FLASH ABSORPTION SPECTROSCOPY. PRINCIPLE AND APPLICATIONS IN PLANT PHOTOSYNTHESIS RESEARCH

Klaus BRETTEL

1. Introduction

Flash absorption spectroscopy is used to monitor the kinetics and spectra of flash-induced absorption changes, which may provide information on molecular events in the sample. Usually, the system is perturbed by a light pulse (actinic flash) which is short compared to the characteristic time of the reaction(s) to be studied. The absorption changes induced by the actinic flash are probed by a usually continuous light beam (measuring light) passing through the sample. This technique has proven particularly useful in photosynthesis research, because the stimulus, light, is the natural driving force of photosynthesis, and because many of the photosynthetic light reactions are accompanied by rather characteristic absorption changes in the visible, near UV and near infrared spectral regions.

Flash absorption spectroscopy and its application in photosynthesis research has been reviewed in detail by Junge in 1976 [1]. In this contribution, I will first summarise the principle and the instrumentation of flash absorption spectroscopy and then focus on practical aspects, some of which are related to the use of lasers as measuring light sources and were not yet treated in the review by Junge [1].

2. Principle

Suppose that the sample to be studied is contained in a cuvette with an optical path l (Fig. 1) and that the actinic flash induces a reaction $X \rightarrow Y$. This reaction will be accompanied by an absorbance change ΔA (at wavelength λ)

$$\Delta A = \Delta\varepsilon \cdot \Delta c \cdot l \qquad (1)$$

where $\Delta\varepsilon = \varepsilon_Y - \varepsilon_X$ is the difference of the molar decadic absorption coefficients (at wavelength λ) of Y and X, and Δc is the change in concentration of the product Y (Eq. 1 can easily be extended to more complicated reactions). The cuvette is crossed by a beam
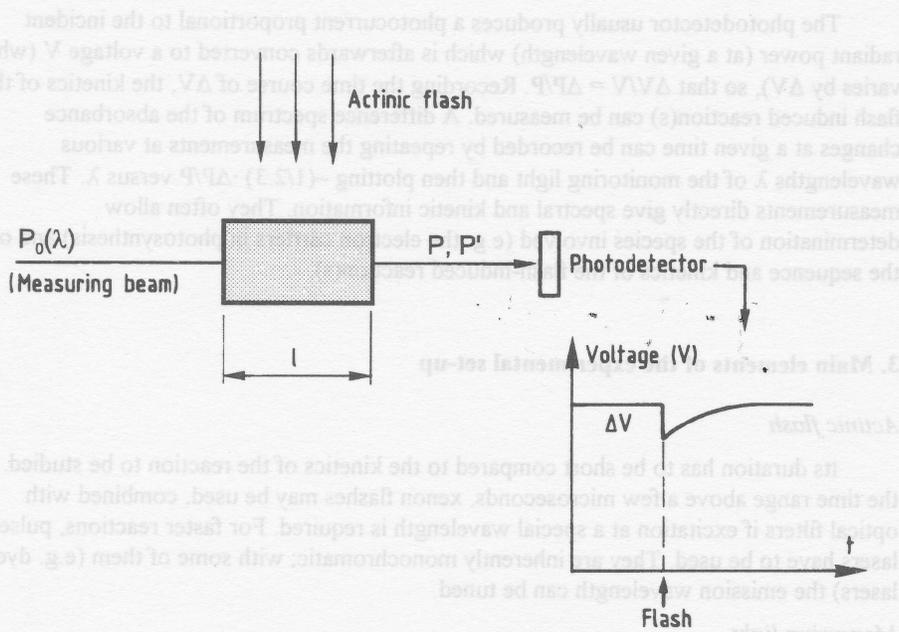
Fig. 1. Principle of measurement of flash-induced absorption changes

of continuous monochromatic light of wavelength λ (monitoring or measuring light) with a radiant power P_0 at the entrance of the cuvette. The measuring light is attenuated by the absorption due to the molecules in the sample (including molecules which do not participate in the flash induced reaction), the windows of the cuvette and possibly other optical elements between the cuvette and the photodetector. The radiant power seen by the photodetector is P prior to the actinic flash and P'(t) at time t after the actinic flash. $\Delta P = P' - P$ can be positive or negative, depending on whether the reaction is accompanied by a bleaching or by an absorption increase. Using the definition of absorbance, $A = \lg(P_0/P)$, we can write

(before the flash) $A_{sample} + A_{other\ elem} = \log(P_0/P)$

(after the flash) $A'_{sample} + A_{other\ elem} = \log(P_0/P')$

Substituting $A'_{sample} - A_{sample} = \Delta A$, one obtains

$$\Delta A = \log(P/P') = \log[P/(P+\Delta P)] = -\log(1+\Delta P/P) \qquad (2)$$

In the limit of small absorbance changes (i.e. $\Delta P/P \ll 1$), Eq. 2 can be approximated by

$$\Delta A = -(1/\ln10) \cdot \Delta P/P \approx -(1/2.3) \cdot \Delta P/P \qquad (3)$$

The error due to these approximations is below 1% for $|\Delta P/P| < 0.01$ and approx. 5% for $|\Delta P/P| = 0.1$.

