BIOELECTROCHEMISTRY

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Contents

Summary

Some aspects of bioelectrochemistry that may provide useful information on the role played by biomolecules in biological processes are overviewed and assessed. Electrochemical reactions of water soluble redox proteins, either immobilized on bare or functionalized electrode surfaces or free to diffuse toward the electrode, are briefly reviewed. The preparation and architecture of experimental models of biomembranes (biomimetic membranes), both freely suspended and noncovalently or covalently tethered to a metal surface, is described. A description of the profile of the electric potential across membranes and of their electropermeabilization by electric fields is provided. The structure of self-organized phospholipid monolayers interposed between a metal surface and the aqueous phase is examined. Particular attention is focused on the preparation of biomembrane models capable of incorporating channel-forming peptides and integral proteins in a functionally active state. The use of biomimetic membranes for investigating the function of integral proteins, either incorporated in

biomembrane fragments or proteoliposomes adsorbed on metal-supported mixed alkanethiol | lipid bilayers, or directly incorporated in metal-supported lipid bilayers, is described. The potential of metal-supported lipid bilayers with a hydrophilic "spacer" interposed between the metal surface and the bilayer, for fundamental studies on the function of integral proteins and for reliable and rapid drug screening, is emphasized.

1. Introduction

Bioelectrochemistry can be defined as that area of science in which electrochemical foundations and techniques are exploited to investigate processes of biological relevance. The electrochemical behavior of biomolecules on electrodes does not necessarily pertain to bioelectrochemistry: this is true only if it provides some useful piece of information on the role played by these molecules in biological processes. Among biomolecules, nucleic acids and proteins are the most significant: nucleic acids carry genetic information and represent the project of life, proteins execute this project.

By far the most important electrified interfaces in living systems are biological membranes. They consist of a bimolecular layer of lipids (the bimolecular leaflet) incorporating proteins (see Figure 1). Lipid molecules are "amphiphilic", i.e. consist of a hydrophobic section (the hydrocarbon tail) and a hydrophilic section (the polar head). In biological membranes the two lipid monolayers are oriented with the hydrocarbon tails directed toward each other and the polar heads turned toward the aqueous solutions that bath the two sides of the membrane. The resulting lipid "bilayer" is a matrix that incorporates different proteins performing a variety of functions. Biomembranes form a highly selective barrier between the inside and the outside of living cells. They are highly insulating to inorganic ions, and large electrochemical potentials can be maintained across them. The permeability and structural properties of biological membranes are sensitive to the chemical nature of the membrane components and to events that occur at the interface or within the bilayer. For example, biomembranes provide the environmental matrix for proteins that specifically transport certain ions and other molecules, for receptor proteins and for signal transduction molecules. The lipid and protein portions of biomembranes are also sensitive to the presence of lipophilic perturbants. Anaesthetics, for example, readily partition into lipid membranes, altering their electrical and permeability characteristics. The various responses observed in biomembranes are concentration-dependent, usually very rapid and reversible, and frequently voltage-dependent.

