Identification of Chl-binding proteins in a PS II preparation from spinach

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Received 5 June 1986

Using a mild solubilization procedure we have been able to detect several Chl-binding proteins by SDS-urea-PAGE. Their molecular masses were 46, 42, 34, 32, 29–24 and approx. 110 kDa (presumably a CPI contamination). The stability of the Chl-protein complexes depended strongly on experimental conditions. A very faint band at 34–30 kDa was resolved in visible light (in 40–50% of the experiments). With a more sensitive fluorometric technique (illumination at 366 nm, fluorescence detection in the red) we identified two Chl-containing bands in the 30 kDa region. Limited proteolysis studies with trypsin revealed that the extent of the enzymatic degradation of some Chl-binding proteins in vitro is obviously dependent on pH and the presence of Ca^{2+}.

Chlorophyll-binding protein *Photosystem II* *Solubilization* *(Spinach)*

1. INTRODUCTION

The structural organization of the PS II reaction center polypeptides and their functional role is a central problem of current photosynthesis research activities. Among different questions, the identification of the apoprotein of the photoactive Chl *a* component P680 and its associated functional redox groups (pheophytin, plastoquinone Q_A) is of special relevance. Basically, two entirely different approaches were used to solve this problem: (i) analysis of Chl-binding proteins and (ii) homology studies of polypeptides from PS II and purple bacteria chromatophores [1–8].

Three Chl *a*-containing protein complexes have been unambiguously identified in PS II of spinach chloroplasts: 47 and 40 kDa proteins [9,10] and the subunits of the light-harvesting complex with molecular masses of 25–30 kDa [11]. Low-temperature fluorescence emission studies indicated that the 47 kDa protein is the PS II reaction center apoprotein and that the 40 kDa polypeptide acts as a core antenna [9]. On the other hand, results on the functional and structural organization of purple bacterial reaction centers revealed some remarkable similarities to components of PS II from higher plants [12]. The L- and M-subunits of the bacterial reaction center show a high degree of sequence homology to intrinsic membrane proteins, the 32 kDa herbicide-binding protein (D-1) and a 34 kDa polypeptide (D-2) [13–17]. D-1 and D-2 span the thylakoid membrane in 5 helices as their counterparts in bacteria chromatophores. From X-ray data of the crystallized reaction center from *Rhodopseudomonas viridis* the exact positions of its prosthetic groups were deduced [14,18]. The amino acid residues involved in their binding are conserved in the 32 and 34 kDa polypeptides from higher plants [1,4,15]. Based on these striking results, a new model for the PS II reaction center of higher plants has been proposed, in which D-1 and D-2 take part

Abbreviations: Chl, chlorophyll; PS, photosystem; PAGE, polyacrylamide gel electrophoresis

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00145793/86/$3.50

in P680 binding rather than the 47 kDa protein [2–4].

So far there has been no direct experimental evidence for the detection of Chl-binding proteins in the 30 kDa region in PS II of higher plants, in contrast to investigations of prokaryotes [5–7].

Here, we report on the development of a mild solubilization procedure for integral membrane proteins that permitted us to discover Chl *a*-containing polypeptides in spinach PS II with molecular masses in the region of 30–34 kDa. Additionally, limited proteolysis was applied to obtain information about the topography of Chl-containing membrane proteins of PS II.

2. MATERIALS AND METHODS

2.1. *Preparation of PS II*

PS II particles were isolated from spinach chloroplasts according to Berthold et al. [19] with some modifications [20].

