EPR Characterization of an Oxygen-Evolving Photosystem II Preparation from the Transformable Cyanobacterium *Synechocystis* 6803†

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ABSTRACT: The transformable cyanobacterium *Synechocystis* 6803 has a photosynthetic apparatus that is similar to that of plants. Because of the ease with which this organism can be genetically manipulated and isotopically labeled, *Synechocystis* has been used extensively in recent studies of electron transfer in the water-splitting complex, photosystem II. Here, we present the first EPR characterization of a highly active oxygen-evolving preparation from this organism. This preparation shows oxygen-evolution activities in the range from 2400–2600 μmol of O_2/(mg of chlorophyll·h). We show that this preparation is stable enough for room temperature EPR studies. We then use this assay to show that the lineshapes of the D+ and Z+ tyrosine radicals are identical in this preparation, as has been observed in photosystem II complexes from a wide variety of photosynthetic species. We also present the first multiline EPR spectrum that has been observed from the *Synechocystis* manganese cluster.

Photosynthetic electron transfer in plants, green algae, and cyanobacteria involves the concerted action of two photosystems that act together to transfer electrons from water to NADPH. Photosystem II is the chlorophyll–protein complex that carries out the light-driven oxidation of water and the reduction of bound plastoquinone (Babcock, 1987; Babcock et al., 1989). Light absorption by the primary donor, P680+,[1] initiates light-induced electron transfer; a pheophytin acts as an intermediate electron acceptor. In addition to the two molecules of plastoquinone, Q_A and Q_B, that act as terminal electron acceptors, the reaction center contains a non-heme iron, a cluster of manganese, which is believed to be the catalytic site of water oxidation, and a cytochrome b_{559}. The minimum size of a functional reaction center appears to be at least seven polypeptides; one of these proteins of 33 000 molecular weight, is an extrinsic polypeptide; two hydrophobic proteins, called D1 and D2, bind P680 and pheophytin. Two other hydrophobic chlorophyll-binding proteins, of 47 000 and 43 000 molecular weight, are also associated with the reaction center and are thought to play a light-harvesting role.

Two redox-active groups are associated with the donor side of photosystem II. One of these species, D+, is a stable radical (Commoner et al., 1956). The other redox-active species, Z+, acts as an electron carrier between the manganese and P680+ (Babcock, 1987). A combination of isotopic labeling and EPR spectroscopy has been used to show that D+ is a tyrosine radical (Barry & Babcock, 1987). Because of the similarity of the D+ and Z+ EPR spectra in photosystem II particles from a variety of organisms, it was suggested that Z+ must also be a tyrosine radical (Barry & Babcock, 1987). Ultraviolet absorption studies of Z+ have supported this idea (Gerken et al., 1987). Since it is known that the EPR spectra of tyrosine radicals are sensitive to tyrosine conformation (Barry & Babcock, 1988), it was proposed that D+ and Z+ are found in conserved protein environments (Barry & Babcock, 1987; Debus et al., 1988a). On the basis of a model that assumes C_2 symmetry on the donor side of photosystem II, it was proposed that the D+ radical is tyrosine-160 of the D2 polypeptide and that Z+ is tyrosine-161 of the D1 polypeptide (Debus et al., 1988a). Site-specific mutagenesis was then used to confirm this assignment for the stable radical, D+ (Debus et al., 1988a; Vermaas et al., 1988). More recently, genetic experiments have been reported that are consistent with the assignment of Z+ to D1 tyrosine-161 (Debus et al., 1988b; Metz et al., 1989).

Z+ mediates electron transfer between P680 and the manganese cluster, where water oxidation occurs. Four sequential charge separations in the reaction center advance the manganese cluster through five oxidation states, called *S* states (Joliot et al., 1969; Kok et al., 1970). However, little is known about the chemistry of this process. At cryogenic temperatures, two types of EPR signals are observed from the S_2 state; this state is prepared by a single turnover of the reaction center from its long-term dark-adapted S_1 state. One of these EPR signals is the multiline signal centered at $g = 2.0$; it shows complex hyperfine splittings arising from a multinuclear, multivalent manganese cluster (Dismukes & Siderer, 1981; Hansson & Andreasson, 1982; Brudvig et al., 1983). The other signal, at $g = 4.1$, shows no resolved hyperfine structure (Casey & Sauer, 1984; Zimmerman & Rutherford, 1984); it may arise from an alternate conformation of the multinuclear manganese cluster or from mononuclear manganese (de Paula et al., 1985; Beck & Brudvig, 1986; Zimmerman & Rutherford, 1986; Hansson et al., 1987). The amino acids that provide ligands to the manganese cluster have not been identified. Site-specific mutagenesis may provide a means by

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[1] Abbreviations: BBY, spinach photosystem II particles, isolated according to the procedure described in Berthold et al. (1981); D1 and D2, polypeptides of the photosystem II reaction center; DCBQ, 2,6-dichlorobenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; LDAO, lauryldimethylamine oxide; MES, 2-(*N*-morpholino)ethanesulfonic acid; P680, primary chlorophyll donor of photosystem II; P700, primary chlorophyll donor of photosystem I; PEG, polyethylene glycol 8000; PMSF, phenylmethanesulfonyl fluoride; PSII, photosystem II; TES, *N*-[tris(hydroxylmethyl)-methyl]-2-aminoethanesulfonic acid; SB-12, sulfobetaine 12; SDS, sodium dodecyl sulfate.

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which protein ligands to the metal cluster can be identified, thus yielding structural information about the mechanism of water oxidation.

Site-directed mutagenesis experiments have been very useful in elucidating the role of redox-active tyrosines in photosystem II, and, as discussed above, site-directed mutagenesis, aimed at the identification of manganese ligands, may help to describe the chemistry of water oxidation. Genetic manipulation of photosystem II can be performed in the unicellular cyanobacterium *Synechocystis* 6803. This organism can grow photoheterotrophically, is transformable, and incorporates DNA into its genome by homologous recombination (Williams, 1988). These characteristics make *Synechocystis* an ideal system to use in genetic studies. Moreover, auxotrophs of *Synechocystis* can also be used to specifically isotopically label photosystem II (Barry & Babcock, 1987). Such isotopic labeling is an important tool in the interpretation of spectroscopic studies of photosynthetic electron transfer.

