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Protein Electronic Conductors: Hemin–Substrate Bonding Dictates Transport Mechanism and Efficiency across Myoglobin

Sara Raichlin, Israel Pecht, Mordechai Sheves,* and David Cahen**

Abstract: *Electron transport (ETp) across met-myoglobin (m-Mb), as measured in a solid-state-like configuration between two electronic contacts, increases by up to 20 fold if Mb is covalently bound to one of the contacts, a Si electrode, in an oriented manner by its hemin (ferric) group, rather than in a non-oriented manner. Oriented binding of Mb is achieved by covalently binding hemin molecules to form a monolayer on the Si electrode, followed by reconstitution with apo-Mb. We found that the ETp temperature dependence (>120 K) of non-oriented m-Mb virtually disappears when bound in an oriented manner by the hemin group. Our results highlight that combining direct chemical coupling of the protein to one of the electrodes with uniform protein orientation strongly improves the efficiency of ET across the protein. We hypothesize that the behavior of reconstituted m-Mb is due to both strong protein–substrate electronic coupling (which is likely greater than in non-oriented m-Mb) and direct access to a highly efficient transport path provided by the hemin group in this configuration.*

The potential incorporation of biomolecules not only in highly sensitive biosensors[1] but also as active components in bioelectronics[2] (at times termed "all-natural" electronics), particularly in solid-state devices, is driving the investigation of proteins as potential electronic materials. One approach explores the use of proteins in a solid-state-like environment, that is, where only strongly bound water molecules that maintain the natural conformation of the protein remain. Resolving the parameters that govern protein electron transport (ET) efficiency in solid-state electronic junctions is central to understanding and, subsequently, controlling such junctions and the devices that contain them.

Proteins capable of electron transfer are prime candidates for the study of ETp in solid-state electronic junctions. We have studied the ETp properties of various proteins, including azurin (Az)[3,4] and cytochrome C (Cyt C).[5] We also explored ETp in human serum albumin (HSA) by doping it with hemin, which made HSA redox-active.[6] Our results reveal that ETp is greater via proteins with an embedded cofactor than for those without. But, remarkably, even those without a cofactor show significantly higher current densities than saturated alkyl chains.[3]

Myoglobin (Mb), a biological oxygen-carrier present in all mammals, is also amenable to redox processes.[7] Its O_2 binding and electron transfer involve its prosthetic group, heme, a conjugated macrocycle with a redox-active Fe ion.[8] In our system (where the protein is placed in a buffer solution before forming a dry monolayer), Mb is in its oxidized form, met-Mb (m-Mb), and contains a hemin, in which the iron is in the ferric (3+) state, instead of a heme, where the iron is in the ferrous (2+) state. The hemin/heme group of Mb is quite exposed to the environment, representing a potential protein-immobilization anchor for ET and ETp studies. In this respect, the hemin/heme group resembles the exposed disulfide bond of Az, which can serve as a binding anchor. Mb, therefore, offers a means to evaluate whether the unique behavior, temperature-independent ETp, observed via holo-Az,[4] is due to the covalent binding of the protein to one of the electrodes, to its defined orientation to the electrodes or both. Moreover, Mb may also be used, at a later stage, to study the effects of chemical reactions on protein ETp by way of ligand binding to Mb. Here, we report the marked impact of the chemical linkage of Mb and orientation to the substrate/electrode on ETp across m-Mb.

Since the isoelectric point (pI) of Mb is about 7, the protein is neutral in solution under our experimental conditions. Mb can be made to covalently bind, by its carboxylic side residues, to an amine-terminated electrode surface with the aid of EDC/NHS cross-linking molecules (EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, NHS = *N*-hydroxy-succinimide).[9] As Mb contains 19 amino acids with carboxylic side residues,[10] EDC/NHS cross-linking yields a non-oriented m-Mb monolayer, with the proteins bound to the Au surface via only a few amino acids (instead of electrostatic binding via multiple interaction sites). We, therefore, assumed that this type of binding to the surface helps preserve the secondary structure of Mb. Figure 1a shows the absorption spectrum of the m-Mb solution versus that of the m-Mb monolayer. The Soret band of hemin at 409 nm did not significantly change following binding of the protein to the surface and drying it. Further characterization of the monolayers by IR spectroscopy (Figure 1b) was performed in order to monitor the amide I & II peptide-bond IR peaks. In many cases, the amide I band (at 1656 cm^{-1}) reflects the secondary