2.2. *Limited proteolysis*

Limited proteolysis was carried out with trypsin (trypsin/total protein weight ratio from 0.01 : 1 to 1 : 1) in buffers A (20 mM Mes-NaOH, pH 6.3, 15 mM NaCl, 5 mM $MgCl_2$), B (20 mM Hepes-NaOH, pH 7.0, 15 mM NaCl, 5 mM $MgCl_2$) or C (20 mM Hepes-NaOH, pH 7.6, 15 mM NaCl, 5 mM $MgCl_2$). PS II particles were thawed and sedimented for 10 min at 12000 rpm in a microfuge (Eppendorf). The supernatants were carefully removed and stored frozen, the pellets being suspended in either buffer A, B or C. Aliquots of PS II particles (equivalent to 100 μg protein) were preincubated in Eppendorf tubes for 5 min at 25°C in the presence or absence of 10 mM Ca^{2+}. After addition of trypsin the samples were incubated at 25°C for either 15 or 30 min. After 5 min Ca^{2+} was added to the non-Ca^{2+}-preincubated samples to a final concentration of 10 mM. Digestion was stopped with benzamidine (final concentration 2 mM). Samples were immediately put on ice and spun down at 12000 rpm for 10 min. The supernatants were removed and stored frozen, and the sediments washed once with buffer A, B or C. Approx. 10 μl of 87% (w/w) glycerol were added to the pellets and cautiously solubilized in electrophoresis sample buffer on ice (14.4 mM β-mercaptoethanol instead of 0.5%, v/v) [21].

2.3. *Analytical electrophoresis*

The polypeptides and their proteolytic cleavage products were analyzed on SDS-urea-polyacrylamide gels (6% stacking, 14% resolving gel). Both gels contained 0.1% (w/v) SDS and 5 M urea. The gel and running buffers were exactly as described by Laemmli [21]. All samples were not boiled prior loading onto the gels. The gels were run at 10–15°C in the dark. The proteins were localized by Coomassie R250 staining.

2.4. *Identification of Chl-binding proteins*

After running the gels the relative mobility of the Chl-containing bands were determined prior to staining. Chl-containing band were either photographed in visible light or detected by their red fluorescence after excitation by UV light (λ = 366 nm). We identified the Chl-binding proteins after Coomassie R250 staining of the gels using molecular mass markers in the range of 94–1.6 kDa.

2.5. *Determination of protein and Chl concentrations*

Protein concentrations were determined according to Bradford using bovine serum albumin as a standard, and Chl as described in [22,23].

2.6. *All chemicals used here were of the highest purity available.*

Trypsin was from Boehringer Mannheim, benzamidine from Sigma, and electrophoresis chemicals from LKB. Marker proteins were purchased from Pharmacia and LKB.

3. RESULTS AND DISCUSSION

3.1. *Detection of Chl-binding proteins*

Here we report on a mild solubilization procedure which allows the identification of several Chl-protein complexes after electrophoretical separation on SDS-urea-polyacrylamide gels using either visible- or UV-light transillumination (λ = 366 nm). Their molecular masses were estimated from the gels. Proceeding from top to bottom on the gels we observed the following Chl-containing protein bands:

(i) A slow migrating component of ~110 kDa, which is probably CPI due to a small PS I contamination in our PS II preparation. This sugges-

tion is supported by the fact that this protein is non-fluorescent when illuminated with long-wave UV light [8]. The Chl binding to CPI is of high stability because we identified it in all of our experiments as a sharp green band near the border of the stacking gel (R_F values 0.015–0.02).

(ii) Proteins with molecular masses of 46 and 42 kDa (as estimated from SDS-urea-PAGE) were detected. They are supposed to be the so-called CP47 and CP43 as described in [9,10,24].

(iii) The most interesting result was the detection of a very faint diffuse band at 34–30 kDa under visible illumination. Surprisingly, with a fluorometric technique two weak fluorescent bands at approx. 34 and 32 kDa could be resolved which were clearly separated from each other and the LHC complex (see fig.1, lanes 1,2). It should be mentioned that these Chl-protein complexes are very sensitive to oxidation and loss of their chromophores. We assume that this could be the reason for visualizing them in only 40–50% of our experiments under illumination with visible light. This is the first report on two Chl-containing polypeptides in the 30 kDa range in higher plants. Several Chl-binding polypeptides with molecular masses of 36–30 kDa were identified in prokaryotes, e.g. 36 kDa proteins from the cyanobacterium *Aphanocapsa* 6714 [6] and *Anacystis nidulans* R2 [7], and 33 and 30.5 kDa polypeptides from *Chlamydomonas reinhardtii* [6]. Antibodies against the PS II core polypeptides 5 and 6 (CP47 and CP40 analogues) from *C. reinhardtii* [25] showed no cross-reaction with the 36 kDa protein from *Aphanocapsa* 6714, demonstrating that it is probably not a proteolytic cleavage product [6]. We also do not refer the 34 and 32 kDa Chl-containing polypeptides to degradation products of the 110, 46 and 42 kDa proteins, because they both appeared in the presence of protease inhibitors as well (1 mM PMSF, 2 mM benzamidine, 1 mM EDTA). Immunological detection is impossible at the moment, because antibodies against them are not yet available in our laboratory. It also seems unlikely that they are due to aggregates of lower molecular mass components. Our results do not directly support the model for the reaction center of PS II proposed by Trebst and Deisenhofer et al. ([2–4]; review [5]) because we do not

