PROTON TRANSFER IN BIOENERGETIC PROTEINS

by

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Ph.D. Thesis

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INTRODUCTION

The external energy sources (food) and stimulations (sound, light, etc) are converting to metabolic energy forms and neural responses, respectively, almost exclusively by chemiosmotic mechanisms in which oxidation-reduction (redox) free energy is changed into transmembrane proton (H^+ ion) and electrical gradients (Mitchell's chemiosmotic theory, Nobel Prize 1978) (1). The vast majority of these reactions occur not in solution but in proteins bound to membranes. About one third of all proteins are redox active and an overlapping one third are membrane proteins. The chemistry is performed by various redox and photobiological cofactors (hemes, metal clusters, quinones, flavins, pterins, etc) that are invested with extraordinary properties by the proteins that bind them. The protein-cofactor interactions are the same as those that operate on a substrate in an enzyme active site. The membrane proteins of respiration, photosynthesis, methanogenesis, etc provide ideal systems for studying the catalysis with astonishing specificity and revealing concepts, realities and design that can be related to similar systems of higher complexity. The present dissertation is inspired by that concept and will demonstrate the abundance of this approach.

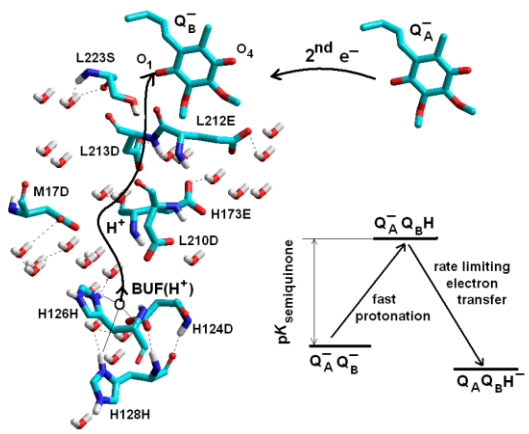
The transfer of protons in living organisms can be classified into two (not fundamentally different) categories: reactions of 1) acid-base catalysis and 2) proton transport. In the former, the proton transfer events are highly localized and occur generally pair wise between adjacent groups (e.g. an amino acid and a substrate) in the active site. The importance of acid-base catalysis in enzyme activity is well known and documented in several fields of medical sciences including the control of pH balance of blood or gastric juice. In the latter, the transport of protons is usually coupled to electron transfer or conformational changes induced by light (e.g. vision) or hydrolysis of ATP (e.g. gastric proton

pump). It is effective over long distance and typical in bioenergetics. The primary purpose of the proton transfer is to translocate protons into and across the membrane, e.g., of the mitochondrion or cells. The transport necessarily involves many elementary proton transfer steps constituting proton delivery pathway in permanent or transiently formed structure of the protein where proton donors and acceptors line up and form bucket-brigade mechanism to transport H^+ ions (2). There are not only energetic constraints (e.g. pathway should be formed) but also kinetic limitations, because usually high rate of proton delivery is needed to reduce the dissipation (losses) of the available free energy by competing additional processes. Below, the transfer of protons in some selected channels and bioenergetic proteins will be introduced to demonstrate the design of functionality of long distance proton transport of medical (biological) interest (3,4). The criteria of natural design of long distance proton transfer pathways include the need to provide kinetic competence, high selectivity and also the overarching criterion of evolutionary stability or robustness. A comparison of diverse proton conducting materials, from gramicidin to cytochrome oxidase, led to the conclusion that rotationally mobile water is a major constituent of proton pathways, for energetic (especially entropic) reasons, and because it provides substantial immunity to mutational catastrophe.

There are numerous examples for proton transport in bioenergetic proteins of medical interest. *Human carbonic anhydrase* catalyzes the rapid interconversion of carbon dioxide and water to bicarbonate and protons to maintain acid-base balance in blood and other tissues, and to help the transport of carbon dioxide out of the tissues (5). *The H^+/K^+ ATPase proton pump* causes the exchange of a proton against a potassium ion through the membrane (6). This pump is present in the colon, the kidney, but especially the stomach where it is particularly active:

controls the secretion of protons into the gastric fluid which becomes acid. It generates a gradient of pH of more than 6 pH units: whereas the blood pH is 7.3 that of the gastric fluid is about 1. The protein *bacteriorhodopsin* relates to vertebrate rhodopsins that sense light in the retina. Although rhodopsins also contain retinal, the functions of rhodopsin and bacteriorhodopsin are different. Bacteriorhodopsin captures light energy and uses it to move protons across the membrane out of the cell (7). The *respiratory cytochrome oxidase* catalyses the reduction of the oxygen molecule in cell respiration and pumps hydrogen ions simultaneously out of the mitochondrion. The overall process leads to generation of an electrical membrane potential and a pH gradient across the membrane, which may be used to form ATP by another enzyme in the same membrane (8).