[*] S. Raichlin, Prof. D. Cahen
Department of Materials and Interfaces
Weizmann Institute of Science
Rehovot 76100 (Israel)
E-mail: David.Cahen@weizmann.ac.il
S. Raichlin, Prof. M. Sheves
Department of Organic Chemistry
Weizmann Institute of Science
Rehovot 76100 (Israel)
E-mail: mudi.sheves@weizmann.ac.il
Prof. I. Pecht
Department of Immunology, Weizmann Institute of Science
Rehovot 76100 (Israel)
E-mail: israel.pecht@weizmann.ac.il

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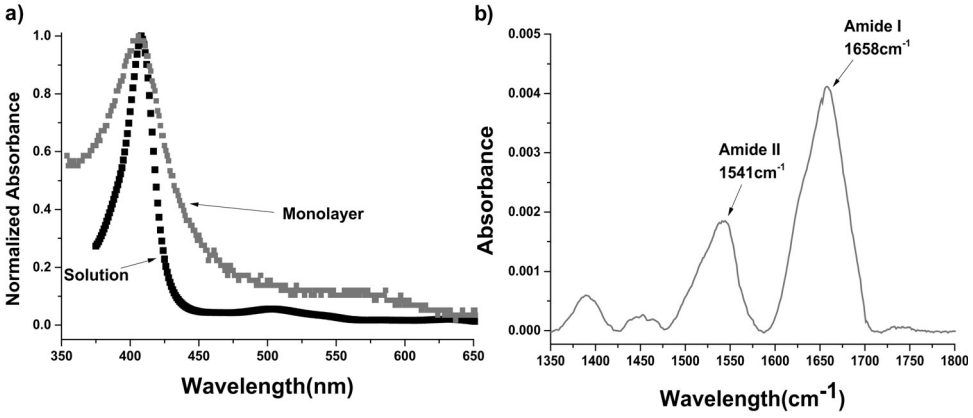

Figure 1. a) UV/Vis spectra of myoglobin in solution and as a dry monolayer on quartz. b) IR (PM-IRRAS) spectrum of Au-coated Si surface, covered with a m-Mb monolayer. For the IR measurements the m-Mb monolayer was formed by first binding mercapto-propionic acid to a Au surface and covalently binding the amine side groups of m-Mb to the carboxylic acids of the modified Au surface.

structure of proteins.[11] As the amide I position of m-Mb in its monolayer and solution forms[12] was similar, we deduced that the secondary structure of the protein was preserved in the monolayer.

Here, we present our ET results for native m-Mb and apo-Mb covalently bound to the electrode by amide bonds, and of reconstituted m-Mb covalently bound to the electrode by the hemin group (see below). We characterized m-Mb and apo-Mb in solution by measuring optical absorption and circular dichroism spectra (see the Supporting Information). As expected, the absorbance measurements of apo-Mb in solution showed the disappearance of the Soret band, whereas in native m-Mb a strong Soret band was present (cf. Figure S1 in the Supporting Information), indicating that the desired derivative was obtained. The circular dichroism spectra (Figure S2) led to the conclusion that removing the hemin group does not disrupt the secondary structure of the protein. The reconstituted m-Mb monolayer was characterized both in solution, by fluorescence, and in the solid state, by optical absorption of the monolayer. As can be gathered from the fluorescence measurements, adding hemin to the solution quenched the apo-Mb emission signal of the two tryptophans in the protein (Figure S3). Our absorbance measurements revealed the successful reconstitution of apo-Mb with the hemin monolayer. The Soret band in both the solution and in the solid state (monolayer) is highly sensitive to the hemin surrounding.[13] The absorbance measurements further show a clear shift in the Soret band of the hemin-modified surface (and also in solution) when incubated with apo-Mb (Figure S4 B + C). This shift indicates that the apo-Mb was reconstituted with the hemin molecules.

