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Inhibition of *Escherichia coli* ATP Synthase by Polyphenols and Their Derivatives

A thesis
presented to
the faculty of the Department of Biological Sciences
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biology

by
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May 2010

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Keywords: F₁F_o-ATP synthase, F₁-ATPase, ATP synthesis, *E. coli*, polyphenols, resveratrol, quercetin, piceatannol, quercitrin, quercetin-3-β-D glucoside, biological nanomotor

ABSTRACT

Inhibition of *Escherichia coli* ATP Synthase by Polyphenols and Their Derivatives by

Prasanna Keerthi Dadi

We have studied the inhibitory effect of natural and structurally modified polyphenols on *Escherichia coli* ATP synthase to test (I) if the beneficial dietary effects of polyphenols are related to their inhibitory actions on ATP synthase, (II) if inhibitory effects of polyphenolic compound could be augmented through structural modifications, and (III) if they can act as antimicrobial agent through their actions on ATP synthesis. X-ray crystal structures of polyphenol binding sites suggested that polyphenols bind at a distinct polyphenol binding pocket, at the interface of α,β,γ -subunits. We found that both natural and modified polyphenols inhibit *E. coli* ATP synthase to varying degrees and structural modifications resulted in augmented inhibition. Inhibition was reversible in all cases. Both natural and modulated compounds inhibited *E. coli* cell growth to varying degrees. We conclude that dietary benefits of polyphenols may be in part due to the inhibition of ATP synthase.

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CHAPTER 1

INTRODUCTION

ATP synthase is the fundamental means of cellular energy production in animals, plants, and almost all microorganisms by oxidative or photo phosphorylation. The energy from ATP is used for various biochemical reactions like transport of nutrients, muscle contraction, nucleic acid synthesis, protein synthesis, active transport, etc. ATP synthase is a membrane bound enzyme that is found in the inner mitochondrial membrane and in the thylakoid membrane of chloroplasts of eukaryotic cells. In bacteria it is found embedded in the plasma membrane. ATP synthase has homology in amino acid sequence and is exceptionally conserved throughout evolution resulting in similar mechanism in ATP synthesis and hydrolysis in most of the organisms. The simplest of all is that of *E. coli* with 8 subunits in 2 main portions. A membrane embedded F_o and a catalytic F_1 . F_1 consists of $\alpha_3\beta_3\gamma\delta\epsilon$ subunits and F_o has ab_2c_{10} as shown in Fig. 1. ATP hydrolysis and synthesis occur on three catalytic sites in the F_1 sector, while 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. The “stator” composed of $b_2\delta$, prevents co-rotation of catalytic sites and the a subunit with the rotor (3,4). Proton gradient-driven clockwise rotation of γ (as viewed from the membrane) leads to ATP synthesis while anticlockwise rotation of γ results from ATP hydrolysis. The mechanism

is essentially a rotary motor that is in fact the smallest known biological nanomotor. Detailed review of ATP synthase structure and function has been reviewed (5-11).

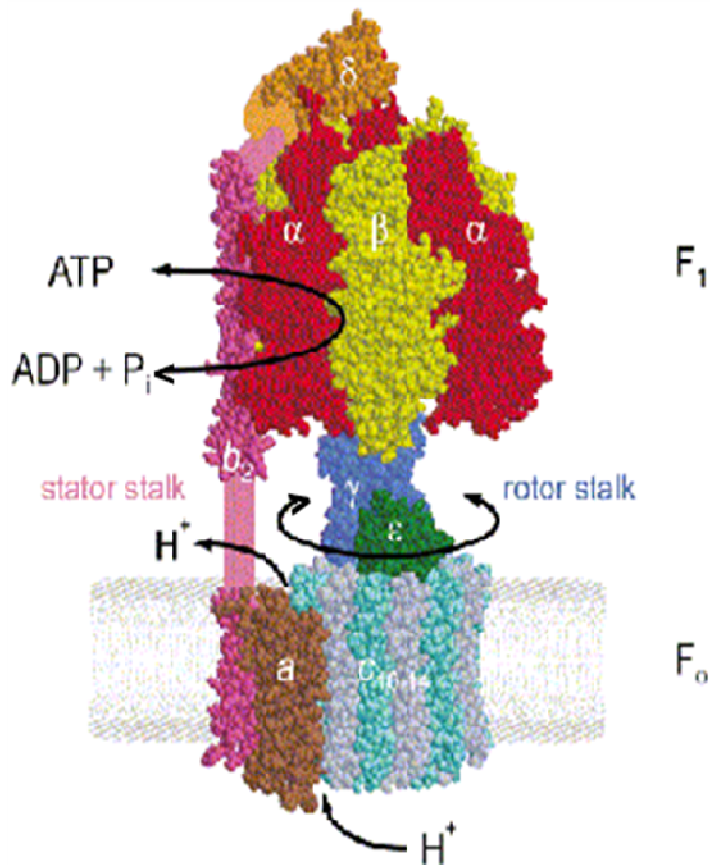


Figure 1. The ATP Synthase of *E. coli* (Reproduced from (9) with permission; copyright Elsevier). The enzyme consists of two sectors catalytic F₁ and membrane bound F₀. F₁ consists of $\alpha_3 \beta_3 \gamma \delta \epsilon$ and F₀ consists of ab_2c_{10} . In mitochondria and chloroplasts additional subunits are present. The rotor stalk indicates the helical coiled-coil extension of the γ subunit into the central cavity of the $\alpha_3\beta_3$ hexagon. The rotor is composed of γ,ϵ , and a ring of c subunits. The “stator” is composed of $b_2\delta$. The proton pathway lies between a and c subunits.

ATP Synthase and its Role in Disease Conditions

ATP synthase malfunctioning has been documented as a basis for many human diseases such as, cancer, heart disease, mitochondrial diseases, immune deficiency, cystic fibrosis, diabetes, ulcers, and tuberculosis (5,12). Alteration of ATP synthase biogenesis may cause 2 types of defects: qualitative, when the enzyme is structurally altered and does not function properly, and quantitative, when it is present in insufficient or adequate amounts (13). Most of these alterations are due to variation in the biosynthesis of the enzyme that can be due to mutations in subunit genes or in ancillary proteins essential for the enzyme assembly or in the areas responsible for gene regulation.

Mutation in subunit α of ATP synthase causes severe impairment of ATP synthesis resulting in a neurodegenerative disease known as Leigh syndrome (14). A mutation in the same subunit causes a dysfunction of ATP synthase causing the neuropathy, ataxia, retinitis pigmentosa syndrome, and the familial bilateral striatal necrosis (15,16). In Alzheimer's disease a deficiency of ATP synthase β subunit and the cytosolic accumulation of the α subunit has been observed (17,18). The intraneuronal cytosolic build up of the α subunit is suspected to be involved in the neurodegenerative process (16,18). Batten's disease belonging to a group of disorders called neuronal ceroid lipofuscinosis is caused by buildup of lipofuscins in the body's tissues and subunit c of ATP synthase has been found as a predominant storage protein (15,16). Circulation of the ATP synthase F6 subunit in the blood has been identified to be involved in the

increase of blood pressure (19,20). It is also reported that the ATP synthase on the cell surface of endothelial cells are associated with the angiogenesis process required for tumor growth (21-23).

As ATP synthase is implicated in many diseases it has been suggested and demonstrated as a good molecular target for drugs in the treatment of various diseases and the regulation of energy metabolism (24-28). One of the drugs developed for the treatment of tuberculosis, was shown to be active against a number of drug-resistant strains of *Mycobacterium tuberculosis*. Interestingly, it was found that 2 mutations in the mycobacterium ATP synthase F_o sector C-subunit, namely D32A and A63P, are responsible for the bacterial resistance to diarylquinoline drugs and the new drug R207910, has been reported to block the synthesis of ATP by targeting subunit *c* of ATP synthase and thus treating the disease (24,29,30). Another drug, Bz-423, which was developed to treat the autoimmune disorder systemic lupus erythematosus, kills pathogenic lymphocytes selectively by inducing apoptosis in lymphoid cells (31). It was found that the apoptosis of lymphoid cells is due to the inhibition of the mitochondrial ATP synthase by Bz-423 through binding to the subunit known as oligomycin sensitivity-conferring protein (OSCP) (26).

