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An electrochemical study of hemoglobin in water–glycerol solutions

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Abstract

The effect the composition of a water–glycerol mixture has on the electrochemical properties of hemoglobin (Hb) is studied. With the increased glycerol concentrations, the peak-to-peak separation of hemoglobin is found to increase from ~ 40 to 200 mV, with the apparent standard potential of hemoglobin negatively shifted, which demonstrate that the electron-transfer activity of hemoglobin will decrease at relatively high glycerol concentrations and the oxidized state of hemoglobin will be more stable with the increasing glycerol concentrations. Meanwhile, the electrocatalytic activity of hemoglobin to hydrogen peroxide, as well as the binding of ligands or effectors to hemoglobin in the presence of glycerol, are also been investigated. Our studies indicate that glycerol will decrease the electrocatalytic activity of hemoglobin, while have little effect on the microenvironment around the heme site.

Keywords: Glycerol; Hemoglobin; Cyclic voltammetry; Hydrogen peroxide; Imidazole; 5′ATP

1. Introduction

Interests in studying proteins in organic solvents increase greatly since it was shown that enzymes were capable of functioning in non-aqueous media [1]. A fundamental question is an elucidation of the effect of the nature of organic solvents on the structure and the functional properties of proteins. Until now, only isolated papers have been devoted to studying the electrochemical properties of redox proteins in organic solvent. Bogdanovskaya et al. [2] have examined the bioelectrocatalytic and enzymatic activity of laccase in water–ethanol solutions. Some groups have investigated the effect of dimethyl sulfoxide (DMSO) on the structure and the functional properties of heme-proteins, such as hemoglobin (Hb), myoglobin (Mb), cytochrome b_5 (cyt b_5) and horseradish peroxidase (HRP) [3–6]. The literature data indicate that there is no consensus on the mechanism of the effects of organic solvents, though it is proved that there are some parameters induced by organic solvents considered to have effects, including the hydration, hydrophobicity and dielectric constant of the solvents.

Alcohols are kinds of organic solvents extensively used in biochemical process. The effect of alcohols on the structural and functional properties of proteins has been widely investigated in literatures [7]. In particular, glycerol is known to act as a strong stabilizing agent on protein structure by the preferential hydration of water-soluble proteins in aqueous–glycerol mixed solvents. Some researches demonstrate that glycerol will order the solvent and make unfolding of a protein more unfavorable than in aqueous solution alone [8]. Therefore, glycerol is commonly employed to minimize the denaturation of proteins during extraction, purification, and analysis.

Recently, many researchers have been devoted to investigate the influence of glycerol on the structure and stability of redox proteins. Grealis and Magner [9] have made a comparison of the redox properties of cytochrome *c* (cyt *c*) in aqueous and glycerol media. Other researches have revealed the effect of glycerol on the structure and thermodynamic stability of Mb [10]. Hb is a tetramer heme protein with functions as an oxygen vehicle, as well as some

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intrinsic peroxidase activities [11]. Researches have been taken to observe the effects on its electrochemical properties by organic solvents such as DMSO [12]. As far as we know, no studies of the influence of glycerol on the redox properties of Hb have ever been reported.

The aim of the present paper is to investigate the effect of glycerol on the structure and the functional properties of Hb. We study the direct electron-transfer and electrocatalytic activities of Hb in glycerol/water mixture (0–90 vol.%). UV/Vis spectra are employed to observe the heme microenvironment of Hb. Furthermore, the interaction of ligand (imidazole) or effector (5′ATP) with Hb in the presence of glycerol is also been studied. The former can coordinate with the heme ring of the protein, while the latter interacts with the globin part of Hb, as indicated in the literature [13,14].

