

A SYNTHETIC SYSTEM MIMICS THE ELECTRON TRANSFER IN MEMBRANES: DEFINITION OF THE PROTEIC SCAFFOLD

Cristina Cantale¹, Mariastella Oriolo¹,
Maria Sperandei¹, Maurizio Paci²

¹ ENEA INN/BIOAG-BIMO Via Anguillarese, 301, 00060 S. Maria di
Galeria Rome, Italy

² Department of Chemistry, University "Tor Vergata", Via della Ricerca
Scientifica, 00133 Rome, Italy

Abstract: A great effort in the research world has been devoted to mimic the electron transfer of biological systems. The assembling of simplified proteic structures, molecular maquettes, with photosynthetic cofactors (chlorophylls, carotenoids, quinones) is considered a promising tool to gain relevant informations, allowing to study the main interactions, without the natural systems complexity. The pioneer works of Dutton, DeGrado and coworkers (ranging from 1984 up today) have largely contributed in developing this approach, with a series of peptides binding up to four haem redox groups, which exhibit native-like properties in aqueous solution. We intend to carry out such an approach in simulated membrane systems (liposomes and Langmuir-Blodgett monolayers). A *de novo* designed peptide was built, as the scaffold for a simple donor-acceptor system to be put inside a lipid film. A wide selection of the available literature concerning protein structures in membranes was examined, including structural motives like coiled coil and leucine zipper, the signal peptides, large structures like the light harvesting complex and the photosynthetic reaction center, together with studies of the aminoacid propensity in the different membrane portions. The designed peptide is 16 aminoacids long and it is planned to contain an histidine as linker for the porphyrine donor and it is covalently bound by its N-term portion to a loop system incorporating the acceptor, namely a substituted quinone. The peptide was synthesised by SPPS methodology, using *Fastmoc*, HBTU protocol. The product was evaluated by mass spectrometry and high-performance liquid chromatography (HPLC). Subsequently, it was purified to be used in NMR and CD experiments, in order to investigate the structure of this element of the whole system.

Keywords: Bioelectronics, *de novo* design protein, electron transfer

INTRODUCTION

Biosensors represent a powerful new evolutionary thrust in analytical measurement technology. However the high potentiality of this new sector still suffers of important limitations which smother a wider commercial development.

The main bottlenecks include stability of biological components and the complex interface between biological and electronic components. For designing future chemical and biochemical sensors, it is necessary to investigate more fully this interface and to understand the electrical interactions at molecular level in biology.

So, progress in biosensor development are strictly related to bioelectronics. Bioelectronics is concerned with the study of the interface between biological and electronic systems, particularly at or below nm scale. There are three particular aspects of this subject that can be considered a future challenge, namely biological sensing, biological microcircuits and bioelectronic information storage.

On the other hand, the miniaturization of electronic devices still is the driving force in the research and development of new materials for the future and it is inevitable that the new frontiers are going towards the mimic of biological systems.

From this point of view, the photosynthetic reaction center, due to its characteristics, represents the ultimate limit for a man-made intelligent biomolecular structure:

- a molecular structure controlled in the nm scale,
- switched by energy differences in the eV range,
- at a time scale of picoseconds.

The study of simplified prototype systems characterized by less structural complexity can be a way to approach this limit, so the future trend is towards the use of engineered proteins or protein-like chemical.

In this context one can place the successful results obtained by Dutton, DeGrado and coworkers, who were able to obtain a native-like system able to produce an electron transfer in solution. They have been protagonists in the experiments aimed at obtaining designed proteins that self-assemble into a protein with native-like properties and finally they communicated the construction of a soluble four-helix bundle protein that incorporate four heme redox sites and two cofacially coupled coproporphyrin I.

Our intention was to design a minimal system based on a peptidic scaffold, able to produce an electron transfer by selected cofactors in Langmuir-Blodgett films.

PROCEDURE

Peptide design

The possibility to design a peptide able to fold in a defined way is a complex problem which solution is not obvious and only recently molecules sharing properties with natural proteins have been produced.

To design the peptide sequence, we refer to the available knowledge concerning the characteristics and the behaviour of natural and designed alpha helix, bundles and coiled coils.

