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ABTS OXIDATION REACTION AS A MODEL OF CYTOCHROME *C*-DRIVEN ELECTRON TRANSFER

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Cytochrome c, as an electron carrier within the mitochondria, plays a crucial role in the electron transport chain. To meet the demand for rapid methods that assess the electron transport properties of cytochrome c, we used the electron donor 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) as a substrate and suitable spectrophotometric reporter of cytochrome c peroxidase-like activity. ABTS and cytochrome c from bovine were purchased from Sigma-Aldrich Inc. The time course of the cytochrome c-driven ABTS oxidation reaction was studied using H_2O_2 as a second substrate. It was demonstrated that CytC addition is a prerequisite for the transfer of electrons from ABTS to H_2O_2. The reaction kinetic analysis with determination of V_{max}, K_m, k_{cat}, and k_{cat}/K_m values for both substrates was performed. Our results demonstrate that the cytochrome c-catalyzed ABTS oxidation reaction can be effectively employed as a model for studying the functional role of cytochrome c in various conditions.

K e y w o r d s: cytochrome c, ABTS reaction, peroxidase activity, electron transport rate, Michaelis-Menten kinetics parameters.

Electron transport (ET) is a fundamental step in redox reactions, encompassing a broad range of processes. It is essential to various biological processes such as photosynthesis, metabolic regulation, and redox reactions [1-3]. The mitochondrial electron transport chain (ETC) is one of the electron transport mechanisms in the cellular signaling network [4,5]. Cytochrome *c* (CytC), as part of this unique alliance, plays an important role in mediating electron transfer between complexes III and IV of the respiratory chain [6].

Due to its function as an electron carrier, CytC acts as an extremely versatile protein [5-7]. Under normal physiological conditions, CytC is localized in the intermembrane space of mitochondria, and its distribution can extend to the cytosol, nucleus, and extracellular space under certain pathological or stress conditions. CytC may interact with apoptotic peptidase-activating factor 1 (APAF1) to form the apoptosome, which initiates caspase-dependent apoptotic cell death, a process highly relevant in cancer. [8, 9].

Thus, CytC plays a vital role in intrinsic apoptosis, making it a key target in the signaling pathways of cancer cells [10-12]. Research has shown that the inhibition and induction of apoptosis are correlated with decreased and increased serum levels of CytC, respectively. In patients with various cancer types, elevated serum levels of CytC have been associated with a greater likelihood of survival [13]. Although high levels of serum CytC may also indicate increased tumor content, their rise during cancer treatment has been linked to more favorable prognoses [14]. Moreover, several commonly used chemotherapeutic drugs promote the release of CytC into the cytoplasm, thereby inducing apoptosis.

As a result, both the quantification of CytC levels and the characterization of its main functions, electron transport properties, in serum could hold significant prognostic value. This study aimed to develop a rapid and straightforward method for assessing the functional state of cytochrome *c* under physiological and various pathological conditions. We focused on its primary function of cy-

tochrome *c* – the ability to transfer electrons. This was achieved through the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) oxidation reaction, which utilizes the peroxidase-like activity of cytochrome *c* [15].

Materials and Methods

Cytochrome *c* from bovine was purchased from (Sigma-Aldrich, Inc., St. Louis, MO., United States) with a purity of ≥ 95% and was used without further purification. These characteristics were confirmed using SDS-PAGE. We also performed spectral analysis of cytochrome *c* using electronic absorption spectroscopy to verify the optical properties of cytochrome *c*. The concentration of oxidized cytochrome *c* was determined spectrophotometrically at 410 nm (ε ≈ 106,100 $M^{-1}\cdot cm^{-1}$).

