PARAMETERS OF HETEROGENEOUS ELECTRON TRANSFER FROM HB TO PYROLLITIC GRAPHITE IN AQUEOUS AND NON-AQUEOUS MEDIA: RATE CONSTANTS AND DISPERSION OF ELECTRON HOPPING DISTANCES

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Introduction

The ability of a living to exchange electrons with a solid surface could be a basis for the creation of a "living" power-cell; therefore, understanding the mechanism of a hetergenous electron exchange between protein and an electrode surface is of paramount importance.

The overall rate of the electron transfer process (ET) depends on the electrode potential, which is regulated within certain frameworks [1]. According to the Butler-Volmer model [1, 2], the rate of interfacial ET increases exponentially with the overpotential, η, which is limited by the reorganisation energy of the redox agent, evaluated within the Marcus [3, 4] theory, which means that the rate of the interfacial ET starts to level off once the overpotential becomes higher than the reorganisation energy of the reaction ($\eta>\lambda$). With these considerations the resulting cyclic voltammogramm is expected to show the current increasing with applied potential and levelling off to the limited value (plateau) [5].

The current study addresses an important extention to the model, which takes into account the disorder of the immobilisation of the molecule to the surface, and made an allowance for the fact that since the orientation of a protein molecule on the surface varies, so does the distance between the redox site and the electrode surface [5]. The current study aimed to develope a model which would factor in the variety of the protein orientation and conformation.

In order to understand the role of the protein conformation in the electron transfer, we tested the rate of heterogeneous electron transfer in a model system used for the study of electron transfer. System Hb on pyrolitic graphite is a model system for studying direct electron transfer to/from redox proteins [6, 7]. Furthermore, it was shown that Hb immobilised on a pyrolitic graphite electrode exhibited complicated behaviour, which allowed us to use this system as a model for different types of protein behaviour on the surface: Hb in water is a mobile protein, Hb in methanol is a rigid protein, and Hb and ethanol is a disordered melted globule.

Here, we report on a methodology of quantitative estimation of the dispersion of kinetic parameters using the reduction of Hb on a pyrolitic graphite electrode as a model and estimation of electron hopping distances within the protein matrix.

Materials and Methods

Hemoglobin (human) and tris[hydroymethyl]aminomethane were obtained from "Sigma", methanol and hydrogen peroxide from "Riedel-de Haën", and ethanol and $LiClO_4$ were from "Aldrich". All chemicals were analytical grade and used as received. All solutions were prepared using water from an Elag-Stat system (18 MΩ sm). Electrochemical experiments were performed using CH–802 or CH-602 potentiostats. A cell containing the working electrode (3 mm diameter edge plane pyrolytic graphite (BAS)) and a platinum wire counter electrode was connected through a flexible 1 M KNO_3 salt bridge to a second cell containing the Ag/AgCl reference electrode (CHI Instruments). The temperature of the cell was maintained at 23°C in a Lauda E100 water bath. Pyrolitic graphite electrodes were polished with 1.0 μm, 0.3 μm and 0.05 μm alumina slurry and rinsed with water. Protein solutions were prepared by dissolving 0.048 g of hemoglobin or 0.012 g of Mb in 1 ml of tris-HCL buffer, at a pH of 7.0. The obtained solution was filtered using a non-pyrogenic filter with a pore size of 0.45 μm (Sarstedt), spread onto the PG electrode (30 μl on 0.28 cm^2) and allowed to dry.

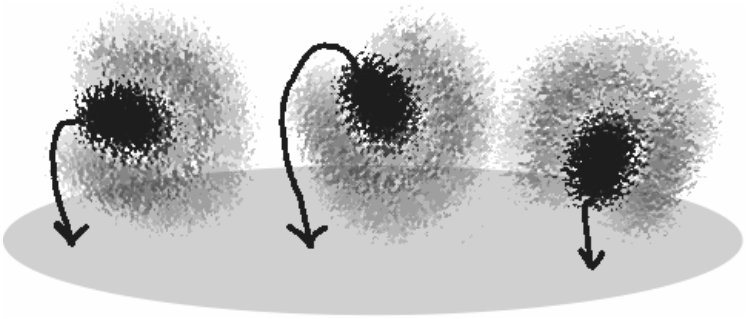
Cross-linked haemoglobin was prepared by mixing 500 μl of 3% glutaraldehyde and 500 μl Hb solution, prepared as described above. The resulting solution was then used in the immobilization procedure. All experiments were carried out under a nitrogen atmosphere and solutions were purged with nitrogen for at least 20 minutes prior to each experiment. Electrochemical measurements in aqueous media were carried out in 10 mM tris-HCl buffer at pH 7.0 in the presence of 50 mM KBr. 0.1 M $LiClO_4$ was used as a supporting electrolyte for solvents methanol and ethanol.

