Genetic Manipulation of the Cyanobacterium Synechocystis sp. PCC 6803

Development of Strains Lacking Photosystem I for the Analysis of Mutations in Photosystem II

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We have taken a genetic approach to eliminating the presence of photosystem I (PSI) in site-directed mutants of photosystem II (PSII) in the cyanobacterium Synechocystis sp. PCC 6803. By selecting under light-activated heterotrophic conditions, we have inactivated the psaA-psaB operon encoding the PSI reaction center proteins in cells containing deletions of the three psbA genes. We have also introduced deletions into both copies of psbD in a strain containing a mutation that inactivates psaA (ADK9). These strains, designated D1"/PSI- and D2"/PSI-, may serve as recipient strains for the incorporation of site-directed mutations in either psbA2 or psbD1. The characterization of these cells, which lack both PSI and PSII, is described.

The study of structure and function relationships in the PSII reaction center has been aided in recent years by the resolution of the three-dimensional structure of the bacterial reaction center (Allen et al., 1987; Deisenhofer and Michel, 1989). The conservation of overall structural and functional components has enabled accurate predictions of the corresponding architecture of PSII (Michel and Deisenhofer, 1988). However, the nonoxygen-evolving bacterial reaction center provides few clues about the organization of the OEC of PSII or the mechanism of water oxidation. In contrast, a wealth of biochemical, biophysical, and physiological data have been collected describing the enzymic and chemical properties of the four Mn atoms that form the active site of the OEC (Diner and Joliot, 1977; Ghanotakis and Yocum, 1990). This Mn cluster serves to accumulate oxidizing equivalents needed for the oxidation of water in a linear series of S-states driven by four single photon events (Kok et al., 1970; Forbush et al., 1971). By using molecular genetics, precise determination of the protein scaffold that coordinates this unique and crucial structure may be possible.

Structure and function studies on the photosystems has been accelerated by the use of the transformable unicellular cyanobacterium Synechocystis sp. PCC 6803 (Debus et al., 1988a; Vermaas et al., 1988b). This strain is naturally competent and has an active homologous recombination system, making it very amenable to molecular genetic manipulation (Williams, 1988). Synechocystis 6803 may also be grown photoheterotrophically, in the presence of DCMU and Glc (Williams, 1988). These conditions eliminate the selective advantage conferred by the presence of wild-type PSII and have allowed for the segregation of mutations in PSII reaction center genes (Debus et al., 1988a; Vermaas et al., 1988b; Metz et al., 1989; Nixon and Diner, 1992).

Synechocystis 6803 lacks a trait found in chloroplast thylakoid membranes that allows for easy purification of PSII, lateral heterogeneity of PSI and PSII (Anderson and Anderson, 1980). In chloroplasts, PSII is concentrated in the stacked membranes and PSI is confined to the unstacked regions (Anderson and Anderson, 1980). Current techniques for the purification of PSII from Synechocystis 6803 rely on selective detergent solubilization, Suc density centrifugation, and/or a series of chromatographic separations (Burnap et al., 1989; Rögner et al., 1990; Noren et al., 1991), techniques that can compromise OEC activity, especially in mutants (N. Bowlby, unpublished results). The present status of PSII purification has served well for certain types of analysis of PSII mutants (Debus et al., 1988a; Nixon and Diner, 1992), and detection of the EPR multiline spectrum from the S1 state of the Mn cluster has been reported (Noren et al., 1991). However, a method yielding highly purified and active PSII with a min-

1 Supported by National Science Foundation Postdoctoral Fellowship in Plant Biology DMB-9148737 (to N.R.B.), by Michigan State University Research Excellence Funds (to L.M.), and by Department of Energy grant DE-FG02-90-ER 20021 (to L.M.).
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Abbreviations: D1", D1 (PsbA) deficient; D2", D2 (PsbD) deficient; EPR, electron paramagnetic resonance; LAHG, light-activated heterotrophic growth; OEC, oxygen-evolving complex; PSI", PSI deficient; PSII", PSII deficient; WT-LAHG, LAHG-grown wild type; WT-MIXO, mixotrophically grown wild type.
imum of biochemical manipulation would be highly beneficial for the analysis of mutants in the OEC.