2. Experimental

2.1. Reagent and apparatus

Bovine heart hemoglobin (Hb) and Polyethyleneimine (PEI) were purchased from Sigma and the protein was used without further purification. 5′ATP (Adenosine 5′ Triphosphate) and MOPS (3-[*N*-morpholino]propanesul fonic acid) were obtained from AMRESO and Phosphatidylglycerol Choline (PC) from Chemical plant of Huadong Normal University in Shanghai. Imidazole was from Huadong Normal University Chemical Plant (Shanghai) and analytical grade glycerol was from Shanghai Chemical. Other reagents were of analytical grade. Water was purified with a Milli-Q purification system to a specific resistance >16 MΩ cm^{-1} and was used to prepare all solutions. All stock solutions were stored at a temperature of 4 °C.

Cyclic voltammetry (CV) was performed with a PAR 263 Potentiostat/Galvanostat (EG&G, USA). A three-electrode configuration was employed. The cell was a 5-ml single electrolyte compartment. The working electrode was a modified pyrolytic graphite (PG) electrode. A saturated calomel electrode (SCE) was used as the reference electrode and all potentials reported here were referred to this electrode. A platinum electrode served as the counter electrode. Prior to the performance, the test solution was bubbled thoroughly with high purity nitrogen. Then a stream of nitrogen was blown gently across the surface of the protein solution in order to maintain the solution anaerobic throughout the experiment.

UV/Vis absorbance spectroscopy was done with a UV-1601 spectrophotometer (Shimadzu, Japan). The pH value was detected by PHS-3D pH meter.

2.2. Electrode preparation and modification

A PG block ($A = 5.35$ mm^2) was inserted in a glass tube and fixed with epoxy resin. A copper rod was used to make electrical contact. Prior to coating, the electrode was polished with rough and fine aluminum oxide paper. It was then polished to mirror smoothness with an alumina (particle size of about 0.05 μm)/water slurry on silk. Finally, the electrode was thoroughly washed and treated in an ultrasonic bath for about 5 min.

An aqueous mixture of equal volume 8.0 mg ml^{-1} Hb and 1.0 mmol l^{-1} PC was spread evenly onto the surface of the substrate PG disk electrode. Similarly, a mixed solution of 1% PEI and 8.0 mg ml^{-1} Hb was prepared. The film was then dried overnight at room temperature. Finally, the modified electrode was thoroughly rinsed with pure water and was ready for use. The buffer solutions were confected by adding glycerol into 0.05 mol l^{-1} MOPS buffer, and 0.1 mol l^{-1} NaAc was introduced into every buffer solution to maintain the ionic strength constant. The pH values of buffer solutions were detected by pH meter and the pH values were kept consistent by adding a little amount of HAc.

3. Results and discussion

3.1. Electron-transfer activity of hemoglobin in water–glycerol solutions

Fig. 1 shows the CV of Hb entrapped within a PC membrane at a PG electrode, running in a glycerol/water mixture (0–90 vol.%) of pH 7.2, at a scan rate 200 mV s^{-1}. A well-defined electrochemistry is observed in the glycerol/water mixture ranging from 0 to 60 vol.%, during which the cathodic and the anodic peaks are symmetric and the i_a/i_c is about unity, with the peak-to-peak separation ~ 60 mV (inset A in Fig. 1). The almost constant peak-to-peak separation demonstrates that the presence of glycerol at this concentration does not substantially alter the redox properties of the embedded protein.

By contrast, when the concentration of glycerol is over 60 vol.%, a differently shaped CV is achieved, characterized by a much larger peak-to-peak separation, indicating the sharply decreased heterogeneous electron-transfer rate of Hb. These data reveal that glycerol might inhibit the electron-transfer activity of Hb at relatively high concentrations. The inhibiting effect of solvents is possibly due to the saturation of glycerol. In that case, there are no enough free water molecules to assist the electron-transfer process.