To ascertain that the coupling and/or orientation of the protein to the electrodes affect ETp across m-Mb, we compared the ETp of non-oriented and oriented m-Mb. In earlier work, we demonstrated that the position of the cytochrome c prosthetic group on the substrate and the orientation of the protein relative to the electrodes can affect ET efficiency, and even its mechanism.[14–16] A defined orientation can be achieved by binding the protein by a specific group on its surface to a suitable substrate, as was found for Az.[14,17–20] We took advantage of the fact that the hemin group of Mb is non-covalently bound and partially exposed to form an m-Mb monolayer with a controlled orientation and to couple the protein by its hemin group to the surface. Protein binding to surfaces, mainly those of enzymes,[21] is a well-studied subject. In the case of myoglobin, the literature contains examples of apo-Mb reconstitution, mostly in solution,[22] but also on surfaces.[13,23,24]

For ETp measurements, native and apo-Mb were adsorbed on highly doped Si, onto which a SiOx layer of about 1 nm was regrown, to which subsequently APTMS was bound. Next, Mb or apo-Mb was bound to the APTMS-terminated surface, using EDC/NHS, via amide bonds (see Materials and Methods section in the Supporting Information). The formed monolayers were then characterized by AFM for topography, roughness, thickness and coverage density and by ellipsometry for thickness estimates (see Figures S5 and S6). To bind the hemin covalently to the surface we employed the EDC/NHS cross-linking reagents in order to form covalent bonds between the carboxylate residues of hemin and the amine-terminated Si surface. The final step in the reconstitution process was achieved by incubating the apo-Mb solution with the hemin-terminated surface overnight, as illustrated schematically in Figure 2. In this manner, we achieved specific binding of the hemin to the protein by its specific binding site in an oriented form. We found the thickness of the reconstituted surface to be almost identical (about 20 Å) to that of the native m-Mb monolayer, as deduced from ellipsometry analysis (see Table on page 1 of the Supporting Information). An Hg drop mechanically placed on the protein monolayer served as the top electrode; the geometric contact area was about 0.02 mm^2, as determined by optical microscopy. Ready-made Au pads, deposited by the lift-off float-on (LOFO) method,[25] were used as the metal top electrodes in control measurements, and were employed specifically to check for the possible contribution of Hg to the measured currents caused by possible Hg penetration via pinholes. The currents varied within a factor of 2.5, which is within the margin of error of the effective contact area (Figure S8). Packing densities of both native m-Mb and the hemin monolayer were deduced from analyzing AFM images of the m-Mb monolayer (Figure S5) and from optical absorbance measurements of a single layer of hemin (Figure S4B, inset) to be 80 % and 55 %, respectively (see detailed calculations on page 6–7 of the Supporting Information).

Measurements of ETp across the m-Mb monolayers were carried out as detailed in the Supporting Information. Measurements at low temperatures revealed that the currents