To facilitate such studies, we have embarked on a genetic approach to the elimination of PSI, a detrimental contaminant of PSI preparations, in Synechocystis 6803. Because there is no effective inhibitor of PSI activity, cells must be grown heterotrophically to eliminate the selective advantage conferred by wild-type PSI. Synechocystis 6803 may be grown in complete darkness using Glc as a carbon source, requiring only a brief (5 min) pulse of light every 24 h (Anderson and McIntosh, 1991). By selecting under these LAHG conditions (Anderson and McIntosh, 1991), we have successfully inactivated the genes encoding the reaction center proteins of PSI, psaA and psaB (Smart et al., 1991; Smart and McIntosh, 1993). These mutants were devoid of any detectable PSI complex but still accumulated functional PSII at wild-type levels (Smart et al., 1991; Smart and McIntosh, 1993).

Here we describe the development of recipient strains for the mutagenesis of the PSI reaction center genes psbA and psbD. These strains, D1'/PSI- and D2'/PSI-, have mutations in psaA that prevent assembly of PSI. To avoid problems associated with the segregation of a mixture of wild-type and mutant psbA or psbD sequences, the regions of the chromosome targeted for mutagenesis have been deleted. In Synechocystis 6803, psbA is present in three copies (Jansson et al., 1987; therefore, deletions were introduced into all three copies by replacing those regions of the chromosome with three different drug-resistance cassettes (I. Sithole, N.R. Bowby, J. Sinclair, G.T. Babcock, L. McIntosh, unpublished data). Likewise, psbD is present in two copies (Williams and Chisholm, 1987), and deletions were introduced into both of them. The rationale for attempting particular mutations in the psbA or psbD genes will be discussed elsewhere (I. Sithole, N.R. Bowby, J. Sinclair, G.T. Babcock, L. McIntosh, unpublished data). Characterization of the strains D1'/PSI- and D2'/PSI- also gives us insight into the biogenesis of PSI and aspects of cyanobacterial physiology.

MATERIALS AND METHODS

Materials

Chemicals and antibiotics used were of high purity and were obtained from Sigma or Research Organics (Cleveland, OH). Restriction and other enzymes were purchased from New England Biolabs (Beverly, MA). Radioactive isotope ([32P]dATP) was purchased from Amersham, nitrocellulose membrane from Schleicher & Schuell, and Bacto-Agar from Difco (Detroit, MI).

Strains and Growth Conditions

All studies were performed using a Glc-tolerant strain of the cyanobacterium Synechocystis sp. PCC 6803 (Williams, 1988). The mutant ADK9, an insertional inactivation mutant of psaA, was previously described (Smart et al., 1991). The mutant 3apsbA, with all three copies of psbA deleted, will be described elsewhere (I. Sithole, N.R. Bowby, J. Sinclair, G.T. Babcock, L. McIntosh, unpublished data). Cells were grown in liquid BG-11 medium with 5 mM Glc or on solid BG-11 with 5 mM Glc and 1.5% purified Bacto-Agar under LAHG conditions, as previously described (Anderson and McIntosh, 1991). Transformation of Synechocystis 6803 was performed essentially as described (Williams, 1988). For analysis of mutants, cells were grown in carboys containing 15 L of medium, were harvested using a continuous flow rotor (DuPont Sorvall, Wilmington, DE), and were frozen at -70°C in BG-11 with 15% (v/v) glycerol. When appropriate, media were supplemented with antibiotics in the following concentrations: chloramphenicol, 10 mg/L; erythromycin, 5 mg/L; spectinomycin, 20 mg/L; kanamycin, 5 mg/L; and gentamycin, 1 mg/L.

Nucleic Acids

All nucleic acid manipulations were performed using standard procedures (Sambrook et al., 1989) unless otherwise stated. DNA was isolated from Synechocystis 6803 using a modification of the procedure described by Golden et al. (1987) for the isolation of RNA from Synechocystis 6803. The aqueous phase obtained after vortexing the cells with glass beads, buffer, phenol-chloroform, and detergents was extracted twice with chloroform, then purified over a cesium chloride gradient with ethidium bromide by centrifugation in a Beckman VTi50 rotor at 45,000 rpm for 18 h. The ethidium-bromide-stained DNA band was recovered, extracted with isopropanol to remove ethidium bromide, and precipitated using ethanol. Hybridization conditions and preparation of radiolabeled probes were previously described (Smart and McIntosh, 1991). The probe for psbD2 was a 0.5-1 kb KpnI/SmaI fragment from plasmid pRD655 (Debus et al., 1988a). The psbD1 probe was a 3.5-kb HindIII fragment from plasmid pRD1219 (Debus et al., 1988a). The psaA probe was a 1.8-kb KpnI fragment from plasmid pLS18 (Smart and McIntosh, 1991).

