Carboxylterminal deletion mutants of ribulosebisphosphate carboxylase from *Rhodospirillum rubrum*

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The carboxylterminal octapeptide of ribulosebisphosphate carboxylase from *Rhodospirillum rubrum*, which lacks small subunits, shows homology to a highly conserved region near the amino terminus of the small subunits of hexadecameric ribulosebisphosphate carboxylases, which are composed of large and small subunits. Truncations of the *R. rubrum* enzyme, which partially or completely deleted the region of homology, demonstrated that the region is not an important determinant of the catalytic efficiency of the enzyme. A further truncation, which replaced the carboxylterminal 19 amino acid residues with a single terminal leucyl residue, yielded a Rubisco whose substrate-saturated catalytic rate resembled that of the wild-type enzyme but which had weaker affinities for ribulose-P_2 and CO_2.

Ribulose bisphosphate carboxylase/oxygenase; Rubisco; Photosynthesis; Site-directed mutagenesis; *Rhodospirillum rubrum*

1. INTRODUCTION

Rubisco (EC 7.1.1.39) plays a central role in the carbon metabolism of photosynthetic organisms, catalysing both the carboxylation and oxygenation of ribulose-P_2, the initial reactions of photosynthetic CO_2 fixation and photorespiration, respectively.

Rubiscos have complex quaternary structures (for a review, see Andrews and Lorimer [1]). In many bacteria, and all cyanobacteria, algae and higher plants, the enzyme is composed of eight large (50–55 kDa) subunits and 8 small (12–15 kDa) subunits (the hexadecameric, L_8S_8 structure). In some purple, non-sulphur bacteria, Rubiscos are found that are composed of large subunits alone (L_n structures). The simplest quarternary structure known is the large subunit dimer (L_2) from *Rhodospirillum rubrum* [2].

The amino acid sequences of the large subunits of hexadecameric higher plant, algal and cyanobacterial enzymes are highly conserved (>75% identity). The L_2 Rubisco from *R. rubrum* shows lower homology (approximately 25%) to the large subunits of the L_8S_8 enzymes, concentrated in the vicinity of the active site. Comparison of the tertiary structures of the L_8S_8 and L_2 Rubiscos shows that, despite sequence divergence and quarternary structure differences, there is strong similarity in the overall conformations of the large subunits, and in large subunit dimer interactions, between the L_8S_8 and L_2 enzymes [3–5]. The sequences of the small subunits are less highly conserved between species. However, three highly conserved regions have been identified [1,6].

The role(s) of the small subunits of hexadecameric Rubiscos are not well understood. Structural studies indicate that small subunit residues do not directly form part of the active site [5,7]. The L_8 core of the cyanobacterial Rubisco carries out catalysis, albeit with a k_{cat} of 1% of that of the holoenzyme [8], demonstrating that small subunits are not obligatory for catalysis.

In comparing the L_n and L_8S_8 Rubisco types, an important question is whether the L_n enzymes incorporate functions of the small subunit within the L_n structure. Andrews and Lorimer [1] proposed that a region of homology between a highly conserved region near the amino terminus of the small subunits and a heptapeptide one residue removed from the carboxyl terminus of the L_2 Rubisco from *R. rubrum* (Fig. 1) may be relevant to this question. The consensus sequence is E(D)TXSXLP, where the initial residue may be either of the acidic residues and X represents any residue.

A search of all sequences in Release 16.0 of the NBRF protein sequence database revealed no non-Rubisco matches for the sequence ETXSXLP and one non-Rubisco match for the sequence DTXSXLP. For a continuous random amino acid sequence of comparable length (1.4 million residues), the expected frequency of matches to either sequence is 0.44.

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Abbreviations: ribulose-P_2, D-ribulose-1,5-bisphosphate; carboxyarabinitol-P_2, 2′-carboxy-D-arabinitol-1,5-bisphosphate; carboxypentitol-P_2, unresolved isomeric mixture of carboxyarabinitol-P_2 and 2′-carboxy-D-ribitol-1,5-bisphosphate; Epps, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Therefore, we conclude that neither sequence is over-represented in the database, as might be expected if it produced a generally useful piece of secondary structure found in many proteins. The low probability of the sequence DTXSXLP occurring in *R. rubrum* Rubisco by chance (1.4×10^{-4}), and its carboxylterminal location, heighten suspicion that this homology is present by virtue of fusion of ancestral large and small subunit genes (which are found consecutively on the genomes of other prokaryotes) with subsequent reduction. In this report we have examined the functional significance of this homology by making carboxylterminal truncations of the *R. rubrum* enzyme.