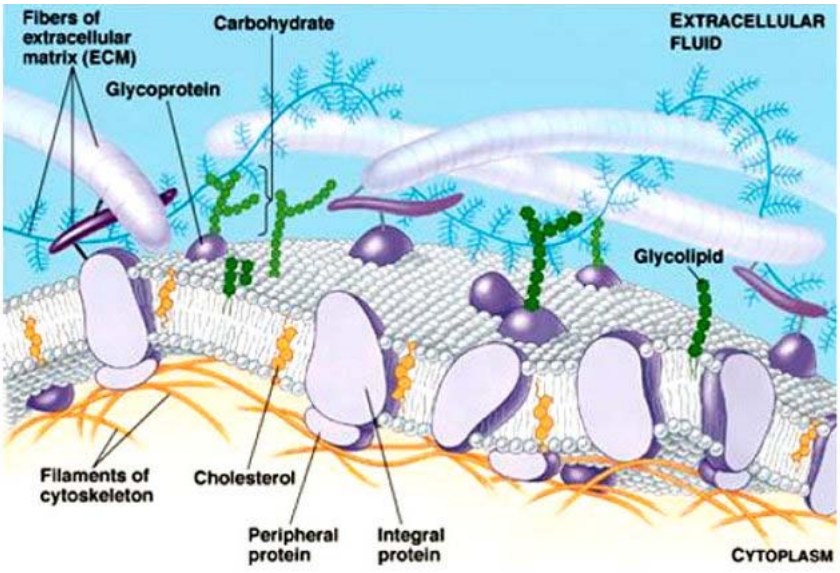

Figure 1 – Schematic picture of a biomembrane, showing the bimolecular leaflet of lipid molecules (including cholesterol), integral proteins spanning the lipid bilayer, peripheral proteins, filaments of cytoskeleton (the cellular "scaffolding" present in the cytoplasm), as well as glycolipids and glycoproteins, which expose their covalently attached oligosaccharide chains (glycans) to the extracellular fluid. [http://telstar.ote.cmu.edu/Hughes/tutorial/cellmembranes/].

Proteins are linear chains of polymers of amino acids linked by peptide bonds (the polypeptide chains). Twenty different amino acids can be found in polypeptide chains: ten are hydrophilic because of their polar, acidic or basic character, while the other ten are nonpolar and hydrophobic. Some of the proteins (the structural proteins) simply support the texture of the membrane. A more important group of proteins (the functional proteins) participates directly in membrane processes such as flow of matter, energy or information. Some proteins (the integral proteins) are embedded in the lipid bilayer with the hydrophobic sections of their polypeptide chain, and protrude from the bilayer surface into the two adjacent aqueous solutions with the extrinsic, more hydrophilic sections of the chain. In other proteins (the globular proteins) the polypeptide chain folds spontaneously in a way that removes the hydrophobic sections from contact with the bulk solvent burying them in the interior of the protein, while the hydrophilic sections remain on the surface of the protein, where they form hydrogen bonds with water and between themselves. These proteins are normally soluble in the “cytosol”, the aqueous medium enclosed by the cell membrane, and therefore in water. The above “native”, or biologically active, folded conformations lie in a shallow free energy minimum and can be relatively easily disrupted when the environment is altered, giving rise to what is called “denaturation”. Two important soluble proteins are contained in the red blood cells of humans: hemoglobin, that transports oxygen, and myoglobin, that stores oxygen in the tissues until it is employed during metabolism. Myoglobin contains a “heme” group, namely a tetrapyrrole ring system that chelates Fe(II): this is a “prosthetic group”, in that it is not part of the polypeptide chain, although it is held tightly by this chain. Hemoglobin contains four heme groups.

Many globular proteins are enzymes, the protein catalysts of the cell. The amino acid residues in polypeptide chains can act as acids, bases or nucleophiles, but they cannot act as electrophiles, i.e. electron-deficient groups that attack electron-rich groups. In "metalloenzymes", metal ions bound to the enzymes, such as Fe(II), Co(II), Cu(II) and Zn(II), provide this function by acting as electrophilic centers in catalytic reactions. These reactions occur in a pocket of the enzyme called the "active site", whose conformation and chemical composition determines the specificity of the enzymatic catalysis. The active sites normally consist of a "binding site", which includes the amino acid residues that come into contact with the reactant molecules, called "substrates", and of a "catalytic site", which includes residues directly responsible for the catalysis. The "turnover number" for an enzyme is the number of molecules of substrate that an enzyme saturated with the substrate through the formation of labile enzyme-substrate complexes, can convert to product in unit time. The turnover number can be determined from the catalytic rate constant, k_{cat}, provided the number of catalytic sites per molecule of enzyme is known.

Other globular proteins (the peripheral proteins) are weakly bound to the surface of the bilayer by electrostatic interactions or by hydrogen bonds and interact with the polar heads of the lipid or with the integral membrane proteins. Some of them (e.g. cytochrome *c*, plastocyanin, ferredoxin) contain electrophilic metal ions and exchange electrons with the integral proteins. In fact, the majority of redox proteins, namely proteins containing one or more redox sites, have no biological function when taken alone; rather, they are associated with other redox proteins.

