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Electrochemical investigation of the chloride effect on hemoglobin

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Abstract

Direct electron transfer between hemoglobin and gold electrode is achieved at both a bare and a 4, 4′-bipyridine-modified gold electrode in the presence of chloride ions. The addition of chloride to hemoglobin solution also increases the reversibility of the direct electrochemistry and shifts the formal potential of hemoglobin to the negative direction. While the existence of chloride does not significantly change the tertiary structure of the protein, it might induce a slight variation of the structure, which is beneficial to the electrochemical response. It is suggested that the chloride binding to hemoglobin is a combination of specific and unspecific bindings.

Keywords: Direct electrochemistry; Hemoglobin; Chloride; Allosteric effect

1. Introduction

Hemoglobin (MW = 64,500), a tetrameric heme protein containing two α chains and two β chains, is the paradigm of allosteric proteins [1]. The studies on its structure–function relationships have intrigued researchers over the years [2–4]. While hemoglobin does not function physiologically as an electron transfer carrier, it does undergo oxidation and reduction at the heme center in certain cases in vivo. For example, it takes part in the erythrocyte met-hemoglobin reduction pathway, a way to keep low levels of physiologically inactive met-hemoglobin in the circulating blood [5]. Therefore, the research on its electron transfer process might lead to a profound understanding of electron flow in biological systems.

Electrochemical studies of hemoglobin may provide meaningful information into the electron transfer and structural characteristics of the protein. Numerous efforts have been made to improve the electron transfer characteristics by using mediators or promoters [6–15]. Alternatively, Rusling et al. [16–18] have developed a technique for incorporating hemoglobin into surfactant film-modified electrode so as to facilitate the direct electron transfer. Here we report the electrochemical behavior of the protein induced by chloride, one of the allosteric effectors of bovine hemoglobin [19].

It is reported that chloride binding to specific sites in the E helix in β subunits of hemoglobin can lead to conformational changes, thus giving rise to significant changes in the oxygen affinity of the heme center [20]. Furthermore, the structure around the heme crevice is a flexible region, sensitive to slight variations of the environment. Perturbations of the structure in this area might occur prior to the collapse of the whole structure of the protein, thereby inducing certain variations of its biological function [21–24]. Meanwhile, the entry of chloride ions will widen the central cavity and reduce the excess positive charges in the cavity, thus reducing the free energy of the T-structure and the oxygen affinity [25]. In this paper, we report the electron transfer characteristics of hemoglobin caused by chloride ions and the information about the allosteric effect obtained by electrochemical techniques.

2. Experimental

2.1. Reagents

Bovine heart hemoglobin was purchased from Sigma (USA) and used without further purification. 4, 4′-Bipyridine (Bpy) was from Jiangsu Huakang Chemical Agent Plant. All other chemical reagents were of analytical grade. Water was

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purified with a Milli-Q purification system to a specific resistance >16 MΩ cm and was used to prepare all solutions. All the test solutions were deaerated by flush bubbling with high-purity nitrogen.

2.2. Apparatus

Electrochemical experiments were performed with a PARC 283 Potentiostat/Galvanostat (EG&G, USA) employing a three-electrode configuration at 25 ± 0.5 °C. The substrate electrode was a gold disk electrode with a diameter of 0.3 cm. The working electrode was the bare gold electrode, or the Bpy-modified gold electrode. A saturated calomel electrode (SCE) was used as the reference electrode and all potentials reported here were referred to this electrode. A platinum wire electrode served as the counter electrode.

Ultraviolet–visible (UV–Vis) spectra were recorded on a UV-2201 Spectrophotometer (Shimadzu, Japan).

2.3. Electrode preparation

The substrate gold electrode was first polished using sandpaper. It was then polished to a mirror smoothness with an alumina (particle size of about 0.05 μm)/water slurry on silk. Then it was thoroughly washed with double-distilled water. After that, it was ultrasonicated in both water and ethanol for 5 min. Finally, it was cycled in 0.1 M H_2SO_4 (scan range 1.8–0.0 V; scan rate: 200 mV/s) for 2 min. To get the Bpy-modified gold electrode, the substrate gold electrode was dipped in 2.0 mM Bpy solution (0.1 M, pH 7.0 phosphate buffer) for 60 min and thoroughly rinsed with double-distilled water.