2. MATERIALS AND METHODS

2.1. *Materials*

Escherichia coli strain JM83 [9] was the host strain in all expression studies. Ribulose-P_2 and [carboxyl-^{14}C]carboxypentitol-P_2 were prepared as described [8]. Oligonucleotides were synthesized on a Beckman System 1 DNA synthesizer. Dideoxy DNA sequencing reagents were obtained from the United States Biochemical Corporation. [α-^{35}S]dATP was purchased from Amersham. Reagents for in vitro mutagenesis were obtained from Biorad Laboratories.

2.2. *Plasmids*

Plasmid pRR1 was constructed by J. Pierce and M. Madden (Central Research and Development Department, E.I. du Pont de Nemours and Company, Wilmington, DE, USA). pRR1 contains the structural gene for *R. rubrum* Rubisco from pRR2119 [10] inserted into the multiple cloning site of pUC118 and engineered by deletion of a 5′ sequence so that the initial ATG codon of the Rubisco gene replaced the translation-initiating ATG codon of the *lacZ* α-peptide. Thus this plasmid expresses authentic *R. rubrum* Rubisco, without any additional residues fused to the amino terminus.

2.3. *Growth and extraction of* E. coli *cultures*

E. coli cells were grown and soluble extracts prepared as described previously [8]. For the electrophoretic experiment (Fig. 2), the insoluble fraction was washed once with extraction buffer and resuspended in a volume of extraction buffer equivalent to the volume of the soluble fraction.

2.4. *Construction of truncated Rubiscos*

Oligonucleotide-directed mutagenesis according to Kunkel [11] was used to construct mutant Rubiscos Δ6 and Δ9 (Fig. 1). A 325 bp

Species	Sequence
Small subunit	<---α1----
Spinacia oleracea [18]	M Q V W P P L G L K K F E T L S Y L P P L T T E Q... 1
Chlamydomonas reinhardii [19]	M M V W T P V N N K M F E T F S Y L P P L S D E Q... 1
Synechococcus PCC 6301 [20]	M S M K T L P K E R R F E T F S Y L P P L S D R Q... 1
Chromatium vinosum [21]	Y S S L E D V N S R K F E T F S Y L P A M D A D R... 7
Large (and only) subunit	--αI--> <--αJ--->
Rhodospirillum rubrum [22]	I Y P G W R K A L G V E D T R S A L P A * 447
Δ6	I Y P G W R K A L G V E D T * 447
Δ9	I Y P G W R K A L G V * 447
Δ18	I L * 447
Rhodobacter sphaeroides [15]	F Y P G W R D R L H R A A * 447

Fig. 1. Comparison of a conserved sequence near the amino terminus of the small subunits of Rubiscos from a higher plant, a green alga, a cyanobacterium and a photosynthetic bacterium with the carboxylterminal sequences of mutant and wild-type *R. rubrum* Rubiscos and the Rubisco of *Rh. sphaeroides*. The numbers below the first residue of the sequences indicates the position of that residue in the mature protein. The conserved sequence is underlined. Secondary structure elements revealed by crystallographic studies [5,14] are indicated above the sequences. Stop codons are represented by asterisks. References are given below the species names.

*Sph*I/*Hin*dIII fragment of pRR1 encompassing the Rubisco carboxyl terminus was cloned into the multiple cloning site of M13mp18. Second strand synthesis was primed by the oligonucleotides 5′ AGGGCGCTGCGTTAGGTGTCCTCGA 3′ (Δ6) and 5′ TGCGGGTGTCCTAGACGCCCAGGGCCTT 3′ (Δ9) (the altered anticodons are underlined). The mutated fragments were cloned into *Sph*I/*Hin*dIII digested pRRI to construct the altered Rubisco genes. Mutant Δ18 was created by ligating the 34 bp *Bam*HI/*Hin*dIII fragment from pUC18 into *Bgl*II/*Hin*dIII digested pRR1, introducing a TAG termination codon 18 codons upstream from the wild-type carboxyl terminus and altering amino acid residue 448, the new carboxyl terminus, from Tyr to Leu (Fig. 1). Dideoxy DNA sequencing confirmed that only the expected sequence changes had been introduced.

