OXYGEN EVOLUTION AT SUB-ZERO TEMPERATURES BY CHLOROPLASTS SUSPENDED IN FLUID MEDIA

Raymond P. COX
Institut de Biologie Physico - Chimique, 13, rue Pierre et Marie Curie, 75005, Paris, France

Received 23 June 1975

1. Introduction

Chloroplasts suspended in fluid media composed of mixtures of polyols (ethylene glycol or glycerol) and water show light-induced electron transport to acceptors reduced after Photosystem I at temperatures as low as −35°C. This was demonstrated both by the measurement of the reduction of artificial electron acceptors [1] and by the observation of the repeated dark reduction of P700 following photooxidation in the presence of methyl viologen [2]. The inhibition of both reactions by 3(3,4-dichlorophenyl)-1,1-dimethylurea provided evidence for the donation of electrons before Photosystem II as did the experiments of Joliot [3] who showed that 6 to 7 electrons could be transferred to the plastoquinone pool by illumination of a frozen chloroplast suspension at −30°C.

However these results all left open the question whether the water oxidising system was operative under these conditions, or whether electrons were being provided from another source. In the case of experiments performed in fluid media the oxidation of the polyol to the corresponding aldehyde cannot be excluded. This point is of considerable interest in view of the possibility of using sub-zero temperatures to study the process of water oxidation by the chloroplast. The experiments reported here show that the production of oxygen can be observed at below −15°C in a fluid medium containing 50% (v/v) ethylene glycol.

2. Materials and methods

One of the most sensitive methods available for the detection of photosynthetic oxygen production is the oxygen luminometer [4]. The principle of this apparatus is that an oxygen-free carrier gas (in this case nitrogen) is passed over a suspension of photosynthetic material and then over a solution of luminol in alkaline dimethylsulphoxide. The measured chemiluminescence is proportional to the oxygen content of the carrier gas and hence to the oxygen concentration in the suspension of photosynthetic material. The oxygen luminometer used was built by Dr. Bruce Diner [5] and was a modification of the design of Burr [4,6].

Chloroplasts from spinach (*Spinacea oleracea*) were prepared and stored frozen as described previously [2]. The thawed chloroplast suspension (about 1.0 mg chlorophyll) was centrifuged and the pellet resuspended in 2.0 ml of a medium containing 50% (v/v) ethylene glycol (pro analysi grade from E. Merck, Darmstadt), 0.3 M sorbitol, 10 mM NaCl, 2.5 mM $MgCl_2$ and potassium phosphate buffer (15 mM K_2HPO_4 − 10 mM KH_2PO_4). The buffer had an effective pH (pH*) of 7.6 in the medium at 20°C, changing little with temperature [7]. The chloroplast suspension was transferred to the luminometer 'cuvette' which was protected from the light while 5 mM NH_4Cl, 25 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine (from a 25 mM solution in ethanol) and 2 mM $K_3Fe(CN)_6$ were added. The cuvette was then

attached to the apparatus for equilibration at room temperature in the dark. The chloroplast suspension was continually stirred by a small 'flea' driven by a rotating magnet beneath the cuvette and its surrounding insulated bath. This small container was connected to two constant-temperature baths containing aqueous ethanol at 20°C and at a variable sub-zero temperature, in an arrangement which allowed either bath to be the source of the circulating fluid surrounding the cuvette. Illumination was from above onto a surface area of 9 cm^2. Light from a slide projector was passed through 15 mm of water and various filters and directed downwards with a plane mirror.

3. Results and discussion

Normally the oxygen luminometer is used to measure the rate of oxygen evolution by an illuminated sample when a steady state has been reached. At sub-zero temperatures with media containing ethylene glycol and water the time taken for the luminescence to reach a maximum was very long (10–30 min). Oxygen production could be observed on illumination with either red light (obtained with a Balzers K7 filter) or yellow-green light (obtained with a Pyrex 3482 Filter and a solution of $NiSO_4$, chosen to get the maximum illumination of the bottom layers of the dense chloroplast suspension) at temperatures at least as low as −19°C. However in view of the probable failure to reach a true steady state no quantitative measurement was possible under these conditions.

In an attempt to circumvent this problem the apparatus was modified to allow an integrated measurement to be taken by isolating the photosynthetic material during illumination. The following experimental protocol was adopted. Following equilibration in the dark at room temperature for at least an hour, the cuvette was isolated (the carrier gas running through a bypass) and the fluid surrounding the cuvette was changed to that from the low temperature bath. After allowing 5 min for temperature equilibration, the light was switched on. After the period of illumination, the fluid surrounding the cuvette was changed to that at 20°C and the valves were opened to allow the carrier gas to pass over the chloroplast suspension.