Short linear chains of polymers of amino acids linked by peptide bonds are called "peptides". The distinction between peptides and proteins is not well defined. This distinction is often based on the number of amino acid residues: peptides are considered to have less that 50 residues, polypeptides from 50 to 100 residues, and proteins more that 100 residues. However, according to a different classification, even polypeptide chains of less that 50 residues are called proteins, provided they have a well defined secondary structure. This is the case of sarcolipin (with 31 residues) or phospholamban (with 51 residues), which modulate the function of Ca-ATPase of the sarcoplasmic reticulum. Many small peptides have biological activity and important functions.

The field of bioelectrochemistry is mainly concerned with the investigation of water-soluble redox proteins capable of exchanging electrons with an electrode, and with the investigation of peptides and proteins that can be incorporated, in a functionally active state, in experimental models of biological membranes, often referred to as "biomimetic membranes". The present contribution will be organized as follows. The electrochemistry of soluble redox proteins will be described first. Subsequently, the various biomimetic membranes, from vesicles to bilayer lipid membranes and to solid supported membranes, will be examined, together with their applications to the study of peptides and proteins.

The electrochemistry of DNA is booming after the discovery of the ability of single-stranded, denatured DNA (ssDNA) to reform (renature) in double-stranded structure (dsDNA). The principle of DNA renaturation and hybridization has been extensively exploited in electrochemical biosensors for DNA hybridization. In these sensors,

ssDNA is used as a probe capable of identifying a nucleotide sequence of a target DNA that is complementary to the DNA probe. Since this subject is dealt with in the "Sensors" Topic of this Encyclopedia , it will not be considered further. Suffice here to mention that some nucleic acid components are electroactive at a dropping mercury electrode. Thus, adenine is polarographically reducible in highly acidic solutions. Cytosine is reducible at both acidic and neutral pH values. Guanine yields an anodic signal, ascribable to the oxidation of a DNA reduction product formed at far negative potentials. In a narrow negative potential range, close to its desorption potential, dsDNA undergoes major changes that are considered to be ascribed to the opening of the DNA double helix.

2. Electrochemistry of Water Soluble Redox Proteins

The electrochemistry of soluble redox proteins can be investigated by using friendly electrode materials that do not cause their denaturation and may exchange electrons with them. The use of an electrode as an electron-transfer partner for redox proteins has the advantage that, by simply varying the applied potential *E*, it is possible to cause the electron-transfer reaction involving the redox protein to proceed in both directions. Thus, by making *E* sufficiently negative, electrons will tend to be released from the electrode to the redox protein, converting it to its reduced form. Conversely, by making *E* sufficiently positive, the electrode will tend to take up electrons from the reduced form of the protein, converting it to its oxidized form. Electron-transfer reactions between two proteins, however, are favored by their natural conformations, which tend to bring the redox sites of the two partners as close together as possible, making almost every collision a successful encounter. This is not normally true between an electrode and a protein, and efforts are made to find electrode materials that, besides retaining the biological activity of the protein, impart to the protein orientations favorable to the electron transfer. For example, cytochrome *c* is electroreduced on bare gold, but only under scrupulously clean conditions. Better results are obtained at a tin-doped indium oxide electrode, whose surface is hydrophilic due to the presence of surface hydroxyl groups. Edge pyrolytic graphite, i.e. graphite cut normally to the plane of the aromatic rings, is also a good electrode material, since its various CO functionalities, which are negatively charged at pH 7, impart notable hydrophilicity and ionic character to the surface and favor binding of the protein prior to electron transfer. Water soluble redox proteins can react at an electrode by diffusing toward it without being appreciably adsorbed on its surface, or else while being immobilized on its surface.