2.5. *Carboxylase assay*

Septum-capped scintillation vials were used to carry out a $^{14}CO_2$ fixation procedure at 25°C. The standard assay solution contained 0.1 mmol of Epps-NaOH buffer, pH 7.8, 20 μmol of $MgCl_2$, 25 μmol of $NaH^{14}CO_3$ (10.2 Bq·nmol^{-1}), 0.1 mg of bovine carbonic anhydrase and extract in a final volume of 1 ml. After preincubation for 5 min at 25°C, catalysis was initiated by the addition of 1 μmol of ribulose-P_2. After 5 min, the reaction was terminated by the addition of 0.5 ml of concentrated formic acid. The mixtures were dried, the residues dissolved in 1 ml of water, and acid stable counts were determined by scintillation counting. To determine K_m(ribulose-P_2), the amount of ribulose-P_2 added was varied at a constant 25 mM $NaHCO_3$. The time courses of the assays were always linear for at least 5 min, even at the lowest ribulose-P_2 concentration. When determining $K_m(CO_2)$, extract was preincubated for 30 min in the presence of 40 mM $MgCl_2$ and 30 mM $NaHCO_3$. Reactions were initiated by the addition of preincubated extract to complete reaction mixtures containing varied concentrations of $NaH^{14}CO_3$ and 1 mM ribulose-P_2. The concentrations of CO_2 present in reaction mixtures were calculated using a value of 6.12 for the pK' of the CO_2/HCO_3^- equilibrium and assuming equilibration of CO_2 between the solution and its headspace. Kinetic parameters were calculated by fitting data to the Michaelis-Menten equation.

2.6. *Molecular weight determinations*

A Pharmacia Superose 6 column was equilibrated with 100 mM Epps-NaOH buffer, pH 7.8, containing 5 mM $MgCl_2$ and 1 mM EDTA. The column was calibrated by determining the elution profiles of the following proteins: carbonic anhydrase (29 kDa), bovine serum albumin (67 kDa), *R. rubrum* Rubisco (101 kDa) and spinach Rubisco (550 kDa). The elution of mutant *R. rubrum* Rubiscos was detected by enzyme assay.

2.7. *Binding of [^{14}C]carboxypentitol-P_2*

Reaction mixtures contained 0.1 ml of the supernatant of an extract of *E. coli* JM83 containing truncated or wild-type Rubisco, 25 μmol of Epps-NaOH buffer (pH 7.8), 10 μmol of $MgCl_2$ and 25 μmol of $NaHCO_3$ in a final volume of 0.5 ml. After 20 min at 23°C, 3 μl of 1.6 mM [^{14}C]carboxypentitol-P_2 (2035 Bq·nmol^{-1}) was added. After a further 30 min, the mixture was applied to a 1 × 27 cm column of Sephadex G-50 fine which had been equilibrated with 50 mM Epps-NaOH buffer, pH 7.8, containing 20 mM $MgCl_2$, 50 mM $NaHCO_3$ and 1 mM EDTA. The column was eluted with a flow rate of 0.5 ml·min^{-1}, fractions of 0.5 ml were collected, and ^{14}C determined by scintillation counting.

3. RESULTS

3.1. *Expression of mutant Rubiscos in* E. coli

Stop codons were introduced within the *R. rubrum* Rubisco gene either 6 (gene product Δ6), 9 (gene product Δ9) or 18 (gene product Δ18) codons 5′ to the wild-type termination codon. In *E. coli* strain JM83,