Results and Discussion

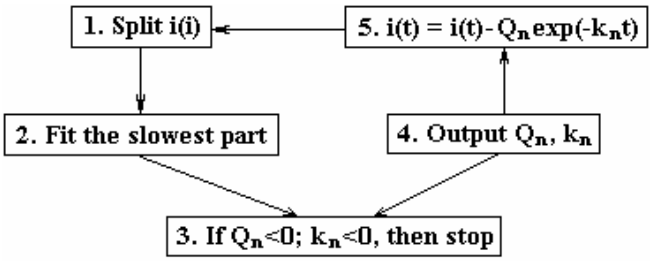
It is general knowledge that the electron transfer rate in protein is established via the electron tunnelling mechanism and directly connected to the distances between the electron pools [8]. The designed experiment aimed to measure the current created by the electron flow from the redox centre of haemoglobin to the electrode surface.

The operational redox potential was chosen as -600 mV vs Ag/AgCl 0.3 mol/kg electrode, which is a reducing potential for Hb [9, 7]. The interfacial electron transfer rate was evaluated from the current time course, and was already independent on the redox potential.

Heterogeneous electron transfer is a first order reaction [10] which is described by a single exponent. However, it is not always possible to active such a signal. The important extension of the model was in consideration of the effect of disorder in the orientation of the protein molecules. We regarded each individual electron transfer event as a Markov process, which occurrence is independent on others. Thus, we suggest that the immobilised Hb exists as a population of several orientations, each of which is capable of transferring electrons with a different rate (fig. *A*).



A



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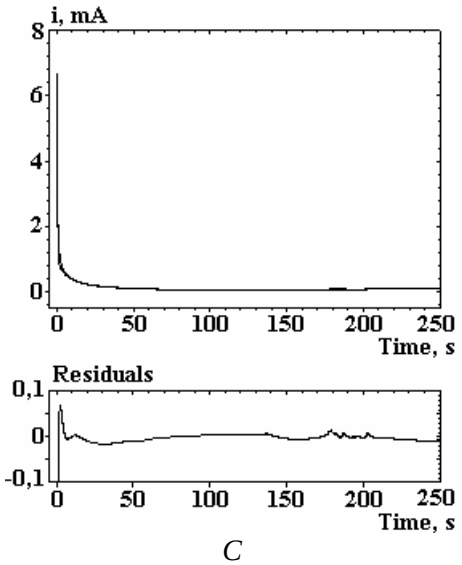

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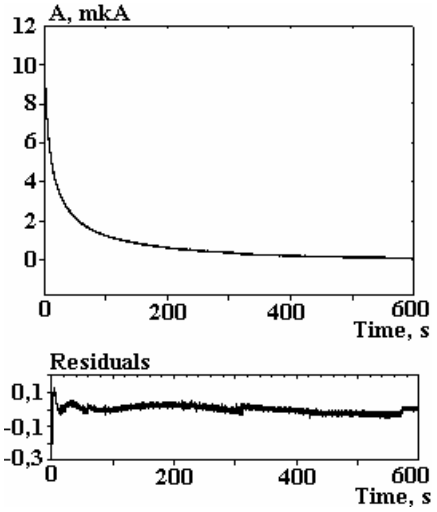

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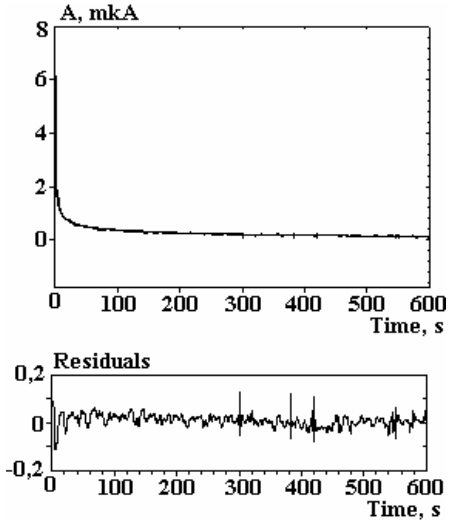

E

Fig. A) Schematic Depiction of different orientations of Hb on the electrode's surface; B) The proposed algorithm; C) Time course of the current of reduction of Hb on pyrolitic graphite in water (black line), and fitted function (red line). The residuals are shown below; D) Time course of the current of reduction of Hb on pyrolitic graphite in methanol (black line), and fitted function (red line). The residuals are shown below; E) Time course of the current of reduction of Hb on pyrolitic graphite in ethanol (black line), and fitted function (red line). The residuals are shown below

The dependence of the ET rate constant *k* on the tunnelling distance is described by exponential decay in eq. 1 [8, 11]:

$$k_0(d) = k_0^{MAX}(\exp(-\beta d). \quad (1)$$

Where, $k_0^{MAX} = k(d_0)$ and β is a decay constant, reported for the α-helical protein 1,25 A^{-1} [8, 11].

It is assumed that during the immobilisation of the protein molecules on the surface of the electrode there are several populations of the molecules with different values of the parameter *d*, where *d* is the distance between the redox centre the electrode surface (fig. *A*). Difference in the parameter *d* values could arouse from the variations in orientation of the protein molecule towards the electrode or due to the different conformations of the protein.

The observed current is representing the sum of the currents, obtained from *n* populations of redox active molecules. Thus, the overall observed current indicates electron hopping from protein to electrode and could be represented by eq. 2 [5]:

$$i(t) = \sum_{i=1}^{i=n} Q_i(\exp^{-k_i t}). \quad (2)$$

Where, $k_i \in [k_0^{MAX}, k_0^{MIN}]$.

Parameter k_0^{MAX} is a rate constant of ET from of population of the protein species with a shortest distance d_0 and parameter k_0^{MIN} is a rate constant of ET from of population of the protein species with a longest distance *d.* Thus the current time course is dependence, described by the sum of the several exponential functions. However, direct fitting of exponential functions sum is impossible, where the amount of exponents is exceeding 3.

Thus the time course of the observed current time course was analysed according to the following interactive algorithm (fig. *B*), which consisted of the following 6 steps: (1) The current time course *i(t)* was split into several intervals $i(t_0\text{-}t_1)$, $i(t_1\text{-}t_2)$, .. $i(t_{n-1}\text{-}t_n)$; (2) the slowest non-linear interval of the time course $i(t_{n-1}\text{-}t_n)$ was fitted to single exponential dependence $f(t)=a+Q_n\exp(-k_n*t)$; (3) Validity of the fit checked, where obtained values of Q_n and k_n evaluated. If the obtained value of k_n were negative, then stop. (4) Output values Q_n and k_n,; $n=n+1$. (5) Deduct the determined component $f(t)$ from the current time course $i(t):g'(t)=i(t)-f(t)$; (6) The obtained data analysed starting from (1) $g'(t):=i(t)$.

The final output gave the number of the populations *n*, values k_n and Q_n for each of the populations. It was found that these parameters are independent on the size of the splitting intervals. The obtained values are presented in table. The time course of the reduction current, together with the fitted function, is shown in figs *C*, *D* and *E*. The residuals (shown below) indicate the validity of the fit.

The obtained data is shown in table, where k_{MAX} is a true heterogeneous electron transfer rate constant, which corresponds to the closest distance between the redox centre of protein and the electrode surface, and *D* is the maximal distance from where the electron is able to reach the electrode surface.

Kinetic parameters of Hb reduction on the pyrolitic graphite electrode

	Media	$k_{MAX,}$ s^{-1}	*d*, A	ΔC_P [9], J/(mol·K)
Hb	H_2O	2.62	2.2	9,6
Hb	MeOH	0.181	1.15	0
Hb	EtOH	0.797	3.18	$-96\times T-6\times T^2$

As can be seen from the data, the fastest electron transfer rate was obtained for the Hb in a native state in water, while the lowest heterogeneous constant was obtained for the protein in its rigid state [9]. So far as the electron transfer distance is concerned, the largest *D* was obtained for the Hb in a melted state, while the shortest distance was obtained for rigid protein.

In the next step, we tried to connect the parameter specific heat capacity changes ΔC_P value of the reaction of haemoglobin reduction with the overall observed electron hopping distance. The reaction, if accompanied by ΔC_P, is a characteristic feature of occurring conformational changes [9]. As we have previously shown, Hb reduction is accompanied by ΔC_P changes, with the largest temperature dependent ΔC_P changes, which were observed in the ethanol, corresponding to the unfolding of the protein, although the high values of the ΔC_P were tightly connected with the higher values of the electron transfer distances. This lack of conformational flexibility of the protein matrix is also shown to decrease the electron transfer distances and also decreases the heterogeneous electron transfer rate constant.

Therefore, we suggest a novel method of evaluating kinetic parameters for systems with a several states, which allows the estimation of the direct electron transfer rate to/from the redox centre onto the electrode surface, and also determines the maximum distance from which electrons could be retrieved.

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Summary

A novel methodology of multi-exponential kinetic data processing was developed and tested for the reduction of hemoglobin on pyrolytic graphite.