nearly zero residual activity. Other inhibitors, resveratrol, quercetin, quercitrin, or quercetin-3-β-D glucoside cause partial inhibition with residual activity of about 25, 25, 40, or 50%, respectively. Addition of extra pulse of compounds to the previously inhibited purified F_1 or membranes did not change the degree of inhibition significantly (Fig. 5A and B). This suggests that the purified F_1 or membrane were fully inhibited with the compounds and the extent of inhibition was the true inhibition. In addition, partial inhibition is not due to uninhibited enzyme or degradation of the compounds with time. The process of inhibition was also found to be completely reversible. A fully reacted F_1 regained activity once they were passed through the centrifuge columns to remove the compounds. Similarly, purified F_1 or membrane regained activity once they were brought back to lower concentrations of compound after exposing them to higher concentrations by dilution with buffer. Readily reversible process also confirms the non-covalent binding of the compounds with no further inhibition on addition of second pulse of the compounds.

Another interesting result is the growth patterns observed in presence of compounds. Resveratrol and piceatannol inhibit both ATP hydrolysis and ATP synthesis. Quercetin, quercetrin or quercetin-3-β-D glucoside prevents only ATP hydrolysis and not the ATP synthesis. This observation is in agreement with the effect of resveratrol and quercetin observed on bovine ATP synthase [15]. We do not have any simple explanation why quercetin, quercitrin, or quercetin-3-β-D glucoside did not inhibit the growth, but one or the other following reasons can not be ruled out at this time (a) the inhibitors could not penetrate into the cells, (b) inhibitors got pumped out by an export pump, or (c) the inhibitors were metabolized by the bacterial cells. More inhibitory studies by new functionally modified polyphenol compounds should help in understanding this difference.

Physiological relevance of dietary polyphenols can be ascribed to their interaction with the mitochondrion in eukaryotic cells. Many degenerating diseases such as cancer, aging, and neurological disorders are attributed to the mitochondrial dysfunction [59,60]. Thus, it is imaginable that the inhibition of ATP synthase by resveratrol or related polyphenols might play a significant role in the physiology of such conditions [15]. Resveratrol is known to induce apoptosis via a mitochondrial pathway [21,61]. Earlier [62] it was shown that oligomycin a highly specific ATP synthase inhibitor induces an apoptotic suicide response in cultured human lymphoblastoid and other mammalian cells within 12–18 h but not in ρ^o cells that are depleted of a functional mitochondrial respiratory chain. Another similar study [63] suggested that inhibition of the components of mitochondrial pathways may lead to marking of some cells, via CD14, for cell death, while allowing commitment to differentiation to occur in the surviving population.

Alteration of cellular bioenergetics is another vital way of triggering the cell death. It was shown that 1,4-benzodiazepine (Bz-423) binds to the oligomycin sensitivity-conferring protein subunit of F_1F_o and inhibits the ATP synthase. This causes a significant decrease in ATP synthesis and increase in the production of free radicals which in turn activates redox regulated apoptosis [64]. Thus, all ATP synthase inhibitors in general and polyphenol inhibitors in particular may be used to target tumor cells without affecting normal cells [15].

The involvement of mycobacterium ATP synthase in conferring resistance against the anti-tuberculosis drug diarylquinoline due to two C-subunit mutations D32V and A63P suggests a need for more potent natural or synthetic inhibitors of bacterial ATP synthase enzymes [13]. The inhibition of biofilm formation and acid production by *S. mutans* through the inhibition of proton-translocating F_1-ATPase activity in presence of a variety of polyphenols [23,24] along with the knowledge of inhibitory effects of polyphenols on *E. coli* ATP synthase could provide starting point to develop inhibitors against bacterial pathogens such as *Mycobacterium tuberculosis* and *S. mutans*.

Interestingly, IC_{50} values for the inhibition of rat brain and liver mitochondrial ATP synthase by resveratrol, piceatannol, and quercetin were 19, 8, and 65 μM, respectively [22]. For the *E. coli* enzyme the IC_{50} values were resveratrol (IC_{50} ~94 μM), piceatannol (IC_{50} ~14 μM), quercetin (IC_{50} ~33 μM), quercetrin (IC_{50} ~20 μM), and quercetin-3-β-D glucoside (IC_{50} ~71 μM). Variation of IC_{50} values between *E. coli* and bovine enzymes may be attributed to the alteration of two amino acids: γK274Q and γI277T in *E. coli* at the proposed binding site. However, identification of more potent polyphenol inhibitors of ATP synthase will surely advance the current understanding about the dietary benefits of polyphenols in combating disease conditions. A detailed study of functionally modified polyphenol compounds through the introduction of additional –OH groups, nitro groups, amino groups, and phenol rings is under way.

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