Experiments to elucidate which aspects of sequence specify a fold are going on since a long time [1-10]. In particular,

some structural motives, like alpha-helical coiled coils and other noncovalently assembled helical bundles - which are largely present in natural proteins, where they serve a variety of functional and structural roles - have been systematically studied [11-14]. The results of these studies have greatly advanced the present understanding of the correlations between secondary structure propensities, binary pattern, buried polar interactions, capping interactions, turns, complementary packing, helix-helix interactions and protein folding and assembling. In solution, GCN4 leucine zipper, Rop protein, signal peptides and de novo designed derived systems are largely studied as model systems [15-21]. In membranes, where the same structural motives are predominant, other model cases, like the TM domain of glycoporphin A, phospholamban and the M2 proton channel from influenza A virus have been currently taken into account, together with studies concerning the partitioning of protein in lipid bilayers and the reciprocal influences on structure [22-31]. Recent advances in NMR, cristallography and some spectroscopic tecniques have been also fundamental for approaching these studies in membrane systems.

Following the overall conclusion, we designed a molecule based on two homologous peptides, which sequence is compatible with an alpha helix inside the lipid monolayer. A general scheme of the system is shown in Fig. 1.



Figure 1 Schematic design of the electron transfer mimic system, with the highlighted histidines complexing the porphyrine

The selected sequence of the proteic scaffold is :

GPSASVFILFILVHAW

The aminoacids were selected taking into account the observed helical propensity [24], the substitution tables for lipid facing residues in membrane environments [26] and the reported thickness. A schematic design of a natural membrane is reported in Fig. 2.

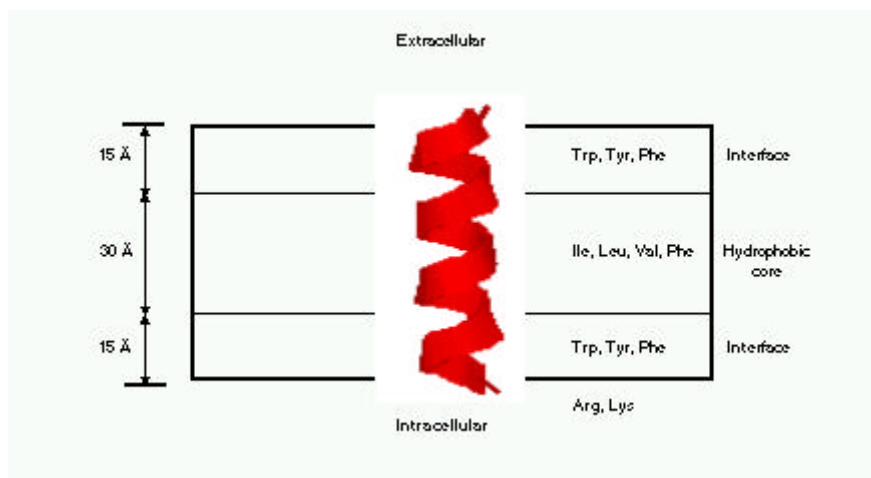


Figure 2 The schematic design shows the dimension of a lipid membrane and the distribution of the aminoacids in TM α -helix.

Mimicking the proposed structure of the cytochrome *b* subunit of the *bc1* complex, where two hemes are sandwiched between two parallel TM helices by two pairs of histidine residues [32], we added an histidine residue to complex a metalloporphyrine. Taking into account the effect of the electric field generated by the the dipole of an alpha-helix on the rate of electron transfer [33], the histidine residue was located near the C-terminus. The metalloporphyrine represents the donor in an electrons donor-acceptor system. As it is reported that the interface region is enriched in aromatic aminoacids, a tryptofan residue was added at the C-terminus. This residue is also necessary in order to accurately measure the peptide concentration and the main residue ellipticity [34].

Due to the sharp requirements for a functional electron transfer protein [35], phenylalanine residues were added in right position to exert a tunneling function with respect to the donor-acceptor

system [36]. Proline is reported as a helix promoter. Serine, which has been identified as a good N-capping residue [1], was used at the first position of the helix. A glycine was added at the junction with the apoteic portion of the system. This was done in view to increase the flexibility in that particular region by exploiting the ability of glycine to adopt several conformations.