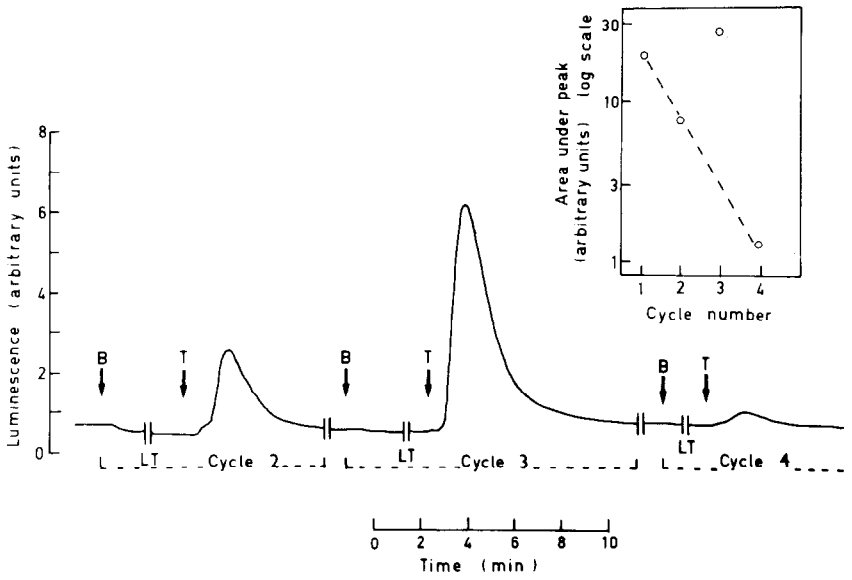

Fig.1. Chemiluminescence produced by the oxygen luminometer during an experiment. The cuvette containing the chloroplast suspension was isolated at the points marked B and the gas allowed to flow over the suspension at the points marked T. During the gaps in the trace marked LT the temperature of the cuvette was lowered to −16.5°C. In the case of 'cycle 3' only, the chloroplasts were illuminated during the last 10 min. of this period. The inset shows the calculated area under each curve. Further details are given in the text.

Under these conditions an artefact was produced by the isolation–cooling–warming–reconnection cycle. This could not safely be regarded as reproducible, but a series of artefacts from successive cooling–warming cycles in the same experiment was always found to decrease regularly in size. The procedure adopted was therefore to place the measurement cycle in which the chloroplasts were illuminated, in a sequence of cycles in which the chloroplasts were kept in the dark. This enabled a reasonable estimate to be made of the size of the artefact.

Fig.1 shows the results of an experiment in which chloroplasts were illuminated for 10 min at $-16^{\circ}C$ with the yellow-green light. It is evident that oxygen is produced under these conditions.

Calibration of the luminometer by the response produced by allowing a known current to flow for various times across an electrolysis cell containing dilute KOH suggested that 150 nmol O_2 had been produced, corresponding to 0.9 μmol/mg chlorophyll · hr. This is equivalent to several hundred turnovers of each reaction centre of Photosystem II during the period of illumination. Hence it seems clear that the 'water dehydrogenase' system is capable of turning over many times in the presence of 50% (v/v) ethylene glycol at $-16^{\circ}C$.

The amount of oxygen produced is rather less than that expected from the effects of solvent and temperature on the reduction of ferricyanide in the presence of 2,3,5,6-tetramethyl-*p*-phenylenediamine (Ref. [1] and unpublished results). However, although this method of measurement is superior to one requiring the production of a steady state, it is still far from satisfactory as a quantitative way of measuring oxygen production at sub-zero temperatures. Sources of error include the effects of ageing in the presence of the solvent during equilibration, the fact that illumination is almost certainly not saturating for the chloroplasts at the bottom of the dense suspension used, and the probable decline in the rate of electron transport during the period of illumination. It is not possible to eliminate all of these problems at once without reducing the amount of oxygen produced to a level where it could not be separated from the artefact.

In view of the various factors which would tend to cause an underestimation of the rate of oxygen production under these conditions, it seems reasonable to suppose that a major part of the electrons donated to photosystem II at temperatures at least as low as $-20^{\circ}C$ come from the oxidation of water. In any case, there is no doubt that the water oxidising system is able to turn over at temperatures inferior to $-15^{\circ}C$.

Acknowledgements

I am indebted to the Royal Society for a Fellowship awarded under the European Science Exchange Programme. I am grateful to Dr. Bruce Diner for advice about the use of the oxygen luminometer.

References

[1] Cox, R. P. (1975) Biochim. Biophys. Acta 387, 588–598.
[2] Cox, R. P. (1975) Europ. J. Biochem., in the press.
[3] Joliot, A. (1974) Biochim. Biophys. Acta 357, 439–448.
[4] Burr, A. H. and Mauzerall, D. (1968) Biochim. Biophys. Acta 153, 614–624.
[5] Diner, B. A. (1972) Ph. D. Thesis, The Rockefeller University, New York.
[6] Burr, A. H. (1966) Ph. D. Thesis, The Rockefeller University, New York.
[7] Hui Bon Hoa, G. and Douzou, P. (1973) J. Biol. Chem. 248, 4649–4654.