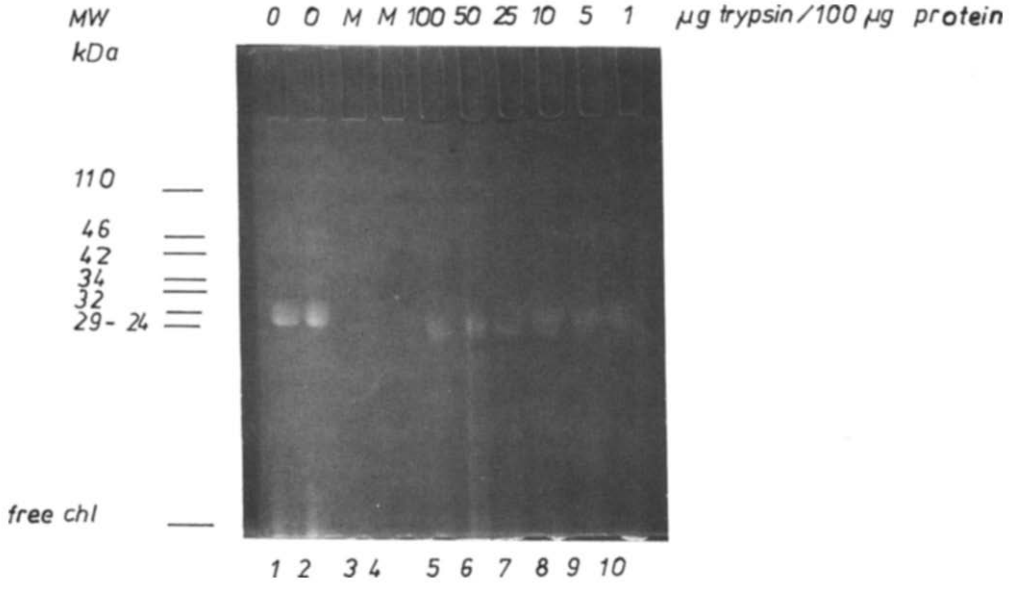


Fig.1. Chl-binding proteins of a PS II preparation. PS II control samples and PS II polypeptides digested with various trypsin concentrations were analyzed using SDS-urea-PAGE and then photographed with transilluminated UV light (without preincubation with 10 mM Ca^{2+}). Lanes: 1, control sample (including 10 mM Ca^{2+}); 2, control sample (without Ca^{2+}); 3–4, marker proteins (not shown); 5–10, samples digested with various trypsin concentrations (see above). Experimental details are described in section 2.

know whether the 34 and 32 kDa Chl-containing proteins correspond to D-1 and D-2. For further conclusions more experimental data are required.
(iv) The fastest migrating complex was the LHC, banding broadly at 29–24 kDa. This complex was identified under both visible and UV illumination in all experiments (see fig.1, lanes 1,2). The Chl *a/b*-binding proteins were highly fluorescent.

3.2. *Limited proteolysis of the Chl-binding proteins of PS II*

To study the topography of the Chl-binding proteins identified we developed a limited proteolysis protocol. Besides lactoperoxidase-mediated radioiodination [26] and immunodetection [27], limited proteolysis is an excellent tool to analyze the functional and structural topography of integral membrane proteins [20]. We further focussed our attention on the effect of Ca^{2+} which has been reported to be a necessary cofactor for the function of PS II and especially the oxygen-evolving system [28–30]. An extrinsic 24 kDa protein which does not contain the high-affinity Ca^{2+}-binding site [31] was shown to play a key role [32,33].