Bacterial reaction center protein as model system for proton transport



In bacterial reaction center (RC), the H^+ ions are taken up from solution by long range proton transfer over a distance of about 15 Å, and a cluster of ionizable residues near the secondary quinone binding site is known to be involved in this delivery pathway. The bacterial RC provides a unique system to understand the principles of long distance proton transfer. The proton-coupled multielectron reactions, i.e., reactions with intermediate redox states like Q_B (but also others including the water oxidizing complex of Photosystem II and hydrogenases), need to protect the cofactors from adventitious electron scavenging reactions. A minimum depth of about 10 Å can be estimated from simple Marcus theory. If the electron transfer is intermolecular, then the Moser-Dutton rule suggests that the distance should not be greater than 15Å, which limits the depth at

which the charge accumulating site can be buried. However, if the electron transfer is intramolecular (as for Q_B), the depth is limited only by biosynthetic cost and functional adequacy. This necessitates long distance proton transfer if H^+ ions are involved in the reactions.

Aims

The RC is ideally suited for studying how protein-cofactor interactions induce unique properties in bound cofactors and substrates. We should like to capitalize on these features to investigate how the protein environment controls proton (and electron) transfer and tunes the functional properties of the cofactors with special interest on Q_B. The rich sructural and functional information about the RC provides a unique (model) system for studying the intraprotein proton transport and dielectric responses of proteins. The kinetics of proton transport if it is the rate limiting step, should be sensitive to deuterization i.e. to change of H^+ ions to D^+ ions in the aqueous cytoplasmic phase and to modification of the p*K* value of the quinone at the Q_B binding site. We hope the results will shed some more light to the principles of electron transport coupled proton uptake in bioenergetic proteins.

Materials and methods

Depending on the particular mutation, the mode of growth of photosynthetic non-sulphur purple bacteria *Rhodobacter sphaeroides* varied between anaerobic, semiaerobic and aerobic conditions. In all cases, the basis of the growth medium was Sistrom's minimal medium but with malate used as the carbon source, in place of succinate. The RC protein was isolated and purified by standard biochemical techniques (9-12).

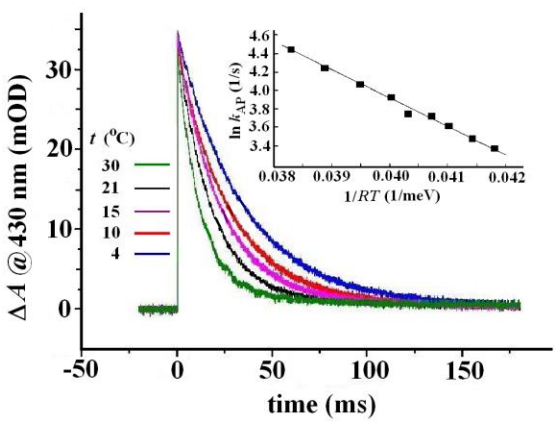
The flash induced electron and proton transfer steps were tracked by optical kinetic absorption spectroscopy. The characteristic optical changes of the

individual processes were detected at the following wavelengths: $P^+Q^- \rightarrow PQ$ charge recombination (P denotes the bacteriochlorophyll dimer of the RC) at 430 nm (or 860 nm), $Q_A^-Q_B \rightarrow Q_AQ_B^-$ first interquinone electron transfer at 398 nm and $Q_A^-Q_B^- \leftrightarrow Q_AQ_B$ production/disappearance of semiquinones at 450 nm (if the semiquinone is deprotonated (ionic)) and at 420 nm (if the semiquinone is protonated (neutral). Ferricenium/ferrocene redox pair was used as external electron donor to P^+ that showed no spectral disturbance to the semiquinone spectrum in the 400-500 nm spectral range.

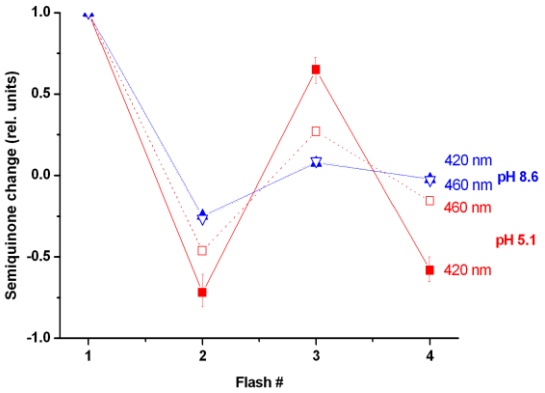
Thesis

1. Rhodoquinone bound to the secondary quinone binding site Q_B ceases the physiological activity of the RC. However, the secondary quinine activity can be re-established by single site mutation of isoleucine to threonine at M265 in the primary quinone binding site Q_A. (II. and IV.)