The photodetector usually produces a photocurrent proportional to the incident radiant power (at a given wavelength) which is afterwards converted to a voltage V (which varies by ΔV), so that $\Delta V/V = \Delta P/P$. Recording the time course of ΔV, the kinetics of the flash induced reaction(s) can be measured. A difference spectrum of the absorbance changes at a given time can be recorded by repeating the measurements at various wavelengths λ of the monitoring light and then plotting $-(1/2.3) \cdot \Delta P/P$ versus λ. These measurements directly give spectral and kinetic information. They often allow determination of the species involved (e.g. the electron carriers in photosynthesis) and of the sequence and kinetics of the flash-induced reaction(s).

3. Main elements of the experimental set-up

Actinic flash

Its duration has to be short compared to the kinetics of the reaction to be studied. In the time range above a few microseconds, xenon flashes may be used, combined with optical filters if excitation at a special wavelength is required. For faster reactions, pulse lasers have to be used. They are inherently monochromatic; with some of them (e.g. dye lasers) the emission wavelength can be tuned.

Measuring light

Conventional lamps (tungsten, xenon, mercury etc.) or continuous lasers may be used. As most conventional lamps have a broad emission spectrum, monochromators or optical filters have to be used to obtain (nearly) monochromatic light. Lasers have the advantage of emitting monochromatic light of high intensity. They can be focused on a small photodetector. Tuning of the wavelength is limited, however.

Detection system

It consists in general of a photodetector (photodiode or photomultiplier), electronic amplifiers and a transient recorder (analog/digital converter and storage element). Photomultipliers provide internal, low-noise amplification of the photocurrent and are the best choice when the measuring light intensity is low. At higher intensities, they become saturated, and a photodiode with subsequent electronic amplifier should be used. The amplifier is often the main source of noise in such a detection system.

In many cases it is necessary to place optical filters in front of the photodetector. They transmit the measuring light, but block scattered light from the actinic flash which otherwise could be misinterpreted as a transient bleaching of the sample.

4. Some practical aspects

4.1. Time resolution

The time resolution of the apparatus can be limited by the duration of the actinic flash, the electronic bandwidth of the detection system or by the sampling rate of the transient recorder. The fastest commercially available transient recorders have an electronic bandwidth from DC to about 1 GHz and sampling rates of a few Gigasamples

per second, allowing a time resolution in the order of 1 ns. It should also be mentioned that the response of fast detection systems is often far from perfect and can falsify the kinetics considerably (e.g. overshooting upon a fast transition; see [2] for a method of detector testing).

Faster reactions (down to the fs range) can be resolved by the "pump and probe" technique where the continuous measuring light is replaced by short laser pulses ("probe") at various delay times after the actinic flash ("pump"). As the time resolution is obtained by delaying the probe pulses, an integral measurement of the energy of the probe pulse behind the sample is sufficient and a fast detection system is not required (see [3] for a recent review on this technique; the same principle can also be used on slower time scales [4]).

4.2. Signal-to-noise ratio

As the flash-induced absorbance changes in photosynthetic samples from plants are usually rather small (rarely larger than 10^{-3}), monitoring by flash absorption spectroscopy is often hindered by a insufficient signal to noise ratio (S/N), expressed as the ratio of ΔV and the noise voltage V_n, i.e. $S/N = \Delta V/V_n$. When the noise is purely random ("white noise"), V_n is proportional to the square root of the electronic bandwidth of the detection system, so that the difficulties to obtain a sufficient signal-to-noise ration increase with increasing rate of the reaction to be studied.

It is possible to improve the signal-to-noise ratio by adding up a certain number (n) of signals measured under identical conditions. Because the accumulated signal amplitude increases proportional to n, but the random noise only proportional to $\sqrt{n}$, S/N increases proportional to $\sqrt{n}$. However, due to practical limitations (time, quantity of sample) it is often not convenient to accumulate a too large number of signals. Therefore, one should first look for other possibilities to improve the signal-to-noise ratio.

The noise originates from three major sources:

(1) From the statistics of the photons of the measuring light and of the photoelectrons created in the detector (shot noise); the shot noise voltage is proportional to the square root of the primary photocurrent in the detector.

(2) From additional fluctuations of the radiant power emitted by the measuring light source; the resulting noise voltage is proportional to the radiant power reaching the detector and hence proportional to the photocurrent.

