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# Preliminary electrochemical studies of the flavohaemoprotein from *Ralstonia eutropha* entrapped in a film of methyl cellulose: Activation of the reduction of dioxygen

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**Abstract**

A flavohaemoprotein (FHP) from *Ralstonia eutropha*, obtained in a pure and active form, has been entrapped in a film of methyl cellulose on the electrode surface and gives a stable and reproducible electrochemical response at pH 7.00 when subject to cyclic voltammetry using a glassy carbon electrode. To our knowledge, no previous direct electrochemistry had been achieved with a bacterial flavohaemoglobin, which possess both a FAD and a haem. A single couple is observed which is assigned to the haem moiety of the protein, since the same result is obtained with a semi-apo form of the protein deprived of FAD (semi-apo FHP). The data collected were further confirmed by potentiometry with a platinum electrode, and the homogeneous electron transfer rate estimated by double potential step chronocoulometry at a bare glassy carbon electrode in the presence of methyl viologen (MV). The presence of FAD in the holoprotein is easily confirmed by UV–Vis spectrophotometry, but its expected electron relay role remains elusive. The protein activates the reduction of dioxygen by about 400 mV, the reduction current being proportional to the concentration of dioxygen up to 10% in volume in the gas mixture.


*Keywords:* Flavohaemoprotein; Cyclic voltammetry; Methyl cellulose; Spectroelectrochemistry; Chronocoulometry; Dioxygen reduction activation

## 1. Introduction

The flavohaemoprotein (FHP) from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) [1,2] is a 43 kDa bacterial haemoglobin whose existence was first mentioned in the 1970s [3,4]. Like all the haemoglobins found in Nature, it possesses a haem moiety that reversibly binds dioxygen ($O_2$) when in its reduced state (i.e. when the haem iron is in the +2 oxidation state) [2,4]. This may easily be checked by UV–Vis spectrophotometry, since the oxidised haem Soret band at 395 nm shifts to 436 nm upon reduction, and is finally found at 414 nm and more intense when reduced haem binds dioxygen [4]. The UV–Vis spectrum of the oxidised FHP has several bands: apart from the Soret band at 395 nm, the ferric haem group gives rise to two more bands, indicative of a high spin iron, at 486 and 645 nm [4]. The band at 456 nm has been assigned to flavine adenine dinucleotide (FAD), which is noncovalently bound to the protein [4]. Finally, there is a band at 277 nm, which is characteristic of the aromatic amino acid residues of any protein, particularly tryptophan and tyrosine [5].

The existence of a haem iron that interconverts between the oxidation states +2 and +3 has several implications regarding the binding affinity of the FHP towards ligands such as nitric oxide ($NO^{\bullet}$), dioxygen ($O_2$) and carbon monoxide (CO). Indeed, while nitric oxide coordinates to haem irrespective of the oxidation state of the iron, dioxygen and carbon monoxide bind just ferrous haem [6].

The determination of the primary sequence [7], composed of 403 residues, and then of the crystal structure of the protein at 1.75 Å resolution confirmed the presence of a haem (axially coordinated to the imidazole of His-85) and of a FAD per

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molecule [8] (Fig. 1). The former is embedded in a hydrophobic crevice, while the latter is partially exposed to the solvent [8]. The shortest distance between the haem and the FAD is 6.3 Å, allowing direct electron transfer between the two sites [8–10]. Apart from these two prosthetic groups, the FHP has been shown to possess a phospholipid adjacent to the haem, and therefore susceptible of modulating the haem's biochemical properties [11].

Even if the biological function of bacterial haemoglobins has not been unambiguously established, they are thought to be $NO^{\bullet}$ dioxygenases (NOD), which render the bacteria resistant to $NO^{\bullet}$ toxicity by converting it into nitrate ($NO_3^-$) in the presence of $O_2$ as co-substrate and with the participation of reduced nicotinamide adenine dinucleotide (NADH), according to the following equation [6]:

$$2NO^{\bullet} + 2O_2 + NADH \rightarrow 2NO_3^- + NAD^+ + H^+ \quad (1)$$

In Eq. (1), the natural reductant, NADH, may in principle be replaced by another source of electrons, which may be another chemical species or an electrode. In the latter case, the biochemical reaction catalysed by the FHP will be modulated by the potential at which the electrode will be poised, provided no complications arise (e.g. electrode fouling, protein denaturation, non-effective electron transfer pathways) [12–15].