The apparent standard potential of Hb ($E^{\circ\prime}$), estimated from its midpoint potential ($E_{1/2}$), is −337 mV (vs. SCE), which is in good agreement with the literature value [15]. With the introduction of glycerol, a noticeable negative shift of $E^{\circ\prime}$ is observed. As displayed in Fig. 1, the redox peak pair continuously shifts with the increasing glycerol concentration (0–90 vol.%), which produces ~ 29-mV shifts. The dependence of $E^{\circ\prime}$ of Hb on the glycerol concentration is illustrated in inset B in Fig. 1. The negative shifts in the redox potential imply that the R-like state of metHb has been stabilized and the ferric state of proteins is more stable in glycerol/water mixture.

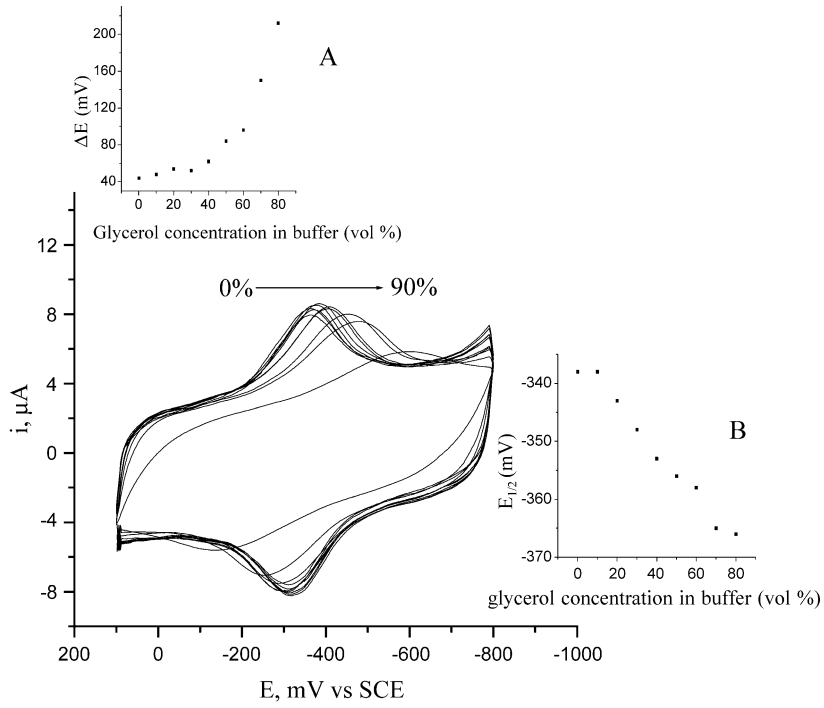

Fig. 1. Cyclic voltammograms obtained at a PC-Hb modified electrode in MOPS-glycerol buffer solution of pH 7.2 at scan rate: 200 mV s^{-1}. Inset: (A) dependence of peak separations of Hb in buffer on the increasing glycerol concentration (vol.%); (B) dependence of the apparent standard potential of Hb in buffer on different glycerol concentrations (vol.%).

Similar results have also been obtained in PEI film. It is well known that PC is a kind of biological lipid membranes, which is almost neutral at pH 7.2 while PEI is a kind of polyelectrolytes, which is positively charged at the same condition. Since the phenomena of Hb displayed in these two films with very distinct characteristics are nearly the same, it can be inferred that the CV results have no relation to the immobilization techniques. It is glycerol that induces the changes of electron-transfer activity and redox potential of Hb.

3.2. Absorbance measurements

Optical measurements are carried out to determine the effect of glycerol concentration on the structure of Hb. The Soret band of Hb is located at 406 nm, which is sensitive to variation of the microenvironments around the heme site. Previous studies have shown that the band will change or diminish if the protein is partially or fully denatured [16,17]. In the 0–90 vol.% glycerol solution range, the heme microenvironments are retained, as the unchanged λ_{max} of the Soret band indicates (Fig. 2).

