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PHOTOACOUSTIC CALORIMETRY OF *HALOBACTERIUM HALOBIUM* PHOTOCYCLE

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SUMMARY

Enthalpy changes that occur during the photocycle of *Halobacterium halobium* purple membrane fragments have been measured on a millisecond time scale, using photoacoustic detection to obtain modulation photocalorimetric data. Details are given on the ways to obtain quantitative thermodynamic and kinetic information by this method.

INTRODUCTION

The sole protein of *Halobacterium halobium* fragments - bacteriorhodopsin - contains a retinal molecule attached to a lysine residue via a protonated Schiff-base linkage, and such membranes operate as light-driven proton pumps, capable of converting light energy into a proton gradient (1). An essential part of this process is a cyclic photoreaction (the photocycle), which is initiated by absorption of light by the retinal and involves at least six photointermediates denoted by their absorption maxima in order bR_{570} (ground state), K_{590}, L_{550}, M_{412}, N_{530} and O_{660} (1). Although the photochemistry of bacteriorhodopsin is being studied intensively, little is known about the thermodynamic parameters of this system, i.e. the enthalpy and entropy changes associated with the photocycle.

We have measured the light-induced heat production by purple membrane fragments, using photoacoustic (PA) detection methods. The important points of this study are: (1) The heat production, following light absorption, reflects enthalpy and internal energy changes in the system (under the experimental conditions, the volume and pressure can be considered practically constant). (2) The above thermodynamic information can be extracted for various times following light absorption and therefore various reaction stages can be differentiated in this respect, especially for milli- and microsecond kinetics. (3) As a by-product of this analysis, kinetic constants for the various reaction changes are obtained. (4) Such

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measurements may detect light-induced processes which do not give rise to absorption changes and therefore cannot be detected by absorption spectroscopic methods.

METHODS

In the PA method a sample situated in a closed chamber of gas is irradiated by a chopped beam of light. Thermal dissipation of the absorbed energy gives rise to modulated heating of the sample inducing modulated pressure changes in the gas phase, which are sensed by a microphone and analysed by a phase-sensitive detector, yielding a signal proportional to the amount of heat released (2). Kinetic measurements are performed by following the chopping frequency dependence of the PA signal. In the case of a single, first-order process, such a frequency spectrum will be a sigmoidal (usually decreasing) curve, the rate constant being equal to the angular frequency at the point of half transition between the plateaus. For several sequential first-order processes (as in the photocycle), a more complex pattern of sigmoid curves is expected.

The PA signal, ρ, generated by absorption, within the thermally-active layer, of fraction α of the incident light, is given by the equation (3):

$$\rho = f\alpha \{1-(\sum_i \phi_i \Delta E_i)/(Nh\nu) \}$$

where ϕ_i and ΔE_i are the quantum yield and internal energy change of any light-driven process i; N, h and ν are Avogadro's number, Planck's number and the frequency of the absorbed light, respectively; f is an instrumental constant which depends also on the sample (its thermal diffusivity, surface area, etc.). By varying the chopping frequency ρ will change because i may change (at high frequency only fast thermal relaxation will be detected, while at low frequency slow events will contribute to the signal too) and/or by affecting α (at higher frequencies the thermally active layer is thinner and the fraction of light absorbed in it smaller (4)).

Extraction of quantitative information from PA measurements requires calibration of the PA signal, i.e. evaluating the value of f and distinguishing between frequency effects on $\sum_i \phi_i \Delta E_i$ (which enable us to calculate rate constants) and on α. We used two calibration approaches. In the first one the PA signal is compared to a reference signal obtained from a sample which has the same optical absorption and thermal properties as the sample studied, but is photo-inactive. A suitable reference was purple membrane bleached by illumination in the presence of NH_2OH (5), to which black ink was added so as to obtain the same optical density as the sample studied. In this case

$$\rho_{ref} = f\alpha \quad \text{and} \quad \rho/\rho_{ref} = 1 - \frac{\sum_i \phi_i \Delta E_i}{Nh\nu}$$

