Electrochemical investigation of the effect of some organic phosphates on haemoglobin

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The effects of DPG, IHP, GTP, GDP and GMP on the structure and stability of haemoglobin were electrochemically investigated with an iodide-modified silver electrode in 0.01 M KNO_3 at pH 7.0. Anodic and cathodic peaks of haemoglobin were observed at 250 mV and 12 mV with a formal potential value of 133 mV *vs.* Ag/AgCl. The effects of different concentrations of DPG, IHP, GTP, GDP and GMP on the anaerobic redox reaction were determined. The results showed that DPG and IHP can lead to a positive shift in the reduction peak of haemoglobin, indicating that the oxidation peak shift of haemoglobin was small as a result of stabilization of the reduced state and destabilization of the R-like state of haemoglobin. GTP elicited a more positive shift in the cathodic and anodic peaks of haemoglobin at a higher concentration, signifying that it has a low-affinity binding site on haemoglobin. The positive shift of the cathodic and anodic peaks revealed a slight variation in the structure and indicated the unfolding of haemoglobin in the presence of high concentrations of GTP. Our study also showed that GDP and GMP did not cause significant shift the cathodic and anodic peaks of haemoglobin even at high concentrations, refuting the existence of specific GDP- and GMP-binding sites on the protein. Moreover, the iodide-modified silver electrode method proved to be easy and useful in investigating the effects of ligands or other effectors on haemoglobin in solution.

[Rezaei-Zarchi S, Saboury A A, Ghourchian H, Hong J, Barzegar A, Norouzi P, Moosavi-Movahedi A A, Ganjali M R and Javed A 2007 Electrochemical investigation of the effect of some organic phosphates on haemoglobin; *J. Biosci*. **32** 271–278]

1. Introduction

Haemoglobin is a hetero-tetramer, consisting of two alpha subunits and two beta subunits (a "dimer of dimers" [β(2): α(2)]). Each polypeptide chain contains a prosthetic haem group that cooperatively binds and releases oxygen (Baldwin and Chothia 1979; Imai 1982; Ajloo *et al* 2002; Pomponi *et al* 2004). Haemoglobin undergoes major tertiary and quaternary conformational changes as it equilibrates between a low-affinity deoxy T-state and a high-affinity oxy R-state (Tsuneshige *et al* 2002). Organic phosphates and some heterotrophic effectors, such as protons, anions and carbon dioxide, although bound spatially at remote sites, could affect the oxygenation process and have been shown to do so (Faulkner *et al* 1994; Poyart *et al* 1994; Marta *et al* 1998; Pomponi *et al* 2004).

Electrochemical studies of haemoproteins may provide structural information on electron transfer and the structural

Keywords. Bioelectrochemistry; organic phosphate; haemoglobin

Abbreviations used: ATP, adenosine-5′-triphosphate; CV, cyclic voltammogram; DPG, 2,3-diphosphoglycerate; GTP, guanosine 3',5'-tetraphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; HbA, human adult haemoglobin; IHP, inositol hexaphosphate; SDS, sodium dodecyl sulphate; SERRS, Surface Enhanced Resonant Raman Scattering

characteristics of proteins (Peng *et al* 2003). Electrochemical investigations of the structural changes of haemoproteins have greatly improved recently.