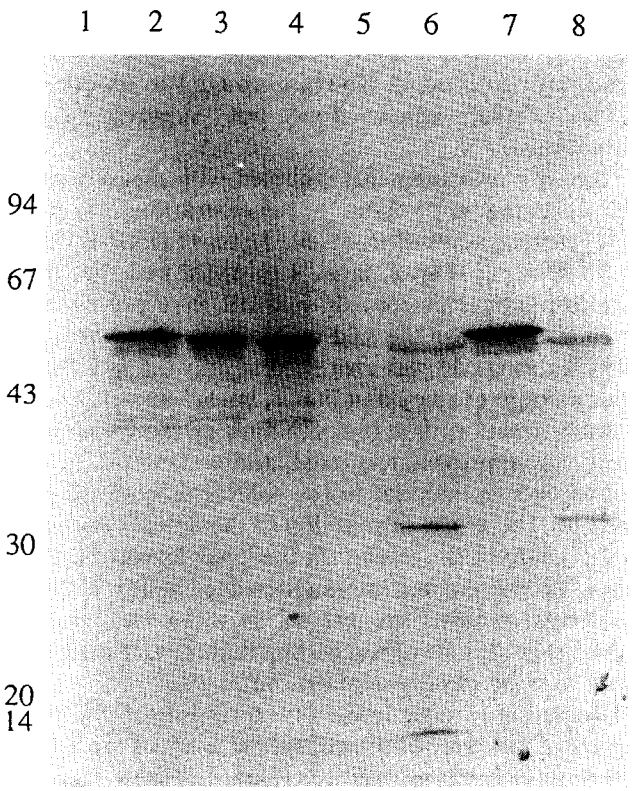

Fig. 2. Immunoblots of an SDS-PAGE gel of extracts of *E. coli* expressing carboxylterminal truncations of *R. rubrum* Rubisco. Crude soluble supernatants were prepared as described [8]. Samples were dissociated in SDS and fractionated according to Laemmli [17] using a 1.5 mm thick 10% acrylamide gel. The samples were: 1, JM83 extract containing 2 μg protein; 2, JM83 (pRR1) extract containing 2 μg protein; 3, JM83 (Δ6) extract containing 2 μg protein; 4, JM83 (Δ9) extract containing 2 μg protein; 5, JM83 (Δ18) extract (soluble fraction) containing 2 μg protein; 6, JM83 (Δ18) extract (soluble fraction) containing 200 μg protein; 7, JM83 (pRR1) extract containing 2 μg protein; 8, a volume of the resuspended particulate fraction of a JM83 (Δ18) extract equal to the volume of soluble extract loaded in lane 6. Proteins were electroblotted onto nitrocellulose. The membrane was blocked with bovine serum albumin, incubated with anti-*R. rubrum* Rubisco antiserum (raised in rabbit and pretreated with *E. coli* extract to suppress non-specific immune reactions) and the bands were visualized using goat-anti-rabbit gold conjugate and silver enhancement reagents obtained from Biorad Laboratories. The mobilities of protein molecular weight standards (obtained from Pharmacia LKB Biotechnology) are indicated adjacent to lane 1.

these truncated genes directed the synthesis of truncated Rubiscos and this is shown by their greater mobilities in SDS-PAGE (Fig. 2). Although the increases in mobility were slight, they were highly reproducible. In JM101, a strain with an amber suppressor mutation, a slight degree of readthrough of the amber termination codon introduced into Rubisco gene Δ9 was observed. This gave rise to an additional faint band on SDS-PAGE immunoblots which was slightly less mobile than the predominant band representing termination at the introduced amber codon (data not shown). For Rubisco gene Δ9, readthrough would pro-

duce a product 9 residues longer than the desired product with termination occurring at the original, wild-type stop codon. No readthrough was observed in strain JM83, which does not suppress amber mutations.

Activity measurements, coupled with measurements of catalytic site concentrations using [^{14}C]carboxypentitol-P_2 (Table I), and SDS-PAGE immunoblotting (Fig. 2, lanes 2 to 4) showed that truncated mutant Rubiscos Δ6 and Δ9 were expressed at greater levels than the wild-type enzyme. However, mutant Rubisco Δ18 was expressed at only 0.5 to 1.0% of that amount (Table I and Fig. 2, lanes 5 and 6). The insoluble fraction of Δ18-containing extracts contained only an approximately comparable amount of immunoreactive material to the soluble fraction (Fig. 2, lane 8). Therefore, insolubility of the Δ18 product does not seem to be the reason for the lower levels of expression. Two prominent immunoreactive bands with lower molecular weights are present in Δ18-containing extracts (Fig. 2, lane 6), suggesting that the Δ18 gene product may be rapidly degraded in *E. coli*.

Remarkably high levels of expression of the wild-type Rubisco were observed and the levels of the Δ6 and Δ9 mutant Rubiscos were even higher (Table I). More detailed studies of expression levels during the growth of the cultures would be required to establish whether the higher expression level of the two mutant enzymes was significant.