2.1. Diffusion Controlled Electrochemistry of Water Soluble Redox Proteins

A versatile procedure for imparting to the protein orientations favorable to the electrode transfer consists in modifying a gold surface by adsorbing on it a bifunctional molecule of the type X-Y, where X is a substituent that anchors the molecule to the electrode surface (e.g., pyridyl, sulfhydryl or disulfide) and Y is a functional group (e.g., a carboxyl or an amino group) that interacts transiently with the protein, imparting a favorable orientation to it. This is often achieved by exploiting attractive electrostatic interactions. Thus, the positively charged cytochrome *c* is favorably adsorbed on hydrophilic surfaces possessing acidic functionalities.

To achieve reversible, diffusion controlled electrode reactions, not only the association of the protein to these organic adsorbates, but also its dissociation must be fast, to avoid accumulation of reactants or products on the electrode surface. These adsorbates, which have no redox properties, are called "promoters" because they promote direct, unmediated electron transfer between the electrode and the favorably oriented protein. They should be distinguished from "mediators", which enter the electron-transport pathway between the protein and the electrode by conveying electrons to or from the bulk solution, thanks to their redox properties. Surface modification can also be realized by species in solution that are only reversibly adsorbed. This is the case of multivalent cations, such as Mg^{2+} and $Cr(NH_3)_6^{3+}$, which can promote the reversible electrochemistry of proteins possessing negatively charged interaction domains, such as plastocyanin and ferredoxins, at negatively charged electrodes.

Reversible, diffusion controlled electrochemistry of water soluble redox proteins allows a direct and relatively simple determination of their formal reduction potentials and diffusion coefficients. As a rule, these formal potentials coincide with those obtained by indirect potentiometric methods, in which small redox mediators are employed to facilitate electrochemical equilibration during spectroscopically monitored titrations of redox centers. One advantage of direct electrochemical methods is the possibility of measuring the redox activity of proteins over a wide potential range without the limitations imposed to indirect potentiometric methods by the properties of the mediators. The formal reduction potential of a redox couple depends on the environment of the couple. This explains the small differences sometimes observed between formal potentials obtained by direct electrochemical methods and those obtained by indirect potentiometric procedures. This occurs when the protein-promoter-electrode interactions involved in the former methods become significant. Differences in adsorptivity between the oxidized and the reduced form of the redox protein may also be responsible for deviations of the formal potential with respect to that obtained in the bulk solution. This is not necessarily a drawback of direct electrochemical methods, because under physiological conditions water-soluble proteins bind with natural redox partners, many of which are incorporated in biomembranes; therefore, they experience an environment somewhat different from a purely aqueous one.

2.2. Electrochemistry of Water Soluble Redox Proteins Immobilized on the Electrode Surface

A different electrochemical approach for the study of water-soluble proteins consists in immobilizing them on the electrode surface by electrostatic or covalent interactions in the form of a stable mono- or submonolayer film. If the film is favorably oriented for electron transfer and retains its native structural and reactivity characteristics, this approach offers some advantages over diffusion-limited electrode reactions. Thus, it is sensitive to trace amounts of the reactant and is more suitable for monitoring complex reactivities and for elucidating some subtle properties of redox enzymes. The functionally active state of immobilized proteins can be probed directly, by in situ spectroscopy, or indirectly, by confirming the intrinsic properties of the protein in the adsorbed state.

A familiar procedure for immobilizing proteins makes use of self-assembled monolayers (SAMs) of functionalized alkanethiols, anchored covalently to the electrode surface via their sulfhydryl group. Thiols terminated with a carboxyl group engage multiple electrostatic interactions with the lysine groups of cytochrome *c* in the vicinity of the exposed heme edge of the protein, thus favoring electron transfer to and from the electrode. Cytochrome *c* was also covalently attached to monolayers of ω-alkanethiol carboxylic acids using carbodiimide coupling to the superficial lysine residues of this protein. Electrostatically immobilized mono/submonolayer films of proteins are unstable at high ionic strengths, due to the well-known decrease in electrostatic interactions, whereas covalently immobilized films are stable indefinitely at all ionic strengths. In some cases, no modification of the electrode surface, apart from normal pre-treatment, is required to induce immobilized adsorption; this is the case for certain redox proteins at carbon and metal oxide electrodes.