Numerous efforts have been made to improve electron transfer characteristics by using mediators, promoters and direct electron transfer for haemoproteins (Eddowes and Hill 1977; Sun *et al* 1997; Ye and Baldwin 1988; Dong *et al* 1989; Chen *et al* 1994; Lu *et al* 1994; Li *et al* 1999; Kuramitz *et al* 1999; Fan *et al* 2001). There are also reports of elucidation of structural information by electrochemical methods on haemoproteins such as cytochrome c, myoglobin and haemoglobin; these methods investigate the folding and unfolding of proteins, and the effects of ligand-binding and inorganic solvents on the structure of proteins (Funk *et al* 1990; Sarma *et al* 1997; Sun *et al* 1997; Li and Mabrouk 2003; Peng *et al* 2003). Many studies on the structure of haemoglobin by electrochemical methods (Funk *et al* 1990; Faulkner *et al* 1994; Peng *et al* 2003; Zhang *et al* 2004; Sun *et al* 2004; Scheller *et al* 2005) have reported that electrochemical methods can be used to determine the structural changes that take place in the folding process of cytochrome c (Moosavi-Movahedi *et al* 2003), and electrochemical methods were applied to investigate the structural properties of haemoglobin in the presence of ligands, adenosine-5′-triphosphate (ATP) and other materials (Peng *et al* 2003). Sun *et al* (2004) investigated the allosteric effect of chloride on haemoglobin by electrochemical methods, which showed that chloride binding to haemoglobin was a combination of specific and non-specific bindings. Dayer *et al* (2002) investigated the structural changes in haemoglobin during the folding and unfolding processes in the presence of different concentrations of sodium dodecyl sulphate (SDS), which showed that electrochemical methods give the same results as fluorescence and turbidity methods.

The globin structure of haemoglobin plays a key role in the redox potential of the haem site of haemoglobin and protects it from rapid oxidation, allowing for reversible oxygen binding. Therefore, the redox potential of haemoglobin is sensitive to the globin alterations surrounding the active haem moiety (Pomponi *et al* 2004; Laberge *et al* 2005). The redox potential of the active site is an important parameter that describes the propensity of the site to donate or accept electrons, and allows us to gain an insight into how effectors, materials and drugs can induce alterations in the equilibration time between the oxidized and reduced forms of the protein, which allows for reversible oxygen binding. Peng *et al* (2003) used haemoglobin immobilized on the surface of a pyrolytic graphite electrode to investigate the electrochemical effect of ATP on haemoglobin. They reported that the changes in cathodic potential of haemoglobin in the presence of those ligands were indicative of the structural changes in the haemoglobin molecule. Zhang *et al* (2004) also studied the structural changes of haemoglobin in the presence of different concentrations of glycerol by using haemoglobin immobilized on the surface of a pyrolytic graphite electrode. Fan *et al* (2000) reported that an iodide-modified silver electrode could show cathodic and anodic peaks for haemoglobin in solution. Cotton's research on Surface Enhanced Resonant Raman Scattering (SERRS) showed that a complex would be formed on the surface of silver after it was treated with iodide (Sibbald *et al* 1996; Sibbald *et al* 1997). Further study revealed that this complex is different from the molecular AgI, though it could be converted to AgI by a suitable electron acceptor in the presence of non-bound iodide in the solution (Sibbald *et al* 1996). Acceleration of the electron transfer process of haemoglobin is therefore not due to the effect of iodide, neither adsorbed nor in solution, but rather due to the complex formed on the electrode. Electrochemical studies support this interpretation, because there was no iodide ion at the surface of this chemically modified electrode, since no peaks corresponding to iodide around 0.295 V could be observed at the modified electrode (Fan *et al* 2000). In this paper, we introduce the electrochemical properties of haemoglobin binding with ligands in solution by using an iodide-modified silver electrode.

Earlier reports have shown that 2,3-diphosphoglycerate (DPG) bound with haemoglobin in a 1:1 stoichiometry at the entrance of the cavity between the two β-subunits contributed positively charged residues to complement the negatively charged DPG phosphate groups, namely Val1, His2 and Lys82 of both β chains (Laberge *et al* 2005). Moreover, inositol hexaphosphate (IHP) and DPG could bind to haemoglobin at the same site (Laberge *et al* 2005). The allosteric function of these materials led to their binding with haemoglobin, inducing the transformation of haemoglobin from the R-like state to the T-like state, resulting in a decrease in the oxygen affinity of haemoglobin (Pomponi *et al* 2004; Laberge *et al* 2005). It is well known that guanosine 3',5'-tetraphosphate (GTP), guanosine diphosphate (GDP) and guanosine monophosphate (GMP) are very important factors in the differentiation of erythroid cells and stimulate them to synthesize haemoglobin and other proteins (Osti *et al* 1997). They have a primary role in the activation and deactivation of some proteins in the cell, such as kinases and G-proteins (Rubin *et al* 1997). Thus, it is very important to investigate the effects of these materials on structural changes in haemoglobin and its affinity to oxygen.

