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Enrichment of bacteriochlorophyll *g'* in membranes of *Heliobacterium chlorum* by ether extraction

Unequivocal evidence for its existence in vivo

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Treatment of *H. chlorum* membrane preparations with diethyl ether of high degrees of water saturation raised the bacteriochlorophyll (BChl) *g'* mole fraction, as determined by HPLC analysis of their acetone extracts, toward a level of 40% of total BChl *g* or higher. Starting from pure BChl *g*, the BChl *g'* mole fraction should never exceed 24.6% which is the equilibrium value in diethyl ether. The existence (and possible functioning) of BChl *g'* in vivo is thus unequivocally demonstrated.

Heliobacterium reaction center; Bacteriochlorophyll *g'*; Epimerization; High-performance liquid chromatography

1. INTRODUCTION

We previously showed the presence of chlorophyll (Chl) *a'*, the 13^2-epimer of Chl *a*, in the core of photosystem (PS) I of higher plants and cyanobacteria [1]. In subsequent work we found bacteriochlorophyll (BChl) *g'* (Fig. 1) at a molar ratio BChl *g*/BChl *g'* = 18 in *Heliobacterium chlorum* and *Heliobacillus mobilis* [2]. This figure, combined with a BChl *g*/P798 (reaction center) molar ratio of 35–40, suggested that P798 might be a BChl *g'* dimer. However, although the appropriate control experiments were done [2], the possibility that some epimerization occurred during extraction of BChl *g* from the membrane could not be entirely excluded.

Treatment of spinach PSI particles with water-containing diethyl ether leads to enrichment in P700 [3] and Chl *a'* [1] relative to the antenna Chls. With increasing degree of water saturation, both P700 and Chl *a'* were enriched from about 0.3% to 12% of the total amount of Chl a, with the ratio Chl *a'*/P700 maintained at unity [1]. This ether treatment was applied here to *H. chlorum* membranes, to observe a BChl *g'* enrichment from an initial (in vivo) value of 5.2% to a value well in excess of 40% at higher water saturation in diethyl ether. This result, together with the observation that the equilibrium BChl *g'* mole fraction in diethyl ether is significantly lower, shows that BChl *g'* is an integral component of the photosynthetic apparatus of *H. chlorum*.

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2. MATERIALS AND METHODS

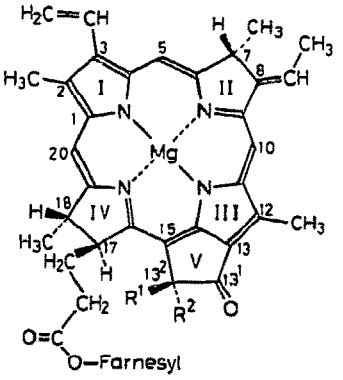
Procedures for the culture of *H. chlorum* and the preparation of membranes were described elsewhere [2,4]. Ether treatment of the membranes was carried out in essentially the same manner as in a previous report [3]. Briefly, about 2 mg of freeze-dried membranes were washed once at 4°C with ca. 35 ml of diethyl ether with water at various degrees of saturation. 2-Mercaptoethanol was added at 1% to prevent BChl *g* degradations. The membranes were precipitated by centrifugation at 13 000 × *g* for 3 min at 4°C, and then the pigments were extracted from the membranes with ca. 10 ml of acetone by sonication for 2 min at 4°C. The acetone extract was filtered through a Teflon filter (Tosoh, H-25-5) and immediately dried in a rotary evaporator at 10^{-3} Torr. The solid material was then dissolved in ca. 20 μl of chloroform, and a 3-μl aliquot was injected into a silica HPLC column (Senshupak 1251-N, 250 × 4.6 mm i.d.) cooled to 4°C in an ice-water bath. The pigments were eluted isocratically with degassed hexane/2-propanol/methanol (100:1.7:0.2) at a flow rate of 0.9 ml/min and monitored with a JASCO 875-UV detector at 400 nm.

The kinetics of BChl *g* ⇌ BChl *g'* epimerization were studied as described elsewhere [5,6]. Briefly, BChl *g* (epimeric purity 99.94%) or BChl *g'* (99.96%) was dissolved in diethyl ether containing 0.1 M triethylamine at 25°C, and the temporal evolution of the epimeric composition was measured by HPLC as described above to obtain the equilibrium as well as rate constants of epimerization.