Reversible (Nernstian) electron transfer at immobilized mono/submonolayers of redox proteins is readily distinguished from reversible electron transfer under diffusion-controlled conditions, by using cyclic voltammetry. Thus, the cyclic voltammograms for reversible *n*-electron electrode reactions controlled by linear diffusion have the familiar shape of Figure 2a, in which the oxidation and reduction peaks are 59/*n* mV apart and their heights are proportional to the square root of the scan rate.

When using a rotating electrode the diffusion layer thickness is kept constant, and the cyclic voltammogram assumes the sigmoidal shape of Figure 2b, with the half-wave potential coinciding with the formal potential of the redox couple; in this case the current is independent of the scan rate but increases with the square root of the rotation rate. In some cases, the inhomogeneity of the electrode surface is such that the protein is able to interact in a productive manner only on certain areas of the electrode.

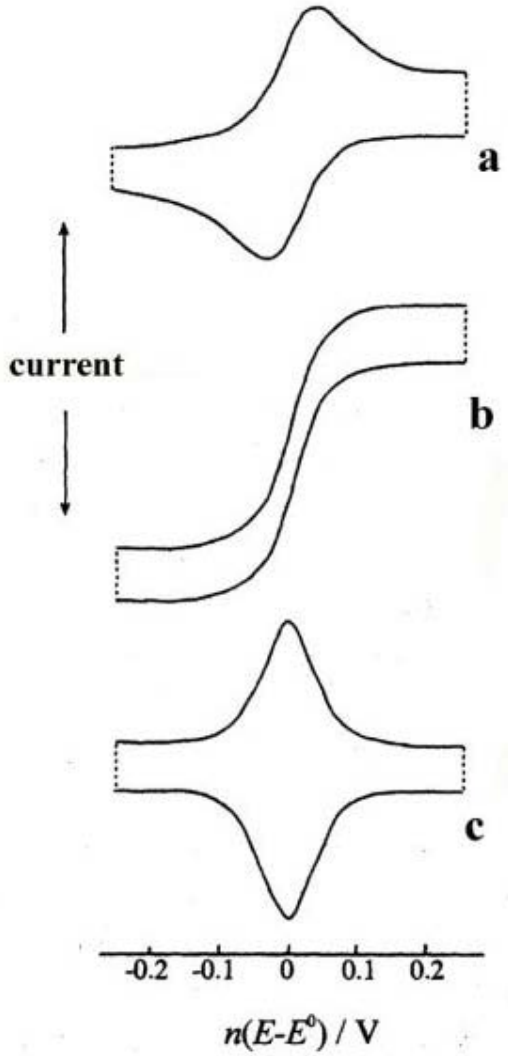

Figure 2. Cyclic voltammogram for a reversible charge transfer process: (a) under diffusion control; (b) under forced convection at a rotating electrode; (c) with an immobilized redox species. [Armstrong F.A. in: Lenaz G., Milazzo G. (Eds.), *Bioelectrochemistry of Biomacromolecules*, Birkhäuser, Basel, 1997].

If the size of these areas is smaller than the time-dependent diffusion-layer thickness while their mutual separation is greater, then protein diffusion toward these active areas becomes spherical rather than linear, and the cyclic voltammogram takes the sigmoidal shape of Figure 2b even in the absence of stirring. Shapes intermediate between those in Figures 2a and 2b are encountered when the diffusion-layer thickness becomes comparable with the size of the active areas and/or with their separation. Conversely, the cyclic voltammogram for reversible charge transfer at an immobilized mono/submonolayer of a redox protein has the bell-shaped form of Figure 2c, in which the oxidation and reduction peaks have the same height and the same peak potential, which coincides with the formal potential of the adsorbed redox couple. Moreover, the peak height is directly proportional to the scan rate.