For a long time, it was believed that ATP synthase was found only in mitochondria and chloroplasts of eukaryotes where most cellular ATP synthesis takes place. However, recent studies indicate that ATP synthase is even expressed on the extracellular surface of several animal cell types such as endothelial cells of cancer tissues, making it an ideal recognition molecule on cancer cells and could possibly be targeted therapeutically in the treatment of such diseases by inhibiting the enzyme. Thus, identification of potent ATP synthase inhibitors may allow the development of lead drugs for therapeutic treatments.

Inhibition of ATP Synthase

Wide ranges of natural and synthetic compounds and molecules are known to bind and inhibit ATP synthase. The inhibitors can be characterized into different groups based on the type of bond formed with the enzyme, inhibitors can be grouped into either covalent or noncovalent inhibitors (32). Covalent inhibitors include NBD-Cl (4-chloro-7-nitrobenzofurazan), DCCD (dicyclohexylcarbodi-imide), and several reactive derivatives of ATP and ADP (33). The noncovalent inhibitors include non-hydrolysable substrate analogues, azide, the natural inhibitor protein IF1, the efrapeptins, the aurovertins, dietary phyto-polyphenols, nonpeptidyl lipophilic cations, and amphiphilic peptides (34).

Another classification is based on the physical and chemical characteristics of the inhibitors (2,35-40).

1) Peptide inhibitors: several types of peptide inhibitors have been identified and can be subdivided into Helical Basic Peptide Inhibitors, Angiostatin, Enterostatin, Leucino-statins, Efrapeptins, Tentoxin and its Derivatives.

2) Phytochemicals: are naturally occurring plant derivatives. They are known to possess chemotherapeutic properties and are known to bind to multiple molecular targets in the body. Phytochemicals are categorized into various groups, and among these are the polyphenols, steroids such as estradiols, estrogen and its metabolites.

3) Polyketide inhibitors: are the polymers of 2-carbon ketide units synthesized by the enzyme polyketide synthases. eg. Macrolides: apoptolidin, cytoovaricin, oligomycin,

ossamycin, and venturicidin.

4) Organotin compounds and structural relatives: are Tin containing organic compounds. They are classified into R_4Sn , R_3SnX , R_2SnX_2 , and $RSnX_3$.

5) Polyenic α -pyrone derivatives: α -Pyrone (or 2-pyrone) is a 6 membered cyclic unsaturated Ester and its derivatives are known to inhibit ATP synthase. Eg. aurovertin, citreoviridin, asteltoxin.

Other inhibitors can be grouped into cationic inhibitors, substrates and substrate analogs, amino acid modifiers, and miscellaneous inhibitors (36).

Polyphenols

Polyphenolic compounds are a distant group of naturally occurring compounds containing multiple phenolic functionalities. These compounds are primarily synthesized by plants as secondary metabolites and are found in abundance in fruits and vegetables. Notable sources of polyphenols can be found in berries, grapes, tea, citrus fruits, cocoa, nuts, and fresh vegetables. Their dietary importance has been linked to their role as anti oxidants. They interact with metal ions such as Fe^{2+} and prevent free radical formation in biological systems that are responsible for the DNA damage (41).

Polyphenols are the subject of interest to many scientists for their medicinal, synthetic, and industrial value. Plant derived polyphenols are known to have numerous biological activities. They are found to be potential candidates for use as drugs and for the treatment of many diseases like cancer, heart ailments, ulcer formation, bacterial infections, mutagenesis, neural disorders, etc. They also block the action of enzymes and other substances that promote the growth of

cancer cells (42-45). Polyphenols are also known to have antimicrobial activity. *Streptococcus mutans* is one of the examples 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 F₁-ATPase activity (46,47).

One important aspect of polyphenols is their ability to selective and unselective binding with biologically important molecules such as proteins. This is made possible by the presence of multiple polar functional groups that interact with proteins strongly and inhibit them (41,48).

Earlier, 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. Fig. 2 shows the recently solved F₁-resveratrol, F₁-piceatannol, and F₁-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 (49). 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 γ Lys-260, γ Ile-263, β_{TP} Val-279, and β_{TP} Ala-278. Polyphenol inhibited mitochondrial ATP synthase (*E. coli* residue numbers used throughout). X-ray structures also show that residues γ Ala-256, γ Thr-259, γ Glu-264, α_{TP} Glu-292, α_{TP} Gly-290, and α_{DP} Glu-292 are within 4Å of the bound compounds thus providing additional non-polar interaction (49).

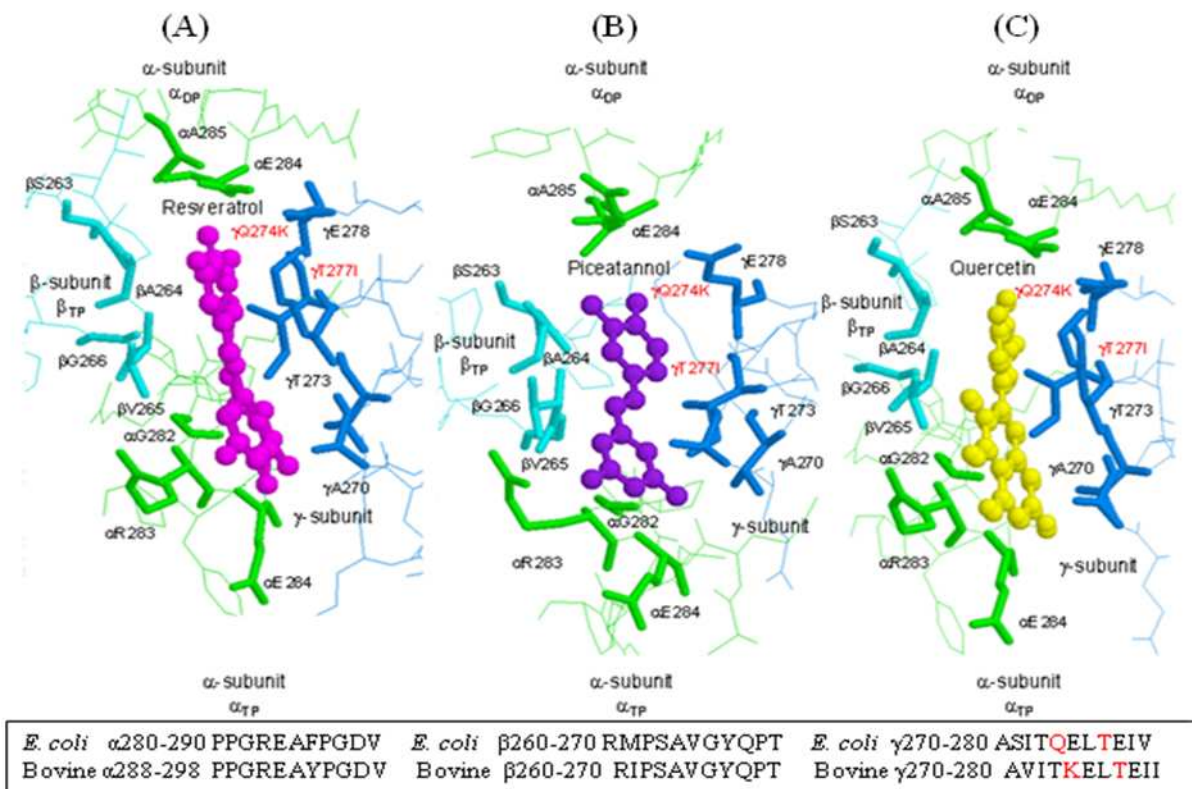


Figure 2. X-ray Structures of Mitochondrial ATP Synthase Showing Resveratrol, Piceatannol, or Quercetin. Rasmol software was used to generate these figures. PDB files used were 2jj1, 2jj2, and 2jjZ (50). (A) Reacted resveratrol in contact with α -, β -, and γ -subunits. (B) Reacted piceatannol in contact 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. γ Q274K, γ T277I 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.