(3) From the thermal movements of the electrons in the detection system (thermal noise); the thermal noise is independent of the measuring light.

In a widely used set-up, a tungsten incandescent lamp serves as the measuring light source, and a photomultiplier as the detector. In this case, noise sources (2) and (3) are usually negligible, and the signal-to-noise ratio increases proportional to the square root of the radiant power P reaching the detector (according to Eq. 3, $\Delta P \propto P$ for a given absorbance change ΔA of the sample, while the shot noise is "only" proportional to $\sqrt{P}$). Because of the limited spectral radiant intensity of incandescent lamps, it is hardly possible to resolve reactions in the sub-microsecond range in photosynthetic samples from plants.

Much higher spectral intensities of the measuring light are now available from laser

sources. As photomultipliers support only weak light, one has to use a photodiode coupled to an electronic amplifier as detection system, which introduces additional (thermal) noise. This disadvantage is, however, largely overcompensated by the gain in signal amplitude due to the higher radiant power. Thus, it became possible to monitor some reactions in plant photosynthesis with a time resolution close to 1 ns (see section 4.5).

Evidently, ΔA can be increased by increasing the concentration c of the sample or the optical path l (see Eq. 1). However, for a given measuring light source, an increase of c or l decreases the radiant power P arriving at the detector, so that the signal-to-noise ratio would decrease when the transmission of the sample becomes too low. In fact, there exists an optimum for the product c·l with respect to the signal-to-noise ratio. For the simplest case, when the shot noise is the only noise source, it has been shown [5] that the best signal-to-noise ratio is obtained when c·l is chosen such that the transmission of the sample is approx. 14% at the wavelength of the measuring light (trace a in Fig. 2). The general case, that all three noise sources mentioned above contribute, has also been analysed ([6], see Fig. 2 for examples) leading to the following conclusions: When the thermal noise of the detection system becomes dominating (trace b), the optimum shifts to a higher transmission (i.e. a less concentrated sample or a shorter optical path). When, however,
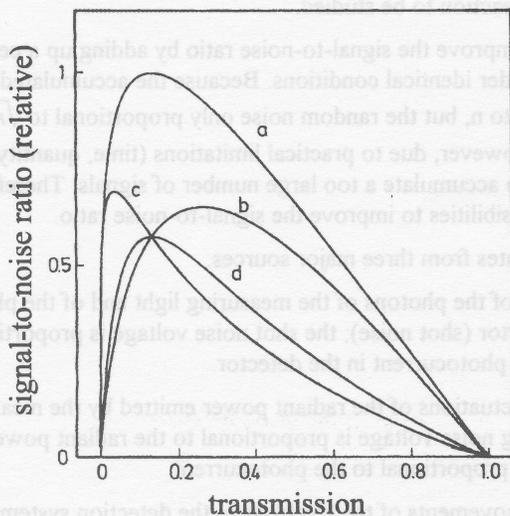

Fig. 2. Dependence of the signal-to-noise ratio on the transmission T of the sample. Different T values represent different optical paths l or different concentrations c of the same sample. The following assumptions about the noise sources were made: Trace a: shot noise is the only noise source. Trace b: thermal noise of the detection system contributes in addition to the shot noise; the ratio of thermal noise to shot noise is $\sqrt{2} : 1$ at T = 0.14. Trace c: fluctuations of the radiant power of the measuring light source contribute in addition to the shot noise; the ratio of these fluctuations to the shot noise is $\sqrt{2} : 1$ at T = 0.14. Trace d: all three noise sources contribute equally at T = 0.14. See text and Ref. [6] for further details. Redrawn from Ref. [6].

fluctuations of the radiant power of the measuring light source are dominating (trace c), the optimum occurs at a transmission below 14%. Whenever the signal-to-noise ratio is critical for a flash absorption experiment, it is worthwhile to check for the optimal concentration and optical path of the sample.

4.3. Actinic effect of the measuring light

The measuring light can have the undesirable effect of inducing the (photo)reaction to be studied already prior to the actinic flash. This effect can be diminished (at the expense of the signal to noise ratio) by decreasing the intensity of the measuring light. Another possibility is to switch on the measuring light only very shortly before the actinic flash. Sometimes the reaction can be studied at a wavelength, where only the product(s) absorb, but not the dark adapted state of the sample. In this case, even high intensities of the measuring light do not disturb the experiment.

4.4. Fluorescence artefact

Similarly to scattered light (see section 3), flash induced fluorescence reaching the photodetector would pretend a transient bleaching of the sample. If fluorescence occurs at the wavelength of the measuring light, it can not be blocked by optical filters. In this case, it can be helpful to increase the distance between sample and detector and to place a small diaphragm in front of the detector. This decreases the fraction of the fluorescence which is "seen" by the detector because the fluorescence of the sample is emitted in all directions in space. In addition, one may record the fluorescence artefact separately (in the absence of the measuring light) and subtract it from the signal recorded in the presence of the measuring light.