In wild type RC, the midpoint redox potential of the ubiquinone at the Q_B binding site is about 60 mV higher that that of the ubiquinone at the Q_A binding site (pH 8). This potential difference drives the $Q_A \rightarrow Q_B$ (interquinone) electron transfer. If, however, the native ubiquinone at Q_B is replaced by the low potential rhodoquinone, then no interquinone electron transfer would occur. As the midpoint redox potential of the rhodoquinone is 80-100 mV lower than that of the ubiquinone, the electron transfer from Q_A to Q_B becomes energetically unfavourable. The electron transfer can be reconstituted if the isoleucine at M265 in the Q_A binding pocket is replaced by threonine. The



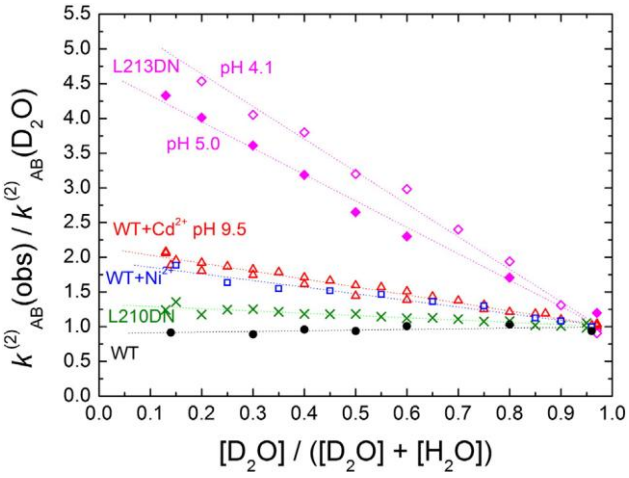
mutation causes a slight conformation change of the alanine at M260 position that reduces the electronegativity and therefore the midpoint potential of the quinone ring of Q_A. The drop is 110 mV (pH 8), that was determined from the temperature-dependence of the $P^+Q_A^- \rightarrow PQ_A$ charge recombination. In contrast to the wild type RC, the rate of the back reaction of the M265IT mutant demonstrated substantial temperature-dependence in the physiological temperature range which proved that the midpoint potential of Q_A became lower. The $P^+Q_A^- \rightarrow PQ_A$ charge recombination did not occur directly (via tunnelling as in wild type RC) but indirectly through one of the relaxed states of P^+I^- (I denotes the bacteriopheophytine in the RC). The drop of the midpoint redox potential generated by mutation at the Q_A site is able to compensate the similar decrease of the midpoint redox potential of Q_B produced by replacement of the native ubiquinone by rhodoquinone at the Q_B binding site. This is the reason why the modified molecular construction can reconstitute the original enzyme activity. Indeed, we could observe all assays that are characteristics of the proper function of the secondary quinone: 1) the $P^+Q_B^- \rightarrow PQ_B$ charge recombination from the secondary quinone becomes slower than that of the $P^+Q_A^- \rightarrow PQ_A$ back reaction and shows characteristic pH-dependence, 2) the semiquinone demonstrates binary oscillation upon a series of saturating exciting flashes. From the damping of the binary oscillation, we could derive the degree of reconstitution of the Q_B activity and the one-electron equilibrium constant in the quinone acceptor complex. The reconstitution of the Q_B activity was complete, it was very close (practically identical) to that experienced in wild type RC.



2. The rate of the second electron transfer shows kinetic and equilibrium solvent isotope effects in proton transfer mutants of RC. (I. és III.)

In wild type RC, the transfer of the first proton is much faster than the transfer of the second electron to Q_B i.e. the electron transfer is the rate limiting step in the electron/proton coupled 2nd electron transfer. Therefore, all phenomena that effect the proton transfer only have not influence on the observed 2nd electron transfer. In wild type or electron transfer mutant RCs where the electron transfer is the bottleneck, no solvent isotope effect (change of H_2O to D_2O) can be experienced. However, in proton transfer mutants, where the rate of proton transfer is significantly reduced (even below that of the electron transfer) by mutations, solvent isotope effects can be expected.