Successful protein electrochemistry has been achieved by confining the biomolecule to a space delimited by the electrode surface and a biocompatible film that retains the protein in its native, active state. Films may be made of lipids [16–18], cellulose derivatives [19–22], silica sol–gels [23], collagen [24], surfactants [25–28], $TiO_2$ and $SnO_2$ nanoparticles [29,30], polyelectrolytes [31–35], polystyrene latex beads [36], Eastman AQ® [37] and Nafion® [37,38].

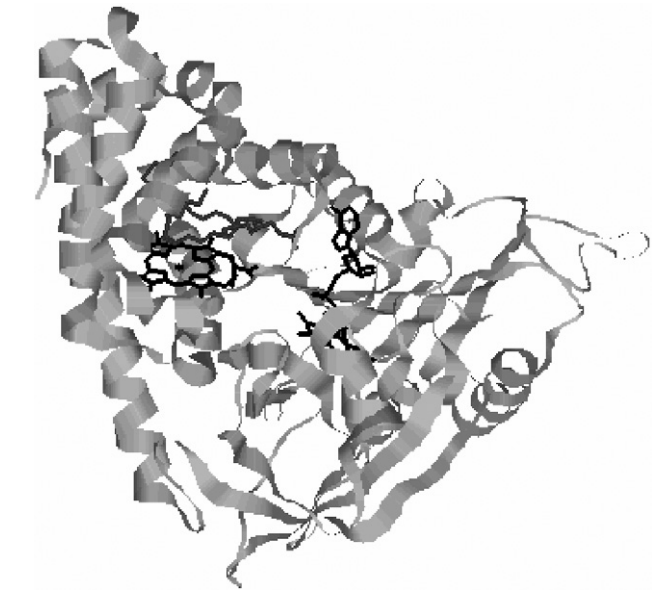

Fig. 1. 3D structure of the FHP from *R. eutropha* (PDB entry code: 1CQX): the haem is shown in black on the left, with the lipid above it in grey, and the FAD, which partially protrudes from the surface, appears towards the upper centre, in black.

In this work, a methyl cellulose film was used to confine the protein to the vicinity of the electrode surface, and the FHP/methyl cellulose modified electrode was studied by cyclic voltammetry. Potentiometry and double potential step chronocoulometry data were also gathered from experiments involving the FHP.

## 2. Experimental

### 2.1. FHP purification

The purity of the protein sample is an essential requirement for any attempt of obtaining meaningful data from electrochemical experiments, and quite often the sole way to aim at getting a good reproducibility. A slightly improved purification method, partially influenced by previous contributions [4,39], is now well established, giving several millilitres of pure samples whose concentration in FHP can reach a few hundreds of micromolars. The purity of each sample was checked by SDS-PAGE, which revealed a single band corresponding to the expected molecular weight of the FHP, and further confirmed by UV–Vis spectrophotometry [4]. The FHP solutions used were prepared by buffer exchange with PD-10 columns filled with Sephadex G-25 M (Amersham Pharmacia).

### 2.2. Materials

Potassium di-hydrogen phosphate, di-potassium hydrogen phosphate, sodium dithionite, safranine, phenazine methosulfate (PMS) and methyl viologen (MV) were from Acros Organics; potassium 1,4-naphtoquinone-2-sulfonate (NQSP) was from Eastman; sodium anthraquinone-2,6-disulfonate (AQDS) was from Ega Chemie; potassium ferricyanide and potassium chloride were from Prolabo; dimethylformamide (DMF) was from Aldrich; methyl cellulose was from Sigma; 99.995% argon and the mixture 80% dinitrogen/20% dioxygen were from Air Liquide. The reagents were used without further purification.