Chl and Protein Analysis

Chl was extracted from whole cells or from membranes using methanol and was quantified using the extinction coefficients of Lichtenthaler (1987). Cells were broken using a bead beater (Biospec Products, Bartlesville, OK), and membranes were prepared essentially as previously described (Smart et al., 1991). Membrane proteins were prepared for electrophoresis as described by Wynn et al. (1989) and were separated by 10% SDS-PAGE using the buffers of Laemmli (1970). Proteins were transferred to nitrocellulose and immunoblotting was performed as described by Towbin et al. (1979) and Smart et al. (1991), respectively. Antibodies raised to the PsaA/B proteins from Synechococcus have been described (Henry et al., 1990), as have antibodies raised to a portion of the D1 protein from Amaranthus hybridus (Ohad et al., 1985). Antibodies raised to the D2 polypeptide from spinach were previously described (Vermaas et al., 1988a).

EPR Spectroscopy

Room temperature EPR spectroscopy was performed using a Bruker ER200D spectrometer as previously described (Smart et al., 1991). Spectrometer conditions were: power, 20 mW; modulation amplitude, 0.4 mT; time constant, 200 ms; and...
RESULTS

Plasmid Constructs and Transformations

We used different strategies to generate the two recipient strains for mutagenesis of \( \text{psbA} \) and \( \text{psbD} \). In the case of the D2 recipient, we started with a PSI\(^{-} \) mutant, ADK9 (Smart et al., 1991), and sequentially introduced deletions into both copies of \( \text{psbD} \). To generate the D1 recipient, we inactivated the \( \text{psaA} \) gene in a strain, 3\( \Delta \text{psbA} \), that had deletions in all three copies of \( \text{psbA} \) (I. Sithole, N.R. Bowbyl, J. Sinclair, G.T. Babcock, L. McIntosh, unpublished data).

The first step in constructing the D2 recipient was the deletion of the \( \text{psbD}2 \) gene. The strain ADK9 was transformed with the plasmid pRD655Cm\(^{R} \) (Debus et al., 1988a), in which most of the \( \text{psbD}2 \) gene had been replaced by a gene encoding resistance to chloramphenicol. Colonies resistant to kanamycin and chloramphenicol were selected and streak purified from single colonies to accomplish gene segregation. DNA was isolated from one of these colonies, ADK9\( \Delta \text{psbD}2 \), and was subjected to Southern analysis using a probe recognizing the 3' flanking region of the \( \text{psbD}2 \) gene that does not recognize \( \text{psbD}1 \) (Fig. 1A). Because these cells were found to be fully segregated for the deletion of \( \text{psbD}2 \) (Fig. 1A), they were then transformed with the plasmid pSLA1219:Gm\(^{R} \) (Fig. 2A). This plasmid was a modification of pRD1219 (Debus et al., 1988a). In pSLA1219:Gm\(^{R} \) most of the \( \text{psbD}1 \) gene had been replaced by a 2.0-kb \( \text{BamH}1 \) fragment, originally purified from the plasmid pRZ1107, which contains a gene encoding resistance to gentamycin (Yin et al., 1988). The Gm\(^{R} \)

gene was also subcloned into the \( \text{BamH}1 \) site of pUC119 (Vieira and Messing, 1987), yielding pUC119-gen. Colonies resistant to chloramphenicol, kanamycin, and gentamycin were selected and streak purified from single colonies to allow for segregation. DNA was isolated from one of these colonies, and Southern analysis using a probe for \( \text{psbD}1 \) revealed complete segregation of the deletion (Fig. 1B). In addition to wild-type bands (3.8, 1.6, and 0.4 kb), the \( \text{psbD}1 \) probe cross-hybridized to higher molecular mass bands representing \( \text{psbD}2 \) (Fig. 1B, lane 3). None of the wild-type \( \text{psbD}1 \) or \( \text{psbD}2 \) bands were detectable in the mutant strain, which was designated D2\(^{-}/\)PSI\(^{-} \) (Fig. 1B, lane 4).