2.4. Electrochemical measurements

Both the bare and modified electrode were cycled in a 0.1 M, pH 7.0 phosphate buffer solution and 0.1 M, pH 7.0 phosphate buffer solutions containing hemoglobin or NaCl or both. The potential range of cyclic voltammetry was between 0.3 and −0.2 V (vs. SCE), scanning in the negative direction. The electrode was put in the test solution for 10 min before scanning. The scan rate was 40 mV/s.

2.5. Spectroscopic measurements

Ultraviolet–visible adsorption spectroscopy was recorded in the range of 370–450 nm. Measurements were performed in a 0.1 mg/ml hemoglobin solution (0.1 M, pH 7.0 phosphate buffer) with a series of chloride concentrations.

3. Results and discussion

As is well known and is shown in Fig. 1a, hemoglobin cannot exhibit direct electrochemical response in a pH 7.0 phosphate buffer solution at a bare gold electrode, since the heme of hemoglobin is deeply buried in the central cavity of each subunit [8]. On the other hand, the surface of a freshly polished gold electrode is positively charged. These excess positive charges will tend to prevent the electropositive heme cavity of hemoglobin from getting close to the electrode through coulombic force, thus the heme crevice of hemoglobin will lie distal to the electrode surface [26]. This orientation, of course, does not favor the electron transfer between hemoglobin and the electrode.

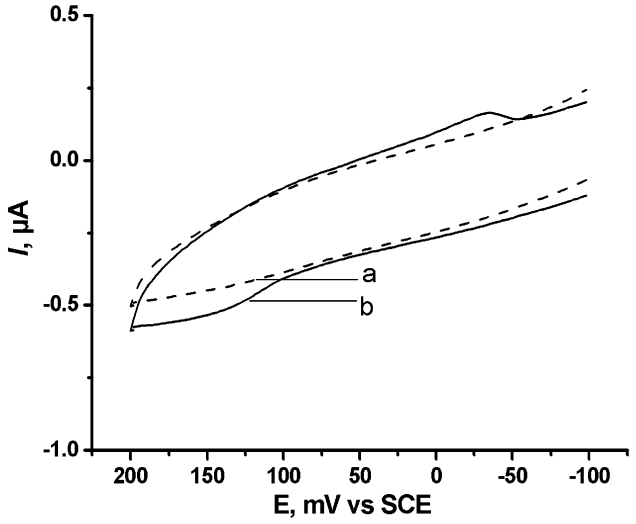

Fig. 1. Cyclic voltammograms obtained at a bare gold electrode for a 2.0 mg/ml hemoglobin solution (0.1 M, pH 7.0 NaH_2PO_4–Na_2HPO_4 buffer): (a) chloride-free, (b) in the presence of 0.05 M NaCl. Scan rate: 40 mV/s.

However, if chloride is added into the buffer, direct electrochemical response can be observed in the cyclic voltammogram (Fig. 1b). Comparatively, no peak can be observed if there is no hemoglobin in the buffer solution although chloride has been added. So, the direct electron transfer between the gold electrode and hemoglobin is facilitated by chloride. Further experimental results reveal that the lowest chloride concentration at which the direct electron transfer can be observed in our tests is 0.05 M when the hemoglobin concentration is 2.0 mg/ml. This chloride concentration is comparable to the concentrations reported in literature in which chloride works as the allosteric effector of hemoglobin [20,25,27].

Ultraviolet–visible spectra have been employed to explore whether hemoglobin is denatured in the presence of chloride. It is well known that the heme Soret absorbance bands around 408 nm can provide information about the structural changes of the protein [28]. Experimental results reveal that the bands around 408 nm for 0.1 mg/ml hemoglobin only change 3 nm even if the chloride concentration is high at 4.0 M. It implies that the heme and the heme environment of hemoglobin will maintain their native forms in the presence of environmental chloride.

