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Site-Directed Mutagenesis of an Energy Transducing Protein:
Bacteriorhodopsin

Final Report

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Introduction

Our objective was to understand at the molecular level how bacteriorhodopsin(BR) transports protons. The work involves the synthesis of mutant BRs, their expression in the natural host, *H. halobium*, and an investigation of their photocycles. Although I am the PI, the original grant was submitted in collaboration with Janos Lanyi (University of California School of Medicine at Irvine) and was awarded to both laboratories. The formal splitting into two grants(i.e., one to Janos and one to me) was made for administrative simplicity, but the work is funded as a collaboration between our laboratories.

Over the period of this final report this collaboration has led to the development of a greatly improved expression system and to an increased understanding of the mechanism of proton transport. At the beginning of the award period a central concern was establishing the details of the photocycle. This phase was essentially complete by mid 1994. We then investigated the energy coupling mechanism which allows uni-directional proton transfer and found that a major determinant was the coupling of the proton release to changes in the pKa of D85.

We present selected excerpts below from our papers; the complete list of publications is listed at the end of the report.

Expression of mutant bacteriorhodopsin

The objective of our genetic studies is to develop vectors that will allow for the rapid construction of site-directed and random mutations in BR. Toward this goal we have constructed new vectors for the efficient production of mutant BR in *H. halobium*. Our previous vectors were integrating vectors which have a low frequency of integration and are unstable requiring the constant purification of strains containing mutant BR. An additional problem is that these vectors use mevinolin resistance as the selective marker in *H. halobium*; mevinolin is unstable, very expensive, and not commercially available in the form used in culture media. In contrast, our new vectors are replicating vectors, transform with frequencies 1000x better, and can be stably propagated using novobiocin resistance as a selective marker. We have also constructed some new fusion vectors for the overproduction of halorhodopsin and other archaebacterial proteins.

The Photocycle

The objective of our spectrophotometric studies of the photocycle is to understand the factors that control the rates of the thermal interconversion of photointermediates.

Nature of the O state

In this endeavor I also worked with J Akio Maeda of Kyoto University.

In the bacteriorhodopsin photocycle the recovery of the initial BR state from the M intermediate occurs via the N and O intermediates. The molecular events in this process include reprotonation of the Schiff base and the subsequent uptake of a proton from the cytoplasmic side, as well as reisomerization of the retinal from 13-cis to all-trans. We have studied the kinetics of the intermediates and the proton uptake. At moderately low pH little of the N state accumulates, and the O state dominates in the reactions that lead from M to BR. The proton uptake lags behind the formation of O, suggesting the sequence $N(0) \rightleftharpoons O(0) + H^+ \text{ (from the bulk)} \rightarrow O(+1) \rightarrow BR + H^+ \text{ (to the bulk)}$, where the superscripts indicate the net protonation state of the protein relative to BR. Together with a parallel study of ours at moderately high pH, these results suggest that the sequence of proton uptake and retinal reisomerization depends on pH: at low pH the isomerization occurs first and O accumulates, but at high pH the isomerization is delayed and therefore N accumulates. Although this model contains too many rate constants for rigorous testing, we find that it will generate most of the characteristic pH-dependent kinetic features of the photocycle with few assumptions other than pH dependency for protonation at the proton release and uptake steps

Our physical understanding of the O state is perhaps the weakest of all the states of the photocycle. O is formed in the last step of the photocycle and is a red-shifted state. The recovery of the bacteriorhodopsin ground state from O requires the loss of a proton from the acceptor of the Schiff base proton, D85 and the gain of this proton by a residue, X, which serves as the release group for the proton on the extracellular side in the next photocycle. Following a clue provided by the work of Ebrey and Balashov, we showed that X could be E204. It was then logical to assume that if E204 was mutated to an amino acid that could not accept a proton--say E204Q--then the lifetime of O would be increased. This was indeed the case, and we published a paper detailing these results (Biochem. 35:4054-62, 1996). Recently we have found that the mechanism is more complicated. While E204 is the acceptor of the Schiff base proton, E194 is the final release group.

Another approach towards understanding the nature of the O state was to use X-ray diffraction to determine the structure of the N intermediate. We used a mutant in which large amounts of N accumulate. The difference Fourier map revealed a major change near helix F. However the structure of N is essentially the same as that of the preceding state M, despite the differences in the protonation state of the Schiff base. The observed structural change near helix F will increase access of the Schiff base and Asp-96 to the cytoplasmic surface and facilitate the proton transfer events that begin with the decay of M.

b. Nature of the M state

These results were reported in our previous Progress Report and are only briefly presented here.