4.5. Examples of set-ups with sub-microsecond time-resolution

- The photoreactions of the chlorophyll-*a* type primary electron donors of photosystem I (P700) and photosystem II (P680) can be studied conveniently with a set-up using a laser diode emitting at 820 nm as the measuring light source and a fast (and small) photodiode as detector. As the chlorophyll-*a* cation absorbs at around 820 nm, but neutral chlorophyll *a* does not, photooxidation of P700 or P680 is accompanied by an absorption increase at 820 nm. High measuring light intensities can be used without actinic effect on the sample, because the absorbance at 820 nm of the photosynthetic apparatus is virtually zero in the dark adapted state (prior to the actinic flash). Furthermore, provided that the sample is optically clear, high concentrations of the sample and a long optical path l can be used without decreasing the radiant power P reaching the detector. As another advantage, the laser light can be readily focused on a small photodiode at a large distance from the sample, thus decreasing the fluorescence artefact. Such a set-up was introduced by van Best and Mathis [7] to study the rereduction kinetics of P680$^+$. With some more recent technical improvements, it is now possible to study P680$^+$ [8-10] and P700$^-$ [11, 12] with a time resolution close to 1 ns.

- To monitor the photoreactions of P680 in the red-most absorption band of its neutral form (around 680 nm; this band is bleached upon photooxidation of P680), a tuneable dye laser (pumped by an Ar ion laser) was used as measuring light source. The fluorescence artefact, which is very strong around 680 nm in a standard set-up, could be largely reduced by using a small photodiode at a large distance (approx. 2 m) from the

sample. The measuring light was pulsed by means of an electro-optical modulator in order to diminish the actinic effect [13, 14, 9]. More recently, red laser diodes (which are rather cheap) were used as measuring light sources [10].

- To study absorption changes in the UV and blue spectral regions with a time resolution of a few nanoseconds, a pulsed measuring light of high radiant power was realised using a Xe flash lamp emitting flashes of approx. 50 µs duration. The top of the flash is sufficiently flat to be used as a quasi continuous measuring light during approx. 1 µs. Such a set-up was applied successfully to monitor the oxidation of the immediate electron donor to $P680^+$ (which turned out to be a tyrosine) [15, 16], and electron transfer through the secondary electron acceptor A_1 (phylloquinone) in photosystem I [17-19].

References

1 Junge W (1976) in *Biochemistry of Plant Pigments* (Goodwin TW, ed.), Vol. II, pp. 233-333, Academic Press, New York.

2 Brettel K and Schlodder E (1988) *Rev. Sci. Instrum.* **59**: 670-671.

3 Van Amerongen H and Van Grondelle R (1995) *Methods Enzymol.* **246**: 201-226.

4 Joliot P, Beal, D and Frilley, B (1980) *Journal de chimie physique* **77**: 209-216.

5 Rüppel H and Witt HT (1970) *Methods Enzymol.* **16**: 316-380.

6 Brettel K (1984) Ph.D. Thesis, Technische Universität Berlin.

7 Van Best JA and Mathis P (1978) *Rev. Sci. Instrum.* **49**: 1332-1335.

8 Brettel K, Schlodder, E and Witt HT (1984) *Biochim. Biophys. Acta* **766**: 403-415.

9 Schlodder E and Brettel K (1988) *Biochim. Biophys. Acta* **933**: 22-34.

10 Van Mieghem F, Brettel K, Hillmann B, Kamlowski A, Rutherford AW and Schlodder E (1995) *Biochemistry* **34**: 4798-4813.

11 Brettel K andSétif P (1987) *Biochim. Biophys. Acta* **893**: 109-114.

12 Brettel K and Golbeck JH (1995) *Photosynth. Res.* **45**: 183-193.

13 Eckert HJ, Renger G and Witt HT (1984) *FEBS Lett.* **167**: 316-320.

14 Schlodder E, Brettel K, Schatz GH and Witt, HT (1984) *Biochim. Biophys. Acta* **765**: 178-185.

15 Gerken S, Brettel K, Schlodder E and Witt HT (1987) *FEBS Lett.* **223**: 376-380.

16 Gerken S, Brettel K, Schlodder E and Witt HT (1988) *FEBS Lett.* **237**: 69-75.

17 Mathis P and Sétif P (1988) *FEBS Lett.* **237**: 65-68.

18 Brettel K (1988) *FEBS Lett.* **239**: 93-98.

19 Sétif P and Brettel K (1993) *Biochemistry* **32**: 7846-7854.

Address of the author: Section de Bioénergétique and CNRS-URA 2096
Département de Biologie Cellulaire et Moléculaire
CEA Saclay
91191 Gif-sur-Yvette Cedex
France