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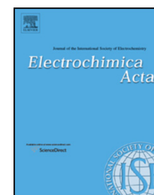

# Correlation between biological activity and electron transferring of bovine liver catalase: Osmolytes effects

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ABSTRACT

Catalase is a crucial antioxidant enzyme that protects life against detrimental effects of $H_2O_2$ by disproportionating it into water and molecular oxygen. Effect of proline as a compatible and histidine as a non compatible osmolyte on the electron transferring and midpoint potential of catalase has been investigated. Proline increases the midpoint potential ($\Delta E_m > 0$), therefore causing the $\Delta G_{ET}$ to be less positive and making the electron transfer reaction more facile whereas histidine decreases the $E_m$ ($\Delta E_m < 0$) and consequently a causes a pronounced increase in $\Delta G_{ET}$, thereby rendering the electron transfer reaction less efficient. These results indicate the inhibitory effect of histidine evident by a −37% decrease in the cathodic peak current compared to 16% increase in the case of proline indicative of activation. The insight paves the tedious way towards our ultimate goal of elucidating a correlation between biological activity and electron transferring.

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## 1. Introduction

Catalase is an important antioxidant homotetramer containing four heme prosthetic groups, to which the enzyme's unique electron transferring characteristics are attributed [1–3]. It is a typical hemoprotein in charge of protecting the biological systems against the detrimental effects of hydrogen peroxide [4]. The enzyme has also found numerous applications in various sectors of industry as an efficient means of remediating the excess $H_2O_2$ [5–7]. Catalase for instance is used in dairy industry to enhance and stabilize diacetyl in lactic starter cultures [8]. Although the electrochemical studies have been conducted on catalase, due to its huge size (∼250 kD), the heme redox center is more or less buried within the macromolecular structure [2–4,7] and therefore direct electrochemical investigations are complicated [9], leading to development of other techniques such as potentiometric titration and spectroelectrochemistry to alleviate those problems [10,11]. Both of these techniques rely on the equilibration of the redox species of the analyte at different potentials within the spectroscopic cell. Those potentials are determined either by applying potential via a potentiostat or alternatively by the help of an appropriate chemical redox agent. Once the equilibrium has been reached, the concentration of redox species could be measured spectroscopically [10]. On the other hand since the oxidized and reduced form of the catalase have two distinctive spectra separated from one another; the enzyme lends itself marvelously to spectroscopic studies monitoring the redox state of the protein [4,12]. Such an approach is much more economical and readily available than the high-end spectroelectrochemical investigations that demand rather expensive experimental setup and mediators such as hexaammineruthenium (III) chloride ($Ru(NH_3)_6Cl_3$) [10].

Spectroelectrochemistry is a common potentiometric hybrid method that combines spectroscopy and electrochemistry. In this technique the bulk of solution is equilibrated whether by using a potentiostat or chemical reductant, to a constant potential ($E_{solution}$). Subsequently the concentrations of oxidized and reduced species are followed spectroscopically at various solution potentials. This method has proven to provide valuable information about the mechanism of electron transfer in various hemoproteins. Interestingly using a coupled reaction (xanthine/xanthine oxidase) can serve as the source of electrons provided by the spectroelectrochemical apparatus during the potential sweep, thereby allowing a conventional UV–vis spectrophotometer to be used for monitoring the oxidized and reduced species. Such an approach has been used successfully in the case of some other hemoproteins such as Cyt c [13] and it is also used extensively in the routine coupled assay for superoxide dismutase [14,15]. In those studies reactant could be either a redox couple such as ferrocyanide/ferricyanide in

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a redox titration or a more sophisticated aforementioned coupled reaction in the scanning kinetics mode. In the coupled reaction a redox indicator e.g. 2,6-dichlorophenolindophenol measures the extent of reduction as is the case for vitamin C [16]. DCIP is deep blue when oxidized and turns colorless upon reduction, additionally its redox potential is well documented in the literature (0.217 V at pH 7.0 versus NHE, i.e. Normal Hydrogen Electrode). It has also shown significant potential as a prooxidant in cancer therapy [17]. Since it does not perturb the biological activity and structural stability of protein at low concentrations it is used as the redox indicator of choice. Both methods are based on the Nernst equation which relates the potential of redox reaction participants to their relative concentrations.

Osmolytes are a group of low molecular weight organic molecules with crucial role in maintaining the osmotic pressure and as an effective countermeasure against environmental stresses [18,19]. Their effect has been extensively studies on various biological systems [18–20]. Osmolytes are generally categorized into compatible and non-compatible ones [18,21]; the former category maintains or enhances the protein stability and biological activity [18,22], whereas the latter perturbs the biological activity and structural stability of the protein to various degrees [18,23]. These effects are generally opposite to those caused by urea [23]. Compatible osmolytes stabilize the folded macromolecular structure indirectly via preferential hydration [24,25] and destabilization of the unfolded conformer [26]. The shift of equilibrium toward native folded conformations by compatible osmolytes is achieved by raising the free energy of the unfolded state. Compatible osmolytes are excluded from the protein backbone and therefore unfolding the protein in their presence is less energetically favorable [27]. Amino acids are an important group of osmolytes that have members in both compatible [18,20] and non-compatible subcategory [28]. Proline was selected as a well-known compatible osmolyte and histidine as a non-compatible one. It was already documented, that proline stabilizes various proteins such as lysozyme [29], lactate dehydrogenase [30], protein L [31], CspTm [31] and catalase [32]; in addition to some typical protein backbone models such as N-acetylglycine amide peptides [26], whereas histidine destabilizes, among others, catalase [32], hen's egg-white lysozyme, RNase [28] and cytochrome c [33].