The strain \( 3\Delta \text{psbA} \) was generated by targeted deletion of \( \text{psbA}2 \) in a strain with \( \text{psbA1} \) and \( \text{psbA3} \) deleted (Debus et al., 1988b), resulting in a strain with genes encoding resistance to chloramphenicol, spectinomycin, and erythromycin in the chromosome (I. Sithole, N.R. Bowbyl, J. Sinclair, G.T. Babcock, L. McIntosh, unpublished data). To inactivate PSI genetically in this strain, we constructed a plasmid, pLS1813G, with a portion of the \( \text{psaA} \) gene replaced by a \( \text{BamH}1-\text{KpnI} \) fragment from the plasmid pUC119-gen encoding resistance to gentamycin (Fig. 2B). The strain \( 3\Delta \text{psbA} \) was transformed with pLS1813G, and colonies resistant to chloramphenicol, erythromycin, spectinomycin, and gentamycin were selected and allowed to segregate by streak purification from single colonies. DNA was isolated from one of the resistant colonies and was subjected to Southern analysis to verify complete segregation of the \( \text{psaA} \) mutation (Fig. 1C). This strain was designated D1\(^{-}/\)PSI\(^{-} \).

Figure 1. Autoradiographs of Southern blots of genomic DNA from
wild-type and mutant cells. Lanes are: 1, 3, 5, wild type; 2, ADK9\( \Delta \text{psbD}2 \); 4, D2\(^{-}/\)PSI\(^{-} \); 6, D1\(^{-}/\)PSI\(^{-} \); 5 \( \mu \)g DNA/lane. A, \text{psbD}2 probe, DNA digested with \( \text{HindIII} \); B, \text{psbD}1 probe, DNA digested with \( \text{BstEII} \); C, \text{psaA} probe, DNA digested with \( \text{KpnI} \). Autoradiographs were exposed for different lengths of time.

Figure 2. Partial restriction maps of plasmids used for mutagenesis
and of portions of the \( \text{Synechocystis} \) 6803 chromosome. A, Map of the \( \text{psbD}1-\text{psbC} \) region and of the insert region from the plasmid pSLA1219:Gm\(^{R} \). B, Map of the \( \text{psaA}-\text{psbA} \) operon and of the insert region from the plasmid pLS1813G. Thick lines represent regions of the \( \text{Synechocystis} \) 6803 chromosome. Hatched boxes represent coding regions. The cross-hatched boxes represent the gentamycin resistance gene cassette. Crosses depict possible sites of homologous recombination. Restriction sites in parentheses were destroyed in the cloning process. Restriction sites are represented by: H, \( \text{HindIII} \); T, \( \text{BstEII} \); E, \( \text{EcoRI} \); B, \( \text{BamH}1 \); N, \( \text{Ncol} \); K, \( \text{KpnI} \).
### Table I. Chl determinations of whole cells and thylakoid membranes

<table>
<thead>
<tr>
<th></th>
<th>WT-MIXO*</th>
<th>WT-LAHG*</th>
<th>D1*/PSI*</th>
<th>D2*/PSI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells (µg Chl mL⁻¹ A₇₅₅⁻¹)</td>
<td>3.66 ± 0.63</td>
<td>0.81 ± 0.31</td>
<td>0.31 ± 0.06</td>
<td>0.15 ± N.D.</td>
</tr>
<tr>
<td>Membranes (µg Chl mg⁻¹ protein)</td>
<td>43.1 ± 7.2</td>
<td>9.9 ± 0.4</td>
<td>5.5 ± 0.1</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
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** Characterization of D1*/PSI* and D2*/PSI**