Another membrane-associated Ca^{2+}-binding protein was isolated and the existence of an intrinsic one has been supposed [34,35]. Besides a specific function Ca^{2+} is probably also involved in grana stacking due to electrostatic interactions with carboxyl groups of Asp and Glu residues [36,37] in LHC II. From preliminary results of column chromatography on a Ca^{2+}-affinity matrix, we know some PS II-Chl-binding polypeptides that unspecifically and specifically bind Ca^{2+}. Therefore, these Chl-protein complexes should exhibit Ca^{2+}-dependent sensitivity to proteolytic attack. On the other hand the surface-exposed proteins which do not interact with Ca^{2+} should be degraded by the protease independently of the presence of Ca^{2+}. In our investigation we found different sensitivities of the Chl-protein complexes to trypsin attack. Digestion of some of the substrates was dependent on pH and on the Ca^{2+} concentration in the assay. Different enzyme:protein ratios were employed at pH 6.3 (see fig.2.1), pH 7.0 (see fig.2.2) and pH 7.6 (see fig.2.3) and the digestion was stopped after incubation times of 15 and 30 min. PS II complexes preincubated with 10 mM Ca^{2+} (see fig.2.1b,2.2b, 2.3b) and without Ca^{2+} preincubation (fig.2.1a,2.2a,2.3a,3a,4a) were investigated with regard to different proteolysis pattern. The data and gels of the 30 min incubations are presented here. Regardless of difficulties in interpreting all the details some general conclusion can be drawn from the data of fig.2.1–2.3:
(i) CPI is completely resistant to limited trypsinization at pH 6.3 and 7.0 in the absence and presence of Ca^{2+}. At pH 7.6 it is digested in the absence of Ca^{2+} and partly protected in Ca^{2+}-preincubated samples. A specific Ca^{2+} effect is not likely (see fig.2.1–2.3). The protein should be, at least to some extent, surface-exposed. These results are in accordance with previous findings [26,38].
(ii) The 47 kDa protein seems to be digested at all pH values tested so far at a trypsin/PS II protein ratio of 50 μg:100 μg (see fig.2.1–2.3). Whether the origin of the pH dependence at lower trypsin concentrations is simply due to trypsin activation or to conformational changes in the PS II or both remains to be clarified. No significant Ca^{2+} effect is observable. The polypeptide is surface-exposed which is supported by radio-iodination studies [26,38].
(iii) The 43 kDa protein is the most sensitive Chl-binding protein to tryptic degradation. The protein is degraded at pH 6.3 at a trypsin/protein ratio of at least 25 μg:100 μg independent of Ca^{2+} preincubation (see fig.2.1, lanes 4–6). At pH 7.0 the protein is barely observable between 100:100 and 25:100 μg protein (see fig.2.2, lanes 1,2,4). At lower ratios the Ca^{2+}-preincubated samples seem to be digested to a lower extent. A cleavage product of 34 kDa is possibly a fragment of the 43 kDa protein (see fig.2.2a, lanes 5–7 and 2.2b, lanes 5,6). At pH 7.6 this protein is cleaved into smaller fragments at all trypsin/protein weight ratios independent of the Ca^{2+} preincubation (see fig.2.3, lanes 4–9). The fragment at 34–32 kDa presumably is a proteolysis product of the 43 kDa protein. UV transillumination of the unstained gels revealed a Chl-containing band (see fig.1, lanes 8–9,10), but it could also be an intact Chl-binding protein in the 30 kDa range (see below). Obviously, the 43 kDa protein is highly surface-exposed. An interaction of the 43 kDa protein with Ca^{2+} is possible. Recently, Isogai et al. [39] claimed that the 33 kDa extrinsic protein of the oxygen-evolving complex shields the 43 kDa protein from tryptic and chymotryptic attack. Using a PS II