All experiments were done in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00. All solutions were prepared with pure water obtained from a Milli-RiOs 8 unit followed by a Milli-Q academic purification set (water resistivity: 18.2 MΩ.cm), and were either thoroughly deoxygenated with argon or set at a desired dioxygen concentration by sparging for 30 min with a convenient mixture of pure argon and 80% dinitrogen/20% dioxygen prior to the experiments.

### 2.3. Electrode modification, electrochemistry and UV–Vis spectrophotometry

Glassy carbon electrodes were conveniently polished [40] on polishing cloths of different roughness with diamond paste (Struers), and thoroughly cleaned with a wet tissue. Cyclic voltammetry and double potential step chronocoulometry were carried out in a three-electrode cell comprising a glassy carbon working electrode, a platinum counter electrode and a saturated

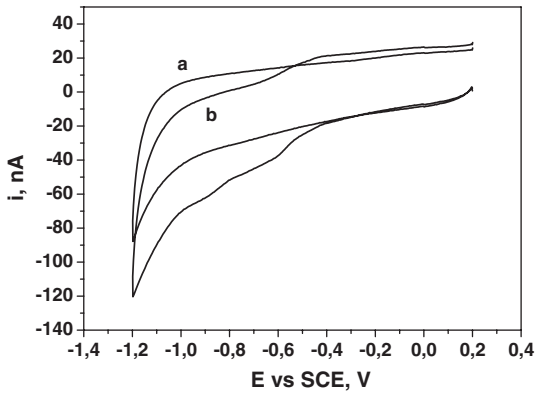


Fig. 2. Cyclic voltammograms obtained in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00, at 2 $mVs^{-1}$: a) blank; b) 300 μM FHP.

calomel reference electrode (SCE). All potentials quoted refer to SCE. The electrodes were connected either to an EG&G PAR Model 273A potentiostat or to an Ametek PAR BiStat potentiostat, both of which were computer-controlled (M270 or EC-Lab software). In the case of cyclic voltammetry, the modified working electrode was prepared by applying a 10 μL drop of a mixture containing 15 μL of a 0.2 $gL^{-1}$ methyl cellulose in water, 5 μL DMF and 20 μL 100 μM FHP in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00. The drop was allowed to dry in air for several hours. For double potential step chronocoulometry, a bare glassy carbon electrode was used in a solution containing roughly 500 μM FHP and 5 μM MV.

Spectroelectrochemistry experiments, in which potentiometry measurements were coupled with UV–Vis spectrophotometric analysis (Lambda 19 UV/VIS/NIR spectrophotometer, Perkin-Elmer), were carried out with a bare platinum electrode and a SCE reference electrode connected to a potentiometer (MVN 83, Tacussel) and inserted in a 1 cm optical path cell under an argon atmosphere. A mediator cocktail was used, consisting of 5 μM each of NQSP, PMS, AQDS and MV.

## 3. Results and discussion

### *3.1. Direct, un-mediated cyclic voltammetry with the FHP free in solution*

All efforts to obtain an unambiguous direct, un-mediated electrochemical response of FHP free to diffuse in solution from which reliable data could be extracted have not been successful so far. When the protein is relatively concentrated ([FHP]> 150 μM), a rather drawn out reduction wave is observed at around −0.900 V, which may be preceded by another one close to −0.600 V, particularly noticeable at scan rates smaller than 10 $mVs^{-1}$, Fig. 2. This poor electrochemical response is not surprising, despite that the FAD is partially exposed to the solvent, according to the crystal structure [8] (Fig. 1). Indeed, the well-behaved electrochemistry of this flavine in aqueous solution has been described in detail [41], but a similar response is not necessarily observed when the FAD is incorporated in a far more complex structure like a protein. In certain cases, its presence in a protein could never be guessed from the electrochemical behaviour of the system, even when directly attached to another easily detected electroactive group [42]. In fact, nothing guarantees that the flavine in the FHP effectively interacts with the electrode surface for electron transfer to ensue, nor that it participates in the main electron transfer pathway leading form the protein surface to its active site, i.e. the iron atom of the haem, when the electron donor is the electrode. Nevertheless, the presence of what seems to be an NADH binding domain interacting through van der Waals contacts with the adenine moiety of the FAD [8], in conjunction with the fact that NADH spontaneously reduces the haem of oxidised holoprotein FHP when they are put together in solution [4], hints that FAD may play a role in the electron transfer in vivo. Important evidence that FAD does participate in electron transfer was gathered from spectrophotometric studies carried out with an FAD-deprived form of FHP (semi-apo FHP) (G. Ollesch and U. Ermler, unpublished work): the semi-apo FHP, unlike the holoprotein form, was not reduced by NADH, but it was reduced by sodium dithionite, a more effective reductant.