Chloride has been known as an effective allosteric effector of bovine hemoglobin, so a low chloride concentration can cause significant changes in its tertiary and heme pocket

conformation. Although hemoglobin does not denature as is illustrated by the UV–Vis study, some conformational changes may have occurred, thus the protein can exhibit direct electrochemical response even at a bare electrode.

The direct electron transfer between hemoglobin and the bare gold electrode can be achieved in the presence of chloride, however, the quick aggregation and adsorptive denaturation of the protein at a metallic surface will inhibit further electron transfer processes. As a result, the peak current of hemoglobin at the bare gold electrode decreases significantly within the subsequent scans. Bpy-modified gold electrodes are therefore employed to investigate the chloride-induced process in more detail.

Bpy is a desirable promoter for cytochrome *c* [29]. But it does not work as a promoter or mediator for hemoglobin, since hemoglobin cannot display electrochemical response at a Bpy-modified electrode if no chloride is added into the protein solution. Nevertheless, Bpy is still an ideal material for this research, because the function of Bpy in this system is just to prevent the protein from directly contacting the substrate gold surface, and thereby to prevent protein aggregation and denaturation, so that stable electrochemical response can be achieved. Meanwhile, UV–visible spectra studies reveal that Bpy will not perturb the structure of the protein since the Soret band will not change after the addition of Bpy in a hemoglobin solution.

The cyclic voltammograms of a Bpy-modified gold electrode in a 2 mg/ml hemoglobin solution (0.1 M, pH 7.0 phosphate buffer) containing different NaCl concentrations are shown in Fig. 2. It can be observed that better waves can be obtained. When the NaCl concentration is 4.0 M, the anodic and cathodic peaks are located at 32 and −64 mV (vs. SCE), respectively. The formal potential (E°) is at −16 mV (vs. SCE). The peak separation is 96 mV, indicative of a quasi-reversible one-electron heterogeneous electron transfer process. Plots of the anodic and the cathodic peak currents

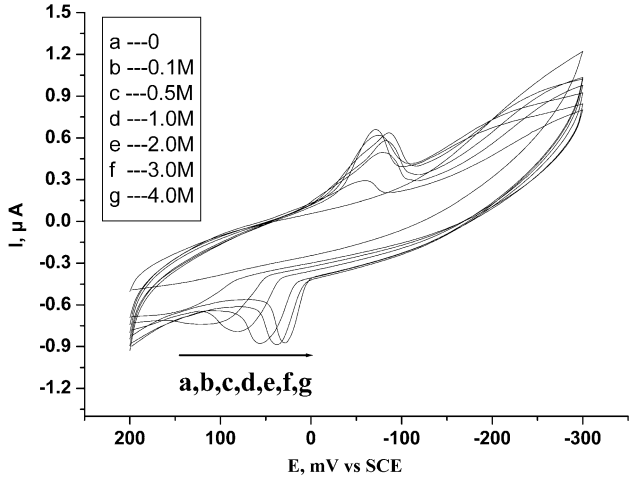

Fig. 2. Cyclic voltammograms obtained at a Bpy-modified gold electrode for a 2.0 mg/ml hemoglobin solution (0.1 M, pH 7.0 NaH_2PO_4–Na_2HPO_4 buffer) containing different NaCl concentrations. Scan rate: 40 mV/s.

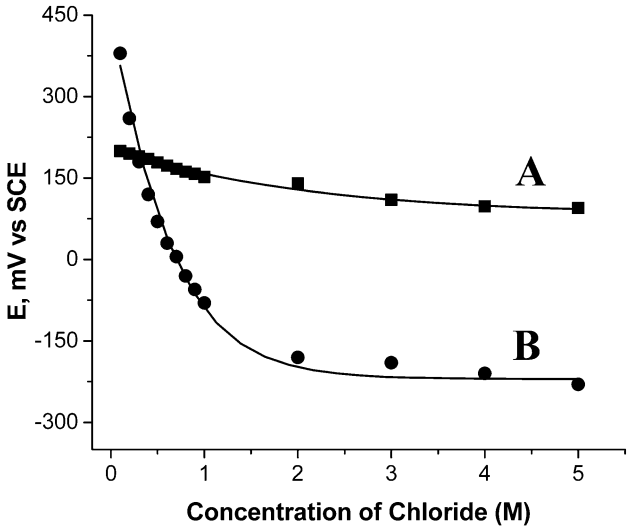

Fig. 3. Relationship between (A) the peak separation, (B) the formal potential and the chloride concentration. Scan rate: 40 mV/s. Other values same as in Fig. 2.