We studied the inhibitory effects of natural polyphenols resveratrol, piceatannol, quercetin, quercitrin, or quercetin-3- β -D glucoside (see Fig. 3), and their derivatives on *E. coli* ATP synthase. Resveratrol is an antimicrobial substance naturally produced by plants when they are under attack by pathogens. Piceatannol is a metabolite of resveratrol. Quercetin is a plant derived flavonoid known to shown anti-inflammatory and antioxidant properties. Quercitrin and quercetin-3- β -D glucoside are both derivatives of quercetin (see Table 1). In the present study our goal was to test (i) if polyphenols resveratrol, piceatannol, quercetin, quercitrin, or quercetin-3- β -D glucoside inhibit *E.coli* ATP synthase similar to bovine ATP synthase (ii) if the beneficial dietary effects of polyphenols are related to their inhibitory actions on ATP synthase, (iii) if inhibitory effects of polyphenolic compound could be augmented through structural modifications, and (iv) if they can act as antimicrobial agent through their actions on ATP synthesis

Hypothesis

We hypothesized that *E. coli* ATP synthase may follow the inhibitory pathway of bovine ATP synthase as the basic structure of both are the same.

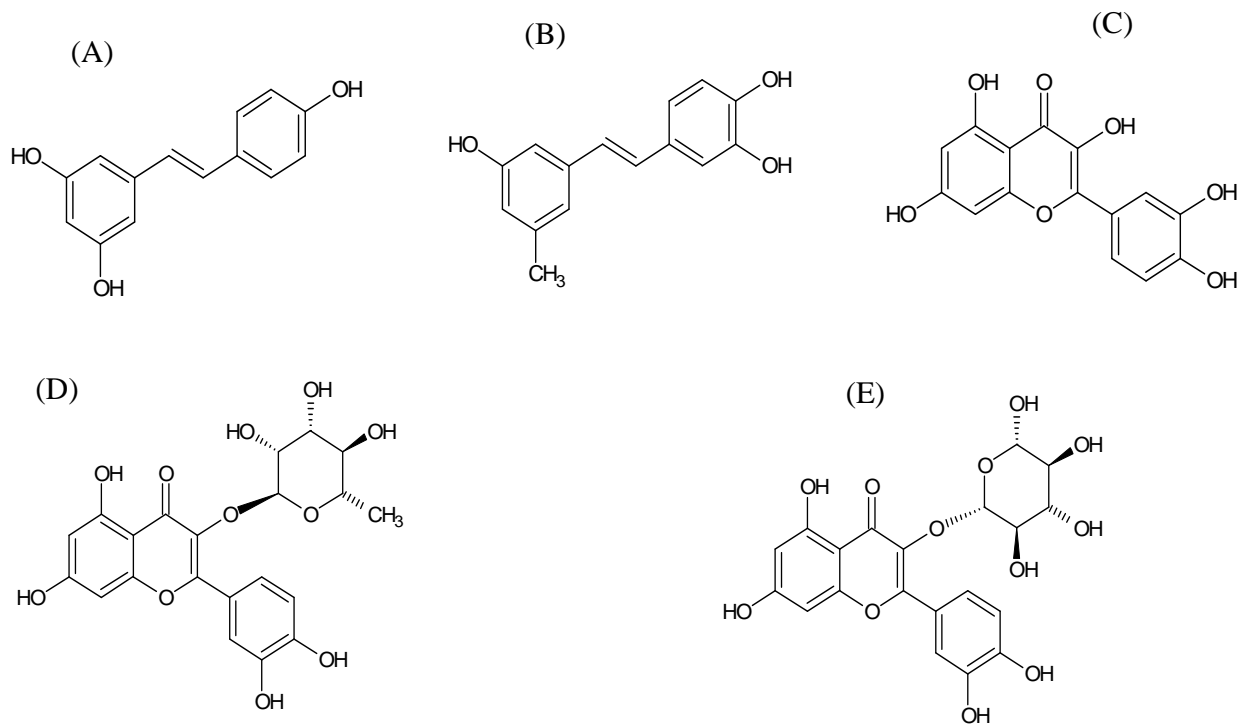


Figure 3. Structures of Polyphenol Resveratrol, Piceatannol, Quercetin, Quercitrin, and Quercetin-3- β -D Glucoside. These structures were drawn using Chem Sketch free version.

Table 1. Polyphenols Their Molecular Formulas and Synonyms

Name	Molecular formula	Synonyms
Resveratrol	$C_{14}H_{12}O_3$	3,4',5-Trihydroxy-trans-stilbene, 5-[(1E)-2-(4-Hydroxyphenyl)ethenyl]-1,3-benzenediol
Piceatannol	$C_{14}H_{12}O_4$	(E)-4-[2-(3,5Dihydroxyphenyl)ethenyl]1,2-benzenediol, 3,3',4,5'-tetrahydroxy-trans-stilbene, 3-hydroxyresveratol
Quercetin	$C_{15}H_{10}O_7 \cdot 2H_2O$	3,3',4',5,7-Pentahydroxyflavone dihydrate, 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one dihydrate
Quercetrin hydrate	$C_{21}H_{20}O_{11} \cdot xH_2O$	3,3',4',5,7-Pentahydroxyflavone-, 3-(6-deoxy-D-mannopyranoside)
Quercetin-3-β-D glucoside	$C_{21}H_{20}O_{12}$	3,3',4',5,7-Pentahydroxyflavone 3-β-glucoside, Isoquercitrin

CHAPTER 2

MATERIALS AND METHODS

Source of Polyphenols

Resveratrol (R5100-50MG), piceatannol (P0453-25MG), quercetin dihydrate (Q0125-100G), quercetrin hydrate (Q3001-50MG), and quercetin-3- β -D glucoside (17793) were purchased from Sigma Chemical Company. Polyphenols were suspended in DMSO to obtain the desired concentrations.

Chemicals

Adenosine 5'-triphosphate disodium salt, ampicillin, glucose, succinic acid, uracil, TES, TRIZMA (Tris[Hydroxyethyl]amino ethane), 4-aminobenzamidine dihydrochloride (PAB), and SDS (Sodium dodecyl sulfate) were purchased from Sigma–Aldrich Chemical Company.

Buffers and Reagents

50 mM Tris-H₂SO₄(pH 8), ATPase assay buffer, T&S reagent (Tuskey and Shorr reagent), 10%SDS, TE(trace elements), 1M MgSO₄, AET (Argenine Ent Thiamine), ILV (isoleucin-valine) STEM, TES 50, and TES5 +PAB were prepared as described in Appendix B.

Culture Media

LB Medium (Luria-Bertani Medium), Minimal media, Limiting glucose, Succinate media. Preparation of culture media (liquid and plates) is as described in Appendix D.

All other standard chemicals used in this study were ultra pure analytical grade purchased either from Sigma–Aldrich Chemical Company or Fisher Scientific Company.

Equipment

Stasar III Colorimeter (Gilford, Oberlin, OH) Evolution-300 UV-Visible Spectrophotometer (Thermo Scientific, Pittsford, NY), French press, cell disrupter (Thermo Scientific, Asheville, NC), C76 Water Bath shaker (New Brunswick Scientific Co. inc, Edison, NJ). Sorvall WX ultra-80, Ultra Centrifuge (Thermo Scientific, Asheville, NC), Sorvall RC-5B-super speed Centrifuge (Thermo Scientific, Asheville, NC), Series 25, incubator shaker (New Brunswick Scientific Co. inc, Edison, NJ).