3.2. *Kinetic properties of mutant Rubiscos*

Mutant enzymes Δ6 and Δ9, which lack part or all of the homologous region, have kinetic properties which are not significantly different from the wild-type enzyme (Table I). Rubisco Δ18 also had a turnover number (k_{cat}) similar to the wild-type enzyme. However, its affinity for CO_2 was twofold weaker and its affinity for ribulose-P_2 was 10-fold weaker. Therefore, the catalytic specificity of this mutant enzyme for CO_2 ($k_{cat}/K_m(CO_2)$) was proportionately poorer (Table I). Since the assay timecourse remained linear, even at the lowest ribulose-P_2 concentration, the lower affinity for ribulose-P_2 of Rubisco Δ18 cannot have been caused artifactually by consumption of the substrate by phosphatases in the extract.

Table I

Kinetic properties and levels of wild-type and truncated *R. rubrum* Rubiscos synthesized in *E. coli* JM83

Enzyme	$K_m(CO_2)$ (μM)	K_m(ribulose-P_2) (μM)	k_{cat}[a] (s^{-1})	$k_{cat}/K_m(CO_2)$ ($M^{-1}\cdot s^{-1}$)	Expression (%)[b]
Wild-type	67 ± 9	20 ± 3	7.3 ± 0.3	1.1×10^5	40
Δ6	51 ± 8	26 ± 6	6.0 ± 0.4	1.2×10^5	66
Δ9	49 ± 4	29 ± 6	5.9 ± 0.2	1.2×10^5	63
Δ18	110 ± 18	225 ± 26	5.4 ± 0.4	4.8×10^4	0.33

[a] Mol of CO_2 fixed per mol of active site under substrate-saturating conditions. Active site concentrations were determined by the binding of [^{14}C]carboxyarabinitol-P_2 as described in section 2.7

[b] Percent of total protein in aqueous extract. Rubisco protein was calculated from the active site concentration

3.3. *Binding of reaction-intermediate analogue*

Carboxyarabinitol-P_2 binds so tightly to the active site of carbamylated *R. rubrum* Rubisco that the complex may be isolated quantitatively by gel filtration [12]. None of the truncated mutant enzymes was obviously impaired in its ability to bind the analogue. The complex formed by each truncated Rubisco with [^{14}C]carboxypentitol-P_2 was readily isolated by gel filtration and the radioactivity returned to background levels between the high-MW (bound analogue) and low-MW (unbound analogue) peaks, indicating that the bound analogue did not dissociate significantly during gel filtration. However, carboxyarabinitol-P_2 binds so tightly that it is possible that a 10-fold reduction in its affinity (parallelling the reduced affinity of the Δ18 Rubisco for ribulose-P_2) would not be revealed by this gel-filtration procedure.

3.4. *Quaternary structure*

The molecular sizes of the mutant enzymes were determined using a calibrated gel-filtration column (data not shown). All three mutant enzymes co-eluted with the wild-type enzyme at a volume expected for the approximately 100-kDa dimer. Therefore, none of the truncations prevented the intersubunit interactions necessary to form the dimer.

4. DISCUSSION

The crystallographically determined structure of *R. rubrum* Rubisco shows that the carboxylterminal sequences deleted in this study are located at the solvent surface of the molecule and are quite distant from the active site [13,14]. The last 9 residues (458–466) yield weak electron density and do not appear to have an ordered secondary structure [13]. The characteristics of mutant enzymes Δ6 and Δ9 show that this sequence does not have an important role in determining the catalytic properties of the *R. rubrum* enzyme. Even if this sequence originated by fusion of a fragment of an ancestral small subunit gene to an ancestral large subunit gene, it clearly no longer serves a structural or activity-enhancing purpose analogous to the function of the small subunit in L_8S_8 Rubiscos [1,8]. This result is supported by comparing the *R. rubrum* Rubisco with the Rubisco from *Rhodobacter sphaeroides* which also lacks small subunits. The amino acid sequences are 89% homologous but the *Rh. sphaeroides* enzyme lacks the carboxylterminal 7 amino acids. Therefore it resembles the mutant enzymes Δ6 and Δ9 in this respect (Fig. 1) [16]. Dimers of the *Rh. sphaeroides* Rubisco

large subunits tend to aggregate to form larger oligomers [16], however, we observed no such tendency with our truncated Rubiscos, indicating that the deletion of the carboxylterminal 7 amino acid residues does not promote dimer aggregation.

