Evidence that a chlorophyll *a'* dimer constitutes the photochemical reaction centre 1 (P700) in photosynthetic apparatus

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Spinach chloroplasts and their various fractions enriched in either photochemical reaction centre 1 (P700) or reaction centre 2 (P680) have been analyzed for pigment composition by high-performance liquid chromatography (HPLC). The results clearly establish a stoichiometry of chlorophyll (Chl) *a'*/P700 = 2, where Chl *a'* represents the C10 epimer of Chl *a*. This is the first demonstration of the association of Chl *a'* with any functional site in vivo. It is further shown that a Chl *a'* dimer prepared in vitro reproduces fairly well the redox and spectroscopic behaviour of P700. These findings provide strong evidence that a Chl *a'* dimer constitutes P700.

P700 *Chlorophyll a' dimer* *Photochemical reaction center* *Spinach chloroplast* *Photosynthetic pigment* *HPLC analysis*

1. INTRODUCTION

The molecular architecture or even the chemical identity of photosynthetic reaction centres 1 and 2 (usually referred to as P700 and P680, respectively) still remains an open question. So far, most workers have assumed, without persuasive experimental evidence, that both P700 and P680 consist of the ubiquitous pigment, chlorophyll (Chl) *a* (fig.1). Of these two, P700 is generally supposed to be a Chl *a* dimer, on the grounds that the ESR signal assignable to photogenerated $P700^{\cdot+}$ exhibits a linewidth roughly $1/\sqrt{2}$-fold narrower than that for monomeric Chl $a^{\cdot+}$ in vitro [1]. In contrast, Wasielewski et al. [2] consider that a monomeric Chl *a* enol could be a more plausible model of P700.

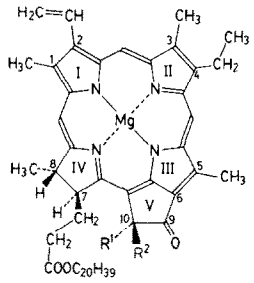

Fig.1. Structural formula for Chl *a* (R^1 = $COOCH_3$, R^2 = H) and Chl *a'* (R^1 = H, R^2 = $COOCH_3$). Replacement of the central Mg atom with 2 H atoms gives the corresponding pheophytins (Phs).

A rigorous analysis of photosynthetic apparatus for pigment composition would be useful, if not sufficient, to identify the building blocks of reaction centres. This has become possible by the introduction of normal-phase high-performance

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liquid chromatography (HPLC) enabling rapid, sensitive, and high-resolution analysis of Chl-type pigments [3,4]. Conventionally recommended analytical methods [5] are rather ineffective for this purpose, as exemplified by the fact that the Mg-free pheophytin (Ph) *a*, present in vivo at a Ph *a*/Chl *a* molar ratio as high as ~1:100 [3,6], was detected only recently. In a previous work we detected Chl *a'* (C10 epimer of Chl *a*) at a molar ratio of Chl *a'*/Chl *a* ~ 1:300 in leaves of different plants [3]. Noticing that this is roughly the P700/Chl *a* or P680/Chl *a* molar ratio [7], we extend the analysis here to spinach chloroplasts and their fractions enriched in either P700 or P680. The results demonstrate the presence of 2 Chl *a'* molecules per P700. This, together with the results from additional experiments, supports a totally unprecedented view that P700 is composed of a Chl *a'* dimer.