Cytochrome c-driven ABTS oxidation reaction. ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate, Sigma-Aldrich, Inc., St. Louis, MO., United States) oxidation in the presence of cytochrome *c* was performed using a colorimetric assay with H_2O_2. The 100 µl reaction mixture in MOPS buffer (25 mM MOPS, 2 mM EDTA, 120 mM KCl, pH 7.2) included 10-50 µM ABTS, 250-1500 µM H_2O_2 and 0.8-2 µM cytochrome *c*. Reduced ABTS displays a characteristic peak at 340 nm; the oxidation of ABTS ($ABTS^{+\bullet}$) forms an absorbance peak at 415 nm [16]. The concentration of $ABTS^{+\bullet}$ was determined spectrophotometrically at 415 nm (molar extinction coefficient [ε], 36,000 $M^{-1}\cdot cm^{-1}$). To determine the concentration of a H_2O_2 solution, we use a molar extinction coefficient of 43.6 $M^{-1}\cdot cm^{-1}$ at 240 nm. The concentration ranges of ABTS and H_2O_2 were selected based on their spectral properties. In the wavelength region relevant to the reaction conditions, this selection is more suitable for analyzing the reaction results.

The electronic absorption spectra were measured by using a 1 cm path-length quartz cuvette in a Shimadzu UV-2700 UV-VIS spectrometer. The absorbance spectra were recorded in the range from 240 and 600 nm.

To characterize the electron transport event, we used cytochrome *c*-driven peroxidase reaction kinetics. The ABTS oxidation reaction kinetic parameters were estimated using the Michaelis-Menten kinetic equation and the Lineweaver–Burk plot at varying concentrations of substrates (ABTS or H_2O_2); equation $1/V = K_m/V_{max}/1/[S]+ 1/V_{max}$.

In which V is the initial reaction velocity, K_m is the Michaelis constant, V_{max} is the maximum reaction velocity, and [S] is the substrate (ABTS or H_2O_2) concentration. kcat (sec^{-1}) values were obtained from the equation: $k_{cat} = V_{max}/[E]$, where [E] is, concentration of cytochrome *c*.

Statistical analysis. Data are presented as mean ± SEM, $n = 6$. Statistical comparisons it was performed using an ANOVA (analysis of variance) test in Excel. Statistical significance was assumed at $P < 0.001$ and indicated by asterisks in the figures.

Results and Discussion

Cytochrome c delivers electrons from ABTS to H_2O2. To demonstrate electron, transfer via cytochrome *c* (CytC) transport, we used ABTS, which is a well-known electron donor reagent for specific enzymes. A suitable electron donor is 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), which can be used as a spectrophotometric reporter of peroxidase activity. The radical produced upon the oxidation of ABTS ($ABTS^{+\bullet}$) has maximal absorbance at ~415 nm, whereas the reduced form of ABTS absorbs maximally at ~340 nm (Fig. 1).

Thus, the time course of ABTS oxidation is ideal for detecting electron transport and quantifying its kinetics as an enzymatic reaction, making it a perfect model for the electron carrier property of CytC.

The reaction catalyzed by many peroxidases may be generalized as:

H_2O_2 + electron donor (2e-) + $2H^+ = 2H_2O$.

We investigated the effect of CytC on the formation rate of $ABTS^{+\bullet}$.

We found that adding CytC to the reaction mix ABTS and H_2O_2 (Fig. 2, *A*, *B*) resulted in a decrease in the absorbance of ABTS at 340 nm with a concurrent increase of the absorbance at 415 nm characteristic of $ABTS^{+\bullet}$ (oxidized form), which indicates that CytC is a prerequisite for the oxidation of ABTS, i.e., for the transfer of electrons from ABTS to H_2O_2 (Fig. 2, *C*).

The time course of ABTS oxidation at different compositions of the CytC reaction mixture is shown in Fig. 3. CytC is required for the peroxidase activity observed in this assay. These experiments demonstrate that CytC exhibits catalytic properties and peroxidase activity due to its electron transport properties [17, 18].