According to natural systems, a quinone based system was chosen [37] as the electron acceptor, covalently bound to the amide group at N-terminus.

The selected sequence was submitted to the Peptide Prediction server AGADIR (<http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html>) [5] which confirmed a good probability to have helical conformation, mainly in the central portion of sequence.

MATERIAL AND METHODS

The peptide was synthesised by solid phase peptide synthesis (SPPS) using Fmoc/NMP chemistry and HBTU protocol for aminoacids activation in a AB 430A synthesizer, modified to allow on line monitoring of deprotection by a coupled UV spectrophotometer. Protocols were modified, too. A resin with good swelling properties and relatively low substitution was selected, to assure disponibility of growing chains (NovaSyn TGA resin from Novabiochem). It was purchased pre-loaded with tryptophan protected Fmoc-Trp(Boc)-OH, that is reported to assure best protection against undesirable reactions during cleavage. The cleavage was carried out by a K reagent containing trifluoroacetic acid (TFA) : thioanisole : 1,2 ethanedithiol : water

(90:5:2.5:2.5) followed by diethylether precipitation at low temperature. The peptide is then repeatedly washed with water and finally freeze dried.

The "crude" has been analyzed by electron spray mass spectrometer at CNR Mass Spectrometer Laboratories, Naples and by LC-Mass at "La Sapienza" University, Rome.

The crude was purified by high pressure liquid chromatography (HPLC) (Perkin Elmer Series 4 with LC-235 diode array detector), using a C4 300Å - 5?m column (Macherey-Nagel) and a gradient of water and isopropyl alcohol/acetonitrile (ACN) (1:4).

The purified peptide was solubilised in deuterated DMSO and in a mixture of water-ACN-TFA-isopropyl alcohol to be respectively analysed by NMR and Circular Dichroism (CD), in view of gaining preliminary informations on its conformation.

RESULTS AND DISCUSSION

The synthesis was carried out without special problems.

The adopted cleavage procedure apparently works well, but following analyses revealed large problems. It appears that the peptide is very sensible to TFA. In fact, after the cleavage, the analysis of "crude" revealed the presence of some fragments besides the entire peptide. That the fragmentation was strictly linked to the cleavage procedure was shown by the mass and NMR results. Two main fragments are present, at 890 and 1037 UMA, that were respectively assigned by NMR to GPSASVFIL and GPSASVFIIF sequences. C-terminal portions of these fragments were not detected, either due to solubilisation during cleavage or to mass limitations. A mass spectrum performed on

volumes recovered after water washings during cleavage, showed the presence of these and other fragments; they were all identified as N-terminus fragments, suggesting that these water washings are very useful as a rough purification for separating the more soluble contaminants and fragments from the peptide (data not showed). Deleted peptides resulting from uncorrect synthesis were not observed. A cleavage with a different protocol using a longer residence-time in the cleavage cocktail was carried out and the mass analysis of this “crude” revealed the absence of the peptide of interest (1757 UMA) and only the presence of the above mentioned fragments (data not shown).

These data suggests the necessity to develop a softer cleavage procedure.

In Fig. 3 mass spectrum of the “crude” is shown, with some corresponding sequences evidenced.