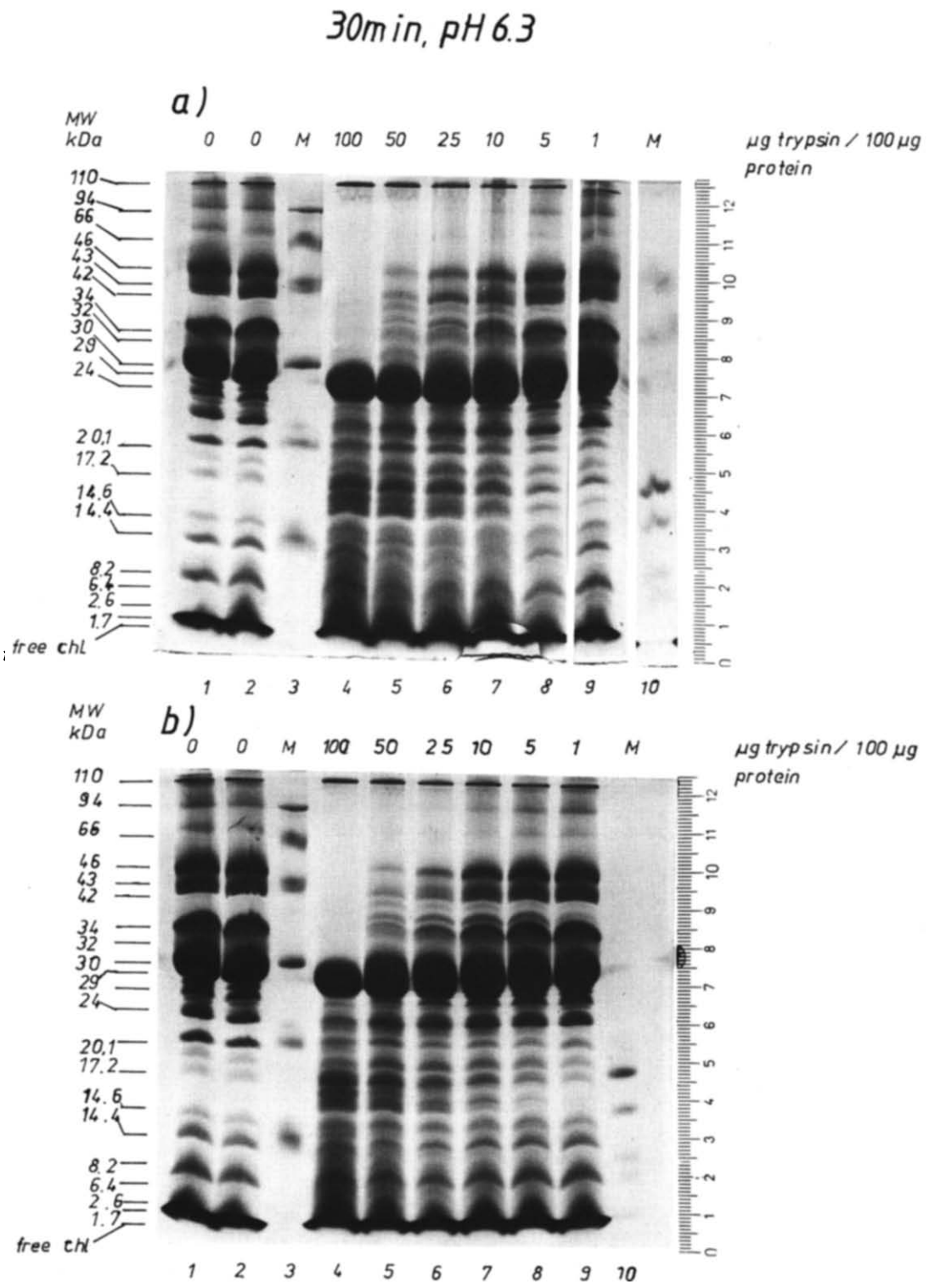


Fig. 2. For legend see page 72.