2. MATERIALS AND METHODS

Spinach chloroplasts were prepared as described [8]. Subchloroplast preparations a (AS-1), b (DT-144), c (DS-1), d (D-50), e (D-10), f (E-516), g (Z-1), h (DS-2) and i (D-144), containing P700 at a variety of levels, were obtained as follows. Digitonin-treated particles (D-10, D-50, D-144) were prepared from spinach chloroplasts according to Anderson and Boardman [9]. The D-144 preparation was subjected to sucrose-gradient centrifugation for partial purification (DS-1, DS-2). The D-144 preparation was further treated with Triton X-100, and a photosystem (PS) I-enriched fraction (DT-144) was separated from other chlorophyll proteins by using sucrose-gradient centrifugation. Triton-solubilized spinach chloroplasts were fractionated to obtain P700-enriched preparations by using 3 different methods (to be published): sucrose-gradient centrifugation (AS-1), DEAE-Bio-gel A column chromatography (Z-1) and gel electrophoresis (E-516). The P700 concentrations in these preparations were determined according to Hiyama and Ke [10] by means of flash spectroscopy. The reaction centre 2 particles were prepared according to Kuwabara and Murata [8], and their P680 concentration was assayed by photosynthetic electron transport activity measurements [11].

The pigment extraction and HPLC analyses of these samples were carried out as in [3]. Briefly, a silica-packed column was employed with hexane/2-propanol (98.6:1.4, v/v) as an eluent, and the detection wavelength was 430 nm unless otherwise noted.

3. RESULTS AND DISCUSSION

HPLC traces for P700 preparations a–e (see above) are displayed in fig.2. The peaks at $t = 3.7$, 4.1, 6.0, and 12.4 min were identified with Chl *a'*, Ph *a*, Chl *a*, and Chl *b*, respectively, from the coincidence of their positions with those of the authentic samples (prepared as in [4]) by using the latter

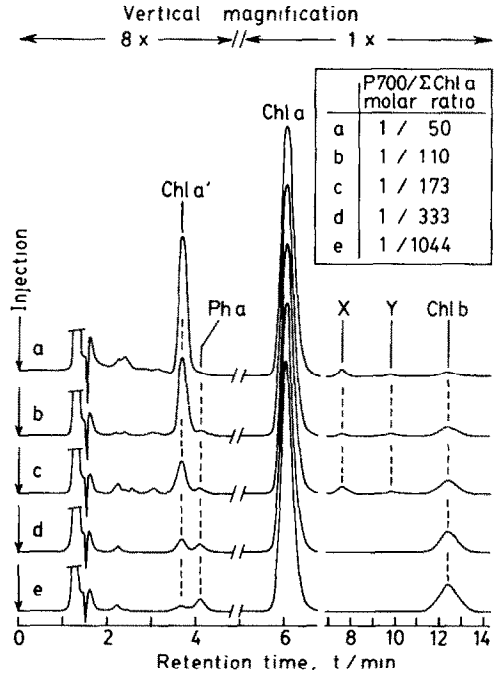

Fig.2. HPLC traces for subchloroplast preparations a–e containing P700 at different levels. The Chl *a* peaks are normalized to a common intensity. Components X and Y have not been identified, but appear to be degradation products of Chl *a* (chlorophyllide *a*, Chl *a* allomer, etc.) since their visible spectra are indistinguishable from that of Chl *a* (λ_{max} = 430.0 and 661.5 nm, with a blue/red absorbance ratio of 1.23 ± 0.01 in acetone). Σ[Chl *a*] denotes the total amount of components having a common Chl *a*-type spectrum (Chl *a* + Chl *a'* + X + Y for samples a–c, and Chl *a* + Chl *a'* for samples d and e). Intensities at $t < 5$ min are magnified 8-fold. The tracing speed at $t > 7$ min is reduced 2-fold.

either as internal or external standards. The identity of the 3.7 min peak with Chl *a'* was further substantiated by observing that (i) the peak intensity changed in perfect proportion to that of Chl *a* by a shift in the detection wavelength from 430 nm to a series of values, as expected from a common molar absorptivity spectrum for Chl *a'* and Chl *a* [4], and (ii) the peak intensity showed a grow-in with time at the expense of the Chl *a* intensity when a basic compound was added as an epimerization promoter to the extract solution. In addition, use of reversed-phase HPLC on a 5 μm ODS column with 100% methanol as an eluent resulted in the reversion of the Chl *a'*–Chl *a* elution order, as confirmed by other workers [12,13]. It is readily seen in fig.2 that an enrichment in P700 is accompanied by an increase in the Chl *a'* content. Denoting by Σ[Chl *a*] the total amount of pigments with a common Chl *a*-type absorption spectrum (see fig.2 legend), we plot the Chl *a'* concentration [Chl *a'*]/Σ[Chl *a*] vs P700 concentration [P700]/Σ[Chl *a*] in fig.3 for all the 9 subchloroplast preparations analyzed. The result clearly demonstrates that 2 Chl *a'* molecules are associated with a single site of P700.