for hemoglobin vs. the scan rates are linear in the range of 20–150 mV S^{-1}, which is characteristic of a thin layer electrochemical behavior [30], i.e., almost all the electroactive met-hemoglobin (oxidized form of hemoglobin) on the electrode surface can be converted to ferrous hemoglobin (reduced form of hemoglobin) on the forward cyclic voltammetric scan and vice versa.

Titration of hemoglobin with chloride ions has been performed. For a 2.0 mg/ml hemoglobin solution, no peak can be observed when the chloride concentration is lower than 0.05 M. When the chloride concentration is 0.05 M or higher, a pair of redox peaks can be observed. The anodic and cathodic peaks are largely separated if the chloride concentration is low, showing a slow heterogeneous electron

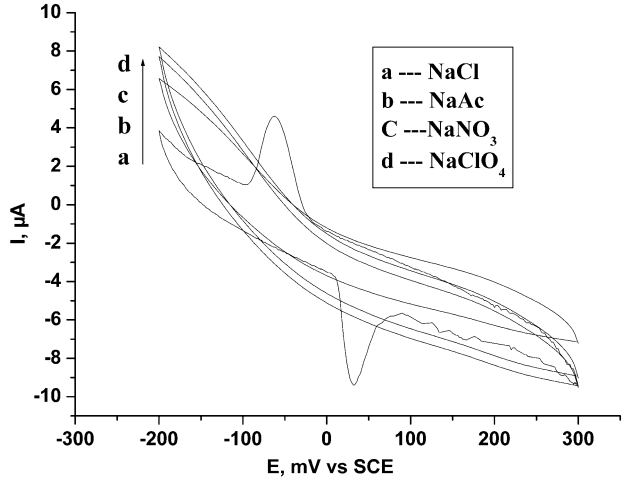

Fig. 4. Cyclic voltammograms for a 2.0 mg/ml hemoglobin solution (0.1 M, pH 7.0 NaH_2PO_4–Na_2HPO_4 buffer) containing 4.0M; (a) NaCl, (b) NaAc, (c) $NaNO_3$, (d) $NaClO_4$, obtained at a Bpy-modified gold electrode. Scan rate: 40 mV/s.

transfer rate. With the increase of chloride concentration, both peaks shift to the negative direction. However, the anodic peak shifts significantly while the cathodic peak just shifts slightly, thus the peak separation decreases, which implies the amelioration of the electron transfer ability. Fig. 3A displays the relationship between the peak separation and the chloride concentration. Fig. 3B is a plot of the formal potential to the chloride concentration. It can be known that the formal potential shifts to the negative direction with the increase of chloride concentration, and reaches a maximum at as high a concentration as 4.0 M.

The shift of the formal potential is not due to the changes of ionic strength. As is shown in Fig. 4, although the concentrations of NaAc, $NaNO_3$ and $NaClO_4$ are as high as 4.0 M, there is no apparent peak observed.

It is noteworthy in Fig. 3 that the peak separation and the formal potential will be largely changed in the lower concentration region of chloride compared with the changes in the higher concentration region. This suggests that slight variations of the chloride concentration in the lower concentration region might modify the electron transfer ability of the protein to a relatively higher degree. So, when the chloride concentration is very low, chloride may mainly bind to the high-affinity and specific chloride binding sites in hemoglobin [19–21,25,31]. When more and more chloride ions are added, they may mainly bind unspecifically in low-affinity or just increase the ion intensity of the solution, which may, to a greater extent, widen the central cavity and stabilize the tertiary structure, and therefore increase the reversibility of the direct electron transfer between the hemoglobin and the electrode.

In summary, direct electron transfer between hemoglobin and an electrode can be achieved with the help of chloride ions. While the protein is not denatured by the species, the reversibility of the electrochemistry has been greatly enhanced. This work has also provided a new approach to study the allosteric effectors of the proteins.

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