### *3.2. Direct, un-mediated cyclic voltammetry with the FHP entrapped in a film*

The FHP gives a stable, reproducible response in cyclic voltammetry when confined to the vicinity of the electrode surface in a film of methyl cellulose (Fig. 3), either as holo-FHP or as semi-apo FHP. In addition, modified electrodes are stable in air at room temperature for several weeks. The $E^{0\prime}$ value is estimated at −0.320±0.010 V for a scan rate of 0.100 $Vs^{-1}$ ($\Delta E_p$=0.090 V). This result is very close to the response obtained with myoglobin and haemoglobin (−0.298 and −0.312 V, respectively), both penta-coordinate haem-containing proteins like FHP, in similar experimental conditions [19]. It seems, then, that the FAD has no influence whatsoever in the electrochemical behaviour of the FHP, assuming that the protein keeps its native conformation and remains in an active form in the methyl cellulose film. Obviously, the integrity of the protein

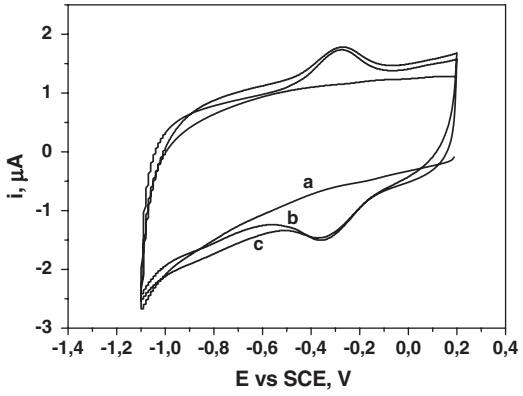


Fig. 3. Cyclic voltammograms obtained in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00, at 100 $mVs^{-1}$ with a differently methyl cellulose-modified glassy carbon electrode: a) no protein in the film; b) FHP in the film; c) semi-apo FHP in the film.

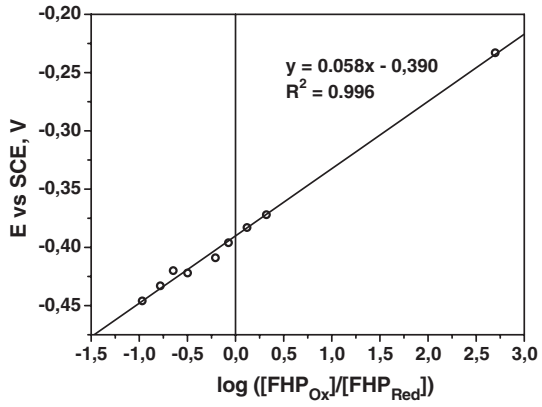


Fig. 4. Plot of the function $E=f(\log([FHP_{Ox}]/[FHP_{Red}]))$ obtained from spectroelectrochemical data.

is essential in order to obtain meaningful results. Several research groups have resorted to spectroscopic analysis of both adsorbed protein films and cellulose film-entrapped proteins to ascertain that the biomolecules gave rise to the same spectra, an indication that their chromophores were present in a similarly folded protein backbone in all circumstances [19,21,22]. The UV–Vis spectra of the FHP solutions containing methyl cellulose and DMF as described in the experimental section, recorded over several hours, coincided with that of a pure FHP buffered solution. This confirms that, in solution, the protein is stable in the presence of methyl cellulose and DMF. However, the falling out of the FAD moiety when the film forms on the electrode surface cannot be ruled out at the present stage. If this were to be the case, it would not be surprising to observe exactly the same behaviour for the holo-FHP and the semi-apo FHP entrapped in a methyl cellulose film, since the protein would be in its semi-apo form in both cases.