However, research using *Synechocystis* has been severely hindered by the lack of an active, spectroscopically well-characterized photosystem II preparation from this organism. The biochemistry of isolation of active photosystem II particles from plants [for review, see Ghanotakis et al. (1987)], thermophilic cyanobacteria (Stewart & Bendall, 1979; Bowes et al., 1983; Smutzer & Wang, 1984; Schatz & Witt, 1984; Koike & Inoue, 1985; Ohno et al., 1986; Dekker et al., 1988; Frei et al., 1988; McDermott et al., 1988), and *Anacystis nidulans* (England & Evans, 1981; Pakrasi & Sherman, 1984) has been described. However, these methods have proved to be ineffective in isolation of the water-splitting complex from *Synechocystis* 6803.

Recently, two methods of purification of photosystem II from *Synechocystis* have been reported (Burnap et al., 1989; Rogner et al., 1990). In the Burnap procedure, the isolated particles evolve oxygen at rates that are rather low for cyanobacterial preparations (Schatz & Witt, 1984); and further, these particles are spectroscopically uncharacterized. Also, cells with unusually high ratios of photosystem II to photosystem I are used as starting material in this preparation; these cells were generated by growth of cultures under red light. In the Rogner procedure, the particles are suitable for EPR and optical experiments that are aimed at observation of the D+ and Z+ tyrosine radicals; however, these particles are inactive in oxygen evolution. Significantly, EPR signals from the manganese cluster have never been observed in a photosystem II preparation from *Synechocystis*.

Here, we describe in detail a method of preparation of photosystem II from *Synechocystis* 6803. The method yields particles with high specific activities [2400–2600 μmol of O_2/(mg of chlorophyll·h)]; we show that these particles are suitable for both room temperature and cryogenic EPR spectroscopy. Moreover, as we will describe, the preparation of these particles is relatively simple, and we can use, as starting material, either photoautotrophically grown or photoheterotrophically grown cells. We use these particles to examine the room-temperature EPR lineshape of D+ and Z+. We also present the first multiline S_2 spectrum obtained from a *Synechocystis* photosystem II preparation.

Materials and Methods

Growth of Cells. A glucose-tolerant strain (Williams, 1988) of *Synechocystis* 6803 was grown as described in Williams (1988) in constant light at 30 °C. The medium employed was BG-11 (Rippka et al., 1979) supplemented with 5 mM TES, pH 8.0. Where appropriate, the medium was supplemented with 5 mM glucose, which was added by sterile filtration.

Preparation of Thylakoid Membranes. Cells from 15-L carboys were harvested by centrifugation when the culture reached 1.5 $OD_{720\ nm}$. The pellets were resuspended in 1.5 L of break buffer (0.8 M sucrose, 50 mM MES, pH 6.0) and pelleted by centrifugation for 10 min at 5000 rpm in a Sorvall GS3 rotor. The cells were resuspended in approximately 300 mL of break buffer and incubated 60–90 min on ice in the dark. They were then pelleted by centrifugation for 10 min at 5000 rpm in a Sorvall SS-34 rotor. As several authors have pointed out, this infiltration with high osmotic strength media assists in cell breakage (Walsby, 1974; Burnap, 1989).

The cells were resuspended in approximately 50 mL of break buffer to which was added 5 mg of PMSF, 1.5 mg of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 0.25 mg of pepstatin A, 1.25 g of bovine serum albumin (Fraction V, Sigma), and 0.5 mg of DNase I (bovine pancrease type IV, Sigma). The cells were added to a prechilled bead-beater chamber (250-mL volume) that was two-thirds full with 0.1-mm diameter glass beads. (Bio-spec Products, OK). The chamber was then filled with break buffer, and the outside jacket was filled with ice water. The cells were broken in darkness in 7–8 cycles; a cycle consisted of a 30-s "on phase" and a 15-min "off phase".

The cellular material was separated from the beads by decantation, and the beads were washed four times with a total of 1 L of break buffer. This solution was then centrifuged for 30 min at 7500 rpm in a Sorvall GSA rotor to pellet unbroken cells and cellular debris. $CaCl_2$ was added to the supernatant of this spin to give a final concentration of 40 mM. Thylakoid membranes were then pelleted by a 30-min spin at 12500 rpm in a GSA rotor. The thylakoid membrane pellet was resuspended in 50 mL of freeze buffer (25% glycerol, 20 mM MES pH 6.0, 20 mM $CaCl_2$, 20 mM $MgCl_2$), and the membranes were pelleted again by centrifugation at 19000 rpm for 30 min in an SS-34 rotor. This additional wash step helped to remove phycobiliproteins from the membrane. The pelleted thylakoid membranes were resuspended in freeze buffer to give a chlorophyll concentration of approximately 1.5 mg of chlorophyll/mL, the membranes were homogenized briefly, and the homogenized mixture was frozen in liquid nitrogen and stored at −80 °C.

Chromatographic Purification of Photosystem II. (A) Fast Flow Q Column, pH 6.0. Fast Flow Q–Sepharose gel was purchased from Pharmacia, and a 30 × 1.5 cm column was poured and equilibrated with two column volumes of high-salt buffer [25% (v/v) glycerol, 0.05% (w/v) lauryl maltoside, 20 mM MES pH 6.0, 20 mM $CaCl_2$, 250 mM $MgCl_2$] and five column volumes of start buffer [25% (v/v) glycerol, 0.05% (w/v) lauryl maltoside, 20 mM MES pH 6.0, 20 mM $CaCl_2$, 15 mM $MgCl_2$].

Thylakoid membranes, up to 20 mg of chlorophyll for the column size described above, were thawed on ice. The membranes were solubilized in freeze buffer with lauryl maltoside for 10 min in the dark at 0 °C; the concentrations during solubilization were 0.6 mg/mL chlorophyll, 0.01 mg/mL PMSF, and 1% (w/v) lauryl maltoside. The solution was then spun at 140000 × *g* for 30 min to pellet unsolubilized material. The resulting supernatant was applied to the Fast Flow Q column. The column was washed with 50 mL of start buffer. A 500-mL linear gradient of 15–120 mM $MgCl_2$ was applied. The flow rate was approximately 1.5 mL/min, and 5-mL fractions were collected. The absorption of the eluant was monitored at 280 nm by using a 2-mm flow cell and a Pharmacia UV-M monitor. Photosystem II activity eluted in the first of two chlorophyll-containing peaks, at $MgCl_2$ con-

centrations between 35 and 55 mM. The visible absorption spectra of each fraction was recorded on a Cary 210 spectrophotometer (1 nm/s, 1-nm spectral band pass, 0.5-s period), and fractions from the first peak with absorption maxima less than 678 nm were pooled. The pooled fractions were precipitated by addition of an equal volume of 30% PEG buffer (30% PEG-8000, 20 mM MES pH 6.0, 20 mM $CaCl_2$) and pelleted by centrifugation for 30 min at 19000 rpm in an SS-34 rotor. The precipitated fractions were then stored at −80 °C.

