Characterization of a gene encoding dihydrolipoamide dehydrogenase of the cyanobacterium *Synechocystis* sp. strain PCC 6803

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The authors previously reported the isolation and partial characterization of a periplasmically located dihydrolipoamide dehydrogenase (LPD) from the cyanobacterium *Synechocystis* sp. strain PCC 6803. In the present work the gene (*lpdA*; database accession number Z48564) encoding the apoprotein of this LPD in *Synechocystis* PCC 6803 has been identified, sequenced and analysed. The *lpdA* gene codes for a protein starting with methionine, which is post-translationally removed. The mature protein contains an N-terminal serine and consists of 473 amino acids with a deduced molecular mass of 51421 Da (including one FAD). The LPD is an acidic protein with a calculated isoelectric point of 5.17. Comparison of the amino acid sequence of the *Synechocystis* LPD with protein sequences in the databases revealed that the enzyme shares identities of 31–35% with all 18 LPDs so far sequenced and published. As a first step in determining the role of this cyanobacterial LPD, attempts were made to generate an LPD-free *Synechocystis* mutant by insertionally inactivating the *lpdA* gene with a kanamycin-resistance cassette. However, the selected transformants appeared to be heteroallelic, containing both the intact *lpdA* gene and the *lpdA* gene inactivated by the drug-resistance cassette. The heteroallelic mutant studied, which had about 50% of the wild-type LPD activity, caused acidification of the growth medium. Growth over a prolonged time was only possible after an increased buffering of the medium. Since it is reported in the literature that inactivation of the pyruvate dehydrogenase complex (PDC) leads to acidosis, a function of the LPD in a cytoplasmic-membrane-associated PDC is conceivable.

Keywords: dihydrolipoamide dehydrogenase, *lpdA* gene, *Synechocystis*, cyanobacteria

INTRODUCTION

Dihydrolipoamide dehydrogenase (dihydrolipoamide: NAD⁺ oxidoreductase, EC 1.8.1.4; LPD) belongs to the enzyme family called pyridine nucleotide disulfide oxidoreductases (Williams, 1991), which consist of lipoamide dehydrogenase (Williams, 1976; Carothers *et*

al., 1989), glutathione reductase (Schulz *et al.*, 1978; Schirmer & Schulz, 1987), mercuric reductase (Fox & Walsh, 1982), thioredoxin reductase (Williams, 1976) and trypanothione reductase (Shames *et al.*, 1986). These enzymes catalyse the electron transfer between pyridine nucleotides and disulfide compounds. All of them contain flavin and two redoxactive cysteines.

LPD is functionally involved in three multienzyme 2-oxo acid dehydrogenase complexes (pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes) which catalyse the oxidative decarboxylation of 2-oxo acids (Williams, 1976). LPD is also a component of the glycine cleavage multienzyme complex (Kikuchi & Hiraga, 1982; Bour-

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Abbreviations: LPD, dihydrolipoamide dehydrogenase; PDC, pyruvate dehydrogenase complex.

The GenBank accession number for the sequence determined in this work is Z48564.

guignon *et al.*, 1992). Besides the well-studied LPDs in the three 2-oxo acid dehydrogenase complexes and the glycine cleavage system, LPDs are present in some archaeobacteria (Danson *et al.*, 1986; Danson, 1988) and parasites (Danson *et al.*, 1987; Danson, 1988; Jackman *et al.*, 1990; Else *et al.*, 1993) in which the above-mentioned multienzyme complexes are absent. The function of these LPDs has remained uncertain. On the basis of results with *Escherichia coli*, which contains a cytoplasmic-membrane-associated LPD besides the LPD in the three 2-oxo acid dehydrogenase complexes (Richarme & Heine, 1986), it was suggested that cytoplasmic-membrane-associated LPDs might have a fundamental role in membrane processes, such as transport of solutes into and out of the cell (Danson, 1988).

Previously, we reported on an LPD in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (Engels *et al.*, 1997). In many respects this LPD belongs to the class of typical LPDs. It contains noncovalently bound FAD, it is a homodimer of 105 kDa and the N-terminus shows significant homologies to the N-termini of other LPDs so far sequenced and also other pyridine nucleotide disulfide oxidoreductases. Immunocytochemical investigations gave evidence that the enzyme is present in the periplasmic space, between the cytoplasmic membrane and the peptidoglycan layer. The only other report on an LPD in a cyanobacterium is that by Serrano (1992), who isolated and characterized an LPD from the filamentous *Anabaena* sp. strain PCC 7119. The function of LPDs in cyanobacteria has so far remained uncertain. Serrano (1992) suggested that the LPD in *Anabaena* might be involved in a glycine cleavage system. On the other hand, Bothe & Nolte (1975) detected a low NAD-dependent pyruvate dehydrogenase activity in *Anacystis nidulans* (*Synechococcus* PCC 6301), suggesting the presence of a pyruvate dehydrogenase complex (PDC) in this unicellular cyanobacterium and thus implying the involvement of an LPD in such a complex. The detection of a cyanobacterial LPD located in the periplasmic space adds a new aspect to research on LPDs in cyanobacteria. To obtain further information about the possible function of the LPD in *Synechocystis* PCC 6803, the gene encoding this LPD was identified, sequenced and analysed. Moreover, an attempt was made to construct an LPD-free *Synechocystis* PCC 6803 mutant.