### 3.3. Potentiometry coupled to UV–Vis spectrophotometry (spectroelectrochemistry)

It is interesting to compare the abovementioned $E^{0\prime}$ value ($-0.320\pm0.010$ V) obtained with the entrapped protein with another one arrived at by spectroelectrochemistry, in which the FHP freely diffuses in the solution. The potential of a dithionite-reduced FHP solution was increased by adding small aliquots of a ferricyanide solution and measured by potentiometry with a bare platinum electrode. A redox mediator cocktail was added to the FHP solution in order to attain equilibrium more rapidly. Redox mediators are electroactive species that shuttle electrons between the electrode surface and the protein [43]. The cocktail consisted of 5 μM each of NQSP ($E^{0\prime}=-0.090\pm0.010$ V), PMS ($E^{0\prime}=-0.380\pm0.010$ V), AQDS ($E^{0\prime}=-0.430\pm0.010$ V) and MV ($E^{0\prime}=-0.690\pm0.010$ V). The ratio between oxidised and reduced protein was easily calculated by spectral analysis, since the haem Soret bands of oxidised and reduced FHP are well apart (395 vs. 436 nm, respectively). A plot of $E=f(\log[(FHP_{Ox})/(FHP_{Red})])$ gives a straight line from which $E^{0\prime}$ is immediately obtained [44], (Fig. 4): $E^{0\prime}=-0.390\pm0.010$ V. This result is close to the one obtained by cyclic voltammetry ($-0.320\pm0.010$ V), an indication that the FHP has probably retained its integrity when entrapped in the methyl cellulose film. In addition, the slope of the straight line is close to 0.060 V per decade, consistent with a one-electron redox process, as expected for haem iron that interconverts between the oxidation states +2 and +3. It is not unusual to see a difference between results obtained with different techniques: for example, horseradish peroxidase, a haem-containing enzyme roughly of the same size as FHP, has an $E^{0\prime}=-0.349$ V when entrapped in a methyl cellulose film and an $E^{0\prime}=-0.511$ V when free to diffuse in solution [20]. It seems, then, beyond doubt that the $E^{0\prime}$ values obtained may be assigned to the haem moiety of FHP.

### 3.4. Dioxygen reduction activation

The reduction peak of dioxygen, found at $-0.630\pm0.010$ V at a methyl cellulose-modified glassy carbon electrode in the present experimental conditions, shifts to $-0.240\pm0.010$ V when the protein is entrapped in the film (Fig. 5). This behaviour is expected for iron and copper-containing peroxidases and oxidases [45–47], but the overpotential decrease for the reduction of dioxygen is particularly pronounced (roughly 0.400 V) in the present system. Indeed, it performs even better than bilirubin oxidase, a rather effective enzyme system for dioxygen reduction, for which the decrease in the overpotential was estimated at 0.350 V [46]. In addition, the cathodic current corresponding to the reduction of dioxygen obtained with a FHP/methyl cellulose-modified glassy carbon electrode is directly proportional to its concentration up to 10% in volume in the gas mixture used to sparge the buffer solution (Fig. 6). Last but not least, the entrapped protein and the film remain stable after oxygen activation, the whole process being reproducible with the same modified electrode.

### 3.5. Mediated cyclic voltammetry and double potential step chronocoulometry

Another strategy to try to address the redox active sites in freely diffusing FHP implies the use of redox mediators in

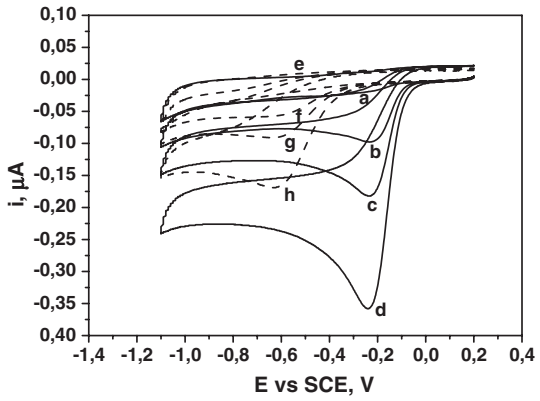


Fig. 5. Cyclic voltammograms obtained at 2 $mVs^{-1}$ in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00, with a methyl cellulose-modified glassy carbon electrode having either no protein (dashed lines) or FHP entrapped in the film (solid lines). The amount of dioxygen in the gas mixture used to saturate the buffer solution was: a and e) 0%; b and f) 0.5%; c and g) 1.0%; d and h) 2.0%.