Experimental Methods

Schematic Representation of Preparation of *E. coli* Membrane Bound ATP Synthase

E. coli (pBWU 13.4/DK8) culture in MM (37°C, 250 rpm)



Pellet at 9500 rpm x 15 min



Resuspend pellet in STEM
Spin at 9500 rpm x 25 min



Resuspend pellet in 2 ml Stem/g wet cells
Add DNase
French press 2 K psi



Spin
22 K rpm x 20 min



Spin supernatant
60 K rpm x 2 hrs at 2°C



Resuspend pellet in TES 50
Spin at 60K rpm x 2hrs at 2°C



Resuspend pellet in TES 5 + PBA
Spin at 60K rpm x 2hrs at 2°C
Repeat this step / store at - 70°C

Membrane bound ATP synthase were purified from *E. coli* strain pBWU 13.4/DK8 (51,52)

Starter Culture

50 ml of Minimal Media was inoculated with a loop full of *E. coli* pBWU 13.4/DK8 bacteria and is grown over night at 37°C, 250 rpm (revolutions per minute).

Bacterial Growth

50 ml of overnight starter cultures is inoculated in to 1 lt minimal media and grown at 37°C, 250 rpm. Growth yield was measured at OD₅₉₅ every hour till late log phase is obtained. Once the required growth is obtained we proceed to the next step i.e. harvesting cells. For all subsequent steps the cells are maintained as close to 0°C as possible.

Harvesting Cells

The cells are harvested by spinning the culture at 4°C in a Sorvall RC-5B refrigerated super speed centrifuge (Fisher Scientific) at 9500 rpm for 15 min. The harvested sample is resuspended in STEM and centrifuged at 9500 rpm for 25 min. The supernatant is discarded and the pellet is resuspended in 2 ml STEM/g wet cells and is stored at -80°C overnight.

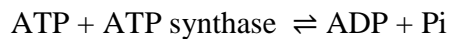
Cell Fractionation and Membrane Bound ATP Synthase Isolation

Cells from overnight are thawed and mixed with DNase to digest nucleic acids. The cells are then disrupted by 2 passages through chilled French press cell fractionator at 2000 psi. Cell debris was pelleted by centrifugation at 18K rpm for 20 min using Sorvall WX ultra-80, Ultra Centrifuge. Subsequently, the membranes were pelleted by spinning the supernatant at 60K rpm

for 120 min. The membrane is then resuspended in TES 50 and centrifuged at 60K rpm for 120 min. The pellet is then washed with TES 5 + PAB by spinning twice at 60K rpm for 90 min. Finally the purified membrane is resuspended in 50mM Tris Sulfate (pH 8) and stored at -80°C.

Membrane Bound ATP Synthase Concentration and ATPase Assay

Membrane concentrations were found by plotting the absorbance at 595 nm using Bradford reagent against standard BSA curve. ATPase activity was measured by adding 1 ml assay buffer containing 10 mM NaATP, 4 mM MgCl₂, 50 mM TrisSO₄ (pH 8) to the purified F₁ or membranes at 37°C and stopped by addition of SDS to 3.3% final concentration. Pi released was assayed by adding 1 ml of T&S reagent containing 10 mM (NH₄)₆Mo₇O₂₄·4H₂O, 250 mM Fe(NH₄)₂(SO₄)₂·6H₂O and 1.176 N H₂SO₄ (53). The color thus developed was measured using Stasar III Colorimeter at 700 nm. A graph is plotted using Sigma plot software with activity on X-axis and OD₇₀₀ on Y-axis. There is a straight line relationship between the calorimetric reading and the concentration of phosphorus. The reaction is as follows



ATP synthase activity was calculated using the formula

$$\frac{(\text{Average Sample OD} - \text{Average Blank OD})}{\text{Amount of protein (mg)} \times \text{Time (min)}} = \text{_____ } \mu\text{mol/min/g protein}$$

Inhibition of ATPase Activity by Resveratrol, Piceatannol, Quercetin, Quercetrin, or Quercetin-3-β-D Glucoside

Membranes or purified F₁ at a concentration of 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 TrisSO₄ pH 8.0. Then 1 ml ATPase assay buffer was added to measure the activity. The reaction is stopped by addition of SDS to 3.3% final concentration. Pi released was assayed by adding 1 ml of T&S reagent containing 10 mM (NH₄)₆Mo₇O₂₄·4H₂O, 250 mM Fe(NH₄)₂(SO₄)₂·6H₂O, and 1.176 N H₂SO₄. The color thus developed was measured using Stasar III Colorimeter at OD₇₀₀ nm. A graph is plotted using Sigma plot software with compound concentration on X-axis and relative ATPase % specific activity on Y-axis. This gives the enzyme activity with different concentrations of the compound in a linear decay fashion. It should be noted that prior to the experiments, F₁ samples (100 μl) were passed twice through 1 ml centrifuge columns with Sephadex G-50 beads equilibrated in 50 mM TrisSO₄ pH 8.0, to remove catalytic site bound-nucleotide.

% Specific activity was calculated using the formula

$$\frac{100}{\text{Specific activity of the blank} \times \text{Specific activity of the test (min)}} = \text{_____ } \mu\text{M}$$

Confirmation of Extent of Inhibition by Extra Pulse Experiment

To check if the inhibition is complete and there is no degradation of the compound and extra pulse experiment was performed. Membranes or purified F_1 at a concentration of 0.2–1.0 mg/ml were preincubated with inhibitory concentration of the compound for 60 min at room temperature, in 50 mM TrisSO₄ pH 8.0. Then another pulse of the compound with inhibitory concentration was added to the tube and incubated for 60 more min at room temperature. 1 ml ATPase assay buffer was added to measure the activity. The reaction is stopped by addition of SDS to 3.3% final concentration. Pi released was assayed by adding 1 ml of T&S reagent. The color thus developed was measured at OD₇₀₀ nm. Control tubes with single dose of compound incubated for 120 min were also performed. A graph is plotted using Sigma plot software with compound concentration on X-axis and relative % specific activity on Y-axis.

Reversal of Purified F_1 or Membrane Bound Enzyme ATPase Activity from Resveratrol, Piceatannol, Quercetin, Quercetrin, or Quercetin-3- β -D Glucoside Inhibition

To check if the inhibition of ATPase activity is reversible or not, reversibility experiments were performed. Reversibility of inhibition was performed on both membrane bound enzyme and on purified F_1 protein. It is assayed by dilution of the membrane enzyme and by passing the inhibited purified F_1 through centrifuge columns. Membranes were first reacted with the highest inhibitory concentrations for 1 h at room temperature. These concentrations were used based on the maximal inhibition of the ATP synthase. Then 50 mM TrisSO₄ pH 8.0 buffer was added to decrease the concentrations to a minimal noninhibitory concentrations, and incubation continued for 1 additional hour at room temperature before ATPase assay. Reversibility was also tested by passing the polyphenol inhibited purified F_1 enzyme twice

through 1 ml centrifuge columns before measuring the ATPase activity. Control samples without the compounds were also incubated for the same time periods as the samples as 2 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. Thus, after passage through centrifuge columns, reactivation is likely a first-order kinetic process that is a function of release of bound inhibitor. After the incubation time regular ATPase assay was performed to check the membrane activity.

Effects of Polyphenols on *E. coli* Growth

Inhibitory effects of polyphenols on *E. coli* cell growth were studied by growing the *E. coli* strain pBWU13.4 on succinate plates, limiting glucose, or LB media in presence of polyphenols. Growth in the liquid media was performed by inoculating LB or limiting glucose in a 16 well culture plate with ATP synthase inhibitory concentrations of polyphenols. The cultures were grown overnight at 37°C at 250 rpm. Growths were measured at OD₅₉₅ nm and are compared with the control cultures without any inhibitors. Plates were prepared by mixing the inhibitory concentrations of polyphenols in the succinate agar and plated into petri dishes. Bacterial cultures are then streaked and incubated at 37°C and the growth was recorded by visually inspection of the number of colonies and size of colonies for 3 days along with the control.