METHODS

Bacterial strains and growth conditions. *Synechocystis* sp. strain PCC 6803 was obtained from the Collection Nationale de Cultures de Micro-organismes, Institut Pasteur, Paris, France. The glucose-tolerant derivative of *Synechocystis* PCC 6803 (*Synechocystis* PCC 6803 Glu^T; Williams, 1988) was a kind gift of Professor M. Rögner, Ruhr-Universität Bochum, Germany. Both wild-type strains and the heteroallelic mutant constructed in this paper were grown as previously described in BG11 medium with slight modifications (Engels *et al.*, 1997). Growth of *Synechocystis* on agar plates was in BG11 medium (with slight modifications as above) containing 1%

(w/v) agar (Gibco-BRL). When indicated, 10 µg ampicillin ml⁻¹ and/or 20 µg kanamycin ml⁻¹ were added to the growth medium.

Escherichia coli DH5αMCR (Grant *et al.*, 1990) was cultivated at 37 °C in LB medium (Miller, 1972) or on PA plates containing, in 1 l distilled water, 17.5 g antibiotic medium no. 3 (assay broth, Oxoid) and 16.0 g bacteriological agar (Oxoid). Where indicated, antibiotics were added at the following concentrations: 150 µg ampicillin ml⁻¹ and 50 µg kanamycin ml⁻¹.

Cell breakage and isolation of the membrane fraction. In general, all *Synechocystis* PCC 6803 cell types were grown for 2 d, harvested by centrifugation and washed once in distilled water. Cells were then resuspended in HMCBS [50 mM HEPES/NaOH, pH 6.5, containing 30 mM CaCl₂, 10 mM MgCl₂, 1 M sucrose, 25% (w/v) glycerol, 25 µM Pefabloc (Merck), 1 mM benzamidinium hydrochloride (Merck), 1 mM ε-aminocaproic acid (Merck) and benzonase (0.4 units ml⁻¹, Merck)] to give a final chlorophyll concentration of 0.3–0.4 mg ml⁻¹. Cell breakage was performed according to the method of Burnap *et al.* (1989) in a Bead Beater (Biospec Products). The membrane fraction, containing the thylakoid, cytoplasmic and outer cell membranes, was separated from the soluble protein fraction by centrifugation for 45 min at 95 000 g and was then resuspended in HMCBS (HMCBS from which sucrose was omitted) to a final chlorophyll concentration of 1 mg ml⁻¹.

E. coli DH5αMCR was grown to a cell density corresponding to an OD₅₈₀ of 0.6–0.8, harvested by centrifugation, resuspended in 29 mM Tris/HCl, pH 7.5, and subsequently broken by treatment in a French press at 138 MPa. The soluble protein fraction from the periplasm fraction and the spheroplast fraction were isolated according to the procedure of Block & Grossman (1988).

LPD activity measurements, protein determination, chlorophyll determination, SDS-PAGE and immunoblotting. LPD activity was determined basically according to Serrano (1992). This assay is based on measurement of the rate of NADH oxidation at 25 °C in the presence of a disulfide substrate for the LPD. The reaction was monitored by the decrease in A₃₄₀. The reaction mixture contained, in a final volume of 1 ml: 100 µmol Tris/HCl, pH 7.0, 3 µmol DL-lipoamide (DL-6,8-thioctic acid amide; added in 30 µl dimethylsulfoxide/acetone, 1:1, v/v), 0.5 µmol EDTA, 10 µmol 2-mercaptoethanol, 0.2 µmol NADH and an adequate amount of enzyme. One unit of enzyme is defined as the amount which catalyses the oxidation of 1 µmol NADH min⁻¹ at 25 °C under the above conditions.

Protein was determined according to Smith *et al.* (1985) and chlorophyll according to Grimme & Boardman (1972). SDS-PAGE was performed according to Laemmli (1970). Protein samples for SDS-PAGE were denatured at 37 °C for 30 min. Immunoblotting was done as previously described, transferring proteins to nitrocellulose (Schleicher & Schüll) and using the antibody raised against the isolated LPD from *Synechocystis* PCC 6803 (polyclonal antibody raised in a rabbit; Engels *et al.*, 1997).

Oligonucleotide synthesis. The following oligonucleotide was designed on the basis of a partial N-terminal amino acid sequence (residues 2–7) of the LPD from *Synechocystis* PCC 6803 previously published (Engels *et al.*, 1997), utilizing a codon usage dictionary of *Synechocystis* PCC 6803: 5'-CA A/G GA T/C TT T/C GA T/C TA T/C GA-3'. The oligonucleotide was synthesized with an Applied Biosystems

Fig. 1. Physical map of the *lpdA* gene region from *Synechocystis* PCC 6803 and insertional inactivation of the *lpdA* gene. (a) The pSVB30 derivative pAE38 contains a 3.8 kb *EcoRI* fragment of *Synechocystis* PCC 6803 DNA carrying the *lpdA* gene. The position of the oligonucleotide probe is shown by the box. (b) pAE26 is a derivative of pAE38 lacking the *SmaI* cleavage site in the multiple cloning site of pSVB30. It represents a 2600 bp *PstI*–*EcoRI* fragment of pAE38 cloned into the *PstI*/*EcoRI*-treated plasmid pSVB30. It contains the *lpdA* gene (ORF1) and ORF2. Size and orientation of the *lpdA* gene are shown by the arrow. (c) pAE26Km^R is a derivative of pAE26. A 2.3 kb *SspI* fragment from pJW31 containing a kanamycin-resistance gene (Km^R) originating from Tn5 was cloned into the single *HpaI* site (905 bp downstream from the putative start codon of the *lpdA*). Size and orientation of the *lpdA* gene and the Km^R gene are indicated by the arrow. This construct was then used to transform *Synechocystis* PCC 6803 Glu⁺.