We found that the rate of the proton transfer was particularly sensitive 1) to the intactness of the proton gate at the entrance and 2) to the amino acids of the acidic cluster in the vicinity of the secondary quinine. Accordingly, proton transfer mutants can be created by inhibition of the histidine ligand of the proton gate by divalent cations (Cd^{2+} or Ni^{2+}) and/or by exchange of some key protonatable amino acids (e.g. Glu L212 or Asp L213) to non-protonatable amino acids of similar size in the proton delivery pathway. In these proton transfer mutants, kinetic and equilibrium isotope effects could be observed which were dependent on the prevailing pH and of the location of the mutations. The rate of uptake of the deuterium ions demonstrated particularly large drop in the L213DN mutant relative to that of H^+ ions. The experimentally obtained kinetic solvent

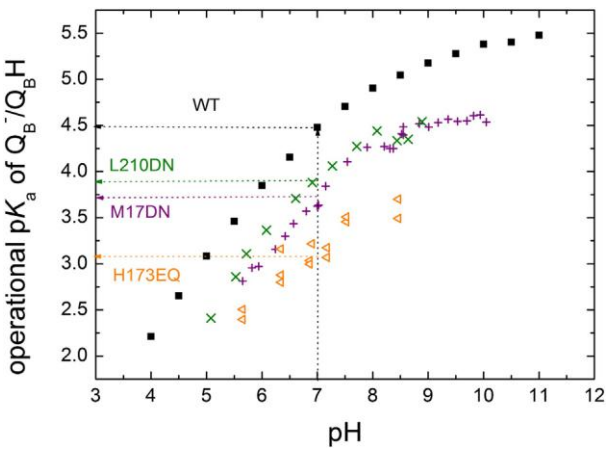


isotope effect (the ratio of the rates of proton and deuterium delivery, respectively) approached the theoretical limit (k_H/k_D ~ 6) determined based on a simplified model of the H/D bond vibrations. In addition to the kinetic isotope effect, an equilibrium isotope effect (the shift of the p*K* values of the protonatable amino acids upon $H^+ \leftrightarrow D^+$ exchange) was also observed with $\Delta pK = pK_D - pK_H < 0.8$ pH unit magnitude.

3. The native secondary ubiquinone bound to the Q_B site is strongly acidic (p*K*<4.5) and the proton affinity of the semiquinone depends on the prevailing pH of the solution and can be modified by exchange of some key protonatable amino acids of the RC to nonprotonatable ones. (I. és III.)

The p*K* value of the UQ/UQH$^\bullet$ redox pair in mixtures of aqueous or organic solvents is low (p*K* ≈ 4.0) and similarly low proton affinity can be predicted in the Q_B binding site of the RC protein. As the standard polarographic or radiolytic measurements do not offer firm results, we applied kinetic spectroscopy combined with solvent isotope and mutational methods to estimate the apparent p*K* value of the $UQ_B/UQ_BH^\bullet$ redox pair. We came to the conclusion, that the UQ/UQH$^\bullet$ redox partners did not follow a simple Henderson-Hasselbalch type pH-titration as commonly used for acid/base titration in aqueous solutions but the titration is complex. To preserve the Henderson-Hasselbalch equation formally even in this case, a pH-dependent p*K* value should be assumed. The extension of the concept of the proton equilibrium constant includes that the protonatable group is located in an environment whose structure and electrostatics is not constant but changes upon pH. Our experiments indicate that the UQ/UQH$^\bullet$ redox couple is located in a similar surrounding in the Q_B binding site. It is in strong and pH-dependent interaction with the amino acids of the neighbouring acidic cluster and its formal consequence is the pH-dependent p*K* value in the Henderson-Hasselbalch

equation. The mainly carboxyl residues of the amino acids in the cluster become more and more negative upon increase of the pH that causes the extension of the pH-titration of the quinine/semiquinone couple. This rationalizes the slight increase of the p*K* of the $UQ_B/UQ_BH^{\bullet}$ redox pair (~4.5) relative to that of 1,4-benzoquinone reported in solution (4.0).



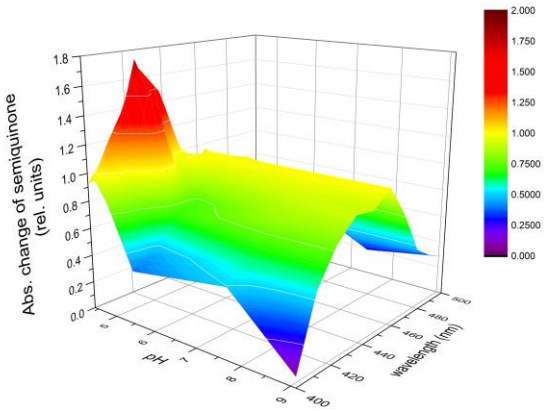
We were able to demonstrate the strong dependence of the p*K* value of the $UQ_B/UQ_BH^{\bullet}$ redox couple on the internal electrostatics the RC modified by mutations. By neutralizing well located negative charge in the protein, significant down-shift of the p*K* values could be detected at pH 7: 4.5 (WT), 3.9 (L210DN), 3.7 (M17DN) and 3.1 (H173EQ).