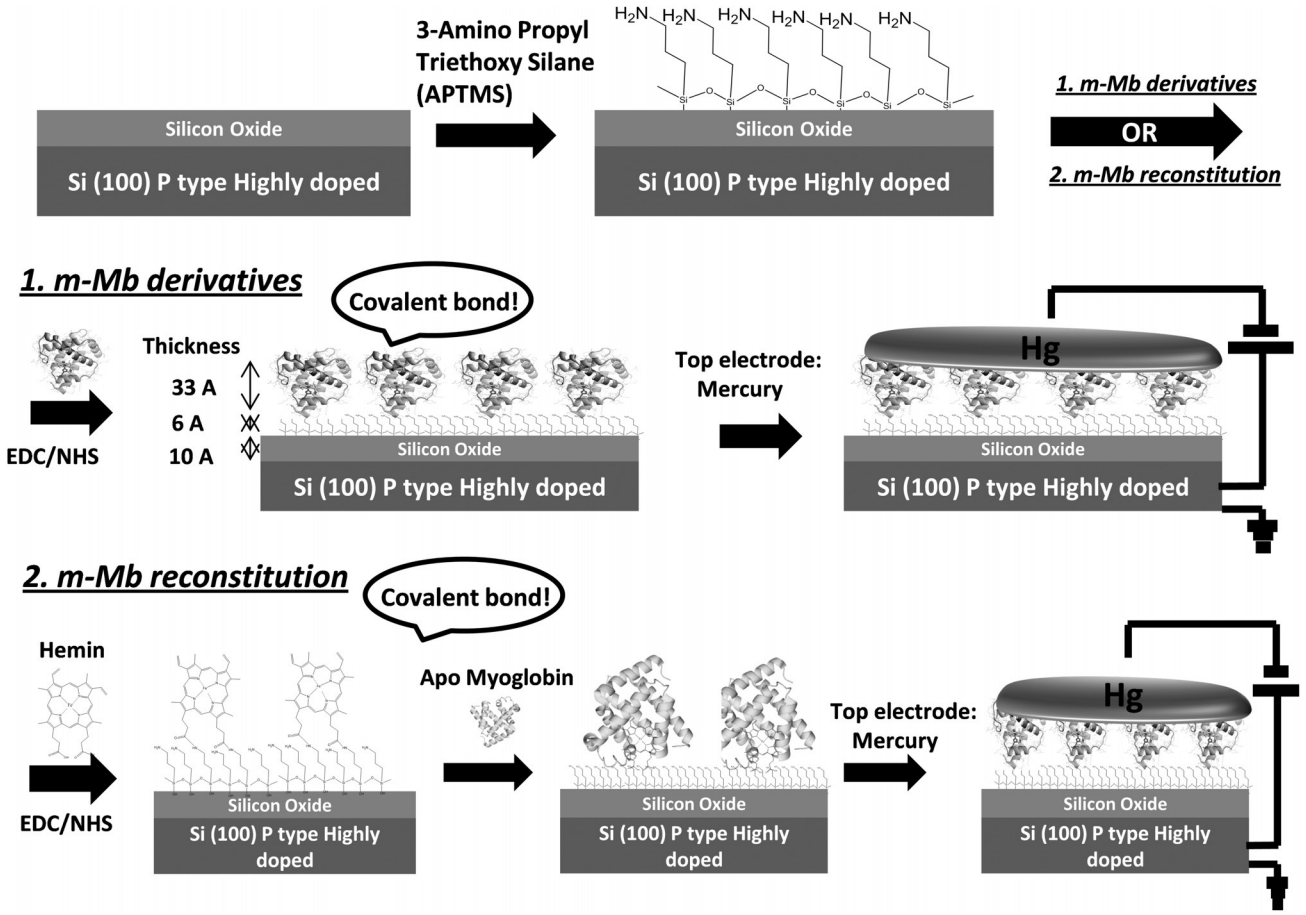

Figure 2. Scheme illustrating the process of preparing native m-Mb monolayers and reconstituting m-Mb samples.

via non-oriented m-Mb and apo-Mb are temperature-independent below 120 K, though currents via these two Mb variants differ markedly in their amplitudes. At higher temperatures, the currents are thermally activated, with small activation energies of approximately 50 meV (calculated from Arrhenius plots of the thermally activated currents). Remarkably, ETp across reconstituted, oriented m-Mb is essentially temperature-independent over the whole temperature range measured (Figure 3, see below). As the temperature was increased, currents via all the layer types tested converge to a particular value range (Figure 3). We found temperature-dependent ETp to be consistent with hopping as the dominant mechanism.[26] m-Mb and, in particular, its α-helices, which comprise about $^{3}/_{4}$ of the protein, can be viewed as a possible bridge with multiple hopping sites, each with vibronic broadening.[27–30] As the temperature increases, so does the degree of phonon–electron coupling and level of broadening. Thus, coupling between adjacent sites suffices to create an efficient conduction path that does not involve the hemin moiety. This scenario is in line with the observed similarity in ETp activation energies of native and apo Mb, as well as their current magnitudes at room temperature, suggesting that the integrated energy barrier for conduction via the protein itself is not strongly dependent on the presence of the hemin group. At low temperatures, the molecular vibrations are weak and, therefore, different pathways can dominate ETp. Judging from the differences in currents at lower temperatures, it appears that those paths strongly depend on the presence of hemin (lower

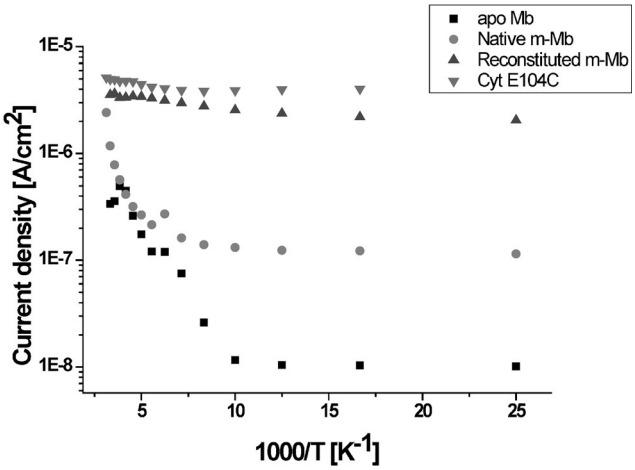

Figure 3. Current–temperature dependence of native m-Mb (●), apo-Mb (■), reconstituted m-Mb (▲) and Cyt E104C (taken from Ref. [14]; ▼) at a constant bias of 0.05 V. A 2–2.5 times increase in the current density occurred from 40 K to 300 K through reconstituted m-Mb (corresponding to an activation energy of 10 meV, i.e., 0.4 kT at RT). The standard deviation of each measurement is about 5 percent of the mean current magnitude.