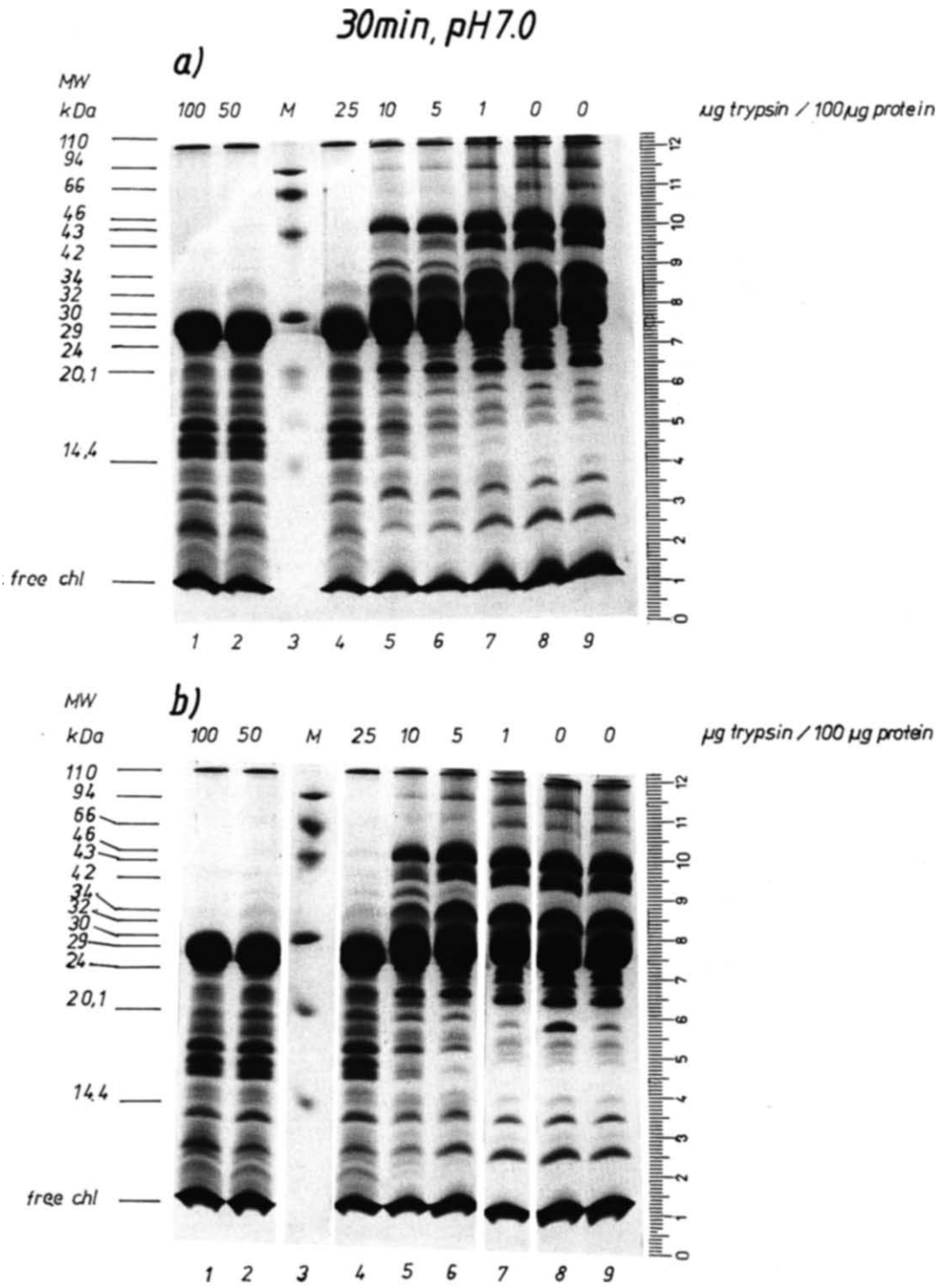