CHAPTER 3

RESULTS

Inhibitory Effect of Resveratrol, Piceatannol, or Quercetin on the Purified F₁ or Membrane Bound *E. coli* ATP Synthase Enzyme

We studied the inhibitory effect of resveratrol, piceatannol, quercetin, quercetrin, or quercetin-3- β -D glucoside (Fig. 3) on the purified F₁ and membrane bound ATP synthase of *E. coli*. Figure 4 shows the inhibition of ATPase activity of purified F₁ or membrane bound enzyme in presence of varied concentrations of resveratrol, piceatannol, or quercetin. Complete, potent inhibition (~100% inhibited; IC₅₀ ~14 μ M) occurs in presence of piceatannol without any residual activity, the maximum amount of inhibition in presence of quercetin was slightly less (~80%; IC₅₀ ~33 μ M) with 20 % residual activity, and resveratrol appear to be the least potent inhibitor (~40% inhibited; IC₅₀ ~94 μ M) with 60% residual activity. We consistently found that the F₁ data and the membrane data were the same for these inhibitors. Earlier studies established that inhibition of ATPase activity can be assayed using either membrane preparations or purified F₁ with same results (54,55).

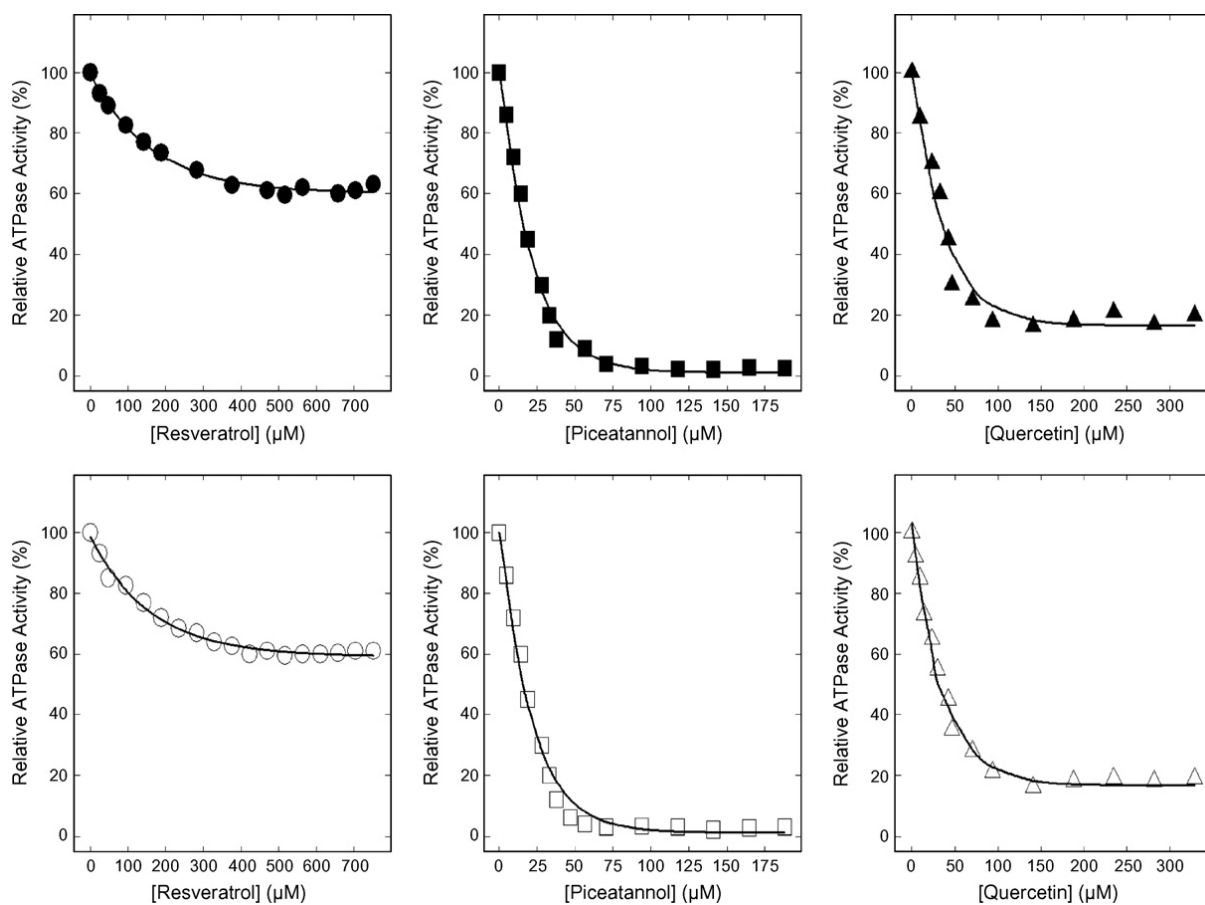


Figure 4. Inhibition of ATPase Activity in Purified F_1 or Membrane Bound ATP Synthase by Resveratrol, Piceatannol, or Quercetin. Membranes or purified F_1 were preincubated for 60 min at 23 °C with varied concentration of resveratrol, piceatannol, or quercetin, and then aliquots added to 1 ml of assay 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 4 experiments done in duplicate tubes, using 2 independent membrane or F_1 preparations. Results agreed within $\pm 10\%$ vv

Inhibitory Effect of Quercetrin or Quercetin-3- β -D Glucoside on the Purified F₁ or Membrane Bound Enzyme

Figure 5 shows the inhibitory effect of quercetrin or quercetin-3- β -D glucoside. Quercetrin shows 40% inhibition with 60% residual activity (IC₅₀ ~120 μ M) and quercetin-3- β -D-glucoside till 50% with 50% residual activity (IC₅₀ ~71 μ M). Again the F₁ data and the membrane data were alike for both the inhibitors.

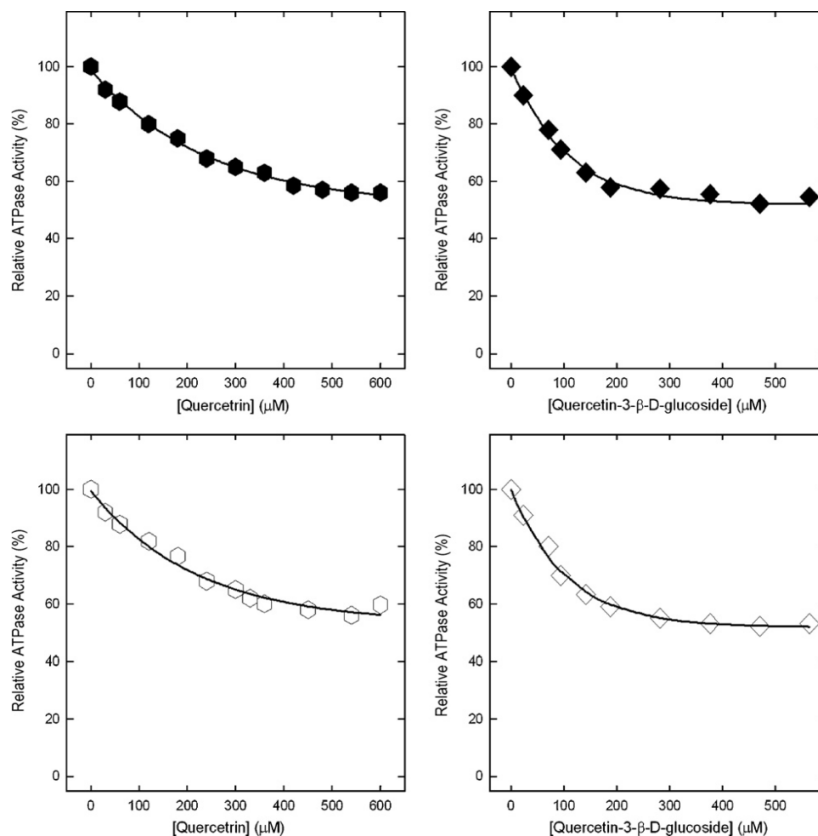


Figure 5. Inhibition of ATPase Activity in Purified F₁ or Membrane Bound ATP Synthase by Quercitrin or Quercetin-3- β -D Glucoside. Purified F₁ or membranes were preincubated for 60 min at 23 °C with varied concentration of quercetrin or quercetin-3- β -D glucoside and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Symbols used are: (◆ and ◇), quercitrin; (● and ○), quercetin-3- β -D glucoside. Filled symbols represent membrane data while open symbols represent data for purified F₁. Each data point represents average of at least 4 experiments done in duplicate tubes, using 2 independent membrane or F₁ preparations. Results agreed within $\pm 10\%$.