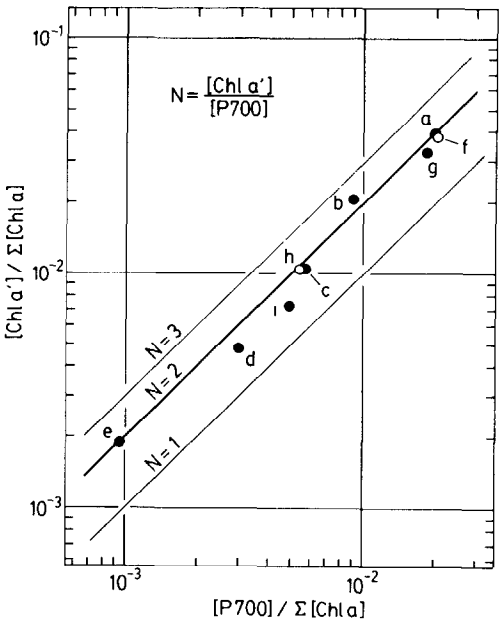

Fig.3. Relationship between the Chl *a'* content and P700 content in 9 subchloroplast preparations submitted to HPLC analysis. Sample notations a–e as for fig.2. See text for samples f–i.

Fig.2 incidentally shows that with increasing P700 concentration the contents of Ph *a* and Chl *b* tend to decrease. For a P700-enriched sample g (not displayed in fig.2), the Σ[Chl *a*]:[P700]:[Chl *a'*]:[Ph *a*]:[Chl *b*] molar ratio was 54:1:1.8:0.13:0.45. Thus, Ph *a* and Chl *b* are evidently not the integral components of P700.

We then analyzed spinach chloroplasts and reaction centre 2 particles. The pigment composition for the chloroplasts was found to be [Chl *a*]/Σ[Chl *a*] ~1:290 and [Chl *b*]/[Chl *a*] ~ 1:2.7; these figures are practically identical with those found for green leaves [3]. The reaction centre 2 particles, with a P680 concentration of [P680]/Σ[Chl *a*] ~ 1:130 [11], gave the following analytical result: [Chl *a'*]/Σ[Chl *a*] ~ 1:1400 and [Chl *b*]/[Chl *a*] ~ 1:2. It follows that [Chl *a'*]/[P680] < 0.1, which is too small a figure to regard Chl *a'* as one of the ingredients of reaction centre 2. This low level of Chl *a'* probably arises from slow epimerization of Chl *a* during preparation of reaction centre 2 particles. We could thus exclude the possibility that Chl *a'* functions at sites other than the reaction centre 1, P700.

Dörnemann and Senger [14–16] and Scheer et al. [17] recently described the isolation of a Chl *a* derivative, named Chl RCI, from algae and spinach chloroplasts at a molar ratio Chl RCI/P700 ~ 1. The Chl RCI, reportedly, absorbs at somewhat longer wavelengths compared to Chl *a*, shows in acetone a blue/red absorbance ratio of 1.61–1.69 which is quite different from that of Chl *a*, and exhibits an R_f value similar to that of Chl *a* on silica gel TLC. From the present HPLC analysis, however, we cannot conclude the presence of such a pigment in spinach chloroplasts unless the 'Chl RCI' possesses strictly the same retention time as that for Chl *a*.