(B) Mono-Q Column, pH 6.5. The pooled fractions from the Fast Flow Q column were resuspended to a concentration of 0.8–1.2 mg of chlorophyll/mL with Mono-Q buffer A [25% (v/v) glycerol, 0.05% (w/v) lauryl maltoside, 50 mM MES, pH 6.5, 20 mM $CaCl_2$, 15 mM NaCl]. The sample was filtered through a Gelman (0.45 μm) Acrodisk filter, and then 1 mg of chlorophyll was loaded onto a Pharmacia HR 5/5 Mono-Q FPLC column. The column was washed with 5 mL of buffer A, and then a 50-mL linear gradient of buffer B was applied [25% (v/v) glycerol, 0.05% (w/v) lauryl maltoside, 50 mM MES, pH 6.5, 20 mM $CaCl_2$, 500 mM NaCl] by using a Pharmacia FPLC system. The flow rate was 0.4 mL/min, and 0.5-mL fractions were collected. The elution profile was recorded as described above. Fractions with absorbance maxima less than or equal to 674 nm were pooled and precipitated as described above. Such fractions eluted at 150 mM NaCl.

Several Mono-Q columns, with 1 mg of chlorophyll loaded per column, were run for each pooled sample from the Fast Flow Q column. Because of the speed and reproducibility with which the particles can be purified by using a Mono-Q column and an FPLC system (1–2 h), multiple Mono-Q columns could easily be run in one afternoon. The precipitated PSII particles from several Mono-Q columns were pooled and were resuspended to a concentration of 0.4–0.6 mg chlorophyll/mL in buffer A and frozen at −80 °C.

Oxygen Evolution Assays. Oxygen assays were performed by using a Clark-type electrode, a water-jacketed cell maintained at 25 °C, and 10–20 μg of chlorophyll. Saturating light was supplied by use of a red-filtered fiber-optic light source (Dolan-Jenner, Model 180). The assay buffer contained 1 M sucrose, 50 mM MES, pH 6.5, 25 mM $CaCl_2$, and 10 mM NaCl, unless otherwise noted. Air-saturated water was used as an oxygen standard. When DCBQ (Kodak) was used as an acceptor, it was recrystallized from methanol.

Chlorophyll Assays. Chlorophyll concentration was determined by extraction of chlorophyll into methanol. The extinction coefficient for chlorophyll in methanol was taken from Lichtenthaler (1987).

Room Temperature EPR Studies. Room-temperature EPR spectra were recorded at the X band on a Varian E-4 spectrometer equipped with a Varian TE cavity. The modulation frequency was 100 kHz. Other spectral conditions are given in the figure legends. Illumination in the cavity was performed by using a microscope illuminator. Purified cyanobacterial photosystem I was oxidized with 1 mM ferricyanide, and the resulting dark-stable chlorophyll, P700+, was used as a *g*-value standard [$g = 2.0025$, Hoff (1987)]. In experiments designed to observe the long-lived Z+ radical, photosystem II particles were Tris washed to remove extrinsic polypeptides and manganese (Cheniae & Martin, 1970; Babcock & Sauer, 1975; Yamamoto et al., 1981). The sample was diluted with an equal volume of 2× Tris buffer (1.6 M Tris, pH 8.0, 4 mM EDTA, 0.1% lauryl maltoside) and incubated for 20 min in the light at 0 °C. After incubation, the particles were PEG precipitated as described above.

Cryogenic EPR Studies. EPR spectra were obtained at 10 K through the use of a Varian E109 spectrometer equipped with an Oxford ESR-910 helium flow cryostat and an ITC4 temperature controller. The system was also equipped with a Hewlett Packard 5350B microwave frequency counter and a 436A power meter. The field modulation was 100 kHz, other spectral conditions are given in the figure legends. Samples were treated as described in de Paula et al. (1985), except that the initial dark adaption was for 1–2 h. Samples were illuminated at 210 K in the presence of 50 μM DCMU (Serva), and the illumination source was a 150-W Dolan-Jenner model 180 illuminator equipped with an infrared filter and a fiber-optic light guide.

Manganese Quantitation. Manganese quantitation was performed with the room-temperature EPR assay described in Yocum et al. (1981). Photosystem II particles in Mono-Q buffer A were diluted with an equal volume of 1 N HCl, and the Mn^{2+} six-line spectrum was recorded with the following conditions: 10-G modulation; 100-mW microwave power; 1000-G field sweep; 1-s time constant; 2-min sweep time. $MnCl_2$ standards in Mono-Q buffer A and 0.5 N HCl gave a linear plot from 0 to 54 μM Mn^{2+}. Spinach photosystem II (BBY) particles, prepared by the method described in Berthold et al. (1981), with specific activities of 700 μmol of O_2/(mg of chlorophyll·h) were used as an additional control and gave the expected result of 4 Mn^{2+} per approximately 200 chlorophylls (Ghanotakis et al., 1984).

Optical Assays. P700+ and cytochrome b_{559} contents were quantitated by using the optical difference spectrum: ferricyanide-oxidized minus ascorbate-reduced. A Perkin-Elmer Lambda 5 spectrophotometer was used to record the data. The extinction coefficient for P700+ was taken as 64 mM^{-1} cm^{-1} (Hiyama & Ke, 1972), and the extinction coefficient for cytochrome b_{559} was taken as 21.5 mM^{-1} cm^{-1} (Cramer et al., 1986; Cramer & Whitmarsh, 1977). Values of cytochrome b_{559} and P700+ content were averages of duplicate measurements, and the agreement between measurements ranged from 1% to 9%.

Denaturing SDS Gel Electrophoresis. Denaturing SDS gels were run as described in Piccioni et al. (1982). The resolving gel contained 12% acrylamide; the stacking and resolving gel contained 6 M urea. Samples were solubilized in 1% SDS sample buffer by incubation for 1 h at room temperature. The gels were stained with Coomassie blue to visualize the polypeptide pattern.

Western Blot Analysis. Extrinsic proteins from spinach BBY particles were purified as described in Kuwabara et al. (1986). Antibodies against the 33-kDa protein were produced in New Zealand rabbits by standard techniques (Harlow & Lane, 1988). Antibodies against the 24-kDa protein were produced in chickens and purified from egg yolks by the methods described in Gassmann et al. (1990) and Jensenius et al. (1981). For western blot analysis, proteins were transferred to nitrocellulose through the use of a Semiphor semidry transfer apparatus (Hoefer, CA), and the blot was developed with either a protein A–alkaline phosphatase conjugate or an anti-chicken IgG–alkaline phosphatase conjugate (host animal–rabbit) according to standard procedures (Harlow & Lane, 1988).