3.3. Electrocatalytic activity of hemoglobin in water–glycerol solutions

Hb has been known to have some intrinsic peroxidase activities and some kinds of biosensors for hydrogen peroxide (H_2O_2) have been fabricated based on the direct electron transfer of Hb. In the present paper, the electrocatalytic activity of Hb for H_2O_2 was studied in glycerol/water mixture, which may provide additional implications for the function of enzyme-based biosensors in organic solvents. Fig. 3 is the electrocatalytic curves of Hb in different concentrations of glycerol with the constant amount of substrate (90 μmol l^{-1} H_2O_2). The electrocatalytic detection limits are listed in Table 1. The results indicate that the electrocatalytic activity of Hb decreases considerably upon the addition of glycerol, which is probably due to the exclusion of water molecules from the heme

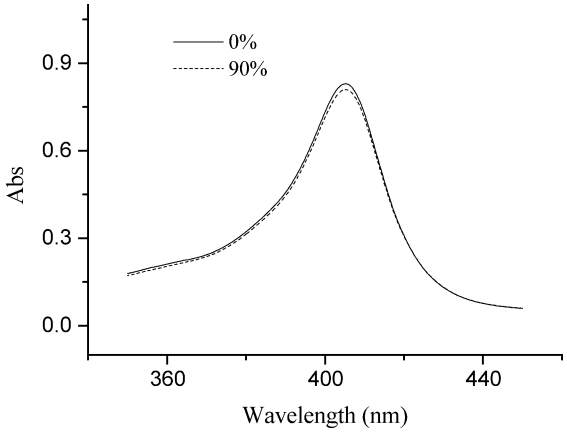

Fig. 2. Absorption spectra of 0.26 mg ml^{-1} Hb in solutions of 0% (solid line) and 90% (dash line) glycerol concentration (vol.%).

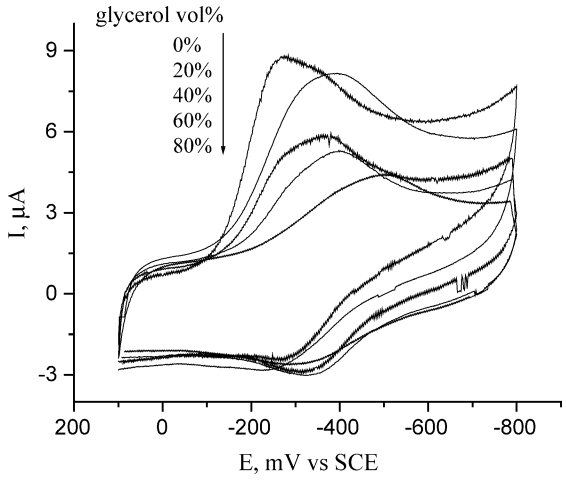

Fig. 3. Cyclic voltammograms of Hb in PC membrane catalyzing 90 μmol l^{-1} H_2O_2 in buffer with 0%, 20%, 40%, 60% and 80% glycerol concentration (vol.%).

environment or the reduced conformational flexibility of the protein in glycerol, as demonstrated in laccase [2].

3.4. Interaction of hemoglobin with other molecules in water–glycerol solutions

Imidazole is a kind of ligands coordinating with heme ring of Hb, and making the redox peaks of Hb shift negatively [12]. The shift of $E^{\circ\prime}$ (ΔE) can therefore be an index to study the liganding activity of Hb upon imidazole, as demonstrated in Mb and microperoxidase-11 [18,19]. Fig. 4A displays the shifting degree under different concentrations of glycerol with the constant amount of imidazole (40 mmol l^{-1}). It is clearly demonstrated that glycerol has no influence on the liganding activity of Hb upon imidazole, which is consistent with the above spectral results that glycerol has no influence on the heme microenvironments of Hb.