The existence of 2 Chl *a'* molecules per P700, and the ESR evidence [1] for the possible dimeric nature of P700, together suggest that a Chl *a'* dimer might constitute P700. This prompted us to prepare and characterize, for the first time, a Chl *a'* dimer in vitro (to be published). In a methanol/water mixed solvent Chl *a'* forms a dimer with a sharp, double (692 and 715 nm)-peaked visible spectrum. Redox titration with the Fe^{2+}/Fe^{3+} couple leads to oxidation of the Chl *a'* dimer accompanied by a spectral change shown in fig.4A. The midpoint potential, being around

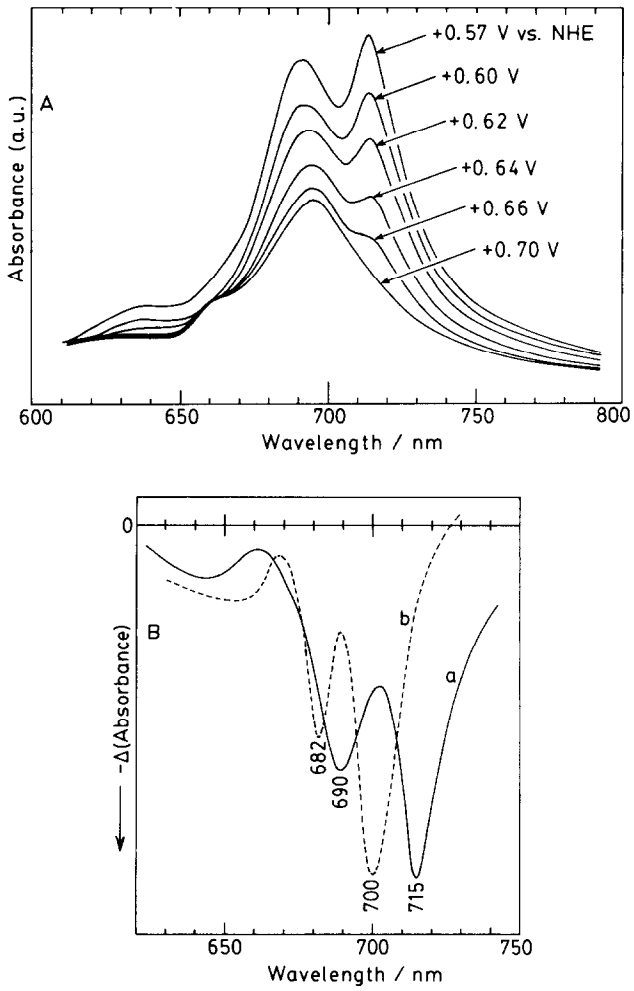

Fig.4. (A) Evolution of the visible absorption spectrum for a Chl *a'* dimer on raising the potential in a methanol/water (3:2, v/v) mixed solvent at 25°C. Chl *a'* (containing ~1.5% Chl *a*) was prepared according to [4]. That the initial species (at potentials negative of 0.57 V) is indeed a Chl *a'* dimer was verified from the slope (2.0) of the log[aggregate] vs log[monomer] plot. The potential was regulated by stepwise increase in the Fe^{3+} concentration, and monitored electrochemically (to be published). (B) (a) The oxidized-minus-neutral difference spectrum for the Chl *a'* dimer in aqueous methanol and (b) the light-minus-dark difference spectrum for P700 of spinach chloroplasts [10].

620 mV vs NHE (normal hydrogen electrode), is shifted substantially from the oxidation potential of monomeric Chl *a'* or Chl *a* (860 mV) [2] in the direction of the estimated P700 oxidation potential (370 to 520 mV) [2]. Though such a negative shift of the oxidation potential from the monomer potential is one of the criteria in ensuring the validity of a 'P700 model compound' [1,2], it cannot be a sufficient condition since redox potentials often shift greatly with a change in environment (e.g. from inside a protein network to aqueous methanol).