Cytochrome c mediated the ABTS oxidation reaction, as a biological enzymatic process. We

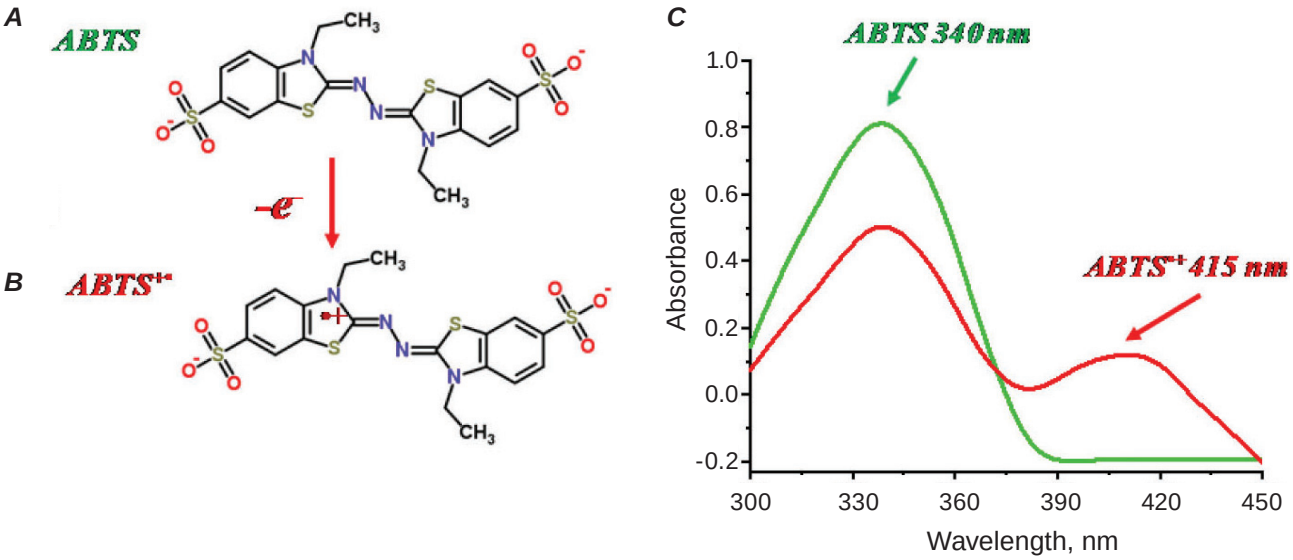

*Fig. 1. (**A**) and (**B**) show the molecular structure of ABTS and oxidized ABTS ($ABTS^{+\bullet}$), respectively. (**C**) Absorption spectrum of oxidation ABTS: reduced ABTS displays maximal absorbance at 340 nm, while the radical produced upon the oxidation of ABTS [$ABTS^{+\bullet}$], absorbs at ~415 nm*

