# Immunological Identification of Polypeptides in Photosystem II Complexes from the Cyanobacterium *Anacystis nidulans*

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Photosystem II complexes from the cyanobacterium *Anacystis nidulans* have been investigated by Western blots with antisera raised against four photosystem II peptides from plants and with an antiserum raised against the soluble L-amino acid oxidase protein from *A. nidulans* to achieve an identification of the polypeptides – especially of the L-amino acid oxidase related protein – in isolated photosystem II complexes. *Anacystis* photosystem II complexes which were solubilized with lauryldimethylamine N-oxide and purified by sucrose cushion and sucrose gradient centrifugation, contained as major Coomassie brilliant blue stained polypeptides a 71 kDa band of unknown identity, a 62 kDa band, which partly contained D-1, a 55 and 49 kDa band which were immunoreactive with an antiserum to the 47 kDa peptide of tobacco PS II complexes, and three distinct bands in the 30 kDa region. These latter bands could be identified as the extrinsic Mn stabilizing peptide (27–30 kDa), D-1 (30–33 kDa) and a 36 kDa peptide (35–38 kDa) which crossreacted with the antiserum raised against the soluble L-amino acid oxidase protein of 50 kDa. These results suggest that the 36 kDa peptide present in purified photosystem II complexes from *A. nidulans* might be a processed form of the soluble 50 kDa L-amino acid oxidase protein.

## Introduction

PS II complexes have been isolated from a number of organisms and relatively well characterized with respect to polypeptide composition as well as organic and inorganic cofactors required for photochemical charge separation and water oxidation (see recent reviews [1, 2]). Although the mechanism of charge separation [3–5] now seems to be fairly well understood, one of the major unanswered questions concerning PS II remains the identity of the "water oxidizing enzyme" [1]. Recently it has been shown that a minimal complex which was isolated from $O_2$ evolving spinach PS II complexes and which only consisted of D-1, D-2, the two cytochrome $b_{559}$ binding peptides (9 and 4.3 kDa) and a 4.8 kDa peptide, can still catalyze photochemical charge separation [6, 7]. In a hypothetical model, Barber [8] suggested that all additional cofactors required for water oxidation might also be associated with this complex and that only the extrinsic 33 kDa peptide is required in addition for stabilization of Mn at the reaction center (although it is not required for catalytic activity) [9]. This model has not yet been experimentally proven, but would imply that a separate mangano enzyme does not exist and that the peptides which early in evolution could only catalyze charge separation, might have acquired additional cofactors necessary for water oxidation [1].

We recently isolated and described a PS II complex with high activity for $O_2$ evolution from the cyanobacterium *Anacystis nidulans* [10, 11]. This complex consisted of six major polypeptides with apparent molecular weights of 57, 49, 43, 36 (broad diffuse band), 27 and 10 kDa. A number of experiments with this cyanobacterium gave indirect evidence that a flavoprotein, which has an L-amino acid oxidase activity under certain conditions, might possibly have a function in photosynthetic water oxidation [10–13]. Those results indicated that besides the peptides required for charge separation an additional enzyme which might interact with the inorganic cofactors (Mn, $Ca^{2+}$ and $Cl^-$), is necessary for water oxidation. In this paper we present results of immunological investigations of purified PS II complexes from *A. nidulans* with antisera raised against four PS II polypeptides from plants and with an antiserum raised against the soluble L-amino acid oxidase protein from *A. nidulans* to achieve an identification of

*Abbreviations:* AOX, amino acid oxidase; chl, chlorophyll; CBB, Coomassie brilliant blue; PS, photosystem.

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the polypeptides in PS II complexes from *A. nidulans*.