Although the biological activity and structural stability of catalase have been investigated in detail [1,6,32] and its electron transfer properties are electrochemically studied more or less in form of biosensors [9,34], the literature is unfortunately almost void of integrated studies that try to shed light on the correlation between the electron transferring and biological activity. Our ultimate goal in this research has been discovering of such a correlation. In order to achieve that goal we have used the osmolyte–catalase interaction as the foundation of our model.

## 2. Materials and methods

### 2.1. Chemicals

Catalase (C40) (EC 1.11.1.6, from bovine liver, 2100 units/mg), xanthine oxidase (X1875) (EC 1.17.3.2, from bovine milk, 5 units/ml) and proline were purchased from Sigma (Saint Louis, MO, USA). Catalase was dialyzed extensively against 50 mM phosphate buffer pH 7.0; to remove the thymol preservative and xanthine oxidase against Tris buffer pH 9.0 to remove the ammonium sulfate. Room temperature ionic liquid (RTIL): 1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIM]/$BF_4$), was purchased from Sigma. Multi-wall carbon nanotubes (MWCNTs), prepared by chemical vapor deposition, were purchased from Shenzhen Nanotech Port Ltd. Co. (China). Ethylenediamine ($C_2H_4[NH_2]_2$), sodium dihydrogen phosphate ($NaH_2PO_4$), disodium hydrogen phosphate ($Na_2HPO_4$), histidine, potassium hexacyanoferrate(II)trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$), potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), sodium dithionite ($Na_2S_2O_4$) and 2,6-dichlorophenolindophenol (DCIP) were obtained from Merck. Thionyl chloride ($SOCl_2$) was purchased from Acros Organics. Hydrogen peroxide ($H_2O_2$) stock solutions were prepared by appropriate dilutions of 30% (v/v) $H_2O_2$ in deionized water and their concentrations were verified spectroscopically based on the absorption at 240 nm. Every solution was freshly prepared in double-distilled deionized water. All other chemicals were of analytical grade and were used without further purification.

Functionalized MWCNTs were prepared according to the procedure outlined in [34]. Briefly the MWCNTs were treated with $SOCl_2$ and the filtrate was then stirred at 50–70 °C for 5 days repeatedly. Anhydrous ethyl alcohol was used to wash the excess ethylenediamine and the functionalized MWCNTs were subsequently dried under the vacuum. The functionalization authenticity was confirmed via FTIR (data not shown).

#### 2.1.1. Apparatus and measurements

Spectroscopic measurements were performed using a Varian Cary 100 spectrophotometer equipped with Peltier temperature control (±0.05 °C). The measurements were performed at a constant temperature of 25.0 °C, controlled using a Varian water bath circulator, the quartz cuvette pathlength was 1 cm. The potentials in both spectroscopic methods delineated here are reported versus Ag/AgCl (3 M KCl) reference potential. In that context the standard reduction potentials of hexacyanoferrate (II)/(III) redox couples and redox indictor DCIP, where extracted from literature [17,35] i.e. versus NHE. Subsequently those potentials were converted to the Ag/AgCl scale, henceforth the final calculated potential would be versus Ag/AgCl (3 M KCl) to be consistent with the electrochemical investigations in which Ag/AgCl (3 M KCl) reference electrode is used. Such a notation makes the comparison of potentials between two spectroscopic and one electrochemical method plausible.

Electrochemical studies were carried out using an electrochemical system (EG&G model 263A potentiostat/galvanostat) controlled by a GPIB interface and PowerSuite software package. All electrochemical studies were performed using a conventional three-electrode cell at 25 ± 1 °C. A working modified glassy carbon (GC) electrode (bare or modified with nano-composites film) (from Azar Electrode, Uromia, Iran), a saturated silver/silver chloride (Ag/AgCl) (3 M KCl solution) reference electrode (from Metrohm), and a platinum wire counter electrode were used. All potentials were measured and reported vs. Ag/AgCl (3 M KCl) reference electrode.

### 2.2. Spectroscopic method

#### 2.2.1. Ferricyanide/ferrocyanide redox titration

The midpoint potential $E_{1/2}$ was measured using the ferrocyanide/ferricyanide couple and dithionite as the reactant according to Scheme 1. The experiment is based on the increased absorbance of reduced catalase (ferrocatalase) at 412 nm [12,36]. Catalase concentration was 1.5 μM (0.375 mg/ml) throughout the experiments and it was prepared in 50 mM phosphate buffer pH 7.0. Care was taken to exclude atmospheric $O_2$ and the solutions were $N_2$ purged and kept in stoppered cuvettes during the course of reaction. Based on Scheme 1, tube 1 (catalase + ferricyanide) has the lowest absorbance say $A_1$ (oxidized), tube 3 (catalase + dithionite) has the highest absorbance, $A_3$ (fully reduced), in between falls the $A_2$, absorbance of the sample being titrated (sample gradually being reduced) i.e. titrand (catalase + ferrocyanide), accordingly at the midpoint potential the two redox couples i.e. ferrocatalase/ferricatalase and ferrocyanide/ferricyanide would be

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