Results

The overall strategy for the purification procedure described here is quite simple. Active thylakoid membranes are solubilized in the nonionic detergent, lauryl maltoside, and then subjected to two rounds of chromatography at two different

Table I: Purification of Oxygen-Evolving Photosystem II Particles from *Synechocystis* 6803

	oxygen evolution[a]	chlorophyll/ P700+[b]	total chlorophyll[c]	yield[d]
thylakoids	470[e]	140	20.0	100
Fast Flow Q	1200[f]	190	2.5	12
Mono-Q	2400[f]	960	0.4	2

[a] μmol of O_2/(mg of chlorophyll·h). [b] mol/mol. [c] mg chlorophyll. [d] percent chlorophyll. [e] 1 mM DCBQ and potassium ferricyanide as acceptors. [f] 400–600 μM DCBQ and 1 mM potassium ferricyanide as acceptors.

pH's. A "strong" anion-exchange resin with quaternary ammonium groups is used in both chromatography steps. Strong anion exchangers, in concert with other purification procedures, have been used previously to purify cyanobacterial photosystem II (Dekker et al., 1988; Rogner et al., 1990). In our preparation, salt is removed, and samples are concentrated by polyethyleneglycol precipitation (Burnap et al., 1989).

When assayed in BG-11 medium with 2 mM potassium ferricyanide and 2 mM DCBQ as acceptors, white-light grown cells of *Synechocystis* 6803 gave rates of oxygen evolution from 500 to 600 μmol O_2/(mg of chlorophyll·h). We observed similar rates in cells grown photoheterotrophically and photoautotrophically. In the cell-breakage procedure we describe, the yield of broken cells is greater than 90%, and the thylakoid membranes show approximately the same oxygen-evolution activity as whole cells, with rates of 450–600 μmol O_2/(mg of chlorophyll·h) (Table I). The length of the off phase in the breakage cycles is critical to maintainance of high rates of oxygen evolution, since overheating of the cell-membrane solution is avoided. Addition of $CaCl_2$ helps to maintain activity and also aggregates the membranes so that they can be conveniently pelleted at low centrifugal force without the use of an ultracentrifuge.

Lauryl maltoside solubilization of the membranes gives no differential solubilization of photosystem II reaction centers. That is, the oxygen-evolution activity and P700+ content of the lauryl maltoside extract is approximately the same as that of thylakoid membranes (data not shown). However, *Synechocystis* photosystem II particles are quite stable in this detergent, and it provides effective solubilization for high-resolution chromatography. After the Fast Flow Q column, pooled fractions from the first chlorophyll-containing peak show rates of oxygen evolution of 1200 μmol O_2/(mg of chlorophyll·h) and a decrease in PSI content (Table I). The majority of the oxygen-evolving activity elutes in this first peak; oxygen-evolution rates in the second peak are less than 150 μmol O_2/(mg of chlorophyll·h). In spite of a 2- to 3-fold increase in specific activity, at this point the fractions still contain too much photosystem I contamination to be useful in EPR spectroscopy (data not shown). Therefore, these fractions from the Fast Flow Q column are pooled and subjected to further chromatographic purification.

Elution at pH 6.5 from the Mono-Q column is more complex than elution from the Fast Flow Q column but provides reproducible purification of photosystem II (Figure 1). Table II shows oxygen-evolution activities, visible absorption maxima, and P700+ and cytochrome b_{559} optical quantitation for pooled sets of chlorophyll-containing fractions from the column. Early fractions from the column (18–29) contain photosystem I and very little photosystem II. This is reflected in visible absorption spectra near 680 nm, low oxygen-evolution activities, and high P700+ content. These fractions contain an ascorbate-reducible cytochrome with an absorption spectrum that is different than cytochrome b_{559}, so we were not able to obtain reliable estimates of cytochrome b_{559} content in these pooled fractions. Later fractions contain active photosystem II particles, although some fractions are contaminated with photosystem I. Increases in specific activity and ascorbate-reducible cytochrome b_{559} content parallel each other when the last four sets of pooled fractions are compared. Fractions with absorption maxima less than or equal to 674 nm, eluting near 150 mM NaCl, are pooled (Table II, fractions 35–40). The oxygen-evolution rates exhibited by this pooled sample are typically in the range from 2400 to 2600 μmol of O_2/(mg of chlorophyll·h), and this sample typically contains 1 P700+ per 960 chlorophylls. Table I shows that when specific activity is used as a criterion, we obtain a 5- or 6-fold purification over thylakoid membranes. When P700+ content is used as the criterion, we have achieved a 6- or 7-fold purification. The yield of this purified photosystem II fraction is approximately 2% of the total starting chlorophyll. From the elution profiles, we qualitatively estimate that this corresponds to 50–60% of the total amount of photosystem II present in the membrane.

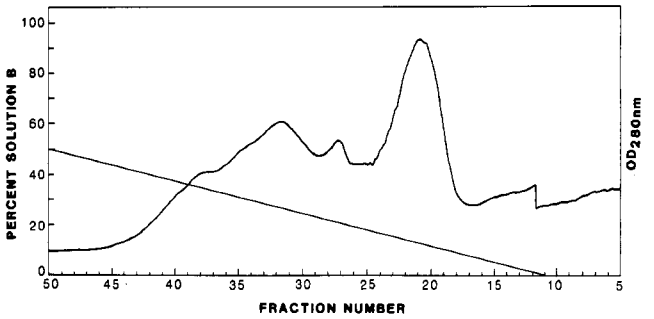

FIGURE 1: Elution profile of Mono-Q anion-exchange column, pH 6.5. Buffer B contains 25% (v/v) glycerol, 0.05% (w/v) lauryl maltoside, 50 mM MES-NaOH, pH 6.5, 20 mM $CaCl_2$, and 500 mM NaCl.

Table II: Elution Profile from the Mono-Q Column, NaCl Gradient, pH 6.5

fraction no.[a]	oxygen evolution[b]	chlorophyll/ cytochrome[c]	chlorophyll/ P700+[d]	λ_{max}[e]
18–25	0		120	680
26–29	350	340	200	678
30–34	1100	90	290	675
35–40	2600	44	960	674
41–44	1600	93	250	675

[a] See Figure 1. [b] μmol of O_2/(mg of chlorophyll·h). [c] mol chlorophyll/mol ascorbate-reducible cytochrome. [d] mol chlorophyll/mol P700+; [e] nm.