A second approach is to assume that $\sum_i \phi_i \Delta E_i$ is independent of ν over a certain range of wavelengths*. Then a plot of ρ/α *vs.* $1/\nu$ will yield a straight line (as indeed was found experimentally in the region of 620 nm - 560 nm) (7) from which $\sum_i \phi_i \Delta E_i/Nh$ can be extracted as the slope/intercept ratio. However, application of this method is possible only if the thermal diffusivity is known as this is essential for calculating α at any chopping frequency. In aqueous solutions we can assume safely a value of $0.001 \pm 20\%$ cm^2/s for this parameter. Each of the approaches described above involves assumptions and therefore includes an ele-

*It was shown (6) that M_{412} is formed from bR_{570} with quantum efficiency of 3 ± 0.03 independent of whether bacteriorhodopsin is excited at 540 nm or 580 nm. It is also not expected that ΔE will vary with the wavelength since it is an intrinsic parameter of the excited state.

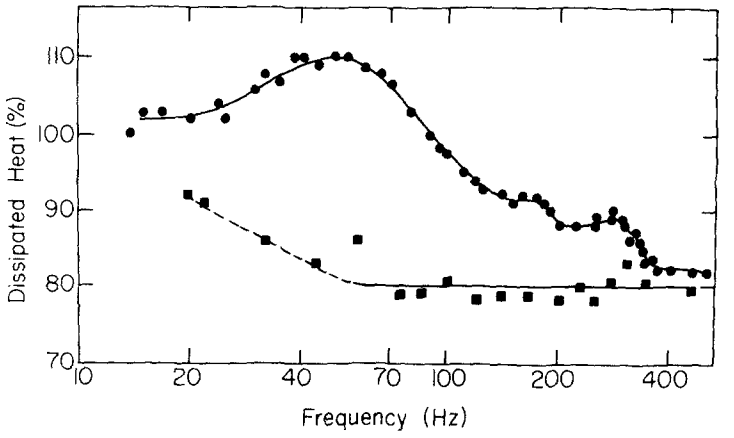

Figure 1: Modulation frequency spectra of purple membrane fragments. (●-●), 30 µl of aqueous suspension containing 0.7 mM bacteriorhodopsin (optical density at 565 nm = 4.0) with 10 mM Tris. (■-■). The same sample as before, after being stored for 48 hrs at 17% relative humidity. Reference signals were obtained from equal amount of bleached purple membrane, which was brought to the same optical density (at 565 nm) by adding a small volume of black ink.

ment of uncertainty; however, it is important to notice that the assumptions involved are different and independent ones. Thus, agreement between the values calculated in both ways is a strong indication of their validity.

The PA signal amplitude ("vector" mode of the Brookdeal 9502 SC lock-in analyzer) was measured at 565 nm. The experimental set-up (7), sample preparation (7) and measuring cells (8) were as described previously.

RESULTS AND DISCUSSION

Fig. 1 shows the frequency spectra of purple membrane fragments in aqueous suspension and dried form (∿17% relative humidity). Aqueous suspensions of membrane fragments exhibit a complex pattern of sigmoid curves which indicates at least four distinct events, three of them associated with heat production (exothermic processes) and one (the slowest) associated with heat consumption (endothermic process). However, more than 80% of the absorbed energy is dissipated as heat at frequencies higher than 500 Hz, i.e. faster than 300 µs). On the other hand, in the dried fragments any frequency dependence was restricted to relatively low frequencies (i.e. long periods after illumination) and above 50 Hz the photoacoustic signal was frequency independent (the standard deviation from the average, constant, value was 2-3%).

These observations were in fact expected. The quantum yield of converting bR_{570} to K_{590} is 0.25-0.30 (6,9), thus at least 70% of the absorbed energy should dissipate as heat within less than 10 ps, both in dried and wet preparations, well beyond our measuring range. Drying the membrane fragments was found to slow down markedly the relaxation processes from the decay of M_{412} onwards (10) and, therefore, any heat production or consumption associated with the remaining part of the photocycle should be delayed and is expected to be observed at low chopping frequencies only.