## Materials and Methods

The soluble L-AOX protein was purified from the supernatant of French press extracts from *Anacystis nidulans* (*Synechococcus leopoliensis* – B 1402-1, Sammlung von Algenkulturen, Universität Göttingen) by two slightly different purification procedures as described in (14 and 15 – purification A of Ref. [15]). An antiserum was raised against the enzyme of both purifications (referred to as anti-L-AOX A and B, respectively). $O_2$ evolving PS II complexes were solubilized from *Anacystis* thylakoid membranes with the detergent lauryldimethyl amine N-oxide as described previously [10]. After solubilization the solution was centrifuged for 30 min at $27100 \times g$. The resulting supernatant was then layered on a sucrose cushion consisting of 0.05 M Hepes-NaOH, pH 6.5, 0.03 M $CaCl_2$, 10% glycerol and 20% sucrose, and centrifuged for 45 min at $80000 \times g$ (at $r_{max}$). This centrifugation gave a pellet which was discarded, and the solution on top of the sucrose cushion was further purified by chromatography on a Sepharose 6B column (here referred to as purification A) as described in [11] or after dilution to reduce the lauryldimethyl amine N-oxide and $CaCl_2$ concentration by sucrose gradient centrifugation (here referred to as purification B) as described in [15]. The purified PS II complexes from both purification procedures had on $O_2$ evolving activity ranging from 800 to 1000 μmol $O_2$ evolved $\times$ $mg^{-1}$ chlorophyll $\times$ $h^{-1}$.

The extrinsic Mn stabilizing polypeptide was extracted from oat PS II complexes by $CaCl_2$ treatment and purified as described in [16]. D-1 and D-2 were purified from oat PS II complexes and the apoprotein of CP-47 was purified from tobacco PS II complexes by cutting the corresponding bands out of SDS polyacrylamide gels, and these gel bands were extracted by electroelution. For further purification the peptides were rechromatographed once. The antisera against the purified polypeptides were raised by injection of the peptides into rabbits. The additional antisera against D-1 and D-2 which were prepared according to the procedures described by Johannigmeier [17] and Geiger *et al.* [18] were a kind gift from Prof. A. Trebst, Ruhr-Universität Bochum.

L-AOX and $O_2$ evolving activities were measured as previously described [10]. Polypeptide compositions were examined by SDS polyacrylamide gel electrophoresis according to Laemmli [19] with slight modifications. For the experiments of Fig. 2 and 3 the samples were denatured by incubation with an equal volume of buffer containing 0.78 M Tris-HCl, pH 6.8, 0.78 M sucrose, 4 mM EDTA, 0.03 M DTE and 3.15% SDS for 15 min at room temperature. The slab gel contained besides the buffer 0.1% SDS, 10% acrylamide and 0.54% bisacrylamide (pH 9.4) in the separating gel and 0.1% SDS, 3% acrylamide and 0.08% bisacrylamide (pH 6.8) in the stacking gel. For the experiments of Fig. 1 LiDS was used instead of SDS and the samples were denaturated for 30 sec at 70 °C. The electrophoresis buffer of pH 8.6 contained 25 mM Tris, 190 mM glycin and 0.1% SDS (or 0.1% LiDS). The immunoblot experiments were performed as previously described [20].

## Results

In Fig. 1 we show a Coomassie brilliant blue stained SDS-polyacrylamide gel and the corresponding immunoblot of the purified L-amino acid oxidase protein. As previously shown, the soluble L-AOX consists of two subunits of 50 kDa [14, 15]. As expected, the 50 kDa band is detected by the anti-L-AOX antiserum. Fig. 1 also shows that the purified L-AOX does not contain a peptide in the 36 kDa

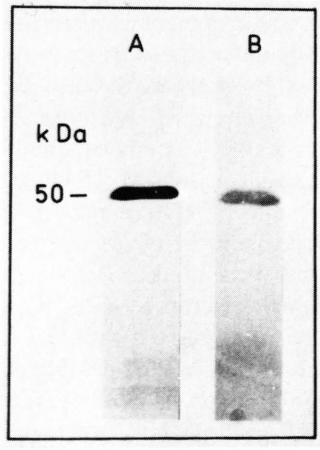


Fig. 1. SDS-PAGE and immunoblot of the purified L-AOX. A. CBB stained SDS polyacrylamide gel with the purified L-AOX protein (6.3 μg protein). B. Corresponding immunoblot with the antiserum raised against the purified L-AOX (anti-L-AOX B, antiserum dilution 1 to 100).