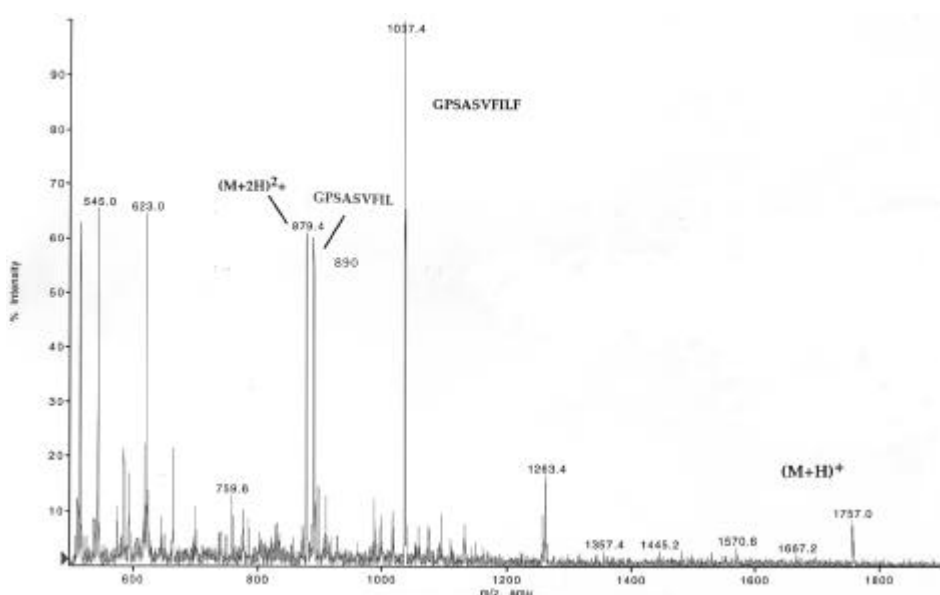


Figure 3 ES mass spectrometry of “crude” dissolved in DMSO

The "crude" is very difficult to manage, probably due to its low hydrophilicity and it is very difficult to purify, as it forms aggregates and it is hardly soluble, even in the most hydrophobic solvents, like dimethylsulphoxyde (DMSO). The purified product seems to be even less soluble.

Analytical HPLC analyses were carried out using different columns and eluents. Finally, a C4 column and a gradient containig water/0.1 % TFA and a mixture of isopropyl alcohol/acetonitrile (1:4, 0.1% TFA) revealed to work properly. A typical chromatogram is shown in Fig. 4, as obtained at 220 nm and 280nm. Peaks named as 1, 2 and 3 have been collected in repeated HPLC runs and analysed by ES mass spectrometry.

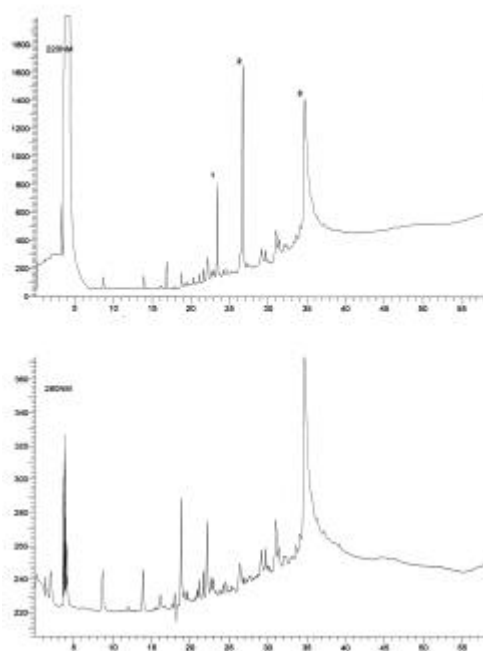


Figure 4 HPLC elution profiles at 220 and 280 nm. Peaks 1, 2 and 3 have been separately recovered and analyzed by mass spectrometry.

In Fig. 5 the mass spectra of these purified peaks are shown.