The data in Fig. 1 were normalized, using the ink reference, but similar values were obtained from calculating the slope/intercept ratios of ρ/α *vs.* $1/\nu$ curves plotted for several selected frequencies; e.g. at 340 Hz this approach predicts dissipation of 77% of the absorbed light (instead of 84% in Fig. 1) and at 15 Hz the obtained value is 98% (compared to 102% in Fig. 1). In both approaches calibration of the signals obtained from dried fragments is not reliable. If the ink reference is used it is hard to assure that both the bleached and unbleached samples will have the same surface area in the dried form (i.e. f may be different in the two cases). If the second approach is taken there is uncertainty with regard to the thermal diffusivity of the dried fragments (which may influence the calculated variation of α with ν). Nevertheless, the results demonstrate that the frequency spectrum clearly distinguishes between photochemically inactive and active samples and give an estimate of the experimental error associated with this measurement.

Approximate life-times of the events detected in the frequency spectrum of wet purple membranes were calculated from the angular frequencies of the points of half transition in Fig. 1 and the numbers obtained were 5 ms (for the endothermic reaction), 2 ms, 1 ms and 0.5 ms. However, since the differences between the various life-times are relatively small, these values can be regarded as rough estimates only. The three largest life-times appear to correlate nicely with those measured for the decay of photointermediates, using modulation excitation spectroscopy (11) : O_{660} = 5 ms, M_{412} = 3 ms, and another life-time of 0.9 ms

which may represent the decay of N_{530}. On the other hand, the smallest life-time, 0.5 ms, cannot be correlated with any known photointermediate and it may reflect a process not associated with distinct absorption changes (e.g. conformational change of the protein).

It should be emphasized that, although the normalized PA signals could be subject to some error due to calibration problems, the shape of the frequency spectrum and the frequencies of the points of half transition are much less influenced by such errors.

The data summarized in Fig. 1 were used to estimate the relative amount of heat released or absorbed in each process. Fig. 2 shows a schematic diagram of the light-induced enthalpy changes, calculated by averaging the data from three different experiments. In constructing this diagram we did not include the fraction of excitation which relaxes immediately and considered only "utilized" photons, i.e. background of 70% has been subtracted. The increase in enthalpy due to light absorption was estimated to be 184 kJ/Einstein. The total energy of a photon at 565 nm is 212 kJ; however, about 13% of the input energy is released during the relaxation of the excited state to its lowest vibrational level* and is not of interest from the energetic point of view.

An important conclusion from the data summarized in Fig. 2 is that the last event in the photocycle involves an increase in enthalpy but, since we deal here with a spontaneous process, it has to be associated with an increase in entropy, i.e. it may involve conformational changes in the protein-lipid complex.

Recently measurements of enthalpy changes in purple membrane fragments, using a capacitor microphone flash calorimeter were reported (12) (the buffer-related volume changes, involved in that study, have a small effect only ($\nu \leq 5\%$, Garty *et al.*, submitted) on our measurements). The scheme they obtained for enthalpy changes resembles the one we get (taking into consideration the different

*We assume that excitation at the tail of the absorption band (ca. 650 nm) will represent excitation to the lowest vibrational level of the excited state. Thus the fraction of electronic energy which is stored upon excitation at 565 nm is 565/650=0.87.

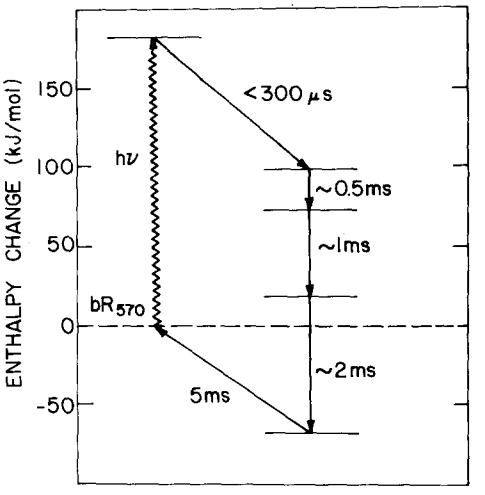

Figure 2: Schematic diagram of light-induced enthalpy changes in purple membrane. For details, see text.

value they assumed for the utilizable light energy) with two distinctions. The value we get for the lowest enthalpy level is much smaller than their value, and they differentiated between only two of the three exothermic events.

The above study demonstrates the use of PA methods for photomicrocalorimetric measurements. This technique is in particular useful for kinetic measurements as chopping frequency scanning of the PA signal appears to be a fast and convenient tool to obtain a detailed picture of thermal relaxation processes.

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