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Biochimica et Biophysica Acta 1623 (2003) 29–32
BIOCHIMICA ET BIOPHYSICA ACTA
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An electrochemical investigation of ligand-binding abilities of film-entrapped myoglobin

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Received 16 April 2003; received in revised form 18 July 2003; accepted 21 July 2003

Abstract

Film-entrapped myoglobin exhibits well-defined electrochemistry which, upon ligand binding, displays a titratable redox potential shift. This effect has been observed to be highly dependent on the charged state of involved films. We have demonstrated that this approach may act as a model system for studies of molecular recognition between proteins and ligands.

Keywords: Protein film voltammetry; Myoglobin; Imidazole

1. Introduction

Studies on the interactions between proteins and ligands in biological systems provide valuable physiological information [1] and lead to rational design of biodevices and biosensors [2]. The protein–ligand interaction has been monitored by a variety of techniques [3–5], including a recently proposed, highly attractive approach, protein film voltammetry (PFV) [6–8]. In the protocol, a surface-confined protein/enzyme produces electronic signals that change upon specific ligand/substrate binding. This method takes advantage in that (1) proteins/enzymes are immobilized at solid electrode surface that significantly reduces the use of valuable samples; (2) the resolution of signal changes is fairly straightforward, which eliminates possible interferences found in homogeneous solutions; (3) it provides a platform where not only proteins can be immobilized in biomimetic membranes, but the environment around proteins can be varied [6–11]. In the present work, we have employed this PFV technique to monitor the binding process of film-entrapped myoglobin (Mb) and its ligand imidazole (Im).

Mb is a well-known oxygen carrier in skeleton muscles. Mb contains a heme group where the iron center is ligated in the porphyrins ring and by a histidine residue in the peptide sequence, leaving a free coordination site. Previous studies have demonstrated that this free coordination site is readily available for binding a variety of ligands such as imidazole and cyanide [12]. This imidazole-binding to heme has been implicated to occur in a variety of biological systems, particularly metallo-enzymatic reactions, and is thus well studied in the literature [13–15]. Here we report the electrochemical investigation of this Mb–Im interaction. Since it is well known that many proteins/enzymes exert their physiological functions only in their membrane-bound state [16], we have also studied the roles of the employed films by evaluating their effects on the ligand-binding events.

2. Materials and methods

Electrochemical experiments were performed with a PARC 263A Potentiostat/Galvanostat (EG&G, USA), using a three-electrode configuration. 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 specified. The working electrode was a homemade pyrolytic graphite (PG) electrode ($A = 4.7$ mm^2). Water was purified with a Milli-Q purification system to a specific resistance >16 M cm^{-1} and used to prepare all solutions.

The modified PG electrode was prepared as follows: it was first polished using rough and fine sand papers. Then it was polished to ‘‘mirror smoothness’’ with an alumina (particle size of 0.05 μm)/water slurry on silk. Finally, the electrode was thoroughly washed with water and then

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doi:10.1016/S0304-4165(03)00156-9

sonified (KQ218 ULTRASONIC CLEANING) in water for about 5 min. The electrode was thus ready for use.

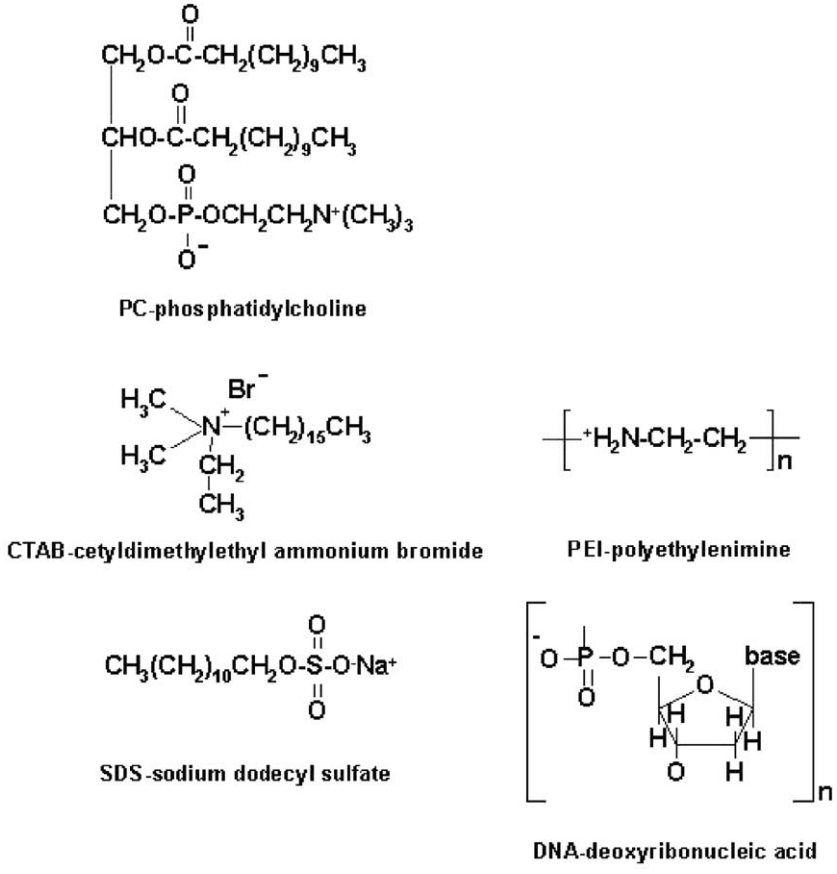
Polyelectrolytes, such as phosphatidylcholine (PC), polyethylenimine (PEI), cetyldimethylethyl ammonium bromide (CTAB), deoxyribonucleic acid (DNA), and sodium dodecyl sulfate (SDS), were dissolved in water to give a 2% solution. They were mixed with 4 mg/ml Mb aqueous solutions at 1:1 ratio. Twenty microliters of such a mixed solution was spread evenly onto the surface of a PG disk electrode with a microsyringe. Films were dried overnight at room temperature. These electrodes were thoroughly rinsed with water and then ready for use. The electrochemical experiments were performed under anaerobic conditions. A 0.1 M phosphate buffer solution with pH 7.0 was used as the electrolyte.

3. Results and discussion

A series of film materials, such as PC, PEI, CTAB, DNA, and SDS (structures are shown in Scheme 1), are employed to entrap Mb by co-deposition at PG electrode surfaces. The electrochemical experiments were performed under anaerobic conditions. In all cases, well-defined redox peaks of Mb can be observed, demonstrating the effective electron-transfer promoting abilities of these films. As an example, Fig. 1 displays a typical cyclic voltammogram (CV) of Mb redox reactions in the presence of PC, a zwitterionic lipid membrane. Mb entrapped in other films demonstrates similar CV curves but with slightly different peak potentials, suggesting interactions between the protein and films. The protein structure might be slightly perturbed upon film formations. However, previous studies have demonstrated that this effect is relatively insignificant [17,18] and more importantly, the partial structural variation possibly mimics the structural reorganization of proteins in their membrane-bound states in vivo. The redox potentials of Mb in different films are listed in Table 1. It's worthwhile to point out that Mb is very stable, over a few weeks, in all films. This extraordinary stability of film-entrapped Mb enables us to investigate the interaction between Mb and its ligands, in the presence of films.

Upon the introduction of imidazole, a noticeable negative shift of the redox potential of Mb was observed. As displayed in Fig. 1, the redox peak pair continuously shifts with the increasing imidazole concentration, up to ca. 0.2 M imidazole, which produces ~50 mV shifts. The dependence of the redox potential of Mb on the imidazole concentration is illustrated in Fig. 2.

Control experiments have shown that the shift in redox potential is not due to the change in either ionic strength or repetitive scan of the Mb-modified electrode (data not shown). So, it is inferred that the shift is induced by the



Scheme 1. Chemical structures of co-deposition biomimetic film materials.

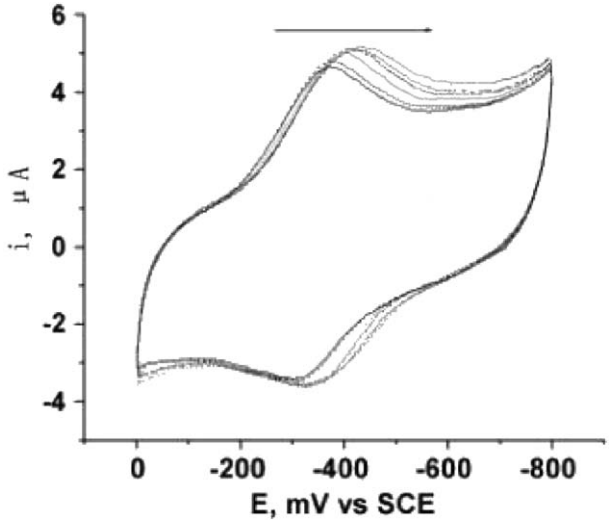


Fig. 1. Cyclic voltammetry of Mb entrapped in PC, in the absence and presence of 0.01, 0.03, 0.05, 0.1 and 0.2 M imidazole (from left to right). 0.1 M Na_2HPO_4–NaH_2PO_4 buffer solution with pH 7.0. Scan rate: 200 mV/s.

interactions between the heme-protein and its ligand. This negative shift in the redox potential reflects the strong preferential binding of imidazole to the ferric state over the ferrous state [15].

The relationship between the dissociation constant of Mb–Im and the redox potential is given by the following equation:

$$E_{1/2}(L) = E_{1/2} + RT/2nF \ln D_M^{n+}/D_{ML}^{n+} - RT/nF \ln K - pRT/nF \ln[L] \quad (1)$$

where $E_{1/2}(L)$ and $E_{1/2}$ stand for the redox potentials of Mb before and after binding ligand, D_M^{n+} and D_{ML}^{n+} represent the diffusion coefficients in the absence and presence of ligand and we assume they have the same values, K represents the apparent dissociation constant, p is the coordination number ($p=1$) and $[L]$ is the concentration of ligand (bulk concentration used). All other symbols have their normal meanings. The PFV approach has provided well-resolved redox potentials, we thus propose that the $E_{1/2}$ related K value may be a useful index for the influence of films.

The films employed in this work fall into three categories: PC is neutral; PEI and CTAB are positively charged;

Table 1
Redox potentials and apparent dissociation constants for Mb–Im in different films ($p=1$, $n=1$)

Deposits	$E^{\circ\prime}$ (mV) vs. SCE (without Im)	$E^{\circ\prime}$ (mV) vs. SCE (with 0.2 M Im)	$\Delta E^{\circ\prime}$ (mV)	K^{app} (M^{-1})
Mb-PC	-342 ± 3	-389 ± 2	47 ± 5	31.1 ± 6.0
Mb-PEI	-328 ± 2	-338 ± 2	10 ± 4	7.4 ± 1.1
Mb-CTAB	-363 ± 2	-374 ± 2	11 ± 4	7.7 ± 1.2
Mb-DNA	-360 ± 2	-401 ± 4	41 ± 6	24.6 ± 5.7
Mb-SDS	-370 ± 2	-409 ± 5	39 ± 7	22.8 ± 6.2

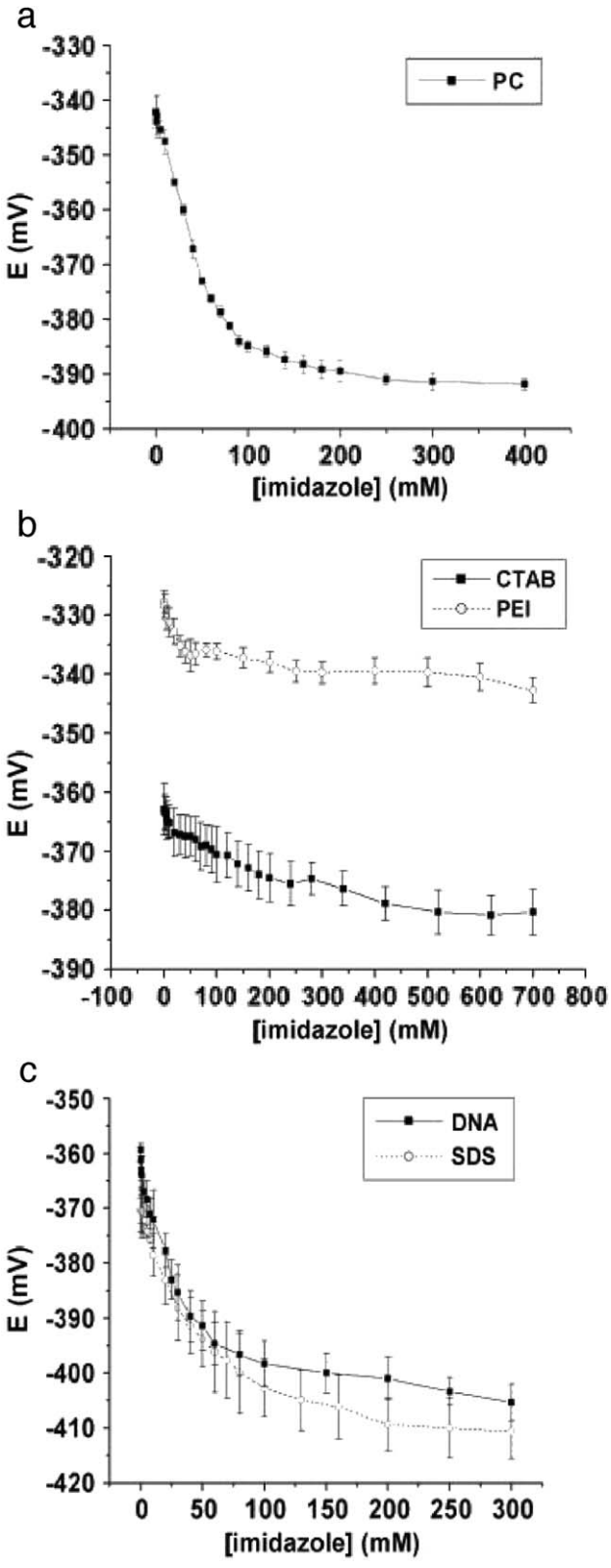


Fig. 2. Titration curves for Mb–Im binding in the presence of (a) neutral PC; (b) cationic PEI and CTAB; (c) anionic DNA and SDS. Error bars are obtained from three independent measurements.

DNA and SDS are negatively charged. In general, all of them induce negative shifts of redox peaks of Mb. However, clearly the behavior of potential shifts varies with different films. The apparent dissociation constants (K^{app}) for Mb–Im in different films are calculated from Eq. (1) and listed in Table 1. Obviously, the presence of polycationic PEI and CTAB significantly decreases the apparent dissociation constant for Mb–Im. We use zwitterionic PC membranes as a reference for further comparison. The K^{app} obtained in either DNA or SDS is slightly smaller than in PC, however, the K^{app} sharply decreases in PEI and CTAB.

Possible explanations for these phenomena are as follows: imidazole has a *p*K of 6.04 which makes it slightly negatively charged at pH 7.0. Therefore, imidazole may be excluded by negatively charged films. This explains the slight decrease of K^{app} in anionic polyelectrolytes (DNA and SDS) compared to neutral PC. In the case of cationic polyelectrolytes, imidazole is attracted by films which facilitate the diffusion of imidazole from solutions to the interior of films. Therefore, there should have been an increase of K^{app} in cationic polyelectrolytes (PEI and CTAB) compared to neutral films. However, according to our observations, the K^{app} sharply decreases in cationic polyelectrolytes. The reasons may be as follows: first, the electrostatic interaction also screens the interaction between Mb and imidazole since many of the imidazole molecules are in contact and thus shielded by films. Second, although the Coulombic interactions between imidazole and polyelectrolytes contribute to the change of K^{app}, it still remains unclear that cationic polyelectrolytes exert significantly higher effect on imidazole-binding of Mb. Moreover, since imidazole is only slightly charged, the electrostatic interaction is not expected to be very strong. Previous studies have demonstrated that the environmental electric field could lead to the changes of energy status of proteins and subsequently the forces, in particular the electrostatic interaction, to maintain the tertiary structure of proteins [19,20]. We thus suggest that cationic films have a larger effect on the microenvironment of the binding site of Mb, which affects the affinity for ligands.

In summary, the binding abilities of film-entrapped Mb with imidazole have been examined with PFV. To our knowledge, it is the first time to evaluate the effect of films on the ligand binding abilities of proteins. This work sheds light on elucidation of physiological roles of membranes. It also provides a convenient and generalizable approach to study protein–ligand interactions.

Acknowledgements

We greatly appreciate the support of the National Natural Science Foundation, and the Ministry of Education, P. R. China for this research.

References

[1] T. Ji, M. Grossmann, I.H. Ji, G protein-coupled receptors: I. Diversity of receptor–ligand interactions, J. Biol. Chem. 273 (1998) 17299–17302.
[2] T.G. Spiro, A.A. Jarzecki, Heme-based sensors: theoretical modeling of heme–ligand–protein interactions, Curr. Opin. Chem. Biol. 5 (2001) 715–723.
[3] J.I. Al-Mustafa, FTIR investigation of the conformational properties of the cyanide bound horse metmyoglobin, Vib. Spectrosc. 31 (2003) 89–99.
[4] M.Y. Hong, H.C. Yoon, H.S. Kim, Protein–ligand interactions at poly (amidoamine) dendrimer monolayers on gold, Langmuir 19 (2003) 416–421.
[5] J.K. Davies, R.M. Hagan, D.C. Wilton, Effect of charge reversal mutations on the ligand- and membrane-binding properties of liver fatty acid-binding protein, J. Biol. Chem. 277 (2002) 48395–48402.
[6] F.A. Armstrong, H.A. Heering, J. Hirst, Reactions of complex metalloproteins studied by protein-film voltammetry, Chem. Soc. Rev. 26 (1997) 169–179.
[7] K. Chen, J. Hirst, R. Camba, C.A. Bonagura, C.D. Stout, B.K. Burgess, F.A. Armstrong, Atomically defined mechanism for proton transfer to a buried redox centre in a protein, Nature 405 (2000) 814–817.
[8] C. Fan, H. Wang, S. Sun, D. Zhu, G. Wagner, G. Li, Electron-transfer reactivity and enzymatic activity of hemoglobin in a SP sephadex membrane, Anal. Chem. 73 (2001) 2850–2854.
[9] C. Fan, G. Li, J. Zhu, D. Zhu, A reagentless nitric oxide biosensor based on hemoglobin-DNA films, Anal. Chim. Acta 423 (2000) 95–100.
[10] C. Fan, X. Chen, G. Li, J. Zhu, D. Zhu, H. Scheer, Direct electrochemical characterization of the interaction between haemoglobin and nitric oxide, Phys. Chem. Chem. Phys. 2 (2000) 4409–4413.
[11] H.M. Goldston, A.N. Scribner, S.A. Trammell, L.M. Tender, A model recognition switch. Electrochemical control and transduction of imidazole binding by electrode-immobilized microperoxidase-11, Chem. Commun. 5 (2002) 416–417.
[12] E. Antonini, M. Brunori, Hemoglobin and Myoglobin in their Reaction with Ligands, North-Holland Publishing Company, Amsterdam, 1971.
[13] J. Lin, L.B. Vitello, J.E. Erman, Imidazole binding to horse metmyoglobin: dependence upon pH and ionic strength, Arch. Biochem. Biophys. 352 (1998) 214–228.
[14] S. Asperger, B. CetinaCizmek, Metalloporphyrins. The nature of ligand bonding and the mechanism of replacements, Croat. Chem. Acta 69 (1996) 1305–1328.
[15] D.J. Cohen, B.C. King, F.M. Hawkridge, Spectroelectrochemical and electrochemical determination of ligand binding and electron transfer properties of myoglobin, cyanomyoglobin, and imidazolemyoglobin, J. Electroanal. Chem. 447 (1998) 53–62.
[16] A. Kotyk, K. Janacek, J. Koryta, Biophysical Chemistry of Membrane Function, Wiley, Chichester, UK, 1988.
[17] J.F. Rusling, Enzyme bioelectrochemistry in cast biomembrane-like films, Acc. Chem. Res. 31 (1998) 363–369.
[18] C. Fan, G. Li, D. Zhu, Curr. Top. Anal. Chem. 3 (2002) 233–251.
[19] J.M. Vanderkooi, The protein state of matter, Biochim. Biophys. Acta 1386 (1998) 241–253.
[20] M. Kohler, J. Friedrich, J. Fidy, Proteins in electric fields and pressure fields: basic aspects, Biochim. Biophys. Acta 1386 (1998) 255–288.