Vol. 100, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1981 Pages 385-390

PHOTOACOUSTIC SPECTROSCOPY OF CATTLE VISUAL PIGMENT AT LOW TEMPERATURE

François Boucher and Roger M. Leblanc,

Groupe de recherche en Biophysique, Département de Chimie-Biologie, Université du Québec à Trois-Rivières, Trois-Rivières, Québec, Canada G9A 5H7

Received March 30,1981

SUMMARY

The non-radiative decay of an excited chromophore can be measured by photoacoustic spectroscopy, but the application of this technique to the study of visual pigments is complicated by the light-sensitivity of the rhodopsin molecule. We succeeded in measuring photoacoustic spectra of native visual pigment. Our results provide experimental evidence that the internal conversion process is an important deactivation pathway in the early intermediates of rhodopsin's bleaching cycle. Moreover, the particular detection system of photoacoustic spectroscopy makes possible measurements on a whole retina.

Despite considerable importance, little experimental attention has been paid to photophysical processes in the visual pigment molecules in recent years. Among dissipative processes which could allow the excited rhodopsin's chromophore to return to the ground state, exciton formation and energy transfer have been ruled out (1, 2). Emission from rhodopsin and some of its intermediates has been observed (3) but the quantum yield of this process, previously reported to be around 5×10^{-3} (4), was recently proposed to lie below 10^{-5} (5). While triplet state population is still a matter of conjecture (6), the possibility remains for the occurrence of non-radiative transitions. The experimental technique for direct measurement of thermal decay of excited molecules was lacking until photo-

---

The abbreviations used are : PAS (photoacoustic spectroscopy); PA (photoacoustic); ROS (rod outer segments).

0006-291X/81/090385-06$01.00/0

acoustic spectroscopy (PAS) was revived and developed (see ref. 7 for comprehensive review). We have measured, for the first time, PA spectra of native visual pigments. Our results provide experimental evidence that internal conversion is an important deactivation pathway in the early intermediates of rhodopsin's bleaching cycle.

PAS does not measure energy absorption by a molecule but only the absorbed energy which undergoes thermal decay. This is achieved by irradiating a sample in a closed chamber with monochromatic light modulated at an acoustic frequency. Due to light modulation, periodic temperature rises are induced upon thermal decay of the excited molecules and result in heat flow detected as pressure waves in the surrounding gas by a sensitive microphone. High light intensity ($\sim$ 1 mW cm^{-2}) is required to produce a measurable photoacoustic signal. Thus PAS measurements on rhodopsin cannot be performed at room temperature as the sample is rapidly bleached. The present report describes the low temperature PA spectra measured with cattle visual pigment.

MATERIAL AND METHODS

In order to perform PAS measurements at low temperature, the photoacoustic cell previously described (8) has been modified in the following manner : The microphone was spaced from the cell body by a hollow Teflon cylinder. It permits to cool the cell body by immersion in liquid nitrogen without changing the microphone performance. Once equilibrated to 77 K, the cell body (2 kg) warmed back very slowly (0,2 degree min^{-1}) and thus permitted recording of PA spectra at any temperature between 77 and 300 K. Temperature was monitored by a Cu-Constantan thermocouple inserted in the sample.

The whole retina can be directly examined by PAS. For these experiments, samples were prepared as follows : Fresh bovine eyes, from a local slaughterhouse, were dissected within a few hours after the death of the animal. A circular quartz slide was placed at the bottom of an hemisected eye ball. The retina was carefully detached from a peripheric point and ripped off at the same time onto the quartz slide, the rod outer segments (ROS)lying then at the top of the sample. The slides were stored for two hours in a dry air chamber at 4°C prior to measurement in order to remove excess water so that signal to noise is enhanced.