Analysis of the strains D1*/PSI* and D2*/PSI* included Chl determination, immunoblotting, and EPR spectroscopy. Chl determinations performed on whole cells revealed very low levels of Chl per cell in strain D2*/PSI*, relative to wild type, grown either mixotrophically (in the light with Glc) or under LAHG conditions (Table I). The Chl levels in strain D1*/PSI* were also lower than in WT-MIXO or WT-LAHG (Table I) but greater than in D2*/PSI*. The ratio of Chl to protein in isolated membranes followed the same trend (Table I). Both strains, D1*/PSI* and D2*/PSI*, grew at near wild-type rates under LAHG conditions but did not grow in continuous light above approximately 3 µmol m⁻² s⁻¹ (data not shown). Also, both cell types exhibited the turquoise-blue color seen previously in cells lacking PSI (Smart et al., 1991; Smart and McIntosh, 1993).

To determine if any PSI or PSII reaction center proteins were accumulating in the recipient strains, membrane proteins were subjected to immunoblotting. Antibodies raised to the PsaA/B polypeptides did not detect the PSI reaction center proteins in membranes from either D1*/PSI* or D2*/PSI* (Fig. 3A). Likewise, antibodies raised against D1 did not detect that protein in membranes from either of the two recipient strains but did display apparent nonspecific cross-reaction to a polypeptide of approximately 33 kD (Fig. 3B). However, when antibodies raised to the D2 polypeptide were used, a reduced amount of D2 protein was detected in membranes from the strain D1*/PSI* (Fig. 3C). No D2 was detected in membranes from the strain D2*/PSI* (Fig. 3C).

EPR spectroscopy is a sensitive and quantitative method for detecting redox components in the two photosystems. We collected room-temperature EPR spectra from membranes of both recipient strains, conditions under which one would expect to detect both signal I (from P₇₀₀⁺ in PSI) and signal II (from Tyr Y₀⁺ in PSII). The spectra from both recipient strains include a prominent feature centered at g = 2.00425 with a linewidth (1 mT) and a lineshape (Fig. 4) that clearly indicate that it does not arise from Tyr Y₀⁺ (g = 2.0045, linewidth ≈ 2 mT) (Barry and Babcock, 1987). This feature is seen under illumination and in the dark and is of approximately equal intensity in samples from the two strains, but is much less intense than one would expect from signal II in membranes of WT-LAHG (Fig. 4). There are no light-induced signals in the spectra from either recipient strain (Fig. 4).

### DISCUSSION

The purification of active PSII is essential for the analysis of site-directed mutations targeted at the OEC. The process of purification of PSII from *Synechocystis* 6803 is complicated by the lack of lateral segregation of PSI from PSII that is found in chloroplasts (Andersson and Anderson, 1980). We have taken a genetic approach to eliminate PSI from mutants with site-directed changes in PSII by generating recipient strains in which PSI has been genetically inactivated and in which deletions have been introduced into the targeted PSII reaction center genes. By deleting the regions targeted for mutagenesis, problems associated with segregating a mixture of wild-type and mutant sequences are avoided. These strains (D1*/PSI* and D2*/PSI*) may be transformed with a plasmid
containing DNA that overlaps the deletion and includes the desired mutation, with an adjoining drug-resistance gene for selection. The mutation will be readily integrated into the genome by homologous recombination (Williams, 1988). This is the first published use of the gentamycin-resistance gene (Yin et al., 1988) for selection of mutations in a cyanobacterium. This gene adds another option for manipulation of the cyanobacterial chromosome.

We have shown previously that cells with mutations that inactivate the PSI reaction center genes exhibit normal assembly of stable PSII complex (Smart et al., 1991; Smart and McIntosh, 1993). However, these strains are extremely light-sensitive, and, therefore, must be grown in very dim light (approximately 3 μmol m⁻² s⁻¹) or under LAHG conditions. The genetic inactivation of PSII does not appear to have relieved that light sensitivity, suggesting that there is some other light-induced component creating toxic elements or that respiration cannot synthesize ATP in light above approximately 3 μmol m⁻² s⁻¹. Our previous analysis of isolated membranes from strains ADK9 or BDK8 yielded EPR spectra with PSII signals of wild-type intensity and essentially free of other contaminating signals (Smart et al., 1991; Smart and McIntosh, 1993). Thus, we are quite confident that the inactivation of PSII does not significantly alter the stable assembly of PSII in Synechocystis 6803.