DNA synthesizer model 380B using the phosphoramidite method (Beaucage & Caruthers, 1981; Matteucci & Caruthers, 1981) and purified as recommended by the manufacturer. The oligonucleotide was labelled with the DIG (digoxigenin) oligonucleotide 3' end-labelling kit and detected with the DIG DNA labelling and detection kit (Boehringer Mannheim).

DNA isolation, cloning, sequencing and analysis. Total DNA of *Synechocystis* PCC 6803 was isolated by the Sarkosyl method and purified by the phenol extraction procedure (Williams, 1988; Sambrook *et al.*, 1989). DNA was digested with *EcoRI* and size-fractionated on agarose gels. Recovery of the fragments was achieved by using the Jetsorb gel extraction kit from Genomed. The size-fractionated fragments of total DNA were cloned into *EcoRI*-linearized vector plasmid pSVB30 (Arnold & Pühler, 1988). The details of plasmid construction (pSVB30 derivatives) are given in Fig. 1.

Cloning procedures, Southern blotting, and recombinant DNA methods were performed using established techniques (Sambrook *et al.*, 1989). Subclones of the resulting hybrid plasmid were made by using the Pharmacia double-stranded nested deletion kit. These subclones were used for sequencing on an ALF Sequencer (Pharmacia). The enzymes used in this study were purchased from Boehringer, Bethesda Research

Laboratories or Pharmacia. All reactions were performed following the recommendations of the corresponding manufacturer.

Computer analysis. Nucleic acid sequences were analysed using the Staden Software package (Staden, 1986). The deduced protein sequence was analysed using the program PC/Gene (Release 6.80, 1993; IntelliGenetics). Related protein sequences were searched for in the SWISS-PROT, PIR and GenBank databases with the BLAST software (Altschul *et al.*, 1990).

Construction of *Synechocystis* PCC 6803 mutant with an insertional inactivated *lpdA* gene. To inactivate the *lpdA* gene, a 2.6 kb *PstI*–*EcoRI* fragment of plasmid pAE38 carrying the *lpdA* gene cloned into vector plasmid pSVB30 was chosen (Fig. 1a, b). For insertional inactivation of the *lpdA* gene a 2.3 kb *SspI* fragment from plasmid pJW31 (a kind gift from Dr W. Jäger, Lehrstuhl für Genetik, Universität Bielefeld; unpublished results) was used. This DNA fragment carries a gene from Tn5 which codes for an aminoglycoside-3'-phosphotransferase mediating resistance to kanamycin and neomycin (Jorgensen *et al.*, 1979). This fragment was cloned into the single *HpaI* site of the *lpdA* gene (905 bp downstream of the start codon). Subsequently, the construct was transferred to *E. coli* DH5αMCR, and clones with the interrupted *lpdA* gene were identified by screening for double resistance (ampicillin resistance encoded by the vector; kanamycin resistance encoded by the Tn5 fragment) and subsequent restriction analysis. Detection was achieved with the synthetic oligonucleotide. This resulted in clones containing the kanamycin-resistance gene inserted either in the same or in the opposite direction as the *lpdA* gene. Only the construct carrying the kanamycin-resistance gene in the same direction as the *lpdA* gene successfully transformed *Synechocystis*. This plasmid was called pAE26Km^R (7.2 kb) (Fig. 1c). The further protocol for construction of the *Synechocystis* mutant was basically the same as previously described for construction of an MSP-free *Synechocystis* PCC 6803 mutant (Engels *et al.*, 1994).

RESULTS AND DISCUSSION

Identification and characterization of the gene encoding the LPD from *Synechocystis* PCC 6803

The gene encoding the LPD from *Synechocystis* sp. strain PCC 6803 was detected by an oligonucleotide designed on the basis of part of an N-terminal amino acid sequence of the enzyme (Engels *et al.*, 1997) in Southern hybridization experiments. Analysis of *Synechocystis* total DNA digested with different restriction enzymes revealed the presence of a single copy of the putative gene (Fig. 2). A 3.8 kb *EcoRI* fragment was cloned in the vector pSVB30, yielding pAE38 (Fig. 1a).

A 2609 bp (*PstI*–*EcoRI*) fragment (Fig. 1b) containing an ORF (ORF2) and the *lpdA* gene (ORF1) was subcloned and sequenced (database accession number Z48564). ORF2 starts at base position 262 with an ATG start codon and terminates at base position 801 with a TAA stop codon. No significant homologies to other so far sequenced proteins were found, and the function of this ORF remains to be elucidated.