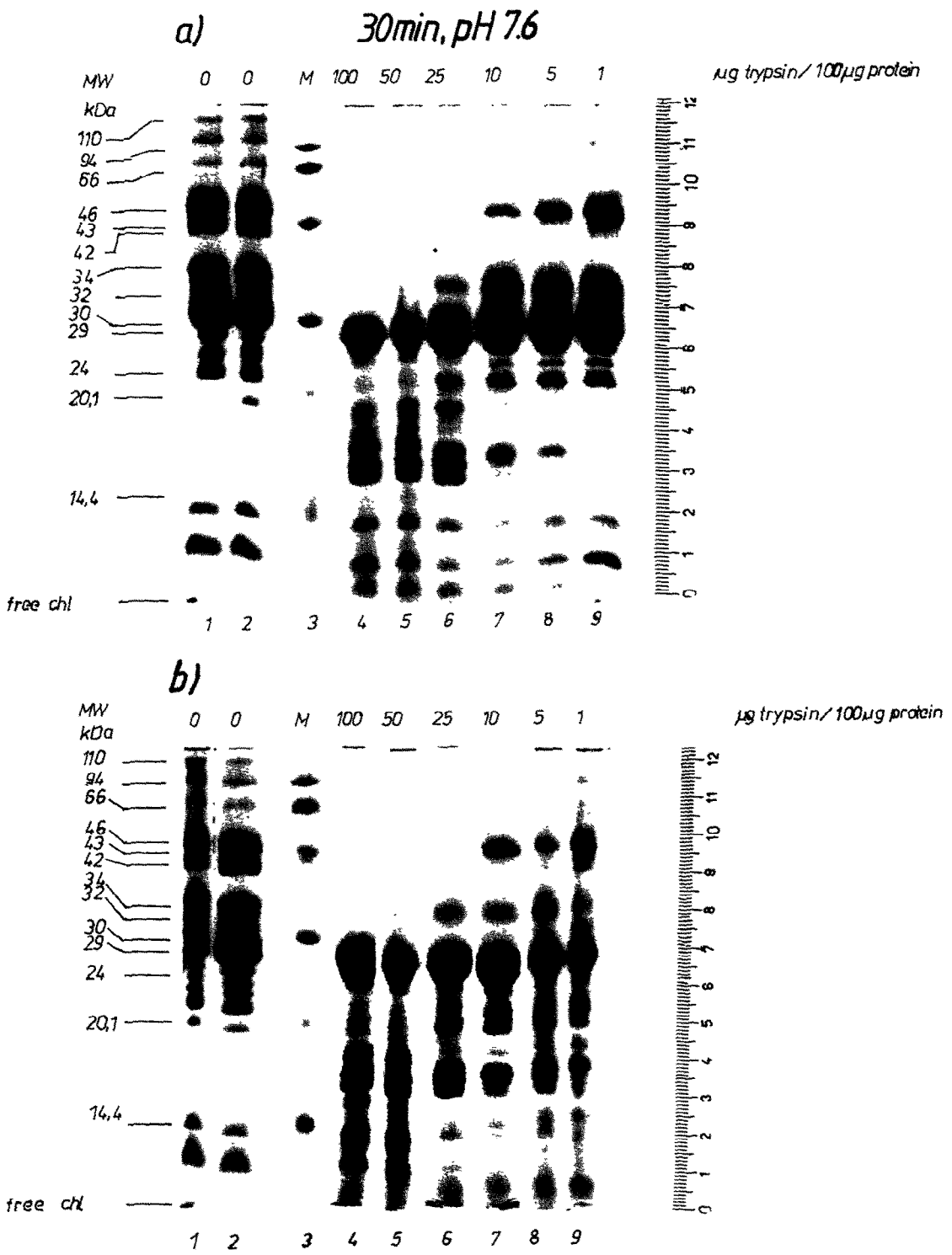