These photosystem II particles require $CaCl_2$ for optimal activity, when assayed at pH 6.5 in our assay buffer. Optimal acceptor concentrations are 1 mM potassium ferricyanide and 400–600 μM DCBQ. Ferricyanide (1 mM) alone sustains rates at 20% of the optimal activity, while DCBQ alone (600 μM) yields 90% of the optimal activity. DCMU (10 μM) inhibits 74% of the activity in these particles.

Manganese quantitation give 4.2 ± 0.3 Mn^{2+} per 60 chlorophylls. A spin count on the D+ tyrosine EPR signal gives us an independent estimate of reaction center size. BBY particles, with 1 D+ per 240 chlorophylls (Babcock et al., 1983) were used as the spin standard. This quantitation gives 1 D+ tyrosine radical per 59 (range of 53–65) chlorophylls. An antenna size of approximately 60 chlorophylls is in good agreement with reaction center sizes that are observed in other cyanobacterial preparations [for example, Bowes et al. (1983)]. Also, we find 1 ascorbate-reducible cytochrome b_{559} per 44 chlorophylls (Table II), suggesting that there are 1–2 cytochrome b_{559} per reaction center [see also, Rogner et al. (1990)].

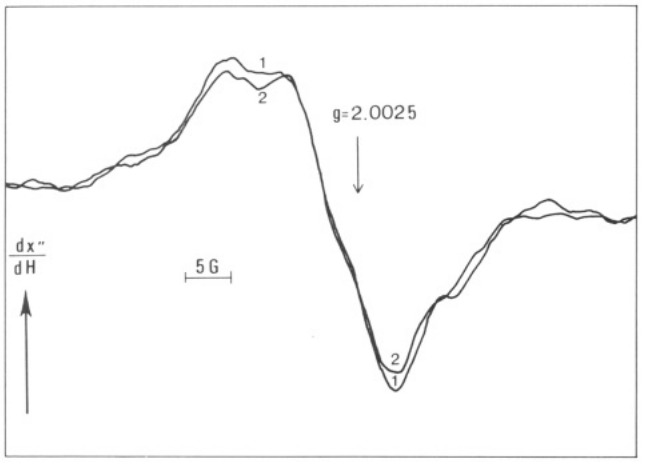

FIGURE 2: Room temperature EPR spectra of purified photosystem II particles from *Synechocystis* 6803. Spectrum 1 was recorded in the dark; spectrum 2 was recorded in the light. Conditions were as follows: microwave power, 6 mW; field modulation, 3.2 G; time constant, 2 s; scan time, 4 min; and gain, 3.2×10^4.

In Figure 2, we present the room-temperature EPR spectrum of purified photosystem II particles. The acceptor concentration is 0.5 mM potassium ferricyanide; the chlorophyll concentration is 0.56 mg/mL. Small light-induced changes in the center of the spectrum are caused by residual photosystem I contamination. In thylakoid membranes from *Synechocystis*, the signal from chemically oxidized P700+ completely obscures the broader tyrosine radical spectrum. In our preparation, however, the amount of P700+ contamination has been reduced to a level that does not interfere with the EPR assay (Figure 2). Moreover, there is no general increase in the amplitude of the spectrum at low field with repeated illumination. This is the expected result if the rereduction of Z+ from the manganese is rapid (D+ is stable over the course of this experiment). Thus, the fact that we see no overall light-induced increase in the EPR spectrum indicates that our preparation is "intact" and quite stable even at room temperature.

In Figure 3 we show a SDS-denaturing gel of Tris-washed and oxygen-evolving photosystem II particles. As expected, the gel reveals that Tris-washing removes, in addition to manganese, a polypeptide of approximately 38 000 molecular weight (Cheniae & Martin, 1970; Yamamoto et al., 1981). This cyanobacterial protein is analogous to the extrinsic 33 000 molecular weight protein from spinach, since western blotting with a rabbit antibody made against the purified spinach 33-kDa protein shows cross reaction (Figure 4). A band at approximately 24 kDa is also removed by Tris washing; we see no cross reaction between this protein and an antibody made against the spinach extrinsic 24-kDa protein (data not shown). Two prominant bands near 60 000 molecular weight are probably subunits of the cyanobacterial ATPase. ATPase subunits have previously been reported to be contaminants of cyanobacterial photosystem II preparations (Bowes et al., 1983). Although photosystem I may make a contribution to bands in this region, the intensity of these two bands does not correlate with photosystem I content, as determined by optical or EPR methods. We also see bands at approximately 47 000, 43 000, 35 000, and 32 000 molecular weights, which must be analogous to the 47 000, 43 000, D2, and D1 proteins found in other photosystem II preparations.

In Figure 5, we present room temperature EPR data on Tris-washed photosystem II particles. The chlorophyll concentration was 0.70 mg/mL; the acceptor concentration was 1 mM potassium ferricyanide. In this experiment, we see a

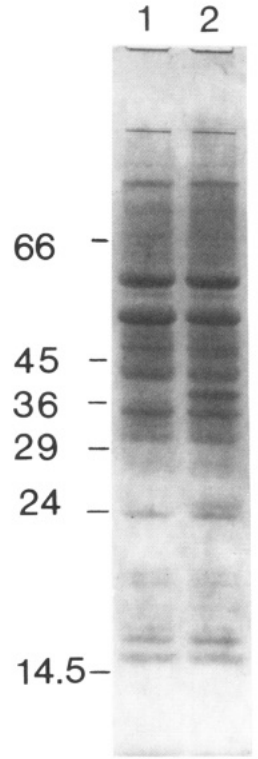

FIGURE 3: SDS–urea denaturing gel of (1) Tris-washed and (2) oxygen-evolving photosystem II particles from *Synechocystis* 6803. For each lane, 2 μg of chlorophyll was loaded.

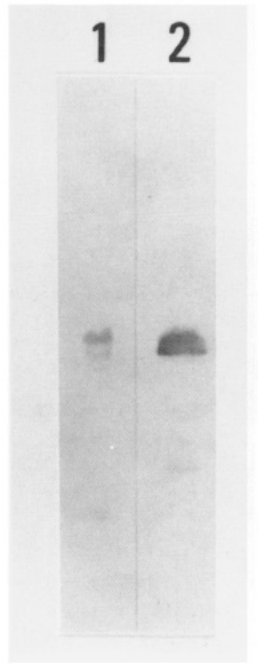

FIGURE 4: Western blot against (1) *Synechocystis* photosystem II and (2) spinach BBY particles. The blot was developed with an antibody against the spinach 33-kDa extrinsic protein. For each lane, 4 μg of chlorophyll was loaded.

light-induced 70% increase in the signal. This increase is caused by the generation of a long-lived Z+ intermediate in centers in which normal donation pathways have been purposely interrupted. A sample with lower chlorophyll concentrations, where the microscope illuminator is saturating, gave light-induced 100% increases (data not shown). Thus, in our preparation, the spectra of D+ and long-lived Z+ are present in approximately a one-to-one ratio and are identical in line width and lineshape.