Previous work, by other investigators, had shown that removal of the proton donor Asp 96 (D96) -> Asn 96 (D96N) greatly increases the lifetime of M. Through a close collaboration with J. Lanyi at UC Irvine we explored in more detail the mechanism by which the Schiff base is reprotonated from the cytoplasmic surface. It was determined that Thr 46 and Asp 96 together mediate proton transfer from the cytoplasmic surface to the Schiff base. One mutant, T46V, was found that accelerated the deprotonation of asp-96 while hindering or slowing proton uptake from the cytoplasmic surface. We proposed that the T46V substitution would retard M decay when it is a second mutation to D96N, even though it accelerates M decay as a single mutation. This was indeed the case, and the M decay is about 6X slower in T46V/D96N than in D96N. To our knowledge this is the first time that a nontrivial property of a BR mutant has been successfully predicted.

c. Comparison of the photocycles of bacteriorhodopsin with halorhodopsin

Halorhodopsin is an inwardly directed chloride pump whose structure is highly homologous to that of bacteriorhodopsin. Indeed we have been able to convert bacteriorhodopsin to a chloride pump with a single amino acid substitution.

The light-driven chloride pump, halorhodopsin, is a mixture containing all-trans and 13-cis retinal chromophores under both light and dark-adapted conditions and can exist in chloride-free and chloride-binding forms. We resolved the multiple photochemical reactions by determining flash-induced difference spectra and photocycle kinetics in halorhodopsin-containing membranes prepared from *Halobacterium salinarium*, with light- and dark-adapted samples at various chloride concentrations. Careful examination of the flash-induced changes at selected wavelengths allowed separating the spectral changes into components and assigning them to the individual photocycles. According to our results, a substantial revision of the photocycle model for *H. salinarium* halorhodopsin, and its dependence on chloride, is required. Unlike in the earlier models, no step in this photocycle was noticeably affected when the chloride concentration was varied between 20 mM and 2 M in an attempt to identify a chloride-binding reaction.

Perhaps the most striking observation made during this period is the essentially unity between bacteriorhodopsin and halorhodopsin. In the light-driven proton pump bacteriorhodopsin, proton transfer from the retinal Schiff base to aspartate-85 is the crucial reaction of the transport cycle. In halorhodopsin, a light-driven chloride ion pump, the equivalent of residue 85 is threonine. When aspartate-85 was replaced with threonine, the mutated bacteriorhodopsin became a chloride ion pump when expressed in *Halobacterium salinarium* and, like halorhodopsin, actively transported chloride ions in the direction opposite from the proton pump. Chloride was bound to it, as revealed by large shifts of the absorption maximum of the chromophore, and its photointermediates included a red-shifted state in the millisecond time domain, with its amplitude and decay rate dependent on chloride concentration. Bacteriorhodopsin and halorhodopsin thus share a common transport mechanism, and determines the ionic specificity.

In *N. pharaonis* halorhodopsin the chloride binding site also binds azide. When azide is bound the Schiff base deprotonates after light absorption. This azide dependent photocycle results in active proton transport in the cytoplasmic to extracellular direction. We conclude that azide fulfills the same role in this halorhodopsin as Asp-85 does in bacteriorhodopsin. Although halorhodopsin is normally a chloride pump, it seems to have all the structural requirements--except for an internal proton acceptor and donor--of a proton pump. This result complements our finding that the replacement of Asp-85 in bacteriorhodopsin with Thr converts bacteriorhodopsin to an inwardly directed proton pump.

d. Energy coupling

All pumps require alternating access of a ligand binding site between the cytoplasmic and external sides of the membrane. In bacteriorhodopsin the Schiff base forms the active site with external access in M1 and cytoplasmic access in M2. We proposed that this change in access is the result of a conformational change of the protein between these two states. Difference density maps from both electron and X-ray diffraction show a large conformational change and examination of the photocycles of 85N as well as 85N/96N led to a model in which these conformational changes are determined by the interaction between the Schiff base and Asp-85. In the ground state there is a positive charge on the Schiff base which is compensated by a negative charge on Asp-85. When the proton is donated to Asp-85, the active site becomes neutral; this state then is similar to that of the mutant 85N. Work with our Japanese colleagues has shown that the conformation of 85N is similar to the M state.

C. List of All Publications and Technical Reports

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