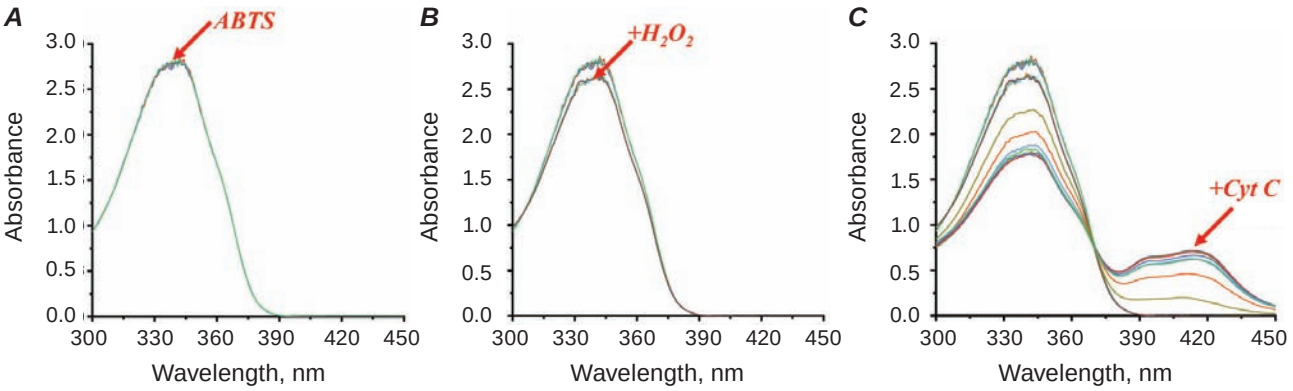

*Fig. 2. Cytochrome c is essential for ABTS oxidation in the presence of H_2O_2. Absorption spectra of ABTS oxidation obtained in the presence of CytC and H_2O_2 at different time intervals. (**A**) Absorption spectra of 10 μM ABTS, which exhibit a peak at 340 nm. (**B**) Absorption spectra of a mixture of 10 μM ABTS and 500 μM H_2O_2. The addition of H_2O_2 to the reaction mixture does not change the spectral properties of the reaction mixture. (**C**) 0.8 μM CytC was added to a reaction mixture containing 10 μM ABTS and 500 μM H_2O_2. After the addition, the spectral properties of the reaction mixture changed dramatically, as a result, of the formation of a band at 415 nm, indicating the oxidation of ABTS. Note that the oxidation of ABTS by CytC in the presence of H_2O_2 is necessary for catalyzing this reaction*

characterized the CytC transport process and estimated the amount of formation of oxidized $ABTS^{+\bullet}$, in the first 60 sec after the addition of CytC, which was defined as the initial rate of the enzymatic event per second. The CytC-catalyzed reaction initial rate (also defined as the electron transfer rate) was estimated by varying H_2O_2 or ABTS concentrations using fixed concentrations of reducing substrate, and vice versa (Fig. 4).

Kinetic parameters of these catalytic reactions were obtained using Lineweaver–Burk, which is one method of linearizing substrate-velocity data so as to determine the kinetic constants K_m and V_{max} (Materials and Methods) (Fig. 5).

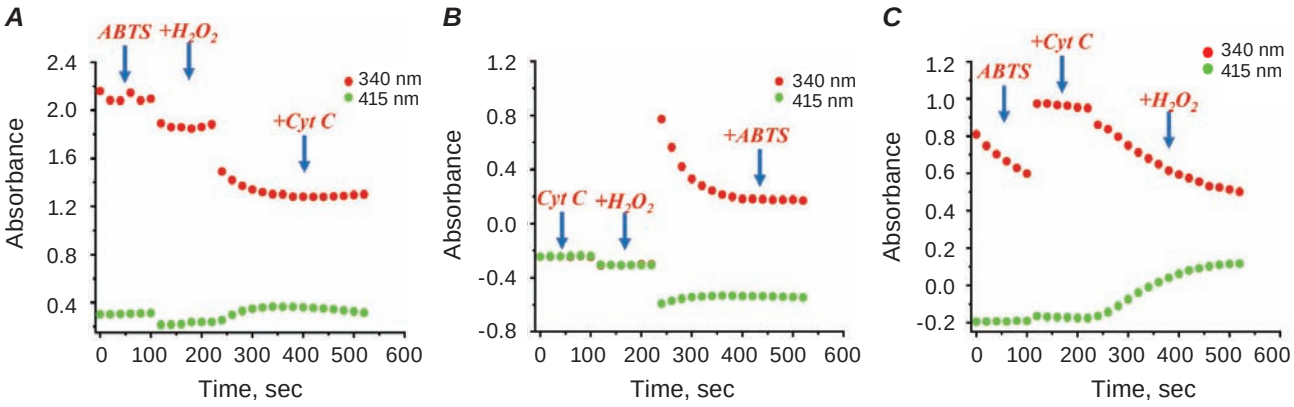

*Fig. 3. Cytochrome c catalyzes the ABTS oxidation reaction in the presence of H_2O_2. (**A**) Time course of ABTS oxidation in the presence of ABTS (10 μM 0-120 sec), after the addition of H_2O_2 (500 μM at 120 sec) and CytC (2 μM at 240 sec). (**B**) as in (**A**), with a different sequence of reagent additions: H_2O_2 was added to the CytC mixture at 120 sec, and then added ABTS 240 sec. (**C**) ABTS mixture was supplemented with CytC at 120 sec, and H_2O_2 at 240 sec*