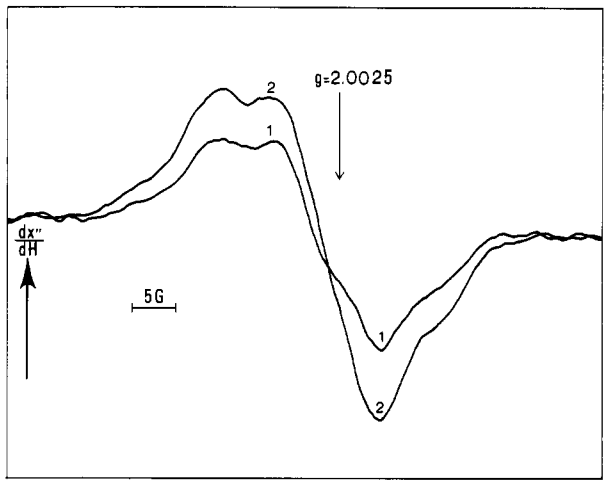

FIGURE 5: Room-temperature EPR spectra of Tris-washed photosystem II particles from *Synechocystis* 6803. Spectrum 1 was recorded in the dark; spectrum 2 was recorded in the light. The microwave power was 3.5 mW; the gain was 2.5×10^4. All other conditions were as described in Figure 4.

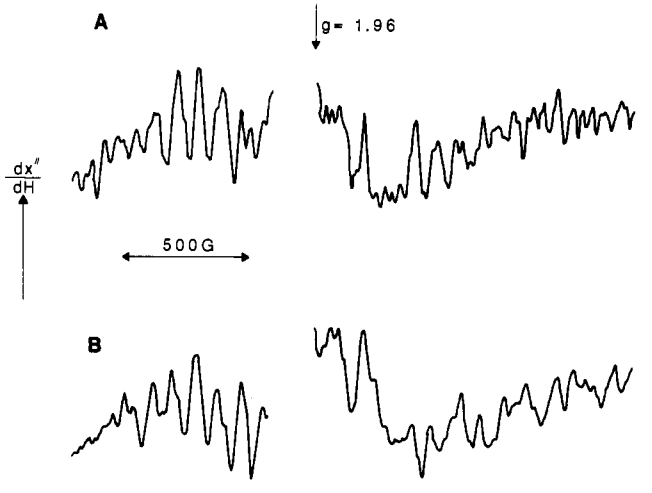

FIGURE 6: EPR spectra of photosystem II preparations from (A) spinach and (B) *Synechocystis* 6803 recorded at 10 K. A dark background has been subtracted from each spectrum. In (A) the chlorophyll concentration was 4 mg/mL, the power was 0.5 mW, the gain was 2.5×10^4, and the scale factor for plotting after data subtraction was 3. In (B) the chlorophyll concentration was 0.9 mg/mL, the power was 10 mW, the gain was 1.0×10^4, and the scale factor for plotting after data subtraction was 5. Other conditions were as follows: microwave frequency, 9.21 GHz; field modulation, 20 G; time constant, 0.25 s; and scan time, 4 min. Each spectrum is the average of four scans. Because of the large D+ signal at $g = 2.004$, a center portion of each spectrum has been removed.

In Figure 6, we show 10 K EPR spectra of (A) spinach BBY particles and (B) *Synechocystis* PSII particles. The PSII preparation from *Synechocystis* was resuspended in Mono-Q buffer A; the BBY membranes were resuspended in Mono-Q buffer A with no added $CaCl_2$. Figure 6A reveals the multiline spectrum in spinach photosystem II particles. These data were taken with 0.5 mW of microwave power; the spectral lineshape of the BBY particles was identical at higher power. Figure 6B shows that a signal with complex hyperfine structure is also observed in the S_2 state of the cyanobacterial preparation.

Discussion

Research on photosystem II in the last few years has demonstrated the utility of genetic manipulation in order to answer structure/function questions about water oxidation. Also, spectroscopic questions can sometimes be most definitively addressed through isotopic labeling. Both types of experiments are possible in the procaryotic cyanobacterium, *Synechocystis* 6803. However, advances in this area have been hindered by biochemical difficulties associated with the generation of active thylakoid membranes and with the isolation of purified complexes.

Progress in this area has been slow because methods that are useful in purifying photosystem II from other cyanobacteria are not useful for *Synechocystis*. The first problem with application of these protocols is breakage of the cells. Methods typically employed to break cyanobacteria are not effective in *Synechocystis*, or they inactivate water oxidation. The procedure described here is similar to that described in Burnap et al. (1989) and gives a high yield of active membranes. However, our procedure does not require the use of an ultracentrifuge or an expensive homogenizer. Moreover, the thylakoid membranes have suffered no loss in the specific activity of oxygen evolution when compared with whole cells. This was found to be an important criterion for starting material for successful photosystem II purification.

Many cyanobacterial preparations rely on differential solubilization of photosystem II from the membrane. *Synechocystis* photosystem II is very unstable in the two detergents that are typically employed, LDAO and SB-12. Moreover, the reproducibility of differential extraction with SB-12 was quite low (data not shown).

Our procedure differs from previous photosystem II preparations from *Synechocystis*. The Rogner procedure employed solubilization in lauryl maltoside, followed by overnight sucrose density gradient centrifugation, ion-exchange chromatography on a Mono-Q column at pH 6.5, and hydroxyapatite chromatography. Oxygen evolution in isolated thylakoid membranes was low and was completely lost after the first purification step. There was no 33 000 molecular weight extrinsic protein associated with the preparation (Rogner et al., 1990). The Burnap procedure used a mixture of detergents, octyl glucoside and lauryl maltoside, to differentially solubilize photosystem II from the membranes of red-light grown cells. This step is followed by ion-exchange chromatography on a weak anion (DEAE) exchanger. Oxygen-evolution rates in the Burnap preparation were approximately 1200 μmol of O_2/(mg of chlorophyll·h) (Burnap et al., 1989).

Our preparation makes use of only one detergent, lauryl maltoside, which does not differentially extract photosystem II from the membrane. However, oxygen evolution is stable in this detergent, and the procedure gives very reproducible results from culture to culture. Moreover, this procedure can be used with either photoautotrophic or photoheterotrophic cells. The fact that our preparation requires no long concentration or sucrose gradient steps is essential to the maintainance of high levels of oxygen-evolution activity and also has the benefit of making the preparation short and easy to perform. Our particles retain manganese and the 33-kDa extrinsic protein and show high specific activities of oxygen evolution. The increase in specific activity that we typically obtain in the purification is approximately the same as the decrease in P700+ content, implying that we have very few inactive complexes in our preparation. Judging from acceptor preference and DCMU sensitivity, the acceptor side of the complex is functionally intact. Also, this procedure is suitable for large-scale preparation of protein for EPR and other spectroscopic techniques.

The room-temperature EPR spectra that are presented here show clearly that the lineshapes of the D+ and Z+ radicals are identical in our photosystem II particles. Tyrosine radical lineshapes are known to be sensitive to their protein environ-

ment (Sjoberg et al., 1978; Barry & Babcock, 1988). This sensitivity comes about because the geometry of the radical at the methylene bond influences the magnitude of the splittings to the methylene protons (Sjoberg et al., 1978; Sealy et al., 1985; Barry & Babcock, 1988). The result that D+ and Z+ have identical lineshapes in our preparation suggests that these two radicals are located in highly conserved protein environments. This has now been shown to be true in photosystem II preparations from a wide variety of photosynthetic species (Ke et al., 1982; Babcock et al., 1983).

Observation of the multiline spectrum in our preparation will facilitate genetic experiments aimed at elucidating the structure of the manganese cluster. Future work will explore the role of other low-temperature donors to P680+ in this preparation.

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Registry No. L-Tyr, 60-18-4; O_2, 7782-44-7; Mn, 7439-96-5.

REFERENCES

Babcock, G. T. (1987) in *New Comprehensive Biochemistry*: *Photosynthesis* (Amesz, J., Ed.) pp 125–158, Elsevier, Amsterdam.

Babcock, G. T., & Sauer, K. (1975) *Biochim. Biophys. Acta 376*, 315–328.

Babcock, G. T., Ghanotakis, D. F., Ke, B., & Diner, B. A. (1983) *Biochim. Biophys. Acta 723*, 276–286.

Babcock, G. T., Barry, B. A., Debus, R. J., Hoganson, C. W., Atamian, M., McIntosh, L., Sithole, I., & Yocum, C. F. (1989) *Biochemistry 28*, 9557–9565.

Barry, B. A., & Babcock, G. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7099–7103.

Barry, B. A., & Babcock, G. T. (1988) *Chem. Scr. 28A*, 117–122.

Beck, W. F., & Brudvig, G. W. (1986) *Biochemistry 25*, 6479–6486.

Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231–234.

Bowes, J. M., Stewart, A. C., & Bendall, D. S. (1983) *Biochim. Biophys. Acta 725*, 210–219.

Brudvig, G. W., Casey, J. L., & Sauer, K. (1983) *Biochim. Biophys. Acta 723*, 366–371.

Burnap, R., Koike, H., Sotiropoulou, G., Sherman, L. A., & Inoue, Y. (1989) *Photosynth. Res. 22*, 123–130.

Casey, J. L., & Sauer, K. (1984) *Biochim. Biophys. Acta 767*, 21–28.

Cheniae, G. M., & Martin, I. F. (1970) *Biochim. Biophys. Acta 197*, 219–239.

Commoner, B., Heise, J. J., & Townsend, J. (1956) *Proc. Natl. Acad. Sci. U.S.A. 42*, 710–718.

Cramer, W. A., & Whitmarsh, J. (1977) *Annu. Rev. Plant Physiol. 28*, 133–172.

Cramer, W. A., Theg, S. M., & Widger, W. R. (1986) *Photosynth. Res. 10*, 393–403.

Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430.

Debus, R. J., Barry, B. A., Sithole, I., Babcock, G. T., & McIntosh, L. (1988b) *Biochemistry 27*, 9071–9074.

Dekker, J. P., Boekema, E. J., Witt, H. T., & Rogner, M. (1988) *Biochim. Biophys. Acta 936*, 307–318.

de Paula, J. C., Innes, J. B., & Brudvig, G. W. (1985) *Biochemistry 24*, 8114–8120.

Dismukes, G. C., & Siderer, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 274–278.

England, R. R., & Evans, E. H. (1981) *FEBS Lett. 134*, 175–177.

Frei, R., Eckert, H.-J., Renger, G., & Bachofen, R. (1988) *Biochim. Biophys. Acta 932*, 258–267.

Gassman, M., Thommes, P., Weiser, T., & Hubscher, U. (1990) *FASEB J. 4*, 2528–2532.

Gerken, S., Brettel, K., Schlodder, E., & Witt, H. T. (1988) *FEBS Lett. 237*, 69–75.

Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1984) *Biochim. Biophys. Acta 765*, 388–398.

Ghanotakis, D. F., Waggoner, C. M., Bowlby, N. R., Demetriou, D. M., Babcock, G. T., & Yocum, C. F. (1987) *Photosynth. Res. 14*, 191–199.

Hansson, O., & Andreasson, L.-E. (1982) *Biochim. Biophys. Acta. 679*, 261–268.

Hansson, O., Aasa, R., & Vanngard, T. (1987) *Biophys. J. 51*, 825–832.

Harlow, E., & Lane, D. (1988) *Antibodies*: *A Laboratory Manual*, Cold Spring Harbor Laboratory, New York.

Hiyama, T., & Ke, B. (1972) *Biochim. Biophys. Acta 267*, 160–171.

Hoff, A. (1987) in *New Comprehensive Biochemistry*: *Photosynthesis* (Amesz, J., Ed.) pp 97–123, Elsevier, Amsterdam.

Jensenius, J. C., Andersen, I., Hau, J., Crone, M., & Koch, C. (1981) *J. Immunol. Methods 46*, 63–68.

Joliot, P., Barbieri, G., & Chabaud, R. (1969) *Photochem. Photobiol. 10*, 309–329.

Ke, B., Inoue, H., Babcock, G. T., Fang, Z.-X., & Dolan, E. (1982) *Biochim. Biophys. Acta 682*, 297–306.

Koike, H., & Inoue, Y. (1985) *Biochim. Biophys. Acta 807*, 64–73.

Kok, B., Forbush, B., & McGloin, M. (1970) *Photochem. Photobiol. 11*, 457–475.

Kuwabara, T., Murata, T., Miyao, M., & Murata, N. (1986) *Biochim. Biophys. Acta 850*, 146–155.

Lichtenthaler, H. K. (1987) *Methods Enzymol. 148*, 350–382.

McDermott, A. E., Yachandra, V. K., Guiles, R. D., Cole, J. L., Dexheimer, S. L., Britt, R. D., Sauer, K., & Klein, M. P. (1988) *Biochemistry 27*, 4021–4031.

Metz, J. G., Nixon, P. J., Rogner, M., Brudvig, G. W., & Diner, B. A. (1989) *Biochemistry 28*, 6960–6969.

Ohno, T., Satoh, K., & Katoh, S. (1986) *Biochim. Biophys. Acta 852*, 1–8.

Pakrasi, H., & Sherman, L. A. (1984) *Plant Physiol. 74*, 742–745.

Piccioni, R., Bellemare, G., & Chua, N.-H. (1982) in *Methods in Chloroplast Molecular Biology* (Edelman, H., Hallick, R. B., & Chua, N.-H., Eds.) pp 985–1014, Elsevier Biomedical Press, Amsterdam.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979) *J. Gen. Microbiol. 111*, 1–61.

Rogner, M., Nixon, P. J., & Diner, B. A. (1990) *J. Biol. Chem. 265*, 6189–6196.

Schatz, G. H., & Witt, H. T. (1984) *Photobiochem. Photobiophys. 7*, 1–14.
Sealy, R. C., Harman, L., West, P. R., & Mason, R. P. (1985) *J. Am. Chem. Soc. 107*, 3401–3406.
Sjoberg, B.-M., Reichard, P., Graslund, A., & Ehrenberg, A. (1978) *J. Biol. Chem. 253*, 6863–6865.
Smutzer, G., & Wang, J. H. (1984) *Biochim. Biophys. Acta 766*, 240–244.
Stewart, A. C., & Bendall, D. S. (1979) *FEBS Lett. 107*, 308–312.
Vermaas, W. F. J., Rutherford, A. W., & Hansson, O. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8477–8481.
Walsby, A. E. (1974) *Methods Enzymol. 31*, 678–686.
Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766–778.
Yamamoto, Y., Doi, M., Tamura, N., & Nishimura, M. (1981) *FEBS Lett. 133*, 265–268.
Yocum, C. F., Yerkes, C. T., Blankenship, R. E., Sharp, R. R., & Babcock, G. T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7507–7511.
Zimmerman, J.-L., & Rutherford, A. W. (1984) *Biochim. Biophys. Acta 767*, 169–167.
Zimmerman, J.-L., & Rutherford, A. W. (1986) *Biochemistry 25*, 4609–4615.

Basis for the Reduced Affinity of β_T- and γ_T-Thrombin for Hirudin

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ABSTRACT: Partial proteolysis of human α-thrombin by trypsin results in the formation of β_T-thrombin and γ_T-thrombin which have a reduced affinity for the inhibitor hirudin and the cell-surface cofactor thrombomodulin as well as reduced activity with fibrinogen. The basis of the reduction in affinity of these thrombin derivatives for hirudin has been investigated by examining their kinetics of interaction with a number of hirudin mutants differing in their C-terminal charge properties as well as with a truncated form of hirudin. The results indicate that the reduced affinity of β_T-thrombin for hirudin is most likely due to a decrease in the strength of nonionic interactions between thrombin and the C-terminal region of hirudin. No decrease in the strength of ionic interactions was observed with β_T-thrombin. In contrast, the reduced affinity of γ_T-thrombin was due to a decrease in the strength of both ionic and nonionic interactions. The N-terminal core region of hirudin, which interacts predominantly with the active-site cleft of thrombin, exhibited similar affinities for α-, β_T-, and γ_T-thrombin, indicating that thrombin–hirudin interactions within the active site are largely preserved in β_T- and γ_T-thrombin.

The serine protease thrombin plays a pivotal role in hemostasis. It interacts with a number of substrates, inhibitors, and receptors, and the specificity of these interactions is essential to the hemostatic process (Fenton, 1981). Thrombin's specificity is determined not only by its active site but also by secondary binding sites (Blombäck et al., 1972). The importance of these secondary binding sites to thrombin's interactions with particular ligands has been studied by using proteolytic derivatives of thrombin (Berliner, 1984). Two such derivatives that have proved useful in these studies are β- and γ-thrombin. Cleavage of human α-thrombin between Arg77A and Asn78[1] by trypsin produces β_T-thrombin (Braun et al., 1988b; Bezeaud & Guillin, 1988). Further cleavage by trypsin of the bonds Arg67–Ile68 and Lys149E–Gly150 leads to the formation of γ_T-thrombin (Bing et al., 1977; Fenton et al., 1977a; Braun et al., 1988b). Similar forms of thrombin also result from autolysis (Fenton et al., 1977a,b; Boissel et al., 1984; Chang, 1986). Studies using these derivatives together with peptide-specific antibodies have identified a region of human α-thrombin between Arg67 and Asn78 as important for the interaction of thrombin with the substrate fibrinogen, the inhibitor hirudin, and the cell-surface protein thrombomodulin (Bezeaud et al., 1985; Lewis et al., 1987; Stone et al., 1987; Bezeaud & Guillin, 1988; Hofsteenge et al., 1988; Noé et al., 1988). The region between residues 67 and 78 contains an unusually high proportion of basic residues and occurs in a surface loop in human α-thrombin (Bode et al., 1989). As it contains the cleavage site for the formation of β-thrombin, this loop is conveniently termed the β-loop. This loop is not present in γ_T-thrombin.

The results from several studies suggest that an ionic interaction occurs between the β-loop of thrombin and a region of hirudin comprising the C-terminal 15 amino acids (residues 51–65). Site-directed mutagenesis has demonstrated that the acidic nature of this region of hirudin is important for its interaction with thrombin (Braun et al., 1988a; Stone et al., 1989). Moreover, studies using fragments of hirudin have indicated that the C-terminal region of hirudin interacts with the β-loop of thrombin (Dodt et al., 1990; Dennis et al., 1990; Chang et al., 1990). Peptides corresponding to residues 48–65 and 53–65 of hirudin were able to protect thrombin from cleavage between Arg77A and Asn78 by trypsin. In contrast, truncated forms of hirudin corresponding to residues 1–47 and 1–52 did not protect against cleavage by trypsin (Dodt et al., 1990; Dennis et al., 1990). Hirudin and the C-terminal fragment of hirudin also protected Lys70 from modification by a lysine-specific reagent (Chang, 1989; Chang et al., 1990).

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[1] The sequence numbering of thrombin is that of Bode et al. (1989); "h-" preceding the three-letter code indicates a residue in hirudin.

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