Tamburrini *et al* (2001) reported that the oxygen affinity of haemoglobin was dramatically reduced in the presence of GTP. However, two issues remain unanswered; how GTP affects haemoglobin, and the effects of GDP and GMP on the structure of haemoglobin. We report for the first time the results of an investigation into the effects on and structural

changes in haemoglobin upon binding with the ligands DPG, IHP, GTP, GDP and GMP in solution, using an iodide-modified silver electrode.

2. Materials and methods

Human adult haemoglobin (HbA), KNO_3, DPG, IHP, GTP, GDP and GMP were purchased from Sigma (UK). Doubly distilled water was used in all the experiments. Stock solutions were stored at 4°C. Electrochemical measurements were carried out with a potentiostat/galvanostat (Model 263A, EG&G, USA) using a single-compartment voltammetric cell equipped with a platinum rod auxiliary electrode, an Ag/AgCl reference electrode (Metrohm) and an iodide-modified silver working electrode with a disk diameter of 1 mm (Azar Electrode Co., Iran), prepared by the method given by Fan *et al* (2000) and Sibbald *et al* (1997). First, the silver electrode was mechanically polished twice with alumina (particle sizes 10 and 0.06 μm) to a mirror finish. Then, the adsorbed alumina particles were further removed by etching the electrode in a 10% HNO_3 solution for 1 min. Finally, the electrode was thoroughly washed with doubly distilled water and then treated in an ultrasonic bath for about 3 min. After this pretreatment, the substrate silver electrode was immersed in a 0.1 mol/l KI solution for 5 min and thoroughly rinsed with doubly distilled water to remove any physioadsorbed material. The iodide-modified silver electrode was then ready for use. All solutions were de-aerated by bubbling with high purity nitrogen for at least 30 min before the experiments.

3. Results

Figure 1 shows the typical cyclic voltammogram (CV) of haemoglobin in 0.01 M KNO_3 solution at pH 7.0, obtained using an iodide-modified silver electrode. The anodic and cathodic peaks were observed at 250 mV and 12 mV *vs.* Ag/AgCl, respectively, from which the formal potential could be calculated as (Ec+Ea)/2= 133 mV *vs.* Ag/AgCl. No redox behaviour was observed for any of the materials used in the ligand studies under the same conditions (data not shown). These results are consistent with those of Fan *et al* (2000). They reported that haemoglobin displayed very good redox properties using an iodide-modified silver electrode.

Figure 2 shows the CV curves of the iodide-modified silver electrode obtained in a haemoglobin solution in the absence and presence of DPG. From this figure, we can see that the cathodic peak potential has shifted in the positive direction. In other words, DPG binds with haemoglobin and leads to conformational changes in haemoglobin. The cathodic peak of haemoglobin shifted by 36 mV in concentrations ranging from 0 to 30 μM DPG. The anodic peak shifted by only 9 mV and then reached equilibration. The formal potential (E°') *vs.* Ag/AgCl in the haemoglobin solution increased with an increase in the concentration of DPG, as seen in the inset of figure 2.