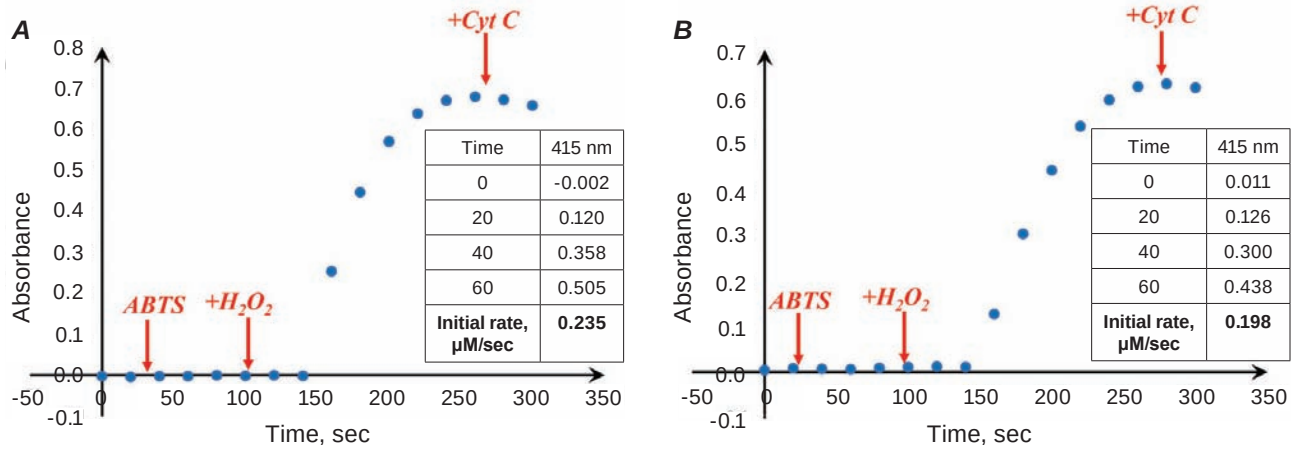

*Fig. 4. Estimation of the initial rate ABTS oxidation reaction in the presence of H_2O_2 driven by cytochrome c. (**A**) Time course of ABTS oxidation in the presence only of ABTS (40 μM 0-60 sec), after the addition of H_2O_2 (500 μM at 60 sec) and CytC (0.8 μM at 140 sec). (**B**) Time course of ABTS oxidation in the presence of ABTS (10 μM 0-60 sec), after the addition of H_2O_2 (750 μM at 60 sec) and CytC (0.8 μM at 140 sec). The initial rate (we defined it as the electron transfer rate) of the cytochrome C-driven oxidation reaction of ABTS in the presence of H_2O_2 is estimated as the initial rate of ABTS oxidation, which is calculated as the amount of $ABTS^{+\bullet}$ formed per second. The amount of $ABTS^{+\bullet}$ was calculated using the extinction coefficient (ε_{420} = 36,000 $M^{-1}\cdot cm^{-1}$)*

The kinetic parameters values obtained using the Lineweaver–Burk plot analysis from 6 independent reactions is shown in the Table.

The kinetic parameters for CytC–driven ABTS were calculated by fitting the experimental initial rate in the Lineweaver–Burk plot equation (Fig. 5). K_m is an intrinsic property of an enzyme; it is a measure of the affinity of the enzyme for the substrate, and it is an inverse measure of affinity of the enzyme for the substrate; a low K_m corresponds to a high affinity and vice versa. The table shows that the Michaelis constant (binding constant, K_m) for ABTS is 14.0 ± 2.65 μM, which is much lower than that for H_2O_2 (571.28± 88.28 μM). Thus, ABTS binds effectively to the enzyme (CytC) active site at a much lower concentration than H_2O_2 according to its small K_m value. Additionally, the turnover number (k_{cat}) or catalytic constant values for both substrates are al-