The strains D1⁻/PSI⁻ and D2⁻/PSI⁻ give us some insight into the biogenesis of PSII. Genetic inactivation of the two copies of psbD, which encode the D2 polypeptide, also prevented accumulation of the D1 polypeptide. However, targeted deletion of the three copies of psbA, which encode D1, caused only partial reduction of the accumulation of D2. The D2 protein that did accumulate did not assemble into functional reaction centers, since we detected no signal II in the EPR spectra of D2⁻/PSI⁻ membranes and no oxygen-evolving activity in the presence of 2,6-dichloro-p-benzoquinone (data not shown), an artificial electron acceptor from PSI. Our data suggest that a lack of PSI assembly does not alter the pattern of PSII biogenesis that has been observed in prior mutagenesis experiments using strains containing PSI. When both copies of psbD were inactivated, the D1 polypeptide failed to accumulate (Vermaas et al., 1988; Yu and Vermaas, 1990). In addition, this strain failed to accumulate some of the other PSII proteins (Vermaas et al., 1988a). In a mutant with the three copies of psbA inactivated, CP43, Cyt b₅₅₃, and the 33-kD protein accumulated to levels slightly lower than wild type, and the accumulation of D2 and CP47 was greatly reduced (Jansson et al., 1987; Nilsson et al., 1990). Our results reaffirm previous observations that both D1 and D2 are crucial for stable assembly and accumulation of PSII complex.

Assays of Chl accumulation per cell serve well in estimating the accumulation of PSI or PSII reaction center proteins (Smart et al., 1991; Smart and McIntosh, 1993) and may be used for initial characterization of mutants. This would be expected, since cyanobacteria do not contain peripheral Chl-binding antennae proteins. The Chl content in the D1⁻/PSI⁻ cells is reduced relative to ADK9 or BDK8, cells lacking only PSI (Smart et al., 1991; Smart and McIntosh, 1993). The Chl content of D2⁻/PSI⁻ is about half that of D1⁻/PSI⁻. The higher Chl levels in D1⁻/PSI⁻ may reflect the accumulation of low levels of D2 and possibly higher accumulation of CP43 and/or CP47. We did not examine the accumulation of CP43 or CP47 in these strains. However, the Chl levels in both D1⁻/PSI⁻ and D2⁻/PSI⁻ seem higher than one might expect for cells that lack both PSI and PSII. This may represent free Chl that is trapped in the membranes or Chl in some stage of synthesis or degradation, which may or may not be bound to a photosynthetic protein.

The EPR spectra from D1⁻/PSI⁻ and D2⁻/PSI⁻ contained neither signal I, from P₆₈₀⁺, nor signal II, from Tyr YD⁺ in D2. The prominent feature, common to spectra from both strains, was not light induced. One possible explanation is that this feature may have arisen from a semiquinone in the Cyt b₅₅₃/f complex (G.T. Babcock, personal communication).

The strains D1⁻/PSI⁻ and D2⁻/PSI⁻ represent the first mutants in a photosynthetic organism with both photosystems genetically inactivated. In addition to serving in the process of site-directed mutation of PSII genes, these strains may be particularly well suited for other studies. In cyanobacteria, the Cyt b₅₅₃/f chain is shared between photosynthesis and respiration (Scherer, 1990). The coordinate regulation of electron flow between these pathways is poorly understood. These strains may be appropriate for the study of respiration in cyanobacteria, since the interaction with the photosystems has been eliminated. The membranes from D2⁻/PSI⁻ are essentially free of photosynthetic proteins, so they may serve as excellent starting material for the purification of the Cyt b₅₅₃/f complex.

ACKNOWLEDGMENTS

We wish to thank G.T. Babcock (Department of Chemistry, Michigan State University) for the use of his spectroscopy facilities and for helpful discussions. The PsaA/B antibodies were the kind gift of J. Guikema (Kansas State University, Manhattan, KS). The D2 antibodies were the kind gift of W. Vermaas (Arizona State University, Tempe, AZ). The plasmid pRZ1107, containing the gentamycin-resistance gene, was the kind gift of C.P. Wolk (M.S.U.-D.O.E. Plant Research Laboratory, Michigan State University.)

Received September 2, 1993; accepted September 27, 1993.

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