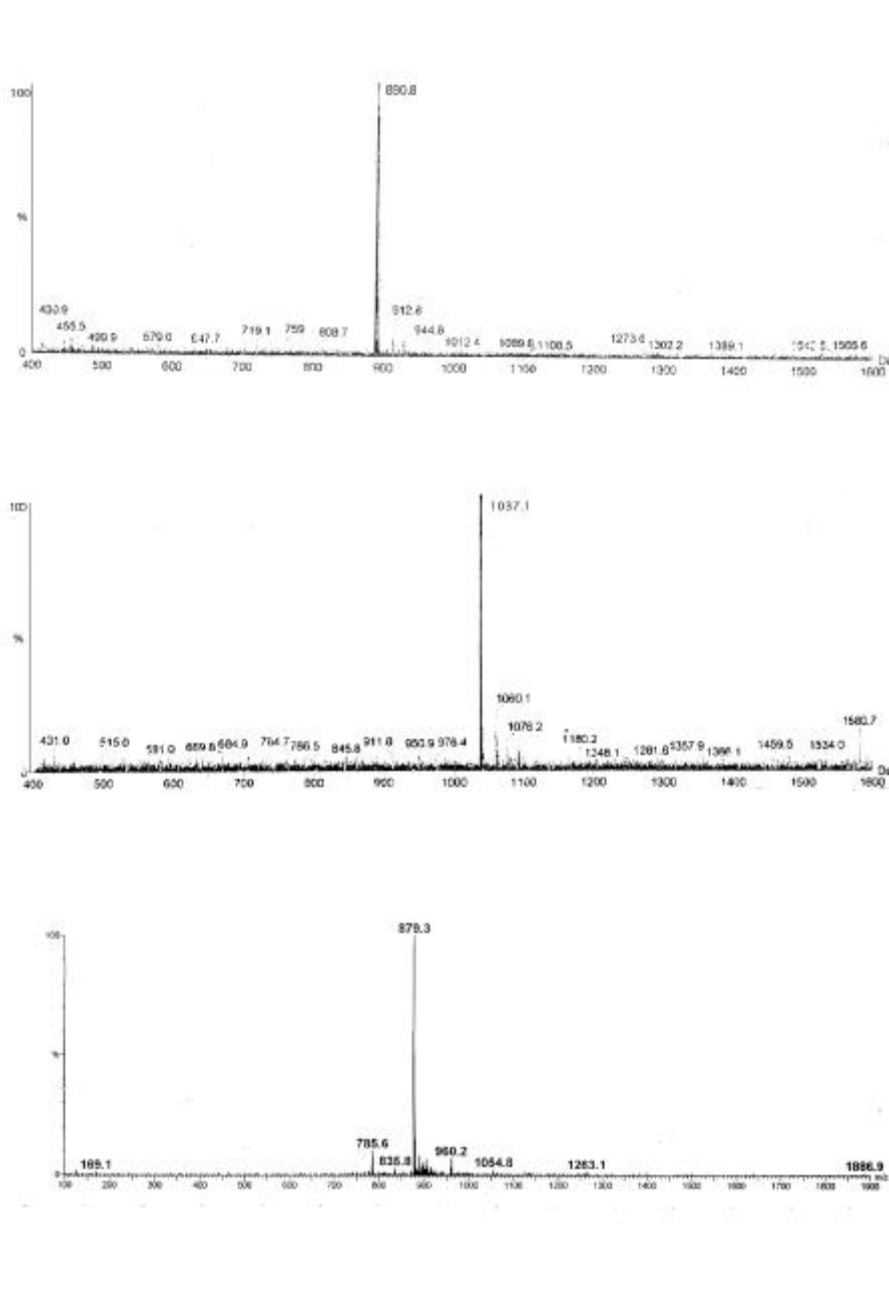


Figure 5 The mass spectra of the three peaks separately recovered by HPLC: peak 1 and peak 2 respectively correspond to 890 and 1037 UMA fragments, peak 3 corresponds to $(M+2H)^{2+}$ UMA of the peptide of interest (1757 UMA).

A preliminary analysis of the purified peptide dissolved in deuterated DMSO was carried out by NMR. An attempt was made to dissolve an other purified sample in a mixture of isopropyl alcohol/water/ACN/TFA for an analysis in CD. The results of these preliminary analyses show that the solubilisation of the purified peptide is a crucial step that has to be solved in order to be able to study the system.

In conclusion, we intend to continue the characterization of this peptide, first of all solving the solubilization problem. So, we intend to test either detergent systems like trifluoroethanol (TFE), sodium dodecyl sulphate (SDS) and esafluoroisopropyl alcohol or liposomes. Secondly, also a softer cleavage protocol has to be studied in order to avoid the fragmentation effect.

At the same time, we intend to carry out a dynamic study both of the peptide and of the complete molecule, using the molecular modeling code GROMOS, with the intent to gain confidence with the theoretical alphahelical structure and with the assembling. Furthermore, it could suggest which substitution configuration of quinone -to be inserted as an acceptor- is better indicated for this particular case.

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