ORF1, encoding LPD, consists of 1745 bp (GC content 52.42 mol %); it starts at base position 900 with an ATG start codon and terminates at base position 2324 with a

Fig. 2. Southern blot of total DNA from wild-type *Synechocystis* PCC 6803 and from the heteroallelic mutant *Synechocystis* PCC 6803 Glu^T KC with various endonucleases. Genomic DNA from *Synechocystis* PCC 6803 was digested with *Hind*III (10 kb fragment, lane A), *Bam*HI (16 kb fragment, lane B), *Eco*RI (3.8 kb fragment, lane C), *Eco*RI/*Hind*III (2.8 kb fragment, lane D), and *Eco*RI/*Bam*HI (3.0 kb fragment, lane E). Genomic DNA from *Synechocystis* PCC 6803 Glu^T KC was digested with *Eco*RI/*Hind*III (2.8 and 4.1 kb fragments corresponding to the native and the cassette-inactivated *lpdA* gene, respectively, lane F), *Eco*RI (3.8 and 5.1 kb fragments corresponding to the native and the cassette-inactivated *lpdA* gene, respectively, lane G), and *Sma*I (fragment of 6.5 kb for both, lane H). The *lpdA*-carrying fragment was detected with a DIG-labelled synthetic oligonucleotide synthesized according to an amino acid sequence of the N-terminal region of the isolated LPD from *Synechocystis* PCC 6803.

TAA stop codon. Potential promoter sequences (a Pribnow box and -35 sequence) are found at base positions 10-14 and 27-32 upstream of the translational start. The presence of canonical promoter sequences for a σ^{70} -like transcription factor was corroborated by expression of the LPD protein in *E. coli* (see below). Amino acids 2-18 of the deduced amino acid sequence of ORF1 are identical to the peptide sequence obtained by direct sequencing of the LPD protein (Engels *et al.*, 1997), demonstrating that the authentic LPD-encoding gene from *Synechocystis* PCC 6803 had been identified. The gene was subsequently called *lpdA*.

The deduced LPD ORF codes for 474 amino acids, starting with methionine. Since protein sequencing gave a serine as the N-terminal amino acid, it has to be concluded that the N-terminal formylated methionine is post-translationally removed. The subsequent computer analysis of the *lpdA* gene product was performed on the remaining 473 amino acids corresponding to a molecular mass of 50701 Da. When FAD is included in the calculation, the molecular mass is 51421 Da. This is in good agreement with the apparent subunit molecular mass of 55 kDa obtained by SDS-PAGE. The calculated isoelectric point of the protein is 5.17. This agrees quite well with the tight binding of the acidic protein to anion-binding exchange columns during purification (Engels *et al.*, 1997).

The hydrophobicity profile on the basis of Kyte & Doolittle (1992) indicated that the LPD is a peripheral and not an integral membrane protein. This is in agreement with our biochemical investigations showing that the mild detergent sulfobetaine 12, which does not release integral membrane proteins, will solubilize the LPD (Engels *et al.*, 1997). In contrast, evaluation on the basis of Rao & Argos (1986) or Eisenberg *et al.* (1984) suggested two potential transmembrane helices, at positions 9-25 and 323-341 or 8-28 and 178-198, respectively. Due to these different results from the

Table 1. Identities and similarities between selected pyridine nucleotide oxidoreductases and the LPD from *Synechocystis* PCC 6803

Similarity includes, besides identical amino acids, the residue groups A/S/T, D/E, N/Q, R/K, I/L/M/V and F/Y/W, respectively.

Enzyme	Identity (%)	Similarity (%)	Reference
LPD			
Human	34.8	48.3	Otulakowski & Robinson (1987)
Pea	33.4	48.1	Turner <i>et al.</i> (1992)
<i>Pseudomonas fluorescens</i>	32.7	43.9	Benen <i>et al.</i> (1989)
<i>Clostridium magnum</i>	31.9	43.2	Krüger <i>et al.</i> (1994)
<i>Alcaligenes eutrophus</i>	31.4	42.6	Hein & Steinbüchel (1994)
Glutathione reductase			
<i>Arabidopsis thaliana</i>	26.6	35.9	Kubo <i>et al.</i> (1993)
Mercuric reductase			
<i>Staphylococcus aureus</i>	27.4	37.1	Laddaga <i>et al.</i> (1987)

Z48564	-SQDFDYDLVIIGAGVGGHGAALHAVKCGGLKTAIEAKD-MG	40
P09622	ADQPIDADVTVIGSGPGGYVAAIKAAQLGFKTVCIENKNETLG	42
Z48564	GTCVNRGCI ^{PSKALLAASGRVREMS} QDHLQQLGIQINGVTF	82
P09622	GTCLNVGCI ^{PSKALLNNSHYH-MAHGTDFASRGIE} MSVRL	83
Z48564	TREAIAAHANDLVSKIQSDLTNSLTRLKVDITRGWGKVSQGPQ	124
P09622	NLDKMEQKSTAVKALTGGIAHLFKQNKVVHVNGYKGTGKN	125
Z48564	EVTVIG-DNETRILKAKEIMLCPGSVFPVPPGIEIDHKTVFT	165
P09622	QVTATKADGGTQVIDTKNLIATGSEVTPFPGITIDEDTIVS	167
Z48564	SDEAVKLETLPQWIAIIGSGYIGLEFSDVYALGCEVTMIEA	207
P09622	STGALSLLKVKPEKVVIGAGVIGVELGSVWQRLGADVTAVEF	209
Z48564	LPDLMP-GFDP ^{EIAKIAERVLK-SRDIETYTG} VFKIKAG	247
P09622	LCHVGGVGIDMEISKNFQRLQKQGF ^{PKLNTKVTGAT} KKSD	252
Z48564	SPVEIELTDAKTKEVIDTLEVDACL ^{VATGRIPATK} NLGLETV	289
P09622	GKIDVSI ^{EAA} SGGKA-EVITCDVLLVCIGRRPFTK ^{NLGL} EEL	292
Z48564	GVETDRRGFIEVNDQM ^{QVIKDGK} EVPHLWAVGDATGKMLAH	331
P09622	GIELDPRGRIPVNT ^{RFQT} -----KIPNIYAIGDVVAGPMLAH	329
Z48564	AASGQGVVAVENICGRKTEVDYRAIPAAAFTHPEISYVGLTE	373
P09622	KAEDGEIICVEGMAGGAVHIDYNCVPSVIYTHPEVAWVGKSE	371
Z48564	AQAKELGEKEGFVVSTAKTYFKGNSKALAEKETDGI ^{AKV} VYR	415
P09622	EQLKE----EGIEYKVGKFPFAANSRAKT ^{NADTDGM} VKILGQ	409
Z48564	QDTGELLGAHIIGIHASDLIQEAAQAIADRKS ^{VRELA} AFVHA	457
P09622	KSTDRVLGAHLGPGAGEMVNEAALALEYGASCE ^{DIAR} VCHA	451
Z48564	HP ^{TLSE} VLD ^{EA} -----YKRAV	473
P09622	HP ^{TLSE} AFREANLAASFGK ^{SINF}	474