region (see below). The enzyme from both purification procedures [14, 15] gave the same results. The antisera against the two L-AOX samples (obtained by the purification procedures described in Ref. [14] or [15]) inhibited the L-AOX activity of the soluble protein maximal 80% (anti-L-AOX A) or maximal 60% (anti-L-AOX B). However, the L-AOX activity associated with the thylakoid membrane was not inhibited by the antiserum. This most likely suggests that the protein, when associated with the thylakoid membrane, is not accessible to the antiserum.

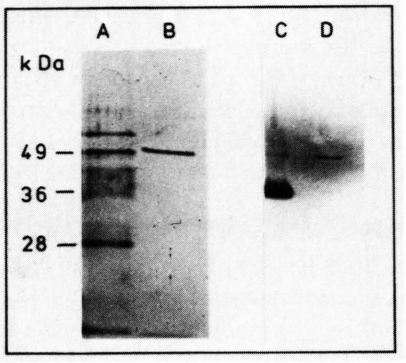


Fig. 2. Identification of the L-AOX related peptide in purified PS II complexes from *A. nidulans*. A. CBB stained SDS polyacrylamide gel with purified *Anacystis* PS II complexes of purification A (1 μg chl) and B. with the L-AOX protein (4 μg protein). C. A corresponding immunoblot of the PS II complexes immunostained with the anti-L-AOX A (dilution 1 to 50) and D. a corresponding immunoblot of the L-AOX immunostained with the anti-L-AOX A (dilution 1 to 50).

In Fig. 2 the polypeptide pattern of PS II complexes obtained by purification A and the corresponding immunoblot with the anti-L-AOX is shown. Originally we thought that the 49 kDa band present in *Anacystis* PS II complexes would correspond to the L-AOX protein [10]. However, this band was only weakly stained by the anti-L-AOX in immunoblots, while a peptide in the 36 kDa region gave a heavy reaction with the anti-L-AOX. To clarify whether the 49 kDa peptide detected by the anti-L-AOX was only a minor impurity of the soluble L-AOX protein which co-purified with PS II complexes on the Sepharose 6B column, we modified the purification procedure for PS II complexes as described under Materials and Methods (purification B). As the results in Fig. 3 show, in PS II complexes purified by

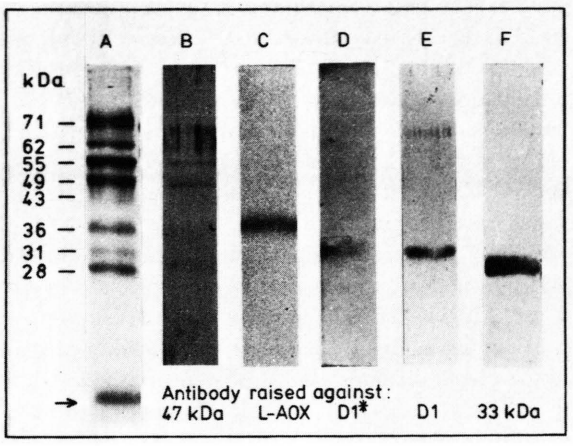


Fig. 3. Identification of polypeptides in *Anacystis* PS II complexes. A. CBB stained SDS polyacrylamide gel of *Anacystis* PS II complexes of purification B (3.6 μg chl). B. to F represent the corresponding immunoblots which were immunostained with: B: the anti-47 kDa (dilution 1 to 10), C: anti-L-AOX B (dilution 1 to 50), D: anti-D-1 prepared according to (Ref. 18 – dilution 1 to 50), E: anti-D-1 raised against the D-1 peptide from oat (dilution 1 to 50), and F: anti-33 kDa (extrinsic Mn stabilizing peptide – dilution 1 to 100).

procedure B the soluble L-AOX protein was totally removed from the complexes (no immunostained band in the 50 kDa region), while the 36 kDa peptide which now was a very distinct and prominent band, again showed a clear and specific reaction with the anti-L-AOX. Both antisera raised against the L-AOX from the two different purification procedures gave identical results.