4. In spite of the very low proton affinity (small p*K*), we were able to observe the stable protonated semiquinone state of the rhodoquinone bound to the Q_B site of the RC. (II. és IV.)

The second electron transfer from Q_A^- to $Q_B^{-\bullet}$ after the second flash will take place only after the $Q_B^{-\bullet}$ semiquinone produced by the first flash has been protonated to $Q_BH^{\bullet}$. In wild type RC, the proton uptake occurs at least one order of magnitude faster (rate $>10^7$ s, pH 8), than the subsequent electron transfer ($\sim10^6$ s). However, the proton uptake by the semiquinone is a free energy demanding process (ΔG = 60 meV·(pH−p*K*)), and therefore the entire protonation of $Q_B^{-\bullet}$ can be never achieved. Although the proton equilibrium between the protonated and unprotonated forms is completed within very short time, the protonation equilibrium is shifted toward the unprotonated form, therefore the observed rate of the second electron transfer is

much (orders of magnitude) smaller than the that of the “net” electron transfer (without protonation, i.e. at very low pH).

The principal assumption of the mechanism of the proton activated electron transfer is the establishment of protonated semiquinone. Its observation is a great challenge because of the low p*K* of $Q_B^{-\bullet}/Q_BH^{\bullet}$. By replacement of the native ubiquinone by rhodoquinone at the Q_B site of the M265IT mutant, we managed to detect the protonated (neutral) form of rhodosemiquinone. The spectra of the optical absorption change of the semiquinone produced after even number of flashes (disappeared after odd number of flashes) were recorded in the 400-500 nm spectral range. Two absorption bands with maxima 420 nm (protonated semiquinone) and 450 (deprotonated semiquinone) could be distinguished below pH 5. The neutral species disappeared completely above pH 5.5. The observation of protonated rhodosemiquinone is a direct proof of the proton-activated electron transfer mechanism of the second electron transfer. This is a nice manifestation of the interaction of the electron and proton transfers occurring in many channel mechanisms and bioenergetic proteins of medical interest.



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Self publications used in the dissertation

I. **Ágnes Maróti**, Colin A. Wraight and Péter Maróti: The rate of second electron transfer to Q_B^- in bacterial reaction center of impaired proton delivery shows hydrogen-isotope effect. *Biochim. Biophys. Acta, Bioenergetics,* 1847 (2015) 223–230.
Impact factor: 4.829.

II. **Ágnes Maróti**, Colin A. Wraight and Péter Maróti: Protonated rhodosemiquinone at the Q_B binding site of M265IT mutant reaction center of photosynthetic bacterium *Rba. sphaeroides. Biochemistry,* Americal Chemical Society (in press).
Impact factor: 3.194.

III. **Ágnes Maróti**, Colin A. Wraight and Péter Maróti: Equilibrium- and kinetic isotope effects of electron transfer in bacterial reaction center of photosynthetic bacteria. COST conference, Visegrád, 2014. talk.

IV. **Ágnes** Maróti, Colin A. Wraight and Péter Maróti: Spectroscopic evidence for protonated semiquinone in reaction center protein of photosynthetic bacteria. Molecular Machines Conference, Ringberg, Germany, 2014. poster.

Other scientific activities not strictly connected to the dissertation

Zita Gyurkovits, **Ágnes Maróti**, Lóránd Rénes, Gábor Németh, Attila Pál, and Hajnalka Orvos: Adrenal haemorrhage in term neonates: a retrospective study from the period 2001–2013

The Journal of Maternal-Fetal Neonatal Medicine, Early Online: 1–4, 2014.

DOI: 10.3109/14767058.2014.976550

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Maróti Ágnes: Inzulinpumpa kezelése és alkalmazása gyermekkorban.

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Training courses

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Seminar in Pediatric Endocrinology and Diabetes, Salzburg, Austria

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2010. 49th Annual Meeting of the European Society for Paediatric Endocrinology (ESPE), Praha

2013. Graz, University Hospital, Intensive Care Unit, Austria

2013. Great Ormond Street Childerens Hospital, Hiperinsulinaemia Group, London

2014. 50th Annual Meeting of European Association for the Study of Diabetes (EASD), Vienna