Fig. 3. Alignment of the LPD from *Synechocystis* PCC 6803 (GenBank accession number Z48564) with the LPD from human erythrocytes (GenBank accession number P09622). Identical and similar (A/S/T; D/E; N/Q; R/K; I/L/M/V; F/Y/W) amino acids are printed bold. A dashed line between amino acid residues (----) indicates an introduced gap. Above the sequence the four domains of pyridine nucleotide disulfide oxidoreductases are indicated by boxes. Box with grid lines, FAD-binding domain; box with vertical lines, NAD-binding domain; open box, central domain; box with diagonal lines, interface domain. Arrows indicate the sequence of the adenosine-binding domain and a broken-line arrow indicates the active cysteine motif.

evaluation programs, no clear conclusion can be drawn. Hydrophobic regions in the FAD-binding domain have also been found in other pyridine nucleotide disulfide oxidoreductases (Thieme *et al.*, 1981; Karplus & Schulz, 1987).

Homology analysis

Comparison of the amino acid sequence of the LPD from *Synechocystis* PCC 6803 with protein sequences in the Protein Information Resource database revealed that the protein has similarities to enzymes belonging to the group of pyridine nucleotide disulfide oxidoreductases, especially to all LPDs so far sequenced. In Table 1 those LPDs with the highest homology to the

Synechocystis enzyme and in addition some oxidoreductases which are relevant for the discussion are listed. A computer alignment of the *Synechocystis* LPD with the LPD from human erythrocytes (Otulakowski & Robinson, 1987) according to the algorithm of Myers & Miller (1988) is shown in Fig. 3. The similarity between the primary structures of these two LPDs occurs in all four domains defined in pyridine nucleotide disulfide oxidoreductases (Carothers *et al.*, 1989) on the basis of those oxidoreductases for which a three-dimensional structure has been proposed, as for example for the LPDs from *Pseudomonas fluorescens* (Mattevi *et al.*, 1993), *Pseudomonas putida* (Mattevi *et al.*, 1992) and *Azotobacter vinelandii* (Mattevi *et al.*, 1991), or for the glutathione reductase from human erythrocytes (Schulz *et al.*, 1978). Those defined domains are the FAD-binding domain (residues 1–147 in the LPD from *Synechocystis* PCC 6803), the NAD-binding domain (residues 148–278), the central domain (residues 279–354) and the interface domain (residues 353–473). The functional motif of the FAD-binding domain (GxGxxG/AxxxG/A; residues 12–21) and of the redox-active cysteines (CxxxxC; residues 43–48: CVNRGC) are present in *Synechocystis* LPD.

Unusual for an LPD is the finding of a valine in position 44 after the first active cysteine in the *Synechocystis* enzyme. All other LPDs have a conserved lysine in this position (Mattevi *et al.*, 1993). So far, only glutathione reductases have been shown to possess a valine in this position (Schulz *et al.*, 1978). However, as in all other LPDs, a cysteine in position 77 is missing in the *Synechocystis* LPD. Such a cysteine is present in glutathione reductase, in which the two subunits are linked covalently by this cysteine, while the subunits in LPDs are not linked covalently. As expected, and as is the case for all other LPDs, within the NAD(P)-binding domain of oxidoreductases the basic amino acid residues which are involved in binding of the 2' phosphoryl group of NADP (for example A²⁰⁷, H²¹⁹ and R²²⁴ in the human glutathione reductase) are replaced by neutral amino acids in LPDs (A²⁰⁷, L²⁰⁸ and P²¹³ in the *Synechocystis* enzyme). This region of sequence is likely to be important in discriminating between NAD (cofactor of LPD) and NADP (cofactor of glutathione reductase and mercuric reductase). In the C-terminal region in position 458 the active histidine is found which according to Holmes & Stevenson (1986) is essential for binding of the lipoyl group in the neighbouring transacetylase. This comparison shows that identity or conservative substitution of amino acids is especially notable for residues which are essential for binding the two cofactors (FAD and NAD), which are catalytically active or located close to the active centre, or which contribute to the interaction between the two subunits. The homologies are not restricted to these active-site regions but extend throughout the protein sequence, and only a few gaps had to be introduced to optimize the alignment.