The additional two peptides in the 30 kDa region could be identified as the D-1 peptide (30–33 kDa) which was detected by both D-1 antisera and the extrinsic Mn stabilizing peptide. Under our conditions this peptide has a molecular weight of 27 to 30 kDa, and this corresponds well with the reported molecular weight of 29.3 kDa deduced from the oligonucleotide sequence from *A. nidulans* R2 [21].

The 49 (46–50 kDa) and 55 kDa (53–57 kDa) bands and a diffuse band in the 65 kDa region were immunologically related to the 47 kDa peptide of higher plants. In SDS-urea gels this antiserum only detected a 49 kDa band (not shown). Obviously, under the conditions which we used here (see Materials and Methods), the 49 kDa peptide has a tendency to aggregate with small peptides or lipids. However, these conditions were chosen because they

gave a very good separation of the peptides in the 30 kDa region (in contrast to SDS-urea gels) and this was our main goal. PS II complexes obtained after purification B also contained a heavy 62 and 71 kDa band which were only minor bands in the PS II complexes obtained after purification A (compare Fig. 2 and 3). The 62 kDa band seems to be an aggregate which partly contained the D-1 peptide (see lane E of Fig. 3). Other groups already reported that the D-1 and D-2 peptide easily form homo or hetero aggregates [22]. The 71 kDa band remains unidentified. It could be an aggregate or possibly the anchor protein of the phycobilisomes, but this is uncertain [23]. Unfortunately, we were not able to identify the D-2 peptide in the *Anacystis* PS II complexes, since both D-2 antisera (raised against the D-2 peptide from oat and against a synthetic peptide) did not show a good crossreactivity to identity D-2. Possibly the D-2 peptide is a component of several of the upper aggregation bands and therefore too dilute to achieve a detection with the available antisera.

## Discussion

The minimal $O_2$ evolving PS II complex of higher plants consists of at least 8 polypeptides: the reaction center peptides D-1 and D-2, the two apoproteins of cytochrome $b_{559}$, (9 and 4.3 kDa) a recently identified 4.8 kDa peptide, two chl a binding peptides of 47 and 43 kDa as the immediate antenna proteins and the extrinsic, Mn stabilizing peptide [1, 2, 6, 7]. PS II complexes from cyanobacteria have been shown to be quite similar in peptide composition to those of higher plants, as *e.g.* shown for the PS II complex isolated from the thermophilic cyanobacterium *Synechococcus sp.* [24, 25]. However, some other highly active $O_2$ evolving preparations from cyanobacteria contain additional bands with molecular weights above 50 kDa [26–28].

The PS II complex which we isolated from *A. nidulans* (purification B) contained as major peptides a 71 kDa band (unknown identity – possibly the anchor protein of the phycobilisomes [23]), a 62 kDa band (an aggregate which partly contained D-1), a 55 and 49 kDa band (immunoreactive with an antiserum to the apoprotein of CP-47 of tobacco) and three bands in the 30 kDa region. These latter bands could be identified as the extrinsic Mn stabilizing peptide (27 to 30 kDa), D-1 (30 to 33 kDa) and a 36 kDa peptide (35 to 38 kDa) which crossreacted with the anti-L-AOX. The peptides of 10 kDa and below were not examined in the paper.

Our immunological experiments show that the antiserum raised against the soluble L-AOX which has a subunit molecular weight of 50 kDa [14, 15] recognizes a peptide of 36 kDa in purified *Anacystis* PS II complexes. These results could possibly be best explained by suggesting that the 36 kDa peptide in PS II might be a processed form of the soluble 50 kDa L-AOX protein. Of course, the variation in the apparent molecular weight could also be partly due to an unusual electrophoretic behaviour either caused by an association of lipids with the peptide or by bound ions which might interfere with the SDS binding to the peptide.