Fig.2. Electrophoretical analysis of limited proteolysis of non-Ca^{2+}-preincubated (a) and Ca^{2+}-preincubated PS II complex proteins (b) at pH 6.3 (2.1), pH 7.0 (2.2) and pH 7.6 (2.3). Limited proteolysis (30 min) of solubilized PS II complex polypeptides with trypsin/protein ratios as indicated analyzed on SDS-urea-polyacrylamide gels using molecular mass markers in the range 94–1.7 kDa (phosphorylase *b*) (94 kDa), bovine serum albumin (66 kDa), ovalbumin



(43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), myoglobin (intact) (17.2 kDa), myoglobin I + II (14.6 kDa), α-lactalbumin (14.4 kDa), myoglobin I (8.2 kDa), myoglobin II (6.4 kDa), myoglobin III (2.6 kDa) and myoglobin (1-14) (1.7 kDa). Control samples are denoted by 0 µg trypsin/100 µg protein.

preparation highly active in O_2 evolution and including the 33 kDa protein we found that CP47 and CP43 exhibit a similar susceptibility to trypsin at pH 6.3, but at pH 7.0 and 7.6 the 43 kDa protein was much more sensitive to proteolysis than the 47 kDa polypeptide. Moreover, Ca^{2+} preincubation slightly affects the proteolysis of the 43 kDa protein. It remains to be clarified whether this pH effect is due to the marked pH dependence of the 33 kDa protein to tryptic attack or whether both proteins indeed interact [20,39].

(iv) If the 32 and 34 kDa Chl-containing proteins are really D-1 and D-2 we could predict possible cleavage products because their primary structures have been deduced from the nucleotide sequences of their genes [2–4,14,16]. An initial cleavage product of D-1 is a 19.5 kDa fragment which is further degraded to a 17 kDa polypeptide [14]. D-2 could be digested to a polypeptide of approx. 20 kDa. However, it was difficult to identify fragments of D-1 and D-2 in this molecular mass region because the bulk of the cleavage products were focused there (see fig.2). At pH 6.3 both proteins were strongly digested at the highest trypsin concentration, but at 50 μg trypsin: 100 μg protein the typical broad diffuse band was again observable (see fig.2a and e, lanes 4–9). In Hepes buffer (pH 7.0) strong proteolysis was detectable down to 25 μg enzyme: 100 μg protein. Below this concentration this diffuse band is difficult to identify (see fig.2.2, lanes 1,2,4–9). At pH 7.6 we could realize a broad band at 34 kDa from 25 to 1 μg enzyme: 100 μg protein which contained Chl (see fig.2.3, lanes 6–9 and fig.1, lanes 8–10).

Nevertheless, there was no recognizable interaction of Ca^{2+} with both proteins. They should be at least partly surface-exposed. Interestingly radioiodination studies revealed two intrinsic proteins surface-exposed in the 30 kDa region [38] which is in agreement with our results.

(v) The largest protein of the LHC complex lost a 2 kDa fragment during proteolysis at pH 6.3, which was not digested more strongly with increasing pH (see fig.2.1 and 2.2, lanes 4–9, fig.2.3, lanes 1,2,4–7). One of the smaller Chl-proteins with a molecular mass of about 24–25 kDa seemed to be involved in Ca^{2+} interaction at pH 6.3 and 7.0. In samples without Ca^{2+} preincubation this protein is degraded to a slightly greater extent (see fig.2.1, lanes 4–9 and fig.2.2, lanes 1,2 and 4–7). At pH 7.6 this polypeptide was cleaved into smaller fragments and a Ca^{2+} effect was not seen (see fig.2.1, lanes 4–9). The Chl-proteins of the LHC complex are partly surface-exposed and at least one component of the complex could take part in Ca^{2+} binding.

Further investigations using other proteases and a 2D PAGE system for better resolution of the cleavage products are in preparation.

4. CONCLUSIONS

The present study provides the first direct experimental evidence for the existence of two chlorophyll-containing polypeptides in the 30–34 kDa region in PS II membrane fragments of higher plants. This result could be of special relevance because a recently proposed model assumes that the 32–34 kDa polypeptides D-1 and D-2 form the apoprotein of the PS II reaction center [2–4]. However, it should be mentioned that our data do not directly support this model because it remains to be shown that the 30–34 kDa Chl polypeptides discovered are identical with D-1 and D-2 and that they are able to perform reaction center photochemistry.

ACKNOWLEDGEMENTS

The authors would like to thank M. Rother for technical assistance. The financial support from the Deutsche Forschungsgemeinschaft (SfB 312) and Bundesministerium für Forschung und Technologie is gratefully acknowledged.

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