Of much more significance is the close resemblance between the oxidized-minus-neutral difference spectrum for the Chl *a'* dimer and the well-established light-minus-dark difference spectrum for P700 [10], as seen in fig.4B. The sharp, double-peaked P700 spectrum is satisfactorily replicated by the Chl *a'* dimer spectrum. The peak wavelengths do not exactly agree between the 2 spectra, but the energetic separations from 700 to 715 nm and from 682 to 690 nm are only 37 and 21 meV, respectively. In aqueous methanol Chl *a* also forms a dimer (or oligomer), but its visible spectrum differs drastically from that of the Chl *a'* dimer. More importantly, chemical oxidation of the Chl *a* dimer generates an essentially single-peaked, broad (half-width > 30 nm) difference spectrum (to be published), which hardly reproduces the salient features of the P700 difference spectrum. All the experimental data obtained here are thus in favour of the idea that a dimer of Chl *a'*, and not of Chl *a*, functions as the primary donor of the photosynthetic reaction centre 1. This in turn means that the P700 content in a given plant material or (sub)chloroplast preparation can be estimated simply by pigment composition analysis.

Chlorophyll *a'*, which has long been neglected in photosynthesis research [3,18], has been detected not only in terrestrial plants [3] but in marine algae submitted to the HPLC analysis (unpublished). We thus suspect the requirement for this pigment as the building block of reaction centre 1 in all the higher photosynthetic organisms. To understand why Chl *a'* is chosen for this particular purpose, further in vivo as well as in vitro studies on this pigment should prove exciting.

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REFERENCES

[1] Katz, J.J. and Hindman, J.C. (1981) in: Photochemical Conversion and Storage of Solar Energy (Connolly, J.S. ed.) pp.27–78, Academic Press, New York.
[2] Wasielewski, M.R., Norris, J.R., Shipman, L.L., Lin, C.-P. and Svec, W.A. (1981) Proc. Natl. Acad. Sci. USA 78, 2957–2961.
[3] Watanabe, T., Nakazato, M., Mazaki, H., Hongu, A., Konno, M., Saitoh, S. and Honda, K. (1985) Biochim. Biophys. Acta 807, 110–117.
[4] Watanabe, T., Hongu, A., Honda, K., Nakazato, M., Konno, M. and Saitoh, S. (1984) Anal. Chem. 56, 251–256.
[5] Svec, W.A. (1978) in: The Porphyrins (Dolphin, D. ed.) vol.5, pp.341–399, Academic Press, New York.
[6] Omata, T., Murata, N. and Satoh, K. (1984) Biochim. Biophys. Acta 765, 403–405.
[7] Melis, A. and Anderson, J.M. (1983) Biochim. Biophys. Acta 724, 473–484.
[8] Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.
[9] Anderson, J.M. and Boardman, N.K. (1966) Biochim. Biophys. Acta 112, 403–421.
[10] Hiyama, T. and Ke, B. (1972) Biochim. Biophys. Acta 267, 160–171.
[11] Murata, N. and Miyao, M. (1985) Trends Biochem. Sci. 10, 122–124.
[12] Braumann, T. and Grimme, L.H. (1979) J. Chromatogr. 170, 264–268.
[13] Shioi, Y., Fukae, R. and Sasa, T. (1983) Biochim. Biophys. Acta 722, 72–79.
[14] Dörnemann, D. and Senger, H. (1981) FEBS Lett. 126, 323–327.
[15] Dörnemann, D. and Senger, H. (1982) Photochem. Photobiol. 35, 821–826.
[16] Dörnemann, D. and Senger, H. (1984) in: Advances in Photosynthesis Research (Sybesma, C. ed.) vol.2, pp.77–80, Martinus Nijhoff/Junk, The Hague.
[17] Scheer, H., Wieschhoff, H., Schaefer, W., Cmiel, E., Nitsche, B., Schiebel, H.-M. and Schulten, H.-R. (1984) in: Advances in Photosynthesis Research (Sybesma, C. ed.) vol.2, pp.81–84, Martinus Nijhoff/Junk Publishers, The Hague.
[18] Strain, H.H. and Katz, J.J. (1969) in: Progress in Photosynthesis Research (Metzner, H. ed.) vol.2, pp.539–546, Laupp, Tübingen.