If two forms of the L-AOX protein are present in *A. nidulans,* then this would explain why part of the protein is readily soluble and the other part is tightly associated with the thylakoid membranes. As previously shown [14, 15], the soluble form of the L-AOX protein has a very high L-AOX activity with $O_2$ as electron acceptor (turnover number of 70,000). Whether the assumed processed form of this protein present in PS II complexes of *A. nidulans* has a measurable L-AOX activity is uncertain at the present time, since PS II complexes obtained after purification B, have practically no detectable L-AOX activity. This could be due to the ions (Mn, $Ca^{2+}$ and $Cl^-$) which are present in PS II complexes and which would suppress the L-AOX activity or could else be due to the detergent preventing the hydrophilic substrate L-arginine to reach the catalytic site of the membrane bound protein.

In some aspects the here presented results might be comparable to those of Gavazzi *et al.* [29] who have recently shown that an antiserum raised against the soluble hog kidney D-amino acid oxidase (being a dimeric flavoprotein of two identical 40 kDa subunits) which uses $O_2$ as electron acceptor, recognizes a D-alanine dehydrogenase in *Escherichia coli* (being a dimeric iron sulphur flavoprotein composed of two unidentical subunits of 45 and 55 kDa). The latter enzyme, being an inducible membrane-bound enzyme of the respiratory chain in *E. coli,* can only be solubilized by detergents and does not interact with $O_2$ [30, 31]. That crossreactivity observed between the anti-D-AOX antibodies and D-alanine dehydrogenase from *E. coli* also raises the question of how these two proteins which differ in their function and localization within the cell, are evolutionary related,

and this might indicate that amino acid oxidases whose functions are generally unknown but which are widely distributed [32], have roles in addition to the normal $O_2$ requiring oxidative deamination reaction. Those results are somewhat related to our previously stated hypothesis which suggests that an L-arginine dehydrogenase type enzyme is modified during evolution so that it could eventually function in water oxidation together with the reaction center complex. This would imply that the L-AOX activity of this protein is a cryptic activity which for unknown reasons is very high in *A. nidulans* and which might play a minor role in L-arginine catabolism under certain conditions [13].

The Sherman group [23, 33] has recently shown that a chlorophyll protein complex (called CP VI-4) increases under iron deficiency in *A. nidulans* R2. This complex consists of a 36, 34 and 12 kDa peptide, and these peptides do not seem to be related to previously identified PS II core proteins. Moreover, a 36 kDa peptide of unknown identity has also been shown to be present in PS II complexes from *Aphanocapsa sp.* [34] and possibly also in *Synechococcus sp.* [25]. Whether the 36 kDa peptide which is a very prominent band in PS II complexes from *A. nidulans* and which is immunoreactive with the anti-L-AOX antibody, is identical to the 36 kDa peptide which is a component of the CP VI-4 in *A. nidulans* R2 [22, 23] remains to be seen.

To prove our hypothesis that the 36 kDa peptide in PS II complexes of *A. nidulans* is a processed form of the soluble L-AOX protein will require at least partial sequencing of the two peptides and/or of the gene(s) and to prove the suggested function of this peptide will require the finding of a comparable peptide in PS II complexes of higher plants. The 36 kDa peptide in *A. nidulans* is difficult to handle, since it has a tendency to aggregate or to give fuzzy bands on polyacrylamide gels, especially on SDS-urea gels, and is difficult to blot. Interestingly, similar difficulties have been reported for the 36 kDa peptide from *A. nidulans* R2 [33]. Therefore, such a peptide might possibly have escaped detection in plants.