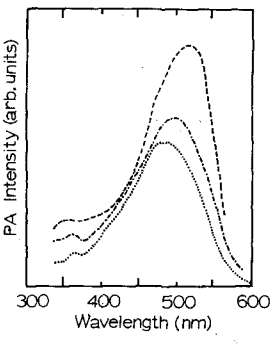

Fig. 1 Photoacoustic spectra of partially delipidated rod outer segments measured at -165°C (----); -105°C (-·-·-·-·-) and -25°C (····). Chopping frequency: 500 Hz. Temperature does not vary by more than 2°C during recording. All spectra are normalized for incident energy at each wavelength by using a carbon black reference spectrum.

The ROS were prepared according to Makino et al. (9). They could be partially delipidated by washing twice in hexane or petroleum ether. The method yields a dry ROS powder from which purified solid rhodopsin can be obtained by a salting out procedure (9). The samples prepared in this manner are highly suitable for PAS measurements.

RESULTS AND DISCUSSION

Figure 1 shows the photoacoustic spectra obtained with partially delipidated rod outer segments. Similar behavior is observed with either purified rhodopsin or fresh rod outer segments. In each case, PA spectra coincide with the known absorption properties of cattle visual pigments. At -165°, the spectrum presents a band centered at 515 nm, similar to the absorption spectrum reported for a photostationary mixture of rhodopsin (498 nm), bathorhodopsin (543 nm) and isorhodopsin (485 nm) which can be attained at this temperature (10, 11). In the PA spectrum, this photostationary state is maintained by the incident chopped light beam. The shape and position of the spectrum remains unaltered even after 3 to 5 scans (~ 45 min). As the temperature rises, thermal reactions become allowed. Above -135°C, lumirhodopsin is formed from bathorhodopsin as the PA spectrum shifts towards 495 nm. This spectrum remains until the temperature reaches -40°C. Even if rhodopsin or isorhodopsin can be photochemically

reformed from lumirhodopsin at these temperatures, their concentrations should remain low as the sample is continuously irradiated by the incident light beam. Above -40°C the spectrum centers at 480 nm, showing the photoacoustic effect in metarhodopsin I.

The spectra recorded with homogeneous samples (rhodopsin or rod outer segments) do not show any particular frequency dependence. Such is not the case when whole retina is measured. Due to anisotropic molecular distribution of the retina, its spectrum should show a frequency dependence because the sample depth probed in photoacoustic spectroscopy corresponds to a thermal diffusion length (μ) defined as $\mu = (2\alpha/\omega)^{\frac{1}{2}}$, where α is the thermal diffusivity and ω the modulation frequency (12). Molecules located within sample's characteristic μ contribute to signal generation while the heat pulses generated upon thermal decay of molecules located deeper than μ are damped out before reaching the sample surface. Retinae are slipped upside down on a quartz slide during preparation and rod outer segments should locate at top of the sample, provided structural integrity is maintained at least partially. One would then expect to measure only the ROS spectrum at high frequency while the low frequency spectra should reveal photoacoustics signals arising from other cellular components. It can be seen in figure 2A that at 465 Hz, the photoacoustic spectrum of a whole retina is different from the spectrum of the ROS. Even if the previously observed rhodopsin bands are the most important, significant signals exist in the 350 and 420 nm region. The appearance of such non-rhodopsin bands at high frequency is probably due to mechanical disruption during preparation since their amplitude varies from sample to sample. However, considerable growth is observed in those non-rhodopsin signals when frequency is lowered at 100 Hz, indicating that they arise from molecular species located below the rod outer segments layer. Many cellular components, including retinal, could be responsible for the appearance of photoacoustic signals in the 350 nm region, so,

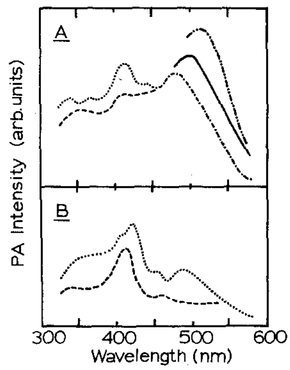

Fig. 2 A) Photoacoustic spectra of whole retina. Only the 500-600 nm region of the photoequilibrium mixture observed at -165°C (-·-·-·-·-·) and of the lumirhodopsin at -100°C (——) is shown. At -25°C, the metarhodopsin I spectra recorded at 465 Hz (----) and 100 Hz (····) are traced. Both metarhodopsin I spectra were normalized at 480 nm.