currents for apo-Mb than for native m-Mb). As hemin is a large conjugated molecule with delocalized electronic states, it can be considered a dopant within the protein's peptide matrix. This view is consistent with super-exchange-mediated off-resonance tunneling being the mechanism governing temperature-independent ETp, where the average energy barrier between the states for tunneling and the electrodes' Fermi levels is smaller for native m-Mb than for apo-Mb. We performed control experiments of temperature dependence of ETp across a monolayer comprising only the linker (metal-linker-metal junction). Our results demonstrate the expected (and earlier reported[31]) temperature independence of the currents, with a magnitude typical for a 1 nm organic layer (see Figure S7).

ETp across the reconstituted, that is, oriented m-Mb monolayer at room temperature is more than ten-fold higher than that via the covalently bound and randomly oriented m-Mb. The likely reasons for this difference are the orientation of m-Mb relative to the electrodes and the direct coupling of the protein through hemin to one of the electrodes. This finding is consistent with our previous ETp results via Cyt C[15] showing a marked difference in ETp efficiency between Cyt C bound in a non-oriented manner to the electrode (native Cyt C) and mutants bound in an oriented manner (cf. Cyt C E104C mutant in Figure 3). Such a change in conduction may be caused by the operation of different ETp mechanisms, namely, tunneling at all temperatures for coupled oriented proteins like azurin, and hopping, above 120 K for other proteins.

The orientation of reconstituted m-Mb relative to the electrodes is analogous to that of the Cyt C E104 mutant (which shows nearly temperature-independent ETp), because in both cases the shortest distance from the porphyrin ring to one of the electrodes is 4–6 Å. Still, there is one important difference: The Cyt C mutant covalently binds to one of the electrodes via a Cys thiolate residue, and, even though the second contact is a physical one, the porphyrin edge of the protein can be close to that electrode, which may allow for efficient coupling to that electrode. In the reconstituted m-Mb, however, it is the hemin that covalently binds to one of the electrodes, while the other contact is only physical (see estimated distances between the hemin and both electrodes in Figure S4 A). This finding, therefore, raises the question of whether the contact between the top electrode and the reconstituted m-Mb is indeed purely mechanical, that is, only by van der Waals interactions, which is an issue for further studies.

We have, thus far, observed ETp that is completely temperature-independent via holo-Az and halorhodopsin.[4,31,32] This type of ETp suggests (coherent) tunneling up to room temperature. Az is oriented in relation to the surface via the covalent bonds between its cysteine thiolate residues and the surface. We hypothesize that its temperature-independent ETp is due to the existence, in addition to the covalent bond between the protein and one electrode, of strong electronic coupling between the other end of azurin and the second electrode. That latter end contains the Cu ion with its first coordination shell being less than 5 Å away from its respective electrode. In such a configuration, the electrode contact may well be more efficient than otherwise.

The striking difference in ETp between randomly covalently bound m-Mb and reconstituted oriented m-Mb may be due to the existence of a highly efficient conduction path involving a specific orientation of the hemin group. We assume that in any other orientation of covalently bound m-Mb, at least part of the conduction will have to pass across the peptide matrix (in order to reach the efficient conduction path). In the latter case, hopping, a thermally activated process, is likely the most efficient mechanism at room temperature. As the temperature drops, the efficiency of thermally activated hopping will decrease, to be eventually replaced by tunneling at temperatures below 120 K.

The efficiency of ETp through a dry m-Mb monolayer covalently bound to an electrode is significantly higher when the m-Mb is oriented in relation to the electrode than when it is not oriented. Even lower ETp is observed via randomly and covalently bound apo-Mb. We found negligible temperature dependence of ETp via the oriented m-Mb monolayer between 40–350 K, suggesting that tunneling is the dominant transport mechanism in this temperature range. This is apparently due to both the covalent binding of the protein via its hemin group to the electrode, which orients the protein, and the conjugated nature of the hemin, which may allow highly efficient protein–electrode electronic coupling.

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