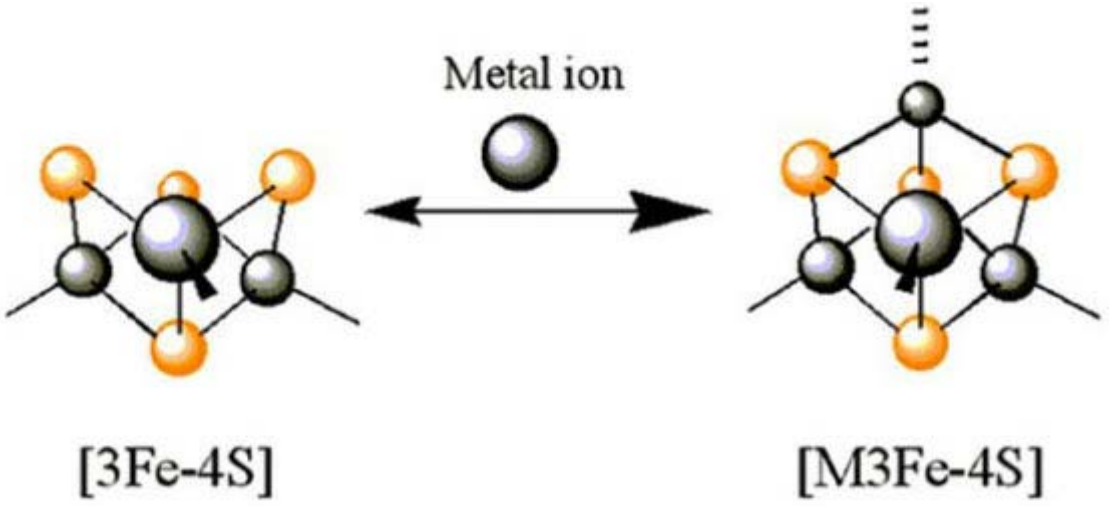

Figure 3. A [3Fe-4S] cluster, which is capable of binding iron or a different metal ion into the vacant position, yielding the "cubane" [M3Fe-4S]. FeS clusters occur in ferredoxins and other electron transfer proteins. [Armstrong F.A., http://www.chem.ox.ac.uk/icl/faagroup/clusters.html].

The special properties of an immobilized configuration have been extensively exploited for the study of [3Fe-4S] clusters in the corresponding iron-sulfur proteins (see Figure 3). In these clusters, which are linked to the residues of the polypeptide chain of the protein, the Fe and S atoms occupy the alternate vertices of a cube, except for one vertex, which is vacant and is open for coordination of a fourth metal ion M (e.g., Fe, Zn, Cd, Tl, Cu). This gives rise to a complex network of elementary electrochemical and chemical reactions, which are revealed by multiple reversible or quasi-reversible cyclic voltammetric peaks. Thus, each iron atom of the [3Fe-4S] cluster can exist as Fe(II) and Fe(III), so that each cluster in the fully oxidized state, +1, can accept up to three electrons in one-electron transfer processes. Each of these three different oxidation states may coordinate a fourth metal M ("metallation"), yielding a "cubane" [M3Fe-4S], linked via M to a ligand X, normally an amino acid residue; the tendency to form cubanes is generally different for the different oxidation states. The resulting cubanes may be converted into each other by exchanging electrons with the electrode. The M atom of the cubane may also substitute the ligand X with an alternative, exogeneous ligand L ("ligation"); again, the propensity for changing ligand depends on the oxidation state. Oxidations or reductions concerted with a metallation or a ligation are not infrequent. Cyclic voltammetry of immobilized mono/submonolayers is particularly suited for studies of these electrochemical and chemical reactions, including those leading to clusters that cannot be generated in the bulk solution by chemical titrants such as dithionite because of their very negative reduction potential and of their sensitivity to oxygen.

The immobilization procedure is also successfully applied to the study of redox enzymes. Flavoenzymes catalyze oxidation-reduction reactions in which the flavin group of flavin adenine dinucleotide (FAD) is the oxidizing or reducing agent. This relatively small molecule (the "coenzyme") is sometimes covalently bound to its host protein, as in the case of fumarate reductase from *Escherichia coli*.

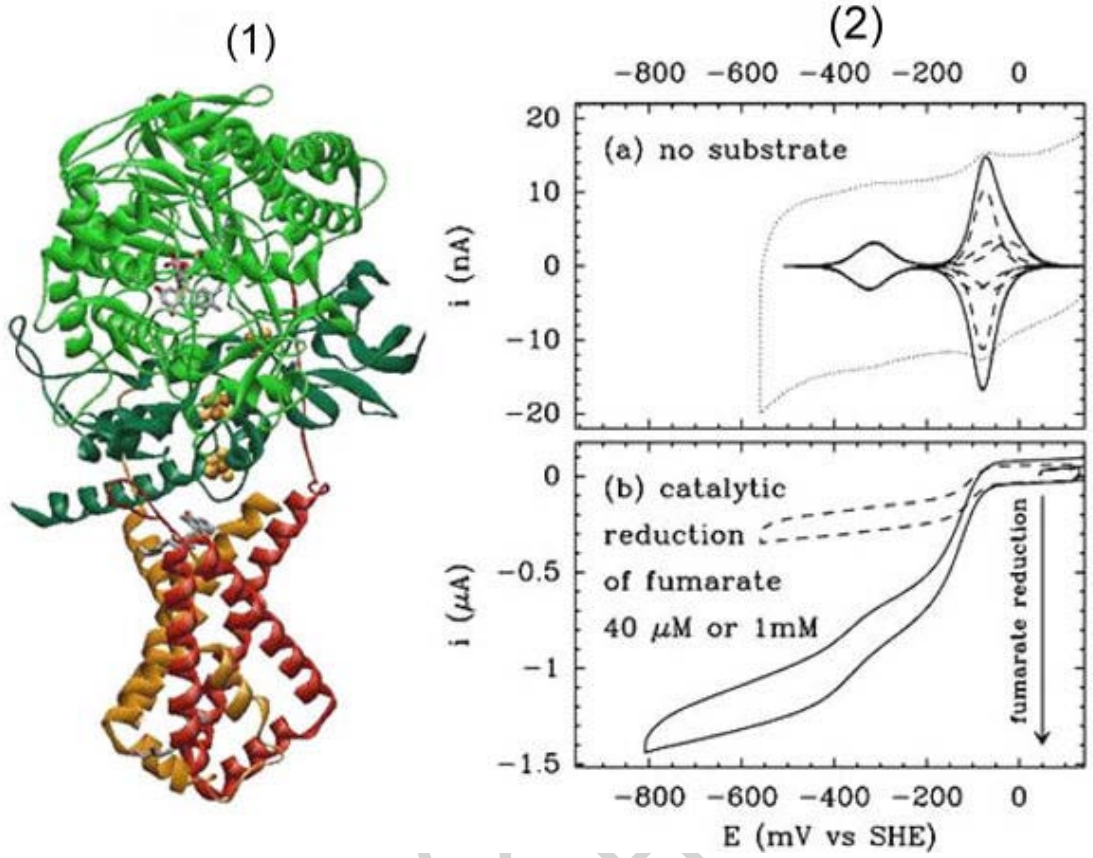

Figure 4. (1) Structure of fumarate reductase. The two water-soluble subunits are: the flavoprotein (in light green), containing a covalently linked FAD; the iron-sulfur protein (in dark green), containing the three iron sulfur clusters [2Fe-2S], [4Fe-4S] and [3Fe-4S]. The two intramembrane subunits are in orange and red. The redox cofactors are arranged in the sequence FAD-[2Fe-2S]-[4Fe-4S]-[3Fe-4S]. (2) Cyclic voltammograms of the FAD/$FADH_2$ redox couple, both in the absence (a) and in the presence of fumarate (b). The solid curve in (2a) was obtained from the corresponding dashed one upon correction for the large capacitive contribution. [Armstrong F.A. http://www.chem.ox.ac.uk/icl/faagroup/clusters.html].

This is an integral protein that catalyzes the reduction of fumarate to succinate (see Figure 4(1)). It consists of four subunits, two of which protrude from the membrane surface and are catalytic, while the other two are incorporated in the membrane and provide a binding site for the natural electron donor metaquinol, which is also membrane-confined. Fumarate (the "substrate") is reduced to succinate by $FADH_2$, the reduced form of FAD, at a substrate transformation site within the enzyme. The liposoluble metaquinol then conveys two electrons to FAD along some electron transfer pathway through the enzyme. This causes metaquinol to be oxidized to the corresponding quinone, while FAD is reduced back to $FADH_2$. The two water-soluble, catalytic subunits can be separated from the two membrane-confined subunits, and can then be strongly adsorbed on a pyrolytic graphite edge electrode. In this case, the electrode replaces menaquinol as the electron donor. In the absence of fumarate, the FAD/$FADH_2$ redox couple gives rise to a small reversible cathodic peak and to the

corresponding anodic one, as shown in Figure 4(2). The electron transfer from the electrode to the electron entry/exit site(s) of the enzyme is, therefore, very fast. Addition of fumarate converts the cathodic peak for FAD reduction into a sigmoidal catalytic reduction wave of shape similar to that in Figure 2b, since the current decay at sufficiently negative potentials is prevented by the continuous FAD regeneration via the chemical oxidation of $FADH_2$ by fumarate at the catalytic site of the enzyme.

The mitochondrial succinate-ubiquinone oxidoreductase (complex II) is an integral protein structurally analogous to fumarate reductase, which can likewise be prepared as a membrane-extrinsic, water soluble form, called succinate dehydrogenase. It also catalyzes both succinate oxidation and fumarate reduction. However, at pH values < 7, the voltammetric curve for fumarate reduction decays toward more negative potentials, instead of increasing. Since this decrease occurs over the potential region of FAD electroreduction, it was tentatively explained by the enzyme favoring a less active conformation in its FAD-reduced form.

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Biographical Sketch

Rolando Guidelli was born in Florence on December 27, 1938. He obtained the Italian Degree in Chemistry at Florence University (Italy) in March 1963, was appointed by Florence University as a lecturer in Electrochemistry in 1970, and then, in 1971, was promoted full professor of Electrochemistry in the Faculty of Science of Florence University.

He has been dean of the Faculty of Chemistry of Florence University, president of the Electrochemical Division of the Italian Chemical Society, national secretary of the International Society of Electrochemistry, director of the Department of Chemistry of Florence University, member of the Advisory Board of the VCH Series "Frontiers of Electrochemistry", member of the Council of the Bioelectrochemical Society, chairman of the "Journées d'Electrochimie 85" (Firenze, May 1985), director of the NATO Advanced Study Institute on "Electrified Interfaces in Physics, Chemistry and Biology" (Varenna, July 1990), chairman of the XVII International Symposium on Bioelectrochemistry and Bioenergetics (Florence, June 2003). He is the recipient of the 2005 "Katsumi Niki" Prize for Bioelectrochemistry of the International Society of Electrochemistry. He has written more than 190 papers and four monographs in important book series in the field of Electrochemistry. His scientific interests were first focused to the kinetics of electrode processes, and then to the structure of the metal |

(aqueous solution) interphase from both a modelistic and a phenomenological viewpoint. In the 1990s he started exploiting his background on interfacial electrochemistry to interpret processes of biological relevance that take place in biomimetic membranes consisting of metal-supported lipid monolayers, bilayers and proteoliposomes.

Prof. Guidelli is member of the Bioelectrochemical Society and of the Biophysical Society, member and fellow of the International Society of Electrochemistry, member of the Editorial Board of the Journal of Electroanalytical Chemistry and associate editor of “Bioelectrochemistry”.