B) Photoacoustic spectra of whole retinae, carefully spread (····) and homogenized (----). Temperature: 20°C. Frequency: 450 Hz. At this temperature, rhodopsin bleaches rapidly and the 420 nm band clearly appears in the spread retina spectrum. Shoulder also exists at 410 nm. In the homogenized retina spectrum, the most important band is located at 410 nm. Purified hemoglobin shows such a 410 nm band.

any band attribution in this spectral region would be only speculative. On the other hand, it appears reasonable to attribute the 420 nm band to mitochondrial cytochromes as it occurs in spectra probing larger depth. According the the equation given above, the 420 nm component occurs at a μ of 20 μm. However little quantitative significance should be paid to this value which is calculated taking α as 10^{-3} cm^2 s^{-1}, an average value for biological material at room temperature (13). The value of α for retinian rods might differ, especially at low temperatures. In no case could the 420 nm band be attributed to the hemoglobin of retinian capillaries which gives rise to band located at 410 nm when spread retina is replaced by homogeneized retina, as depicted in figure 2B.

The importance that should be given to the intensity of a photoacoustic spectrum is somewhat puzzling since it depends on photophysical, thermal and absorption properties specific to each sample. However, it is

convenient to relate a sample's photoacoustic signal amplitude to carbon black's signal amplitude. The relative intensities range from 0,055 to 0,065, depending on temperature and the nature of the sample (rhodopsin, ROS, retina). This might to be due to the variance of any of the photophysical, thermal or environmental factors determining signal amplitude. However, none of the visual pigments photoacoustic spectra can be referred to as having weak intensity. Spectra recorded with the non-fluorescent non-photosensitive hemoglobin show relative intensity comparable to visual pigments spectra. Thus, we can say that non-radiative transitions are very important in cattle visual pigment, whatever the nature of the sample and whatever the intermediate looked at. On this basis, the photoacoustic spectra presented here can be considered as reflecting the absorption properties of the last layers of cattle retina.

REFERENCES

1. Falk, G. and Fatt, P. in "Handbook of sensory physiology-Photochemistry of Vision" (H.J.S. Dartnall Ed.) Springer-Verlag, New York, pp. 209-244 (1972).
2. Brown, P.K., Nature New Biol., 236, 35-38 (1972).
3. Guzzo, A. and Pool, G., Photochem. Photobiol., 9, 565-570 (1969).
4. Guzzo, A. and Pool, G., Science, 159, 312-314 (1968).
5. Bush, G.E., Applebury, M., Lamola, A.A. and Rentzepis, P.M., Proc. Nat. Acad. Sci. U.S.A., 69, 2802-2806 (1972).
6. Bensasson, R., Land, E.J. and Truscott, T.G., Photochem. Photobiol. 17, 53-55 (1973).
7. Rosencwaig, A., Ann. Rev. Biophys. Bioeng., 9, 31-54 (1980).
8. Ducharme, D., Tessier, A. and Leblanc, R.M., Rev. Sci. Instrum., 50, 42-43 (1979).
9. Makino, M., Hamanaka, T., Orii, Y. and Kitō, Y., Biochim. Biophys. Acta, 495, 299 - 311 (1977).
10. Yoshizawa, T. and Wald, G., Nature (Lond.) 197, 1279-1286 (1963).
11. Yoshizawa, T., pp. 146-179 in ref 1.
12. Rosencwaig, A. and Gersho, A., J. Appl. Phys., 47, 64-69 (1976).
13. Touloukian, Y.S., Powell, R.W., Ho, C.Y. and Nicolau, M.C. (eds), Thermophysical Properties of Matter, Vol. 10, 622-648 (IFI/Plenum, New York, 1973).