Confirmation of Extent of Inhibition by Extra Pulse Experiment

In previous studies (54-60), it has been noted in several instances where ATP synthase was incompletely inhibited by potent inhibitors like fluoroaluminate, fluoroscandium, sodium azide, or NBD-Cl. In recent studies also it was found that *E. coli* ATP synthase is partially inhibited by several polyphenols like hesperidin, chrysin, kaempferol, diosmin, apigenin, genistein, or rutin in the range of 40–60% (61). Even several peptides like aurein, carein, magainin, or magainin II-amide inhibited *E. coli* ATP synthase partially (62). Our polyphenols also showed some residual activities so to be sure that the maximal inhibition with resveratrol, piceatannol, quercetin, quercetrin, or quercetin-3- β -D glucoside had been reached we performed extra pulse experiment where we incubated each membrane preparation or purified F₁ with the inhibitory concentrations of the compounds i.e. 376 μ M resveratrol, 50 μ M piceatannol, 100 μ M quercetin, 400 μ M quercetrin, or 376 μ M quercetin-3- β -D glucoside for 1 h as in Figs. 4 and 5 followed by an extra pulses of the compounds doubling the concentrations of the polyphenols in the reaction mixture and continued the incubation for an additional hour and assayed ATPase activity. As shown in Fig. 6A and B very little or no additional inhibition occurred consistent with Figs. 4 and 5 data. This shows that the inhibition by resveratrol, quercetin, quercetrin, or quercetin-3- β -D glucoside was maximal and fully inhibited F₁ or membranes retained residual activity and the inhibition achieved is maximal. Although we used 1 h incubation time, it was observed that the maximal inhibition of purified F₁ or membrane bound enzyme was achieved within 15 min.

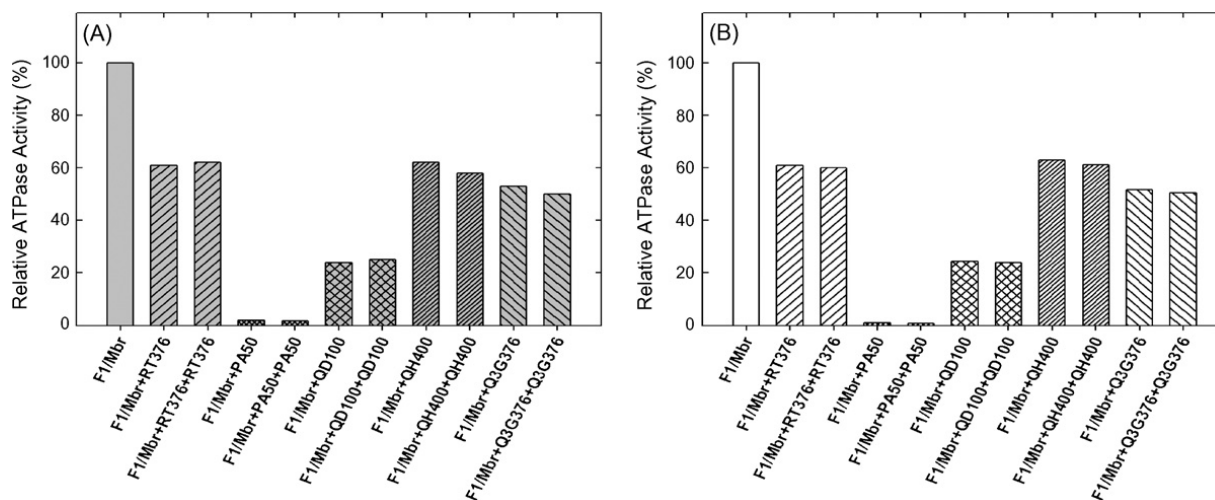


Figure 6. Results of Extra Pulse of Resveratrol, Piceatannol, Quercetin, Quercitrin, or Quercetin-3- β -D Glucoside on Purified F₁ or Membrane Bound ATP Synthase. (A and B) Membrane bound ATP synthase (Mbr, gray background) or purified F₁ (F₁, white background) was inhibited with 376 μ M resveratrol, 50 μ M piceatannol, or 100 μ M quercetin, 400 μ M quercitrin, or 376 μ M quercetin-3- β -D glucoside for 60 min under conditions as described in Fig. 4 and Fig. 5. Then a further pulse of 376, 50, 100, 400, and 376 μ M for resveratrol, piceatannol, quercetin, quercitrin, or quercetin-3- β -D glucoside, respectively, was added and incubation continued for 1 h before assay.

Reversal of ATPase Activity of Purified F₁ or Membrane Enzyme from the Resveratrol,

Piceatannol, Quercetin, Quercitrin, or Quercetin-3- β -D Glucoside Inhibition

Here we examined whether the inhibition of ATP synthase by the polyphenols is reversible or not. This experiment was carried out in 2 ways (i) the purified F₁ or membrane bound enzyme was inhibited with the higher concentration of resveratrol, piceatannol, quercetin, quercitrin, or quercetin-3- β -D glucoside. Then the samples were diluted to a noninhibitory concentration. It was found that the inhibition was totally reversible (Fig. 7A and B). (ii) 20 μ g purified F₁ samples were inhibited with 470 μ M resveratrol, 50 μ M piceatannol, 94 μ M quercetin, 400 μ M quercitrin, or 400 μ M quercetin-3- β -D glucoside for 1 h. Again these inhibitory concentrations were determined based on data from Fig. 4 and 5. Then they were passed twice through 1ml centrifuge columns and ATPase activity was measured. It was found

that in all cases activity was restored back to the near normal level as seen in Fig. 6C and D as in absence of the compounds similar to Fig.7A and B. 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.