A major difference of the *Synechocystis* LPD in comparison to other LPDs so far sequenced is that the C-terminal region downstream of the active histidine is

<i>Synechocystis</i>	458	HPTLSEVLDEAY KRAV	473
<i>Homo sapiens</i>	487	HPTLSEAFREAN LAASFGKSINF	509
<i>Sus scrofa</i>	487	HPTLSEAFREAN LAASFGKAINF	509
<i>Pisum sativum</i> (a)	480	HPTMSEAIKEA AAMA-TYDKPHSLKSWLLSSLVFIFVQGFTLTWRRYFVC	529
<i>Pisum sativum</i> (b)	480	HPTMSEAIKEA AAMA-TYDKPHI	501
<i>P. fluorescens</i>	450	HPTLSEALHEA ALAVNGHAIHIANRKKR	477
<i>B. subtilis</i>	447	HPTLGEITMEA AEVAIGSPIHIVK	470
<i>P. carbinolicus</i>	449	HPTLSEAVKEA ALDVGAAVHC	470
<i>B. stearotherm.</i>	446	HPTLGEIAMEA AEVALGTPIHIITK	470
<i>P. putida</i> (LPD-3)	445	HPTRSEALRQA AMNVDGMAMQI	466
<i>S. aureus</i>	446	HPTLGEITMEA AEKAIGYPIHTM	468
<i>C. magnum</i>	554	HPTVGEAMKEA ALAVNQAIHMMNK	578
<i>A. vinelandii</i>	451	HPALSEALHEA ALAVSGHAIHVANRKK	477
<i>E. coli</i>	445	HPTLHESVGLA AEVFEGSITDLPNPKAKKK	474
<i>H. volcanii</i>	450	HPTLAEAVMEA AENALQQAHTLNR	474
<i>S. cerevisiae</i>	478	HPTLSEAFKEA NMAAYDKAIHC	499
<i>P. putida</i> (LPD-Val)	448	HPTLSEALHEA ALAVNGGAIHVANRKKR	475
<i>P. putida</i> (LPD-Glu)	449	HKAVHEG-HVA AENCAGNKAYFDARVIPGVAYT	480
<i>T. brucei</i>	458	HPTMSEAVKEA CMACFAKTINF	479
<i>A. eutrophus</i>	567	HPTLGESIGMA AEIYEGTCTDVPFPRKR	594

Fig. 4. C-termini of all LPDs sequenced so far. The C-terminus starting from the active histidine residue is given. The listing from top to bottom is according to the degree of similarity of other LPDs to the *Synechocystis* PCC 6803 LPD. Identical amino acids are written in bold letters. The histidine region which according to Mattevi *et al.* (1993) participates in an intermolecular hydrogen bridge to an N-terminal tyrosine of the other LPD subunit is underlined. For the C-terminal region of pea two diverging sequences are given according to Turner *et al.* (1992) (marked a in the figure) and to Bourguignon *et al.* (1992) (marked b in the figure). The sequences are from the GenBank database: *Synechocystis* PCC 6803 (accession number Z48564; this paper), *Homo sapiens* (P09622), *Sus scrofa* (P09623), *Pisum sativum* (a) (S96804), *Pisum sativum* (b) (X63464), *Pseudomonas fluorescens* (A45796), *Bacillus subtilis* (P21880), *Pelobacter carbinolicus* (M01100), *Bacillus stearothermophilus* (X53560), *Pseudomonas putida* LPD-3 (P31046), *Staphylococcus aureus* (S19723), *Clostridium magnum* (L31844), *Azotobacter vinelandii* (P18925), *Escherichia coli* (P00391), *Haloferax volcanii* (Q04829), *Saccharomyces cerevisiae* (P09624), *Pseudomonas putida* LPD-Val (P31052), *Pseudomonas putida* LPD-Glu (A39406), *Trypanosoma brucei* (X70646), *Alcaligenes eutrophus* (U09865).

substantially shorter than in the other LPDs (Fig. 4). On the basis of mutant studies with the LPD from *Pseudomonas fluorescens* (Mattevi *et al.*, 1993), this region is assumed to contribute to the dimer and redox stability of the enzyme. Moreover, two sequences with polar amino acids exist in the *Synechocystis* LPD (region 308–312: I, K, D, G, K and region 379–382: L, G, E, K) which are not found in any other of the LPDs so far sequenced. By comparing the primary structure of the *Synechocystis* LPD with the known three-dimensional structure of the LPD from *P. fluorescens* (Mattevi *et al.*, 1993), a possible location between the β -sheets A5 and A6 and between the α -helix 4·8 and β -sheet E3 can be suggested. Alterations in these regions should result in a modification of loop 5, which is assumed to be involved in the association of the *P. fluorescens* LPD with the transacetylase (Mattevi *et al.*, 1993). These sequence deviations of the *Synechocystis* LPD from other LPDs so far sequenced might be an indication of an altered interaction with neighbouring proteins or of a completely different function of this extracellularly located cyanobacterial LPD.