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[1] P. H. Homann, Plant Physiol. **88,** 1 (1988).
[2] L.-E. Andréasson and T. Vänngård, Ann. Rev. Plant Physiol. Plant. Mol. Biol. **39,** 379 (1988).
[3] J. Deisenhofer, O. Epp, K. Miki, R. Huber, and H. Michel, Nature **318,** 618 (1985).
[4] H. Michel and J. Deisenhofer, Nature **27,** 1 (1988).
[5] A. Trebst, Z. Naturforsch. **41c,** 240 (1986).
[6] O. Nanba and K. Satoh, Proc. Natl. Acad. Sci. U.S.A. **84,** 109 (1987).
[7] M. Ikeuchi and Y. Inoue, FEBS Lett. **241,** 99 (1988).
[8] J. Barber, Trends Biochem. Sci. **12,** 321 (1987).
[9] A.-F. Miller, J. C. De Paula, and G. W. Brudvig, Photosynth. Res. **12,** 205 (1987).
[10] E. K. Pistorius and A. E. Gau, Biochim. Biophys. Acta **849,** 203 (1986).
[11] E. K. Pistorius and A. E. Gau, FEBS Lett. **206,** 243 (1986).
[12] E. K. Pistorius, Z. Naturforsch. **40c,** 806 (1985).
[13] E. K. Pistorius, R. Kertsch, and S. Faby, Z. Naturforsch. **44c,** 370 (1989).
[14] E. K. Pistorius and H. Voss, Biochim. Biophys. Acta **611,** 227 (1980).
[15] G. Wälzlein, A. E. Gau, and E. K. Pistorius, Z. Naturforsch. **43c,** 545 (1988).
[16] T. Kuwabaro, M. Miyao, T. Murata, and N. Murata, Biochim. Biophys. Acta **806,** 283 (1985).
[17] U. Johannigmeier, Z. Naturforsch. **42c,** 755 (1987).
[18] R. Geiger, R. J. Berzborn, B. Depka, W. Oettmeier, and A. Trebst, Z. Naturforsch. **42c,** 491 (1987).
[19] U. K. Laemmli, Nature **227,** 680 (1970).
[20] R. Meyer and E. K. Pistorius, Biochim. Biophys. Acta **975,** 80 (1989).
[21] T. Kuwabara, K. J. Reddy, and L. A. Sherman, Proc. Natl. Acad. Sci. U.S.A. **84,** 8230 (1987).
[22] K. Satoh, R. Dostatni, U. Johannigmeier, and W. Oettmeier, FEBS Lett. **233,** 1 (1988).
[23] H. B. Pakrasi, H. C. Riethmann, and L. A. Sherman, Proc. Natl. Acad. Sci. U.S.A. **82,** 6903 (1985).
[24] K. Satoh, T. Ohno, and S. Katoh, FEBS Lett. **180,** 326 (1985).
[25] J. P. Dekker, E. J. Boekema, H. T. Witt, and M. Rögner, Biochim. Biophys. Acta **936,** 307 (1988).
[26] J. M. Bowes, A. C. Stewart, and D. S. Bendall, Biochim. Biophys. Acta **725,** 210 (1983).
[27] H. Koike and Y. Inoue, Biochim. Biophys. Acta **807,** 64 (1985).
[28] R. Frei, H.-J. Eckert, G. Renger, and R. Bachofen, Biochim. Biophys. Acta **932,** 258 (1988).
[29] E. Gavazzi, N. Malgaretti, and B. Curti, Biochim. Biophys. Acta **915,** 188 (1987).
[30] P. J. Olsiewski, G. J. Kaczorowski, and C. Walsh, J. Biol. Chem. **255,** 4487 (1980).
[31] H. Jones and W. A. Venables, FEBS Lett. **151,** 189 (1983).
[32] A. Meister, Biochemistry of the Amino Acids, 2nd edn., **Vol. I,** p. 294, Academic Press, New York 1965.
[33] H. C. Riethman and L. A. Sherman, Biochim. Biophys. Acta **935,** 141 (1988).
[34] G. S. Bullerjahn, H. C. Riethman, and L. A. Sherman, Biochim. Biophys. Acta **810,** 148 (1985).