3. RESULTS AND DISCUSSION

3.1. *Enrichment of BChl g' by ether treatment of the membranes*

Membranes of *H. chlorum* were treated with diethyl ether at various degrees of water saturation. Fig. 2 shows the mole fraction of BChl *g'* with respect to total



	R^1	R^2
BChl g	H	$COOCH_3$
BChl g'	$COOCH_3$	H

Fig. 1. Structures of BChl *g* and BChl *g'*.

BChl *g* in the membranes after the treatment. Treatment with dry ether (0% saturation) gave a BChl *g'* mole fraction of 5.2%, the same as that in untreated membranes. An HPLC chart for the latter sample is given as trace A in Fig. 3. Upon increasing the degree of water saturation, the BChl *g'* mole fraction increased, first slowly and then abruptly, and finally reached a level of 40% or higher. Trace C in Fig. 3 is the HPLC chart for an acetone extract of the membranes treated with diethyl ether of 90% water saturation. Under these

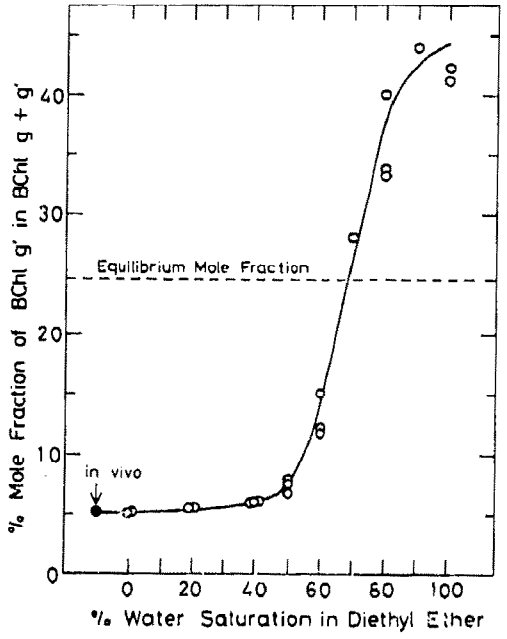


Fig. 2. Enrichment in the BChl *g'* mole fraction by treatment of the *Heliobacterium chlorum* membrane preparations with diethyl ether of various degrees of water saturation. The dashed line shows the equilibrium fraction in diethyl ether (see text).

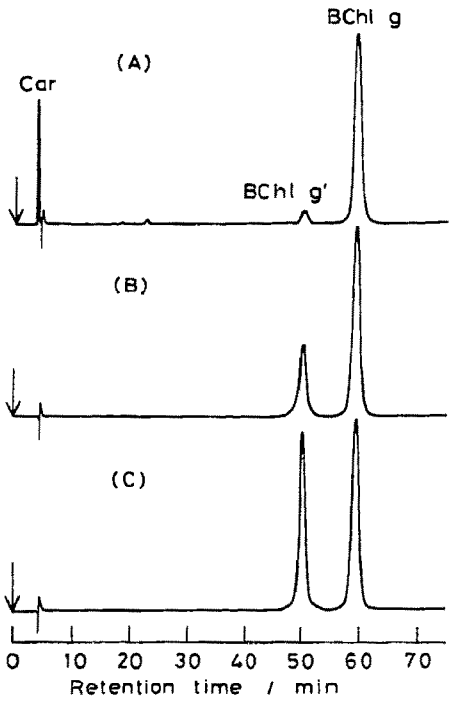


Fig. 3. HPLC traces for the acetone extract of *H. chlorum* membranes before ether treatment (A), the BChl *g*/*g'* mixture at thermodynamic equilibrium in vitro (B), and the acetone extract of *H. chlorum* membranes after treatment with diethyl ether of 90% water saturation (C). The minor peaks at 18 and 23 min (trace A) are due to BPheo *g'* and BPheo *g*, respectively [2].

conditions about 99% of BChl *g* was extracted, while bacteriopheophytin (BPheo) *g* and BPheo *g'*, which are degradation products of BChl *g* [2], and the carotenoid (neurosporene) are completely removed.