Attempt to generate an LPD-free *Synechocystis* PCC 6803 mutant

As a first step in elucidating the possible function of the *Synechocystis* LPD we attempted to generate an LPD-deficient mutant by insertional inactivation of the *lpdA* gene and subsequent homologous recombination into

the wild-type genome of *Synechocystis* PCC 6803. With plasmid pAE26Km^R, containing the *lpdA* gene insertional inactivated by a 2·3 kb *SspI* fragment from pWJ31, which carries a gene mediating resistance to kanamycin and neomycin (for details see Methods; Fig. 1c) we first tried to transform *Synechocystis* PCC 6803 (a strain which is not able to grow on glucose under photoheterotrophic conditions). However, we were not successful in obtaining transformants using either a natural transformation system, electroporation or transformation after CaCl₂ treatment of the cells. Therefore, at this stage we switched to the glucose-tolerant strain *Synechocystis* PCC 6803 Glu^T, which is able to grow on glucose under photoheterotrophic conditions (Williams, 1988). From all information so far available in the literature, these two *Synechocystis* strains are otherwise identical. The hybridization pattern of genomic DNA from these two strains after treatment with various restriction enzymes was identical (not shown).

Transformation of CaCl₂-treated *Synechocystis* PCC 6803 Glu^T cells with pAE26Km^R resulted in Km^R Ap^S transformants. Ninety-five percent of the single colonies had lost the vector-encoded ampicillin resistance, which implies that the desired double recombination event had occurred and that only the cyanobacterial DNA containing the internal drug resistance cassette had integrated into the genome. In an attempt to achieve segregation, repeated subculturing was performed. For this purpose five of the above colonies were transferred to liquid BG11 medium containing kanamycin and

Table 2. Comparison of growth of *Synechocystis* PCC 6803 Glu^T and the heteroallelic mutant *Synechocystis* PCC 6803 Glu^T KC under various conditions

Synechocystis cells were grown under normal growth conditions in BG11 medium with either nitrate or ammonium as nitrogen source. Additionally, the medium was supplemented with 5 mM glucose or with 20 mM HEPES/NaOH buffer, pH 7.5, where indicated. After 2 d the pH of the medium was determined. The last column in the table indicates whether cells grew (+) or did not grow (−) under the chosen conditions.

<i>Synechocystis</i> PCC 6803	Supplements to nitrate-free BG11	pH of medium after 2 d growth	Growth
Wild-type (Glu ^T)	KNO ₃	7.5	+
	NH ₄ Cl	7.5	+
	KNO ₃ + glucose	7.5	+
	KNO ₃ + HEPES/NaOH, pH 7.5	7.5	+
Heteroallelic mutant (Glu ^T KC)	KNO ₃	6.3	−
	NH ₄ Cl	6.5	−
	KNO ₃ + glucose	6.2	−
	KNO ₃ + HEPES/NaOH, pH 7.5	7.5	+

Table 3. Comparison of the LPD activity of *Synechocystis* PCC 6803 Glu^T and the heteroallelic mutant *Synechocystis* PCC 6803 Glu^T KC

Growth was for 2 d in BG11 medium containing 20 mM HEPES/NaOH buffer, pH 7.5. After breaking the cells as described in Methods, LPD activity was determined in total cell extracts, in the membrane fraction and in the soluble protein fraction. One unit of enzyme was defined as 1 µmol NADH oxidized min^{−1} in the presence of 0.2 mM NADH and 3 mM lipamide at 25 °C.

Preparation	LPD activity in <i>Synechocystis</i> [units (mg protein) ^{−1}]		
	PCC 6803	PCC 6803 Glu ^T	PCC 6803 Glu ^T KC
Total cell extract	0.024	0.022	0.012
Membrane fraction	0.019	0.018	0.010
Soluble fraction	0.005	0.004	0.002

grown for several growth cycles. The cell suspension was diluted every 3 d. After three such treatments the cells had died, implying that complete segregation was lethal. After trying a number of other growth conditions (various nitrogen sources and adding glucose to the medium), it became obvious that under all tested growth conditions mutant cells caused an acidification of the culture medium. The pH dropped from about 7.5 to 6.5, suggesting that the *Synechocystis* mutant cells cannot tolerate a slightly acidic medium (Table 2). Such an acidification of the medium was not observed with wild-type cells. To overcome this problem, the buffering capacity of the culture medium was increased by adding 20 mM HEPES/NaOH buffer, pH 7.5. Under such conditions the transformants could be cultivated over

prolonged periods without damage (10–15 dilution cycles of 3 d).

Partial characterization of these transformants showed that all of them were of the same phenotype. Thus, the results of only one transformant, called *Synechocystis* PCC 6803 Glu^T KC, will be presented. Southern blot analysis revealed the presence of both the wild-type *lpdA* gene (2.8 kb fragment) and the insertionally inactivated *lpdA* gene (4.1 kb fragment: lane F of Fig. 2). As shown in Table 3, the mutant had detectable levels of LPD activity, but the activity was only about half that of wild-type cells. The same conclusion could be drawn from the Western blot (Fig. 5). The LPD protein was detected in the mutant but the amount was reduced by at least 50%. Thus, the transformant is heteroallelic. Selection occurred for viability (native *lpdA* gene) and antibiotic resistance (cassette-inactivated *lpdA* gene), implying that the *lpdA* gene product is essential in *Synechocystis* PCC 6803 Glu^T. This condition is possible because of the polyploid nature of *Synechocystis* PCC 6803, which contains six to eight chromosomal DNA copies (Williams, 1988). Other heteroallelic mutants have been obtained, for example after insertional inactivation of the gene encoding phosphoenolpyruvate carboxylase in *Synechococcus* PCC 7942 (Luinenburg & Coleman, 1990; there referred to as a merodiploid mutant) and of the gene encoding the FUR protein in *Synechococcus* PCC 7942 (Ghassemian & Straus, 1996).