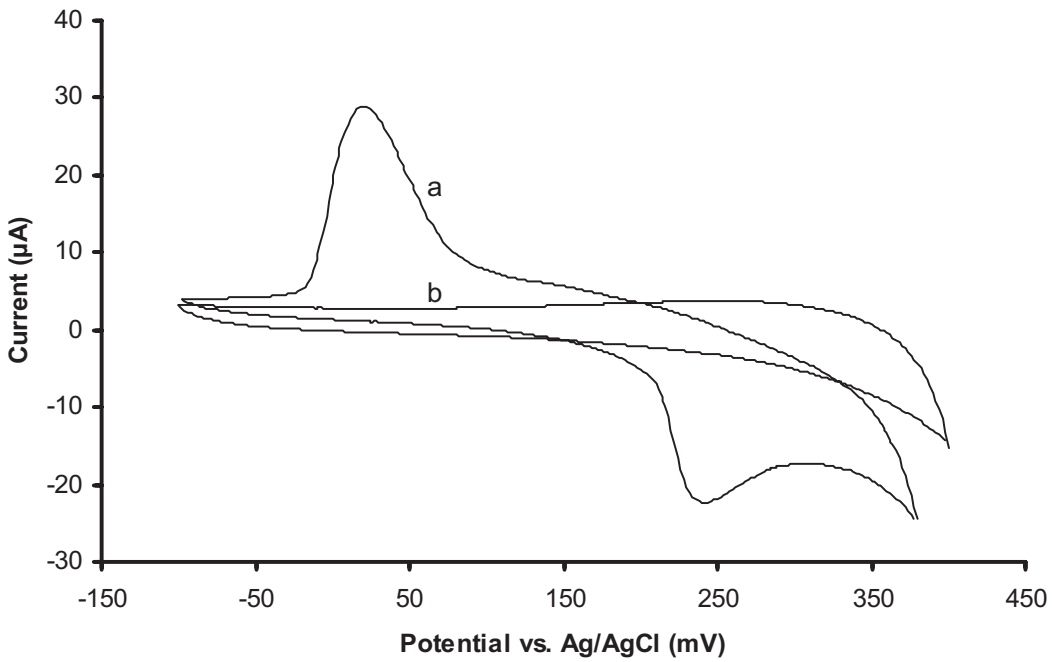

Figure 1. A typical cyclic voltammogram of the iodide-modified Ag electrode in KNO_3 in the presence (a) and absence (b) of haemoglobin.

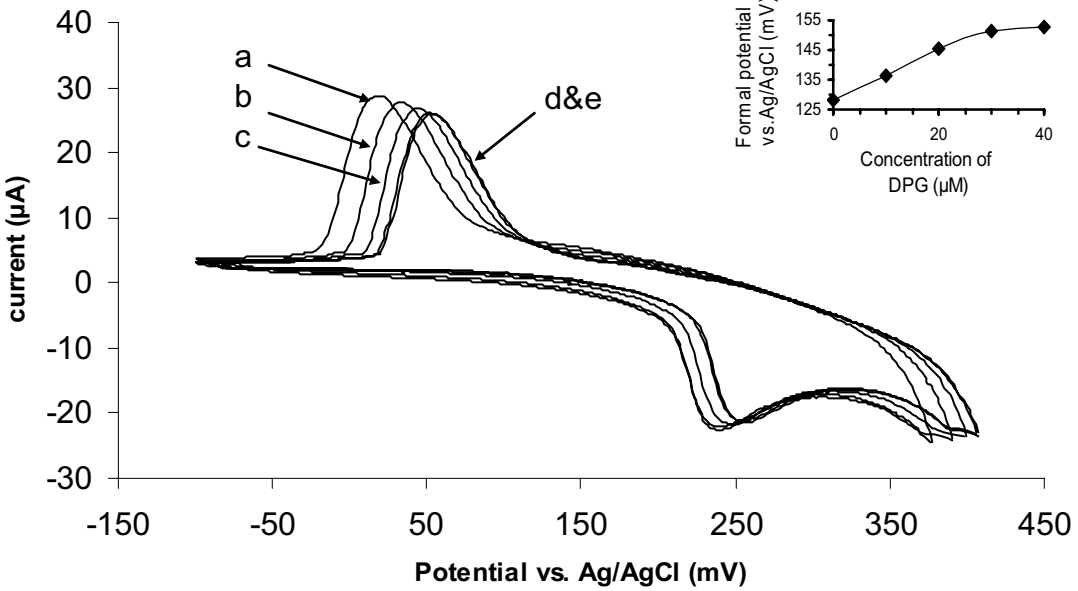

Figure 2. Cyclic voltammograms of haemoglobin in the presence of (a) 0, (b) 10, (c) 20, (d) 30 and (e) 40 μM DPG. Inset shows the plot of [DPG] *vs.* E°′.

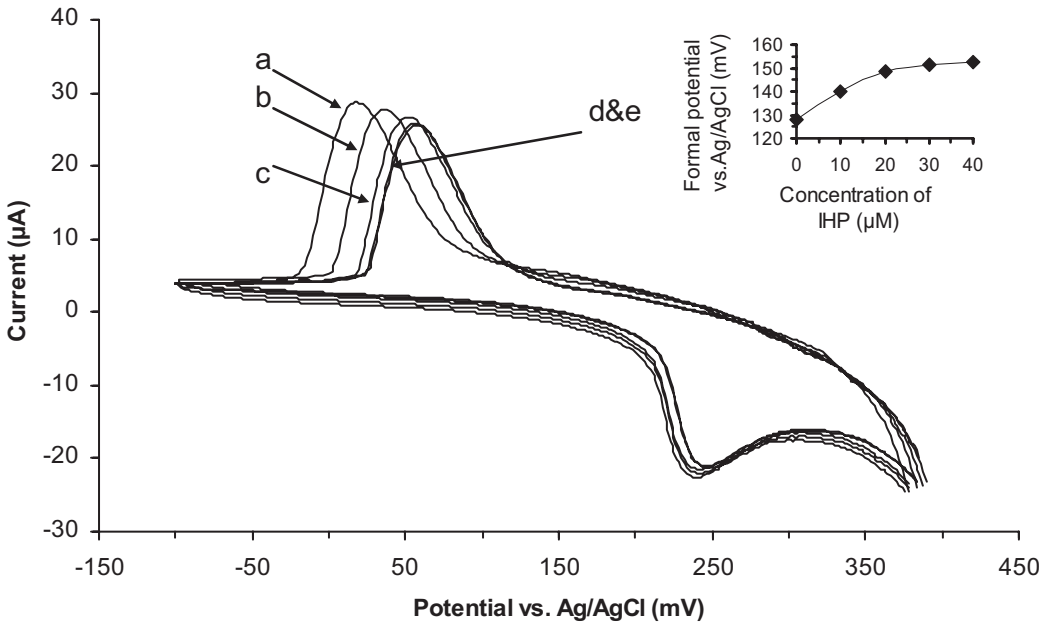

Figure 3. Cyclic voltammograms of haemoglobin in the presence of (a) 0, (b) 10, (c) 20, (d) 30 and (e) 40 μM IHP. Inset shows the plot of [IHP] *vs.* E°′.

Figure 3 shows the CV effects of IHP on haemoglobin using the iodide-modified silver electrode. The inset shows the relationship between the formal potential and the concentration of IHP. The electrochemical effects of IHP on haemoglobin are very similar to those of DPG: the cathodic peak of haemoglobin shifts by 32 mV and the anodic peak by 8 mV in the positive direction with concentrations of IHP ranging from 0 to 30 μM.

Figure 4 shows the CV effects of GTP on haemoglobin using the iodide-modified silver electrode. The inset shows the relationship between E°′ and [GTP]. Though the same potential shift of haemoglobin is seen for GTP as that for

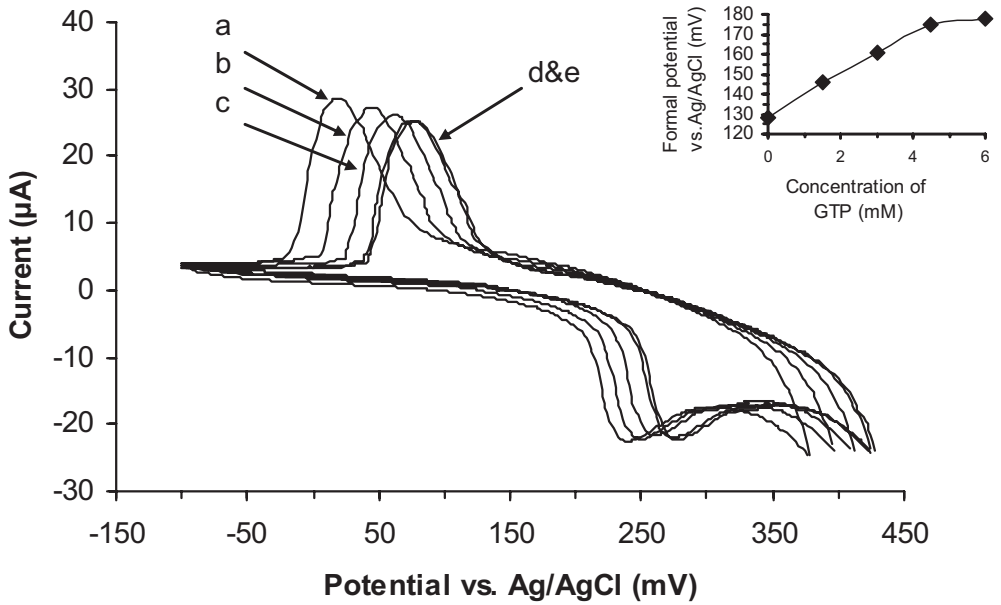

Figure 4. Cyclic voltammograms of haemoglobin in the presence of (a) 0, (b) 1.5, (c) 3.0, (d) 4.5 and (e) 6.0 mM GTP. Inset shows the plot of [GTP] *vs.* E°′.

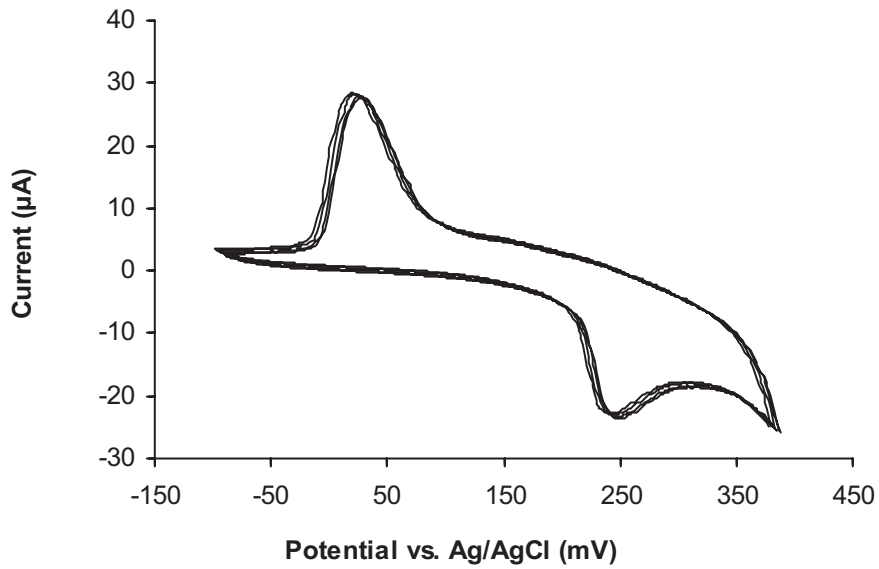

Figure 5. Cyclic voltammograms of haemoglobin in the presence of different concentrations of GDP (*see text*).

DPG and IHP, this potential shift only occurred at high GTP concentrations. The cathodic peak of haemoglobin shifts by 61 mV and the anodic peak by 40 mV in the positive direction with GTP concentrations ranging from 0 to 4.5 mM. Here, we see almost no CV changes in haemoglobin above 4.5 mM GTP. CV studies also show no shift in both the cathodic and anodic peaks in the presence of 4 mM guanosine (data not shown), which indicates that only the phosphate part of GTP can interact with haemoglobin.

The CV effects of GDP (figure 5) and GMP (figure 6) on haemoglobin were also studied. Our results show that there was almost no difference in the CV properties of haemoglobin, even in the presence of 6 mM GDP or GMP.

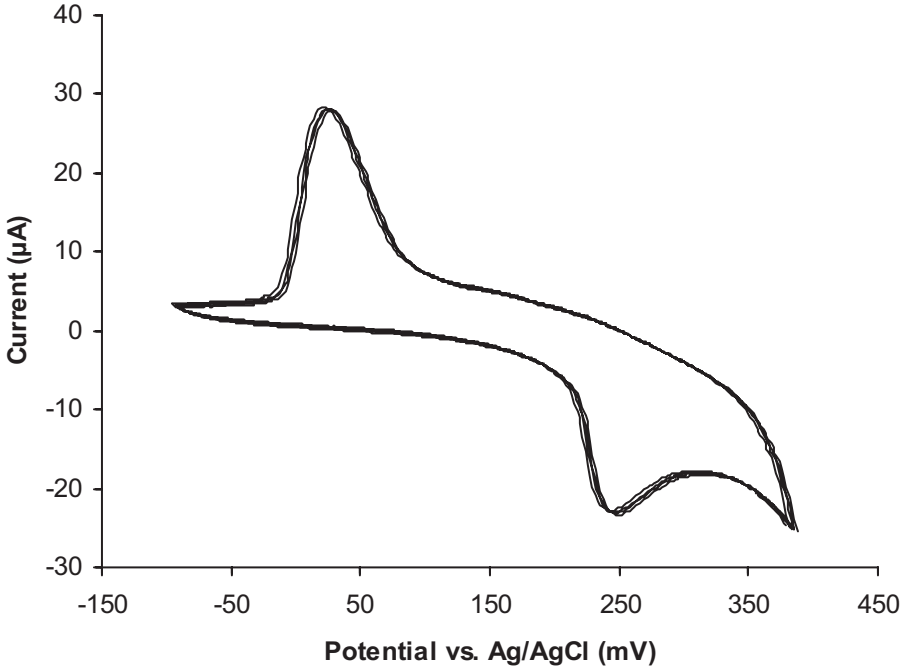

Figure 6. Cyclic voltammograms of haemoglobin in the presence of different concentrations of GMP (*see text*).

4. Discussion

The results of the effect of DPG on haemoglobin are consistent with the works of Peng *et al* (2003), who used haemoglobin immobilized on the surface of the electrode. Moreover, DPG may assist in the conformational changes in structure of haemoglobin from the relaxed (R) state to the tense (T) state, causing a positive potential shift at the cathode, which is indicative of conditions that are conducive to reduction as in the case of ATP (Peng *et al* 2003). In this case, using DPG, the anodic peak has a smaller shift than the cathodic peak. This means that DPG can stabilize the reduced state and destabilize the R-like state of haemoglobin. Furthermore, the cathodic peak and anodic peak shifts of 36 mV and 9 mV, respectively, resulted in a decrease in peak separation of 27 mV, which indicates amelioration of the electron transfer ability and a slight structural change in globin surrounding the active haem.

The results of the electrochemical effects of IHP on haemoglobin show that IHP binds with haemoglobin and facilitates reduction. It also indicates conformational changes in haemoglobin from the R state to the T state in the presence of IHP, as explained by other workers (Laberge *et al* 2005). The positive shift of the cathodic peak potential suggests that the conditions are conducive to reduction, and the decreases in peak separation indicate an improvement in the electron transfer ability and a slight change in the globin surrounding the active haem. These results indicate that IHP may also bind with haemoglobin at the same site as DPG. The electrochemical results show that there is only one high-affinity binding site on haemoglobin for DPG or IHP. This binding site has been reported earlier by simulation and other methods (Laberge *et al* 2005).

We see that GTP affects the CVs of haemoglobin only at high concentrations compared with DPG and IHP, and that GTP has a very low affinity for haemoglobin. However, GTP induces a significant positive shift of the cathodic and anodic peaks, indicating that it facilitates the redox behaviour of haemoglobin. GTP can influence the structure of haemoglobin, causing unfolding, thereby changing the position of the haem of haemoglobin from the interior to the surface of haemoglobin, resulting in a change in its tertiary structure.

Our results on GDP and GMP showed no changes in CVs in the presence and absence of haemoglobin, and no ligand binding between haemoglobin and GDP or GMP was observed. One molecule of GTP has four negative charges at pH 7.0, while GDP has three and GMP two. Therefore, only a small positive shift was observed for the cathodic peak (9 and 7 mV) in the presence of 6 mM GDP and GMP, respectively. Under the same conditions, 4.5 mM GTP will lead to a positive shift of 61 mV. Thus, the effect of GTP on haemoglobin is not a non-specific anion effect, and a specific site(s) for GTP to bind to haemoglobin may exist.

Overall, the effects on haemoglobin of DPG and IHP are much greater than those of GTP. DPG or IHP (30 μM)

or GTP (4.5 mM) could lead to an equilibrium stage for haemoglobin. Similar concentrations of DPG and IHP were used simultaneously (insets of figures 2 and 3); these two materials appear to have the same binding site(s) on haemoglobin in the central cavity between the two *ß*-polypeptide chains, known to be a dominant binding site for DPG or IHP (Pomponi *et al* 2004; Laberge *et al* 2005). The different concentrations of DPG and GTP required to reach the equilibrium E°' indicate that GTP does not bind to haemoglobin at the same site in the central cavity between the two *ß*-polypeptide chains; it seems to act on the non-numeric polypeptide chain. Thus, changes in the globins surrounding the active haem in the presence of GTP are not as drastic as those caused by DPG. Peng *et al* (2003) observed that 3 mM ATP shifts only the cathodic peak, without an anodic peak shift, and a concentration of up to 7 mM shifts only the anodic peak

At low concentrations, ATP stabilizes the reduced state of haemoglobin and, at high concentrations, strongly binds preferentially to the ferrous state over the ferric state (Peng *et al* 2003). However, in our study, we observed cathodic and anodic positive shifts of haemoglobin when the GTP concentration was less than 4.5 mM, after which it reached equilibrium. GTP facilitated both reduction and oxidation such that its effect on the structure of haemoglobin was to transfer haem to the surface of the molecule. Binding of DPG and IHP to haemoglobin under different conditions has been confirmed by X-ray crystallography (Arnone *et al* 1972; Richard *et al* 1993), molecular daynamics (Laberge *et al* 2005) and NMR (Pomponi *et al* 2004), which confirms that our electrochemical method can be used for investigating ligand binding of materials to haemoglobin. We also showed that GTP can bind to haemoglobin but GDP and GMP cannot.

5. Conclusion

Electrochemical measurement by the iodide-modified silver electrode method for obtaining information about ligand–haemoglobin binding is simple. The binding ratio of concentrations of haemoglobin and ligand can indicate the affinity of the ligand for binding with haemoglobin. Furthermore, titrable potentiometric responses of haemoglobin caused by DPG, IHP and GTP revealed that the electrochemical effects of DPG and IHP, using the iodide-modified Ag electrode, confirmed previous reports that used other techniques (Laberge *et al* 2005). DPG and IHP had significant allosteric effects on haemoglobin as effectors that stabilized the reduced state of haemoglobin at low concentrations. In addition, these results show that these two materials have only one specific binding site on haemoglobin and can change the globin structure of haemoglobin. Moreover, GTP affects haemoglobin as a ligand, while GDP and GMP do not. GTP affects haemoglobin as a ligand at high concentrations, showing that there are multiple GTP-binding sites on haemoglobin, but its effect on haemoglobin is weak relative to DPG and IHP. GDP and GMP do not affect the cathodic and anodic peaks of haemoglobin, indicating that they may not bind to haemoglobin.

Acknowledgements

Financial support from the University of Tehran and the Iranian National Science Foundation (INSF) is gratefully acknowledged.

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MS received 24 July 2006; accepted 22 December 2006

*e*Publication: 27 January 2007

Corresponding editor: Vidyanand Nanjundiah