5′ATP is a physiological modulator of Hb. It is known that if the concentration of 5′ATP around Hb is high enough, such as in our experiment, it will interact with Hb non-specially as an anionic effector. When 0.015 mol l^{-1} 5′ATP is introduced in buffer solution without the presence of glycerol, $E^{\circ\prime}$ of Hb shifts positively from -343 to -334 mV. Interestingly, when the concentration of glycerol

Table 1
CV detection limits of H_2O_2 by Hb in buffer solutions with different glycerol concentrations

Glycerol concentration (vol.%)	Detection limit of H_2O_2 (μmol l^{-1})	Glycerol concentration (vol.%)	Detection limit of H_2O_2 (μmol l^{-1})
0	3.3	50	26.0
10	8.3	60	30.0
20	13.0	70	43.0
30	17.0	80	90.0
40	22.0	90	130.0

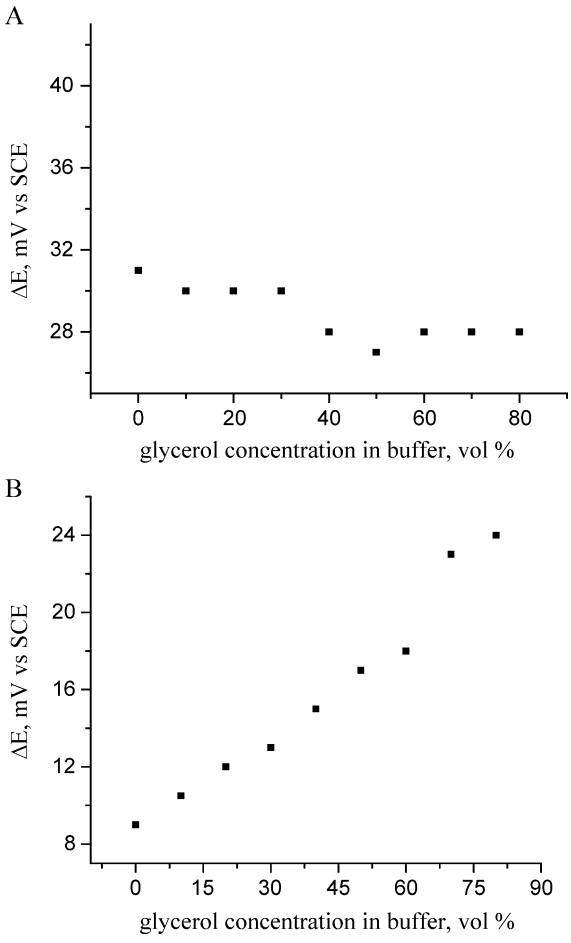

Fig. 4. (A) Dependence of the potential shifts of Hb on different glycerol concentrations (vol.%) in the presence of 0.04 mol l^{-1} imidazole. (B) Dependence of the potential shifts of Hb on different glycerol concentrations in the presence of 0.015 mol l^{-1} 5′ATP (vol.%).

increases in our system, ΔE increases accordingly (Fig. 4B). It has been traditionally explained that by interacting with the charged amino acid group in globin, anionic effectors will break down the salt bridge among peptides [20] and induce the positive shift of apparent standard potential of the protein [14]. Therefore, the increase of ΔE upon glycerol indicates that the high extent of hydration in glycerol solution does not inhibit the change of superior structure of Hb caused by 5′ATP. Instead, glycerol makes the interaction convenient.

4. Conclusions

Our research has investigated the influence of glycerol on the electrochemical properties of Hb. The data in UV–vis spectra and direct electrochemical response demonstrate that glycerol will change the electron-transfer activity of Hb without affecting the heme microenvironment. Accordingly, the electrocatalytic activity of Hb declines. The interaction between the imidazole and Hb indicates that glycerol will

not affect the ligands coordinating with heme ring of Hb, which again proves that glycerol has no influence on the heme microenvironment of proteins. Further experiment demonstrates that glycerol may promote the interaction between Hb and some kinds of anionic effectors, such as 5′ATP.

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