3.2. *Inertness of BChl* g *to ether treatment in vitro*

A 99.94% pure sample of BChl *g*, obtained by preparative-scale HPLC, was treated with diethyl ether of 90% water saturation. The solution was centrifuged, evaporated to dryness, and the residue was redissolved in acetone, sonicated, filtered, and finally injected into the HPLC column, as in the handling of the membranes. In the whole course of this procedure, the epimeric purity of BChl *g* was maintained at 99.94% within an error limit (±0.005%) of the present HPLC system. This indicates that the observed enrichment in BChl *g'* (Fig. 2) is not due to the epimerization of BChl *g* after extraction from the protein matrix.

3.3. *BChl* g/g' *epimerization in vitro*

The temporal evolution of the BChl *g'* mole fraction, starting from either BChl *g* or BChl *g'* in diethyl ether containing 0.1 M triethylamine as base-catalyst [6], is displayed in Fig. 4. The solid curves obey the theoretical equation [6] with forward ($g \rightarrow g'$) and backward ($g' \rightarrow g$) rate constants of 5.1×10^{-6} and 1.7×10^{-6} s^{-1}, respectively. Of more importance in the

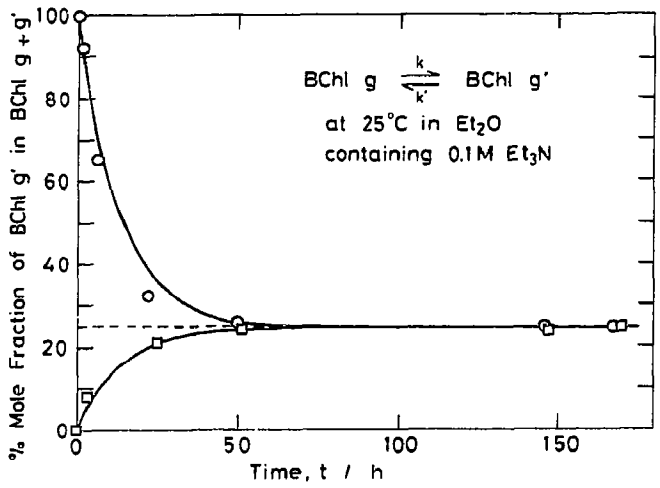


Fig. 4. Kinetic plots showing the BChl *g*/BChl *g'* epimerization in diethyl ether, starting from BChl *g* (□) or BChl *g'* (○).

context of this paper, is that the mole fraction of BChl *g'* is 24.6±0.3% at equilibrium (HPLC trace B in Fig. 3, and the dashed horizontal line in Fig. 2). The BChl *g'* mole fraction therefore should never exceed 24.6% if the starting species is pure BChl *g*. The ether treatment, in contrast, gave a BChl *g'* mole fraction much higher than this (Fig. 2). These results strongly suggest that BChl *g'* resides in the core part of the *H. chlorum* photosynthetic apparatus, and the ether treatment progressively removed antenna BChl *g* to give BChl *g'*-enriched membranes.

There still remains a possibility, even when BChl *g* is the sole chlorophyllous pigment functioning in *H. chlorum*, that the membranes can be enriched in BChl *g'*. Namely, if a small proportion of BChl *g* were converted into BChl *g'* *while bound to the protein* during the ether treatment, and if BChl *g* alone were then washed out with diethyl ether, the apparent BChl *g'* mole fraction could increase. For this to occur, the solubility of BChl *g* should be much higher than that of BChl *g'* in a nonpolar solvent as diethyl ether, i.e. BChl *g* must be much less polar than BChl *g'*. This is, however, in sharp contrast to the observation that BChl *g'* is eluted faster than BChl *g* in silica normal-phase HPLC where a nonpolar solvent is used as eluent (Fig. 3). Similarly, in reversed-phase HPLC (data not shown) BChl *g* was eluted faster than BChl *g'*. The possibility of BChl *g*→BChl *g'* conversion followed by selective BChl *g* depletion as a cause for the observed BChl *g'*-enrichment, can thus be excluded.

The present results strengthen our proposal [2] that the two molecules of BChl *g'* per P798 are indeed key components of photosynthetic apparatus of heliobacteria and, indirectly, would point to a crucial role of its counterpart, Chl *a'* [1], in the photosystem I reaction center of higher plants. At present there is a discrepancy as to the number of these pigments per reaction center: two BChl *g'* molecules per P798, and only one Chl *a'* molecule per P700. Work is currently under way toward reinvestigating the Chl *a'*/P700 stoichiometry and elucidating the roles of Chl *a'* and BChl *g'* in the molecular machinery of reaction centers.

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