Deletion of the carboxylterminal 18 residues and replacement of tyrosine-448 with leucine at the new carboxyl terminus did not alter k_{cat} significantly but it weakened the affinities for both substrates (Table I). The ten-fold increase in K_m(ribulose-P_2) is particularly obvious. This truncation also drastically reduced the level of expression in *E. coli*, perhaps because the truncated product was susceptible to proteolytic attack (Fig. 2). This truncation removes the terminal α-helix (αJ) of the protein [14]. This may influence substrate affinities at the active site by indirect means, perhaps by affecting the position of loop 8 of the α/β barrel which is nearby. Loop 8 contains some of the residues involved in binding the C-1 phosphate group of ribulose-P_2 [14]. Removal of helix αJ might also expose protease-sensitive sites.

Recently, Ranty et al. [16] also constructed three carboxylterminal truncations of *R. rubrum* Rubisco. Their mutant enzymes lacked the terminal 8, 17 and 25 residues. The enzyme lacking 8 residues resembled the wild-type enzyme in all characteristics studied and thus resembles our Δ6 and Δ9 Rubiscos. However, the properties of the enzyme lacking the terminal 17 residues were very different from our Δ18 enzyme. Whereas our Δ18 enzyme had a k_{cat} near to that of the wild-type enzyme and retained the dimeric structure, their 17-residue truncation was virtually inactive and its dimers aggregated to octamers by binding end to end in a circular fashion. The 25-residue truncation had similar properties to the 17-residue truncation. The reason for the differences between our Δ18 enzyme and the Ranty et al. [16] 17-residue truncation must lie in the carboxylterminal two residues. The dipeptide Tyr-Pro of the latter is replaced by the single residue, Leu, in Δ18. However, it is not obvious how this sequence difference leads to such important differences in properties.

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REFERENCES

[1] Andrews, T.J. and Lorimer, G.H. (1987) in: Photosynthesis (Hatch, M.D. and Boardman, N.K. eds) The Biochemistry of Plants, vol. 10, pp. 131–218, Academic Press, New York.
[2] Tabita, F.R. and McFadden, B.A. (1974) J. Biol. Chem. 249, 3459–3464.
[3] Lundqvist, T. and Schneider, G. (1988) J. Biol. Chem. 263, 3643–3646.
[4] Chapman, M.S., Suh, S.W., Curmi, P.M.G., Cascio, D., Smith, W.W. and Eisenberg, D.S. (1988) Science 241, 71–74.
[5] Knight, S., Andersson, I. and Brändén, C.-I. (1989) Science 244, 702–705.
[6] Wasmann, C.C., Ramage, R.T., Bohnert, H.J. and Ostrem, J.A. (1989) Proc. Natl. Acad. Sci. USA 86, 1198–1202.
[7] Chapman, M.S., Suh, S.W., Cascio, D., Smith, W.W. and Eisenberg, D.S. (1987) Nature 329, 354–356.
[8] Andrews, T.J. (1988) J. Biol. Chem. 263, 12213–12219.
[9] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[10] Somerville, C.R. and Somerville, S.C. (1984) Mol. Gen. Genet. 193, 214–219.
[11] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[12] Miziorko, H.M., Behnke, C.E. and Houkom, E.C. (1982) Biochemistry 21, 6669–6674.
[13] Schneider, G., Lindqvist, Y., Brändén, C.-I. and Lorimer, G.H. (1986) EMBO J. 5, 3409–3415.
[14] Andersson, I., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Brändén, C.-I. and Lorimer, G.H. (1989) Nature 337, 229–234.
[15] Wagner, S.J., Stevens, S.E., Nixon, B.T., Lambert, D.H., Quivey, R.G. and Tabita, F.R. (1988) FEMS Microbiol. 55, 217–222.
[16] Ranty, B., Lundqvist, T., Schneider, G. and Lorimer, G.H. (1989) in: Progress in Photosynthesis Research (Baltscheffsky, M. ed.) Kluwer Academic Publishers, Dordrecht, in press.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Martin, P.G. (1979) Aust. J. Plant Physiol. 6, 401–408.
[19] Goldschmidt-Clermont, M. and Rahire, M. (1986) J. Mol. Biol. 191, 421–432.
[20] Shinozaki, K. and Sugiura, M. (1983) Nucleic Acids Res. 11, 6957–6964.
[21] Viale, A.M., Kobayashi, H. and Akazawa, T. (1989) J. Bacteriol. 171, 2391–2400.
[22] Nargang, F., McIntosh, L. and Somerville, C.R. (1984) Mol. Gen. Genet. 193, 220–224.