Expression of the *Synechocystis* LPD in *E. coli*

To answer the question whether an active *Synechocystis* LPD was expressed, *E. coli* DH5αMCR transformants with plasmids pAE38 and pAE26, both containing the intact *lpdA* gene, and also with plasmid pAE26Km^R, containing the inactivated *lpdA* gene, were analysed. As the results of Table 4 show, the *E. coli* cells transformed with pAE38 and pAE26 contained an about 20–30-fold

Fig. 5. Immunoblot for detection of the LPD protein in *Synechocystis* PCC 6803 Glu^T and in the heteroallelic mutant *Synechocystis* PCC 6803 Glu^T KC. *Synechocystis* total cell extracts were separated into a membrane fraction and a soluble protein fraction by centrifugation. After SDS-PAGE, proteins were transferred to nitrocellulose and immunostained with the anti-LPD antiserum (dilution 1:1000). Lanes A and C, *Synechocystis* PCC 6803 Glu^T membrane fraction (lane A) and soluble protein fraction (lane C); lanes B and D, heteroallelic mutant *Synechocystis* PCC 6803 Glu^T KC membrane fraction (lane B) and soluble protein fraction (lane D). Each lane contained 30 µg protein.

increased level of LPD activity. This clearly demonstrates that in *E. coli* an active, FAD-containing *Synechocystis* LPD was expressed. The LPD protein could also be detected by the anti-LPD antiserum (Fig. 6). As expected, *E. coli* cells transformed with pAE26Km^R did not have an elevated LPD level. Western blot analysis, however, revealed that an approximately 35 kDa LPD protein fragment was expressed which cross-reacted with the antiserum but which did not have LPD activity (Table 4, Fig. 6).

Concluding remarks

It was not possible to construct an *lpdA* mutant of wild-type *Synechocystis* sp. PCC 6803. Also in a glucose-tolerant mutant, *Synechocystis* sp. PCC 6803 Glu^T, not

Fig. 6. Immunoblot for detection of the *Synechocystis* LPD in transformed *E. coli* DH5αMCR cells. *E. coli* DH5αMCR was transformed with pAE38 (lane B), pAE26 (lane C) or pAE26Km^R (lane D). Lane A, untransformed *E. coli* DH5αMCR. After growth, the spheroplast fraction was submitted to SDS-PAGE (30 µg protein in each lane). Subsequently, proteins were transferred to nitrocellulose and immunostained with anti-LPD antiserum (dilution 1:1000).

all copies of the *lpdA* gene were eliminated from the polyploid genome. The heteroallelic *Synechocystis* mutant studied, which had a reduced LPD activity, caused an acidification of the growth medium. On the basis of the results presented here and of results previously published by other groups showing that a functional 2-oxoglutarate dehydrogenase complex is absent in cyanobacteria (Smith, 1973), it seems likely that the LPD is a constituent of a PDC (Patel & Roche, 1990). A reduced PDC activity would lead to accumulation of pyruvate and subsequently of phosphoenolpyruvate and 3-phosphoglyceric acid, and after carboxylation of phosphoenolpyruvate also to accumulation of oxaloacetate. If such an accumulation of mono- and dicarboxylic acids should occur in the mutant as a consequence of a limited PDC activity, then it is likely that these metabolites are partially excreted into the medium, causing the observed acidification. An accumulation of acidic metabolites as a consequence of inactive 2-oxoacid dehydrogenases, leading to various forms of metabolic acidosis, has

Table 4. Determination of the LPD activity in *E. coli* DH5αMCR transformed with pAE38, pAE26 or pAE26Km^R

Preparation	LPD activity [units (mg protein) ⁻¹]			
	DH5αMCR	DH5αMCR(pAE26Km ^R)	DH5αMCR(pAE38)	DH5αMCR(pAE26)
Total cell extract	0.13	0.10	2.69	3.68
Spheroplast fraction	0.13	0.09	2.64	3.64
Periplasmic fraction (soluble proteins)	0.00	0.01	0.04	0.04

already been described in the literature (Robinson *et al.*, 1977; Yeaman, 1986; Patel *et al.*, 1995).

Very little is known about the function of PDC in cyanobacteria. Bothe & Nolteernsting (1975) described an NAD-dependent pyruvate dehydrogenase activity in *Anacystis nidulans*, but the enzyme was not further investigated. Since higher-plant chloroplasts contain a PDC (Taylor *et al.*, 1992; Camp & Randall, 1985) and since it is assumed that chloroplasts have evolved from cyanobacteria (or from a precursor form of cyanobacteria) (Douglas, 1994), the presence of a PDC in at least some cyanobacteria is very likely. The inability to clearly detect such a PDC by activity measurements in cyanobacteria might be related to an unusual location of the LPD (and possibly of the total PDC). Our immunocytochemical investigations with *Synechocystis* PCC 6803 (Engels *et al.*, 1997) have indicated that the LPD is located between the cytoplasmic membrane and the peptidoglycan layer, implying that the LPD is associated with the outer (and not with the inner) side of the cytoplasmic membrane. It is well documented in the literature that PDCs can be found associated with the NADH:ubiquinone oxidoreductase (complex I) of the respiratory system of the mitochondrial inner membrane (Sumegi & Srere, 1984). However, if in *Synechocystis* PCC 6803 such an association of the suggested PDC with the respiratