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An electrochemical investigation of effect of ATP on hemoglobin

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Abstract

The titratable potentiometric response of hemoglobin (Hb) induced by adenosine-5′-triphosphate (ATP) is observed. The concentration-dependent effect of ATP on the anaerobic redox reaction of the protein at pH 7.0 reflects that ATP will induce stabilization of the reduced state and destabilization of the R-like (met Fe(III)) state of the metHb, when ATP concentration is lower than 3.0 mM. But when ATP concentration is between 4 and 7 mM, shift of the oxidation potential may also be observed. With reference to the study of adenosine, adenosine-5′-monophosphate, adenosine-5′-diphosphate and 2,3-diphosphoglycerate, the allosteric effect of ATP on Hb is discussed extensively. This study has given an electrochemical approach to the investigation of effect of ATP, an in vivo allosteric effector, on Hb in the physiological concentration range.

Keywords: Hemoglobin; ATP; Allosteric effector; Cyclic voltammetry

1. Introduction

Hemoglobin (Hb) consists of a protein component comprising two α- and β-polypeptide chains, and a nonprotein component, within each polypeptide chain, containing a prosthetic heme group that reversibly binds one oxygen molecule. The globin structure controls the redox potential of the heme site of Hb, protects it from rapid oxidation, and thereby allows for reversible oxygen binding. Therefore, the redox potential of Hb is sensitive to the globin alterations surrounding the active heme [1]. Studying the redox potential of the active site, a parameter that describes the propensity of the site to donate or accept electrons, allows us to gain insight in how effector-induced alteration tunes to the equilibrium between the oxidized and the reduced form of the protein.

The importance of organic phosphates, such as 2,3-diphosphoglycerate [2,3] and inositol hexaphosphate [4–7], which is known to bind at the β–β interface, in allosteric control of Hb is well recognized. In addition to organic phosphates, some heterotropic effectors, such as protons, anions and carbon dioxide, although bound spatially at remote sites, are capable of influencing the oxygenation process and have been shown to affect the oxidation process as well [8–10]. Adenosine-5′-triphosphate (ATP) is an important intraerythrocytic organic phosphate in vivo. The change in the concentration of ATP in red blood cell results in

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modulation of Hb oxygen affinity [11,12]. Bonafe and coworkers have found that ATP can induce a pH-dependent tetramerization of deoxyHb in vertebrates [13]. In physiological pH, ATP exists with four negative charges, which implicates that the effect of ATP on Hb may be similar to that of anionic heterotropic effectors. It has been traditionally explained that anionic heterotropic effectors operate on Hb as inhibitors with the formation of salt bridge between effectors and Hb, and preferentially bind to the low oxygen affinity quaternary conformation [14,15], although recent study has shown no significant binding sites of Hb to some anions [16]. The ATP-binding site on other heme protein like cytochrome *c* is invariant Arg^{91}, and it is nucleotide that induces conformation change in this protein [17,18]. But there are still unanswered questions with regard to how ATP affects Hb. Structural changes of Hb that stabilize either the oxidation or reduction conformation can reflect alternations of T (deoxy Fe(II)) and the R (oxygenated) states processes [1]. Therefore, evaluating trends in the redox potential of Hb, as the ATP concentration is changing, is conceivable to get further understanding on the way that in nature Hb is able to accomplish oxygen transport, oxygen storage and electron transfer reaction in polar environment.

In this work, an attractive technique named protein film voltammetry (PFV), in which an electrode-confined protein produces electronic signals, is employed to study the redox process of Hb. Rusling et al. have developed this technique so as to facilitate the direct electron transfer [19,20]. Armstrong et al. have used PFV to study the interaction between proteins and small molecular effectors [21,22]. Here, we present the results of anaerobic redox potential of Hb with titration of ATP at the physiological pH 7.0, by employing this electrochemical method.

2. Experimental

Bovine Hb was purchased from Sigma and used as received. Stock Hb solution (8 mg/ml) was stored at a temperature of 4 °C. The Na_2 salt of ATP, adenosine-5′-diphosphate (ADP) and adenosine-5′-monophosphate (AMP) (all in ultra pure grade) and 3-(*N*-morpholino) propane sulfonic acid (MOPS) (>99%) were provided from Sigma as well. Kieselguhr and egg-phosphatidylcholine (egg-PC) were supplied from Shanghai Chemical Reagent Co. All the other reagents used were of analytic grade. Water was purified using a Milli-Q purification system (Barnstead; Bedford, MA) to a specific resistance $>16\ M\Omega/cm$ and used to prepare all solutions.

Electrochemical experiments were performed using a PARC 263A potentiostat/galvanostat (EG&G; Princeton, NJ), using a three-electrode configuration. The working electrode was a modified pyrolytic graphite (PG) disk electrode. A saturated calomel electrode (SCE) and a platinum electrode served as reference and counter electrodes, respectively. Potentials are reported with respect to SCE unless specially stated. UV–vis absorbance spectroscopy was performed using a UV-2201 spectrophotometer (Shimadzu; Kyoto, Japan). High-purity nitrogen was used for deaeration before every electrochemical measurement.

Prior to being modified, the substrate PG disk electrode was polished with rough and fine abrasive papers, respectively. It was then polished on silk containing an alumina (particle size of ~0.05 mm)/water slurry. After that, it was ultrasonicated in water for 5 min. The Hb–kieselguhr film modified electrode was prepared as follows. A proper amount of kieselguhr was dissolved in 25% dimethyl sulfoxide to a final 2 mg/ml solution and mixed with 8.0 mg/ml Hb aqueous solution in the ratio of 1:1. (An aqueous mixture of Hb (8.0 mg/ml) and egg-PC (1×10^{-3} mol/l) with the ratio of 1:1 was prepared for Hb–PC film.) Twenty microliter of mixture was dropped on electrode, and then the modified electrode was dried overnight in room temperature and rinsed with pure water before use.

MOPS was selected as buffer for its noncomplexing nature and stability, as well as the absence of electrochemical interference.

3. Results and discussion

Hb will not be denatured after it is incorporated with egg-PC or kieselguhr membrane, as is suggested by UV–vis spectra studies. The Soret band

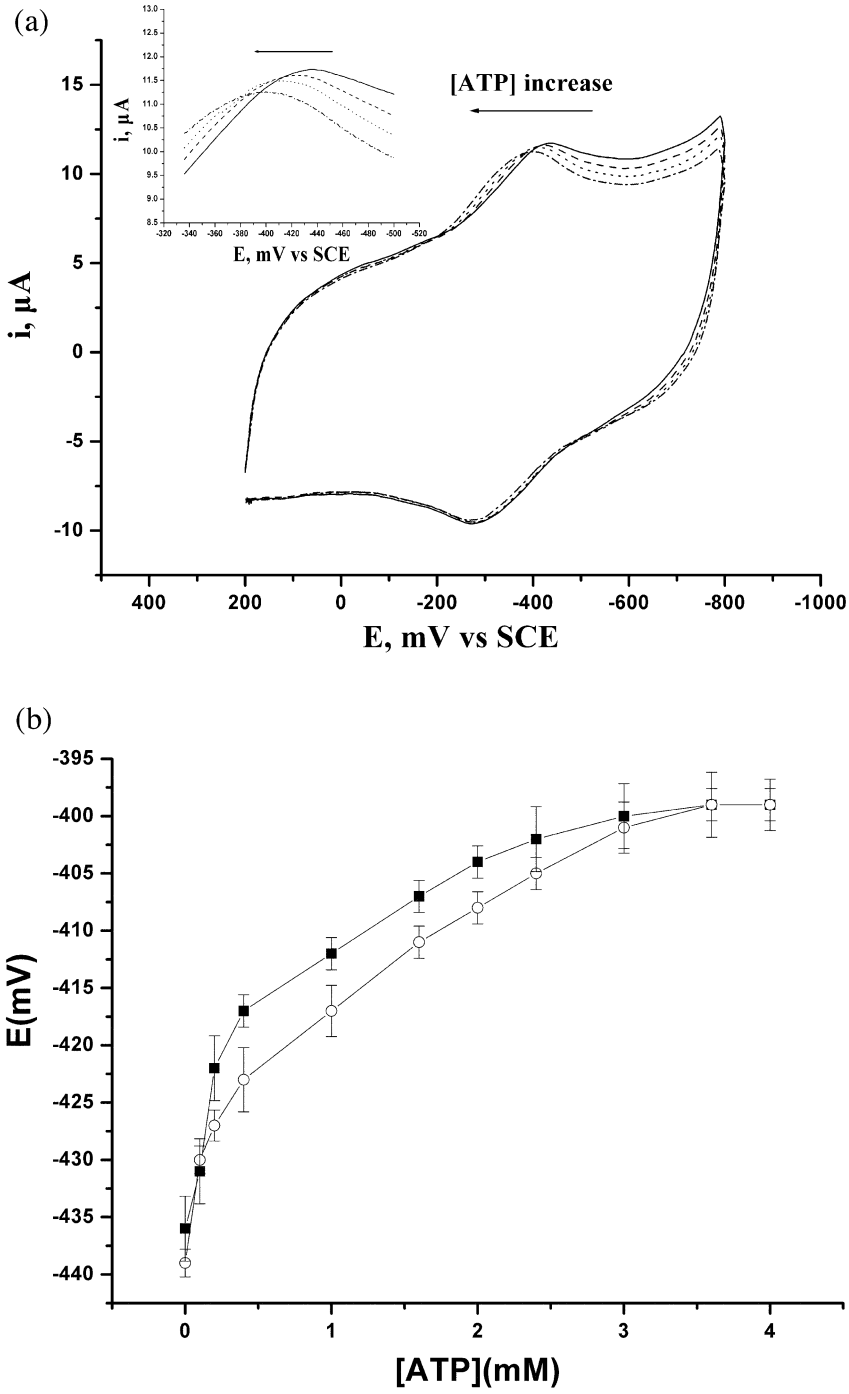

Fig. 1. (a) CV of egg-PC membrane-entrapped Hb for a 0.05 M MOPS solution with pH 7.0 in the absence and presence of 1, 2 and 3 mM ATP (from right to left). Scan rate: 100 mV/s. (b) Voltammetric titration curves of Hb entrapped in kieselguhr (■) and egg-PC (○) film with ATP concentration. Observed formal potentials shown with standard deviation of measurement ($n=3$).

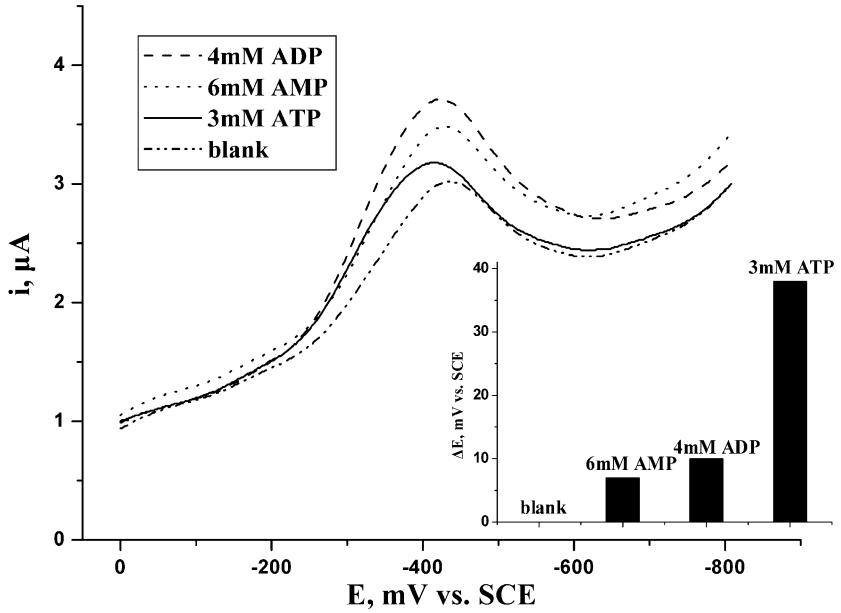

Fig. 2. Fraction of the CV of Hb in the absence and presence of 3 mM ATP, 4 mM ADP and 6 mM AMP. Others are the same as Fig. 1a. The insert graph shows the relationship between ΔE and AMP, ADP and ATP.

of Hb is located at 406 nm, which is sensitive to variation of the microenvironments around the heme site. Previous studies have showed that the band will change or diminish if the protein is partially or fully denatured [23,24]. However, our experimental results reveal that no shift of the Soret band of Hb can be observed after introductions of egg-PC or kieselguhr. This result suggests that no denaturation occurs.

After kieselguhr or egg-PC film has been employed to entrap Hb, the film is further deposited at a PG electrode surface, and nice redox peaks of Hb can be observed. This phenomenon is consisted with the results of our previous work [25,26]. But with the introduction of ATP, a noticeable positive shift of the cathodic peak is observed. And a successive addition of ATP, from absence to presence of 3 mM, will lead to the continuous positive peak shift. Whereas, there is no change of the anodic peak potential. Meanwhile control experiments reveal that the shift of formal potential is not due to the changes of ionic strength or repeated scanning of the modified electrode. Fig. 1a shows a series of typical cyclic voltammograms (CV) of Hb incorporated in egg-PC membrane, with increasing ATP concentration. The CV curves of Hb entrapped in kieselguhr film have also been examined, which are similar to those in egg-PC.

Fig. 1b plots the effect of ATP on the shift of cathodic peak of Hb entrapped in kieselguhr and egg-PC, respectively. The peak of kieselguhr-entrapped Hb shifts from −436 to −399 mV with a concentration range from 0 to 3 mM, while for the Hb entrapped in egg-PC, it will shift from −439 to −399 mV. Such similar results imply that the potential shift has no relation to the film. It is ATP that induces the changes of the peak potential.

The positive shift of the cathodic peak of Hb indicates that the R-like state of metHb has been destabilized and ATP has induced the stabilization of reduced form of protein. Furthermore, since the anodic peak potential keeps unchanged, the peak separation has decreased from 136 to 99 mV, which indicates a melioration of electron transfer ability and a slight change of the globin surrounding at the active heme. However, since there is no trend

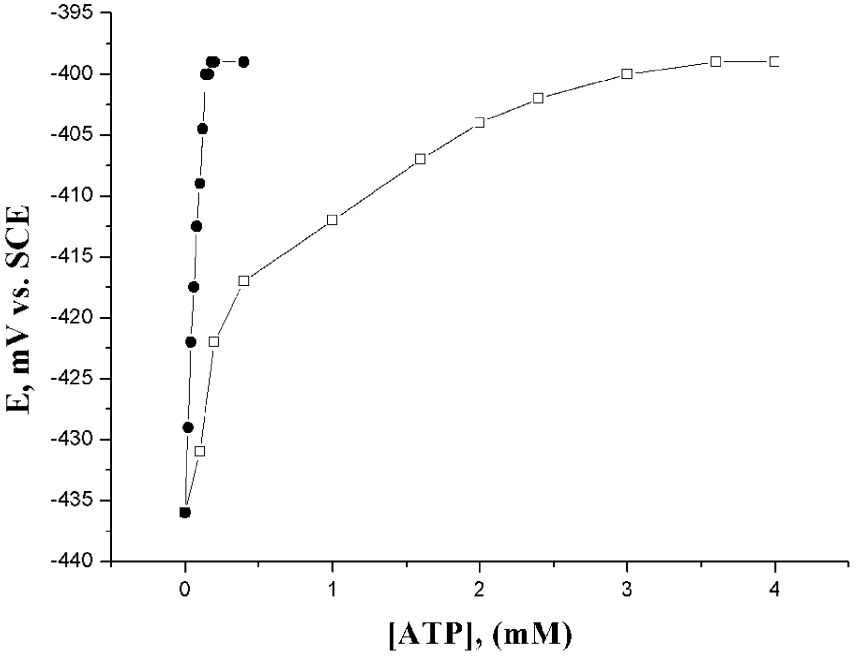

Fig. 3. Voltammetric titration curves of Hb with ATP (□) and DPG (●). Others are the same as Fig. 1b.

of the shift when the ATP concentration is between 3 and 4 mM, a balance stage has appeared if ATP concentration is high to be 3 mM.

Our CV studies show that no shift can be observed in both the cathodic and the anodic peak in the presence of 3 mM adenosine. This result indicates that it is not the adenosine part but the phosphate part of ATP that interacts with Hb.

At pH 7.0, ATP carries four negative charges, ADP carries three negative charges and AMP carries two negative charges. As is shown in Fig. 2, with the introduction of 3 mM ATP, 4 mM ADP or 6 mM AMP, AMP and ADP can only cause a slight shift of the cathodic peak (7 and 10 mV, respectively), but ATP will lead to a 40 mV shift. Therefore, the big influence caused by ATP, but not AMP and ADP, indicates that the ATP effect on Hb is not an unspecific anion effect since the negative charges in the above cases are equal. Specific binding sites of Hb to ATP might exist.

2,3-Diphosphoglycerate (DPG) is a typical allosteric effector to Hb. Our comparative studies reveal that a positive shift of the cathodic peak of Hb will appear as well with titration of DPG. Fig. 3 plots the shift with continuous addition of DPG and ATP, respectively. It is known that the effect of DPG is much stronger than that of ATP. Meanwhile, a 0.16 mM DPG concentration can lead to a balance stage, whereas 3 mM ATP is necessary to be added. The dissimilar concentration of DPG compared to ATP supports a conclusion that ATP effect on Hb is not likely to interact with the central cavity between the two β-polypeptide chains, a dominant binding site to DPG, but on the monomeric polypeptide chain. Thus, the change in globin surrounding at the active heme caused by ATP is not as drastic as DPG.

Continuous addition of ATP after a balance stage will also lead to a shift (from −300 to −286 mV) of the anodic peak potential. For example, if 7 mM ATP is introduced, a noticeably positive shift of wave is observed (the formal potential, $E_{1/2,}$ has changed from −369 to −318 mV). Such phenomenon implicates the strong preferential binding of ATP to the ferrous state over the ferric state [27].

The allosteric effect of phosphate ion has also been examined. Experimental results reveal that phosphate ion can cause a noticeable influence on the redox potential. Introduction of 0.05 M phosphate ion will lead to a positive shift of anodic peak potential from −300 to −278 mV. Phosphate ion is a typical allosteric effector of Hb with unspecific binding sites, whereas MOPS is a zwitterion with absence of allosteric effect on protein [9]. Thus, addition of phosphate ion in the MOPS buffer solution will cause the shift of the redox potential.

As an unspecific binding effector, phosphate can influence the anodic potential distinctly. Therefore, the shift in the anodic peak may be the result of excessive ATP concentration in which special binding has been saturated and unspecific allosteric effect has occurred.

In summary, the titratable potentiometric response of Hb caused by ATP has been obtained. ATP shows significant allosteric effect on Hb with its phosphate part as an effector to stabilize the reduced state of Hb in the physiological concentration range. From 0 to 3.0 mM concentration range, ATP might interact with Hb on specific sites, but when the ATP concentration increases from 4 to 7 mM, unspecific binding will occur. Since ATP exists in erythrocyte, the effect of ATP on the electronic property of Hb is important. This work has given an electrochemical approach to the research on the allosteric effector of Hb.

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