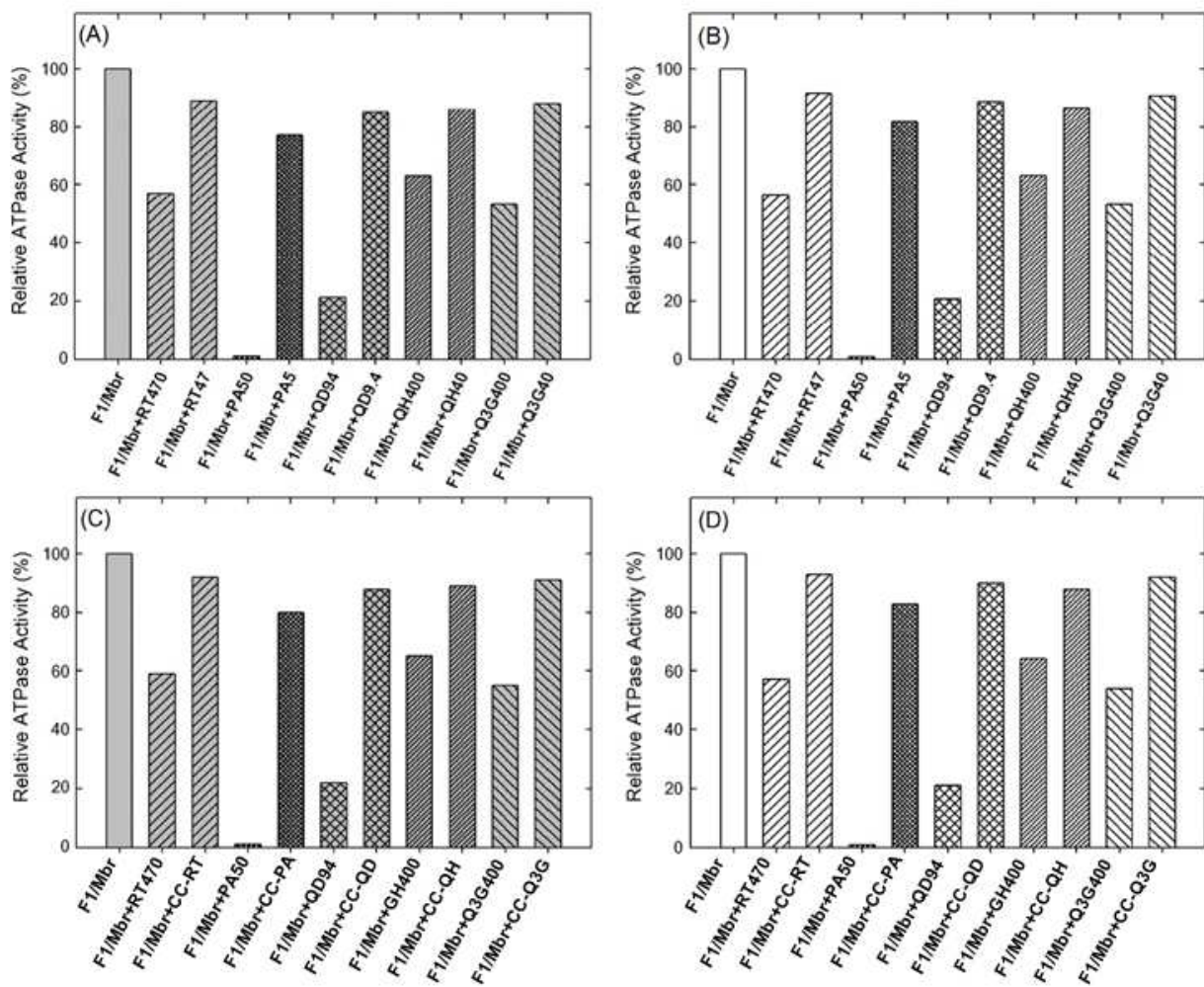


Figure 7. Reversibility of Inhibition. (A and B) Results of reversibility through dilution of membrane bound ATP synthase (Mbr, gray background) or purified F₁ (F₁, white background). Membrane/F₁ was incubated with 470 μM resveratrol, 50 μM piceatannol, 94 μM quercetin, 400 μM quercetrin, or 400 μM quercetin-3-β-D glucoside for 60 min under conditions as described in Figs. 2 and 3. Concentrations were lowered to 10-fold by adding TrisSO₄ buffer and incubation continued for 1 h before assay. The first bars are purified F₁ 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. (C and D) Reversibility through centrifuge columns. 20 μg purified F₁ 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 and were passed twice through 1ml 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.7A and B. The first bars are purified F₁ 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 and CC represents the reaction which is passed through the centrifuge column.

Growth Assays: 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 2).

Table 2. 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. (++++) High growth; (–) no growth.

^b Growth yield on limiting glucose and LB was measured as OD₅₉₅ 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 4 to 6 experiments each at 37 °C. Each individual experimental point is itself the mean of duplicate assays.

CHAPTER 4

DISCUSSION

A variety of inhibitors are known to bind and inhibit ATP synthase enzyme. Recent finding showed that polyphenols: resveratrol, piceatannol, and quercetin inhibit bovine heart mitochondrial ATP synthase by blocking both clockwise and anti-clockwise rotation of the γ -subunit. X-ray crystallography at 2.3, 2.4, and 2.7-Å resolution showed that these polyphenol compounds bind between the C-terminal tip of the γ -subunit and the β_{TP} subunit through hydrophobic interactions and H-bonds. The hydrophobic interaction between the inhibitor compounds and mitochondrial enzyme was shown to involve γ K260, γ I263, 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 γ Ala-256, γ Thr-259, γ Glu-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 Figure. 2. (49). The equivalent residues in the *E. coli* enzyme are γ Q274 (γ K260), γ T277 (γ I263), β_{TP} V265 (β_{TP} V279), and β_{TP} 264 (β_{TP} A278) (63). Parentheses show the bovine numbers. The focus of our study is to examine the inhibitory role of polyphenols resveratrol, piceatannol, quercetin along with quercitrin, and quercetin-3- β -D glucoside on the *E. coli* ATP synthase. The aim of our study is to test (i) if the beneficial effects of polyphenols are related to their inhibitory actions on ATP synthase, (ii) if inhibitory effects of polyphenolic compound could be augmented through structural modifications, and (iii) if they can act as antimicrobial agent through their actions on ATP synthesis.

By using *E. coli* membrane preparations, we have tested polyphenols resveratrol, piceatannol, quercetin, quercitrin, or quercetin-3- β -D glucoside on the activity of F_0F_1 -

ATPase/ATP synthase. We also screened the effect of these polyphenols on purified F₁ protein that is similar to that of the membranes. We found out that polyphenols resveratrol, piceatannol, quercetin, quercetrin, or quercetin-3-β-D glucoside inhibited *E. coli* ATP synthase to varying degrees (Fig. 4 and 5). Piceatannol causes maximal inhibition with 0% residual activity with IC₅₀ values of ~14 μM. Other inhibitors, resveratrol has a residual activity of 25% with IC₅₀ values of ~94 μM, quercetin has a residual activity of 25% with IC₅₀ values of ~33 μM, quercetrin has a residual activity of 40% with IC₅₀ values of ~20 μM, and quercetin-3-β-D glucoside has a residual activity of 50% with IC₅₀ values of ~71 μM. These results are not consistent with earlier findings of bovine ATP synthase whose inhibitory concentrations for resveratrol, piceatannol, and quercetin were 19 μM, 8 μM, and 65 μM, respectively (49). The reason for this might be due to the differences in the amino acids at the binding sites of polyphenols in bovine and *E. coli* ATP synthase.

Though resveratrol and quercetin like piceatannol are composed of 2 phenolic rings did not significantly inhibit the ATP synthase even at higher concentrations. The reason for this might be because of differential numbering / positioning of OH groups on the phenol rings. Like quercetin its derivatives quercetrin and quercetin-3-β-D glucoside did not inhibit the enzyme to the extent as quercetin did. It may be due to the presence of the third phenol ring that might have caused steric hindrance resulting in low inhibition rates. The difference in the rate of inhibition clearly indicates the importance of hydroxyl groups in particular positions and the number of phenolic rings.

Additional dose of compounds to the previously inhibited purified F₁ or membranes did not change the degree of inhibition significantly (6A and B). This suggests that the inhibition of

purified F_1 or membrane by the phenolic compounds was the true and maximum achievable. The process of inhibition was also found to be completely reversible. A completely inhibited F_1 regained its activity when it was passed through the centrifuge columns that resulted in removal of the bound compound with the enzyme. Similarly, purified F_1 or membrane regained complete activity once it was brought back to lower concentrations of compound after exposing it to higher concentrations by dilution with buffer and by passing it through centrifuge columns (Fig. 7A,B,C and D) This shows that the compound and the enzyme share a non-covalent interaction.

Another important aspect observed is the bacterial growth patterns in presence of the phenolic compounds. Resveratrol and piceatannol restricted the growth of *E. coli* suggesting that both ATP hydrolysis and ATP synthesis are inhibited. This finding is consistent with the effect of resveratrol and quercetin observed on bovine ATP synthase (49). Quercetin, quercetrin, or quercetin-3- β -D glucoside prevents only ATP hydrolysis and not the ATP synthesis suggested by the growth of the bacteria in the presence of the compound. The reason for this performance is unknown but can be speculated by the following reasons (a) as observed the inhibition of the enzyme by these compounds is not complete because of which the bacterial are able to survive (b) the inhibitors could not pass the cell wall to react with the membrane (c) inhibitors got pumped out by an export pump, (d) the inhibitors were metabolized by the bacterial cells. More inhibitory studies by new functionally modified polyphenol compounds should help in understanding this difference.

Our studies show that the inhibition of ATP synthase could be a potential mechanism contributing to the many effects of dietary polyphenols. Mitochondrial dysfunction is the cause of a number of degenerative diseases such as cancer, neurological disorders, and cardiovascular

disease (64,65). Therefore, it is acceptable that the inhibition of the ATP synthase by piceatannol and other related compounds might play a significant role in the pathophysiology of such conditions (49). For example, inhibition of mitochondrial F_1F_o -ATPase by dietary polyphenols might be beneficial is by induction of apoptosis selectively in tumor cells. Resveratrol is known to induces cell death in tumor cells via pathways that depend on mitochondria (44,66), and another inhibitor, oligomycin, has similar effects on mitochondrial F_1F_o -ATPase (49), possibly by specifically marking tumor cells for cell death by CD14, while assuring differentiation to occur in the surviving population (49). Alteration of cellular bioenergetics is another vital way of triggering the cell death. The benzodiazepine Bz-423 inhibits the mitochondrial F_1F_o -ATPase by binding to the oligomycin sensitivity-conferral protein, a component of the peripheral stalk resulting in apoptosis. Nontumor cells are not affected by the drug, but the autoimmune lymphocytes with altered mitochondrial bioenergetics, are sensitized to Bz-423-mediated inhibition of ATP synthase (49).

The resistance of mycobacterium against the anti-tuberculosis drug diarylquinoline is due to two C-subunit mutations D32V and A63P. This commends a need for more potent natural or synthetic inhibitors that can specifically target bacterial ATP synthase enzymes (24). 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 (46,47) 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*.

Thus, by identifying and modeling potent polyphenol inhibitors that can selectively induce apoptosis through inhibition of the F_1F_0 -ATPase, we can treat diseases caused by mitochondrial dysfunction and can even design anti microbial drugs.

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APPENDICES

APPENDIX A: Abbreviations

ATP - Adenosine triphosphate

ADP- Adenosine diphosphate

F₁- F₁ protein

Gln – Glutamine

IC₅₀- half maximal inhibitory concentration

Ile – Isoleucine

Lys - Lysine

Mbr- Membrane

PA - Piceatannol

Pi- Inorganic phosphate

Q3G - Quercetin-3-β-D glucoside

QH - Quercetrin hydrate

QD - Quercetin dihydrate

RT - Resveratrol

Thr - Threonine

APPENDIX B: Buffers and Reagents

50 mM Tris-SO₄ buffer

To 90 ml H₂O add

0.61 g Tris

Adjust pH to 8.0 with H₂SO₄

Bring to a final volume of 100 ml with H₂O

ATPase cocktail

In 150 ml H₂O add

10 ml 1 M Tris

0.8 ml 1M MgCl₂

5 ml 0.4 Na ATP (Adenosine 5'-triphosphate disodium salt)

Adjust pH to 8.5 with H₂SO₄

Bring to a final volume of 200 ml with H₂O

Freeze in plastic bottles at -20°C

10 % SDS

100 gm Sodium dodecyl sulfate

Bring to a final volume of 1000 ml with H₂O

T & S reagent / Tuskey and Short reagent

Sol A: 1.2 g Ammonium molybdate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ in 9.8 ml 12 N H_2SO_4)

Sol B: 10 g Ferrous ammonium sulfate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2\cdot 6\text{H}_2\text{O}$ in 70 ml H_2O)

Add sol A to sol B while stirring

Bring to a final volume of 100 ml with H_2O

Store at 4°C

STEM

To 700 ml H_2O add

100 ml 1 M TES

4.29 g $\text{Mg}(\text{CH}_3\text{CO}_2)_2\cdot 4\text{H}_2\text{O}$

85.5 g sucrose

0.0951 g EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid)

5 g EACA (6-Ainocaproic acid6-Ainocaproic acid)

Adjust pH to 6.5 with NaOH

Bring to a final volume of 1000 ml with H_2O

Freeze in plastic bottles at -20°C

TES 50

To 700 ml H₂O add

50 ml 1 M TES

150 ml glycerol

5 g EACA (6-Aminocaproic acid)

1 g PAB (4-Aminobenzamidine dihydrochloride)

Adjust pH to 6.5 with NaOH

Bring to a final volume of 1000 ml with H₂O

Freeze in plastic bottles at -20°C

TES 5 + PAB

To 700 ml H₂O add

5 ml 1 M TES

150 ml glycerol

1 ml 0.5 M DTT (Dithiothreitol)

5 g EACA (6-Aminocaproic acid)

1 g PAB (4-Aminobenzamidine dihydrochloride)

2.5 ml 0.2 M EDTA (Ethylenediaminetetraacetic acid disodium salt dihydrate)

Adjust pH to 6.5 with NaOH

Freeze in plastic bottles at -20°C

AET (Arginine Ent Thimine)

To 60 ml H₂O add

0.617 g 2,3 Dihydroxy Benzoic acid

16.86 g L-Arginine HcL

1 ml 20 mM Thiamine

Add just enough amount of NaOH to dissolve everything

Make final volume to 100 ml with H₂O

Filter sterile

TE (Trace Elements)

To 80 ml H₂O

0.251 g Zinc Sulfate (ZnSO₄.7H₂O)

0.017 g Manganese Sulfate (MnSO₄.H₂O)

0.029 g Boric acid (H₃BO₃)

0.012 g Calcium Sulfate (CaSO₄.2H₂O)

0.037 g Calcium Chloride (CaCl₂.2H₂O)

0.049 g Ferric Chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$)

Make final volume to 100 ml with H_2O .

Filter sterile

ILV (Isoleucin-Valine)

To 95 ml H_2O add

0.394 g Isoleucine

0.352 g Valine

Make final volume to 100 ml with H_2O

Filter sterile

APPENDIX C: Culture Media and Plates

LB liquid medium

12.5 g of LB broth powder

Add H₂O to bring to 500 ml

Autoclave for 30 minutes

Cool the media to ~50°C

Add 500 µl of 100 mg/ml Ampicillin

Minimal Glucose

To 400 ml H₂O add

5.225 g Potassium Phosphate Dibasic Trihydrate (K₂HPO₄)

2.40 g Sodium Phosphate Monobasic (NaH₂PO₄)

0.99 g Ammonium Sulfate ((NH₄)₂SO₄)

Autoclave for 30 min, cool it and add the following additions

10 ml Uracil

10 ml 27 % Glucose

5 ml ILV (isoleucin-valine)

0.5 ml TE (trace elements)

0.5 ml 1 M Magnesium Sulfate (MgSO₄)

0.5 ml AET (Arginine Ent Thimine)

0.5 ml 100 mg/ml Ampicillin

0.312 ml 4X LB

LB-Agar plate with Ampicillin

12.5g of LB broth powder

7.5g of agar

Bring to a final volume of 500 mL

Autoclave for 30 minutes

Cool the media to ~50°C

Add 500 µl of 100 mg/ml Ampicillin

Pour into sterile plates

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- Nagababu Chinnam, Prasanna K. Dadi, Mubeen Ahmad, M Anaul Kabir, and Zulfiqar Ahmad. (2010). “*Dietary bioflavonoids inhibit Escherichia coli* ATP synthase in a differential manner”. *In press doi:10.1016/j.physletb.2003.10.071 In.t, J. Biol. Macromol.*
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