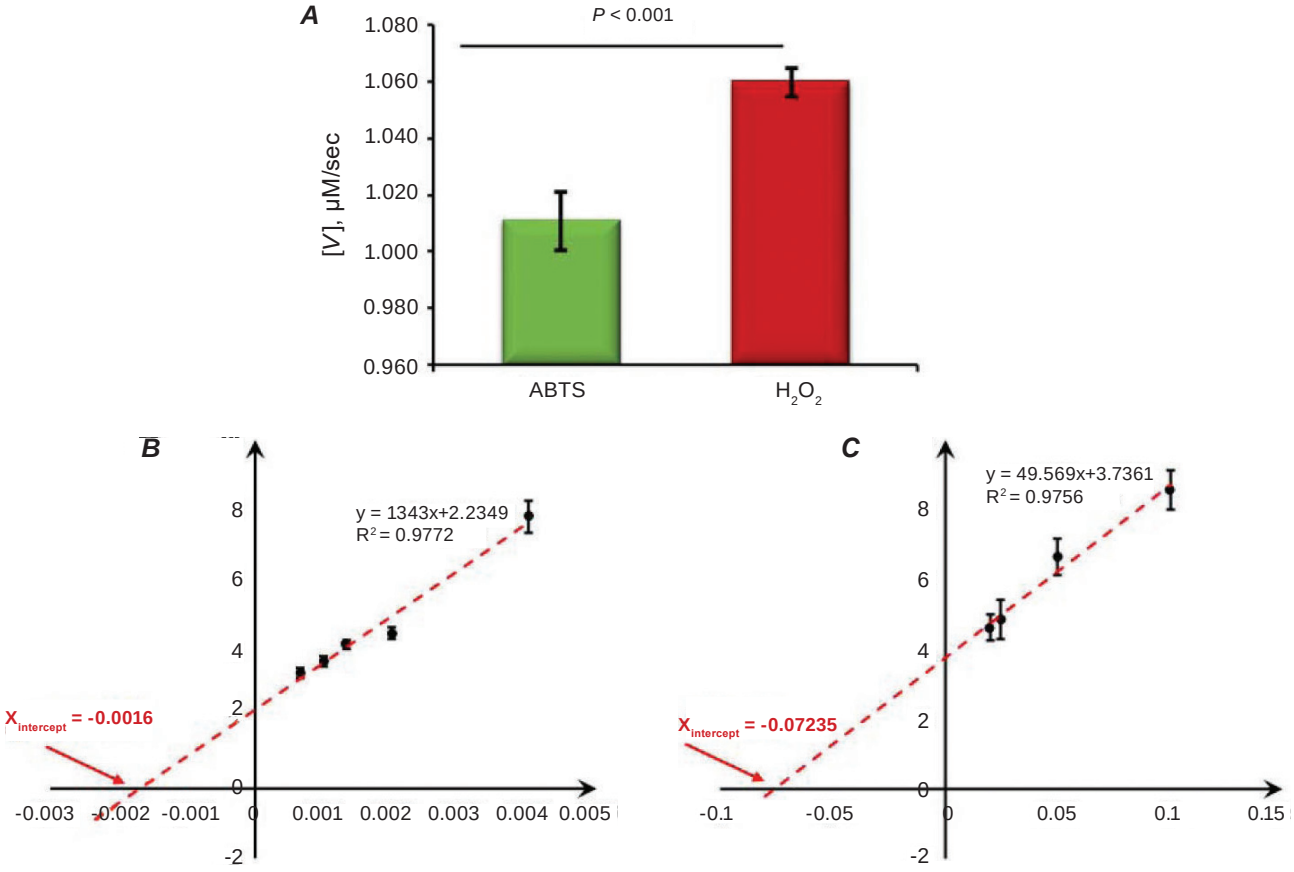

*Fig. 5. Calculation of kinetic parameters of ABTS oxidation reaction in the presence of H_2O_2 catalyzed by cytochrome c. (**A**) The initial rate of the CytC-catalyzed reaction, also referred to as the electron transfer rate, was determined by varying the concentrations of ABTS or H_2O_2, while keeping the concentration of the reducing substrate fixed, and vice versa. (**B**) Lineweaver–Burk plot to determine the kinetic constants K_m and V_{max} of ABTS oxidation reaction by varying [ABTS] while H_2O_2 was used at a fixed concentration (500 µM), and (**C**) vice versa (varying [H_2O_2] while [ABTS] = 50 µM) in the presence of 0.8 µM cytochrome c. The data for this analysis is shown in the bottom Table*

Table. Kinetic parameters of the ABTS oxidation reaction in the results of the electron transport property of the cytochrome c. The kinetic parameters were obtained using Lineweaver–Burk plot analysis from 6 independent reactions (Fig. 5), mean ± SEM, n = 6

Parameters	[ABTS] is constant	[H_2O_2] is constant
V_{max}, 1/sec	0.44 ± 0.04	0.28 ± 0.03
K_m, µM	571.28 ± 88.28	14.08 ± 2.65
$k_{cat,}$ 1/sec	0.56 ± 0.05	0.36 ± 0.043
k_{cat}/K_m, $1/sec^{-1}\cdot\mu M^{-1}$	0.001 ± 0.0001	0.026 ± 0.0037

most similar. Consequently, the catalytic efficiency value (k_{cat}/K_m) for H_2O_2 is relatively higher than that for ABTS. CytC-driven ABTS oxidation reaction kinetic values fall within the typical range of Michaelis-Menten constant and other kinetic parameters for biological catalytic evets and likely represent electron transfer properties of CytC as mechanism this enzymatic reaction (Fig. 6) [19, 20].

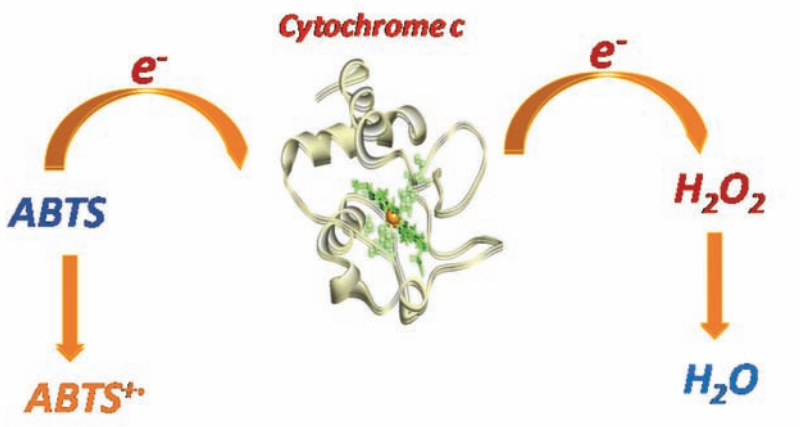

Fig. 6. Proposed cytochrome c-driven electron transport pathway. CytC mediates the oxidation of ABTS in the presence of H_2O_2 by transferring electrons between ABTS and H_2O_2. Electron transfer occurs through the heme iron center of CytC, which undergoes redox reactions to transfer electrons. During the electron transfer process, the coordinated iron of heme C changes from a redox state to a state where the iron accepts and donates electrons, which is the basis of the mechanism of this catalytic reaction

Conclusion. Despite these known roles, the specific contributions of CytC's electron transport properties to its diverse functions in cellular biology – both under normal and pathological conditions – remain unclear [21-23]. Therefore, it is essential to develop assays to investigate these properties, where CytC plays a critical role [24-27].

In this study, we examine the electron transfer event of CytC during the oxidation of ABTS. We analyzed various reaction parameters using the Lineweaver–Burk equation enzyme kinetics model, which enabled us to determine the Km, kcat, and kcat/Km values for both substrates, all of which are characteristic of biological catalysis. The calculated Michaelis-Menten constants, which are an intrinsic property of an enzyme, and it reflect the enzyme's affinity for its substrate, were for ABTS K_m 14.0 ± 2.65 μM, and for H_2O_2 K_m 571.28 ± 88.28 μM. These values are very low, compared to the proteins with peroxidase-like activity, such as hemoglobin and is hemoglobin variants (K_m 30 mM) [28], myoglobin (K_m 296 mM) [29], also monoclonal antibody 13G10 (K_m ABTS 3.48 mM, Km (H_2O_2) 1.52 mM) [30]. K_m values are very close typical peroxidase from plant origins~200-300 μM [31], but much low compare to the artificial peroxidase 13G10 antibody complexed with its Fe(ToCPP) [30].

The k_{cat}/K_m value, also known as the specificity constant, combines k_{cat} and K_m to offer a comprehensive measure of an enzyme's catalytic efficiency. A higher k_{cat}/K_m value indicates a more efficient enzyme capable of catalyzing reactions with speed and specificity. Our results show that the k_{cat}/K_m value for ABTS is 0.026 ± 0.43 $\mu M^{-1} \cdot sec^{-1}$ which is comparable to the k_{cat}/K_m values of highly efficient enzymes such as tRNA synthetase (0.008 $\mu M^{-1} \cdot sec^{-1}$) and pepsin (0.002 $\mu M^{-1} \cdot sec^{-1}$) [32, 33].

Our data demonstrated that the ABTS oxidation reaction, as well as similar biocatalytic reactions, can be a very efficient and technically simple method for characterizing the core function – electron transport features of cytochrome *c*.

In this paper, we present the cytochrome *c*-catalyzed oxidation of ABTS as a method for selectively assessing the functionality of cytochrome *c* under various pathological conditions. We believe this model reaction can effectively be used to investigate the molecular mechanisms underlying the primary functions of cytochrome *c*, particularly in electron transport.

This model system is characterized by its simplicity, speed, and cost-effectiveness, allowing experiments to be conducted with very small volumes in the microliter range. However, certain limitations arise from the concentration ranges of ABTS and H_2O_2, which are determined by their spectral properties.

Overall, the cytochrome *c*-catalyzed ABTS oxidation reaction serves as an effective model for studying the functional role of cytochrome *c* in

various pathological conditions, especially in the context of cancer cell metabolism.

Conflct of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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РЕАКЦІЯ ОКИСЛЕННЯ ABTS ЯК МОДЕЛЬ ЕЛЕКТРОННОГО ПЕРЕНЕСЕННЯ, ОПОСЕРЕДКОВАНОГО ЦИТОХРОМОМ *C*

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Цитохром *c*, як переносник електронів у мітохондріях, відіграє ключову роль у функціонуванні ланцюга перенесення електронів. З метою розробки швидких методів оцінки електронтранспортних властивостей цитохрому *c*, ми використали електронний донор 2,2′-азино-біс(3-етилбензотіазолін-6-сульфонат) (ABTS) як субстрат і зручний спектрофотометричний індикатор пероксидазоподібної активності цитохрому *c*. Кінетику реакції окислення ABTS, опосередкованої цитохромом *c*, досліджували у присутності H_2O_2 як другого субстрату. Показано, що додавання цитохрому *c* є необхідною умовою перенесення електронів від ABTS до H_2O_2. Виконано кінетичний аналіз реакції з визначенням параметрів V_{max}, K_m, k_{cat} та k_{cat}/K_m для обох субстратів. Отримані результати свідчать, що реакція окислення ABTS, каталізована цитохромом *c*, може ефективно застосовуватися як модель для дослідження функціональної ролі цитохрому *c* за різних умов.

Ключові слова: цитохром *c*, реакція ABTS, пероксидазна активність, швидкість перенесення електронів, параметри кінетики Міхаеліса-Ментен.

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