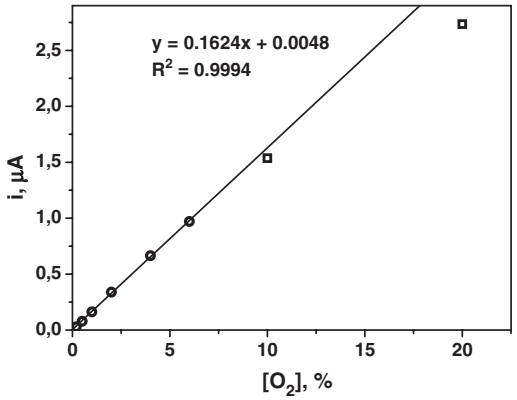


Fig. 6. Dependence of the cathodic peak current (corrected for the background current) on the concentration of dioxygen for a FHP/methyl cellulose-modified glassy carbon electrode, at 2 $mVs^{-1}$, in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00.

transient-state techniques like cyclic voltammetry. Fig. 7 shows an example of an effective electron transfer mediation by methyl viologen (MV): in the presence of oxidised FHP, the reduction wave of the mediator at −0.730±0.010 V is preceded by a wave at −0.620±0.010 V (i.e. before that of MV) for small $\gamma$ values, $\gamma$ being the concentration ratio $\gamma$=[FHP]/[MV]. This wave becomes the dominant and finally the sole one at −0.660± 0.010 V as $\gamma$ increases from 0.5 to 7, and its current magnitude increases linearly with $\gamma$ (Fig. 7, inset). This has an immediate implication: the $E^{0\prime}$ of the FHP redox group that is reduced in the mediation process is more positive than that of MV, i.e. higher than −0.690±0.010 V. Other mediators have been tried, and the outcome matches the abovementioned value arrived at by potentiometry (i.e. $E^{0\prime}$=−0.390±0.010 V): a mediation wave was observed in the presence of safranine ($E^{0\prime}$=−0.530±

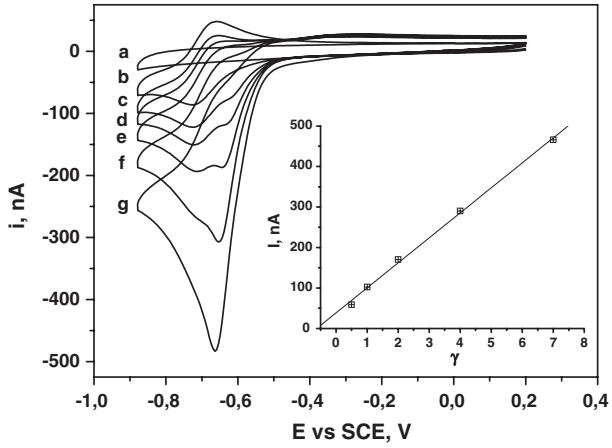


Fig. 7. Cyclic voltammograms obtained with a solution containing FHP and MV in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00, at 2 $mVs^{-1}$. The conditions are: a) blank; b) [MV]=31.5 µM, [FHP]=0 µM, $\gamma$=0; c) [MV]=30.5 µM, [FHP]=14.5 µM, $\gamma$=0.5; d) [MV]=29.5 µM, [FHP]= 29.5 µM, $\gamma$=1; e) [MV]=27.6 µM, [FHP]=55.3 µM, $\gamma$=2; f) [MV]=24.7 µM, [FHP]=98.7 µM, $\gamma$=4; g) [MV]=21.2 µM, [FHP]=148.5 µM, $\gamma$=7. Inset: dependence of the cathodic peak current (corrected for the background current) of a solution containing both FHP and MV in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00, obtained at 2 $mVs^{-1}$, on the concentration ratio, $\gamma$=[FHP]/[MV].

0.010 V), while NQSP ($E^{0\prime}$=−0.090±0.010 V) failed to shuttle electrons from the electrode surface to the protein (not shown).

The kinetics of the mediation phenomenon was studied by double potential step chronocoulometry, after a methodology previously described in detail [48]. Briefly, the second order rate constant, $k$, for the homogeneous one electron transfer reaction

$$MV_{Red} + FHP_{Ox} \rightarrow MV_{Ox} + FHP_{Red} \quad (2)$$

is given by

$$k = \beta^2/(2[FHP_{Ox}]) \quad (3)$$

where $\beta^2$ is a pseudo-first order rate constant valid for $\gamma \geq 10$, and $[FHP_{Ox}]$ is the concentration of oxidised FHP. Values of $\beta^2$ may be readily calculated with a homemade software which relies on the charges consumed during the forward and the backward scans, $Q_f^*$ and $Q_b^*$, respectively, corrected for their electrode–electrolyte double layer capacitance charges. These charges are obtained in a straightforward way with the M270 software from curves like the one shown in Fig. 8, which led to $k=(8\pm2)\times10^3$ $M^{-1}$ $s^{-1}$. This is roughly three orders of magnitude smaller than the hydride transfer rate from NADH to the FAD moiety of the closely related bacterial haemoglobin protein (HMP) from *Escherichia coli*, which was estimated at $k'_H=15\times10^6$ $M^{-1}$ $s^{-1}$ [49,50]. The difference between these two values may be explained by a larger activation energy barrier for electron transfer from MV to the FAD in FHP, which would not be a surprise if NADH were to be undoubtedly confirmed as the natural reductant of the protein, since one would expect this process to be optimised by Nature, and therefore to be particularly performing. Other complications may arise, though, from the fact that MV, unlike NADH, is not involved in hydride transfer as such nor in protonation/deprotonation reactions. FAD takes two electrons and two protons from its immediate surroundings upon full reduction. If protons are not available nearby and have to come from the medium close to the surface of the protein, it is normal that the overall process slows down. It will be interesting to check the effectiveness of other mediators, eventually participating in acid/base reactions, as far as the electron transfer rate to the FHP is concerned.

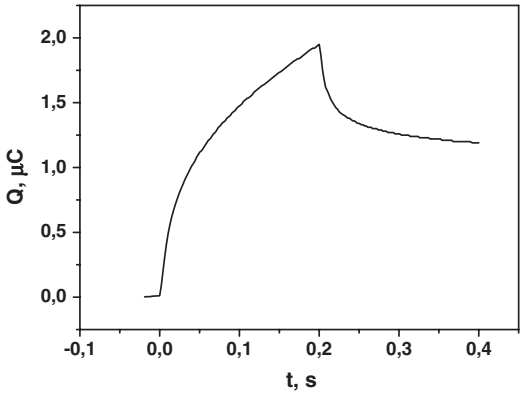


Fig. 8. Double potential step chronocoulometry curve obtained with a bare glassy carbon electrode in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00, containing 557 µM FHP and 6 µM MV; $\tau$=0.2 s.

## 4. Conclusion

A flavohaemoprotein (FHP) from *R. eutropha* in an active state has successfully been confined in a film of methyl cellulose on a glassy carbon electrode surface. The protein gives rise to a stable, reproducible electrochemical response in cyclic voltammetry, allowing the $E^{0\prime}$ value of its haem site to be estimated. This has been confirmed to a reasonable degree by potentiometry coupled to UV–Vis spectrophotometry (spectroelectrochemistry).

The reduction of dioxygen is activated by the FHP/methyl cellulose-modified glassy carbon electrode by 400 mV, rendering it a rather performing system. The modified electrode remains stable after oxygen activation and may be used again several times.

A first attempt at obtaining an estimation of the homogeneous electron transfer rate between the FHP and MV has been made by double potential step chronocoulometry. Although the results are several orders of magnitude smaller than others calculated from stopped-flow data using the assumed natural reductant NADH, the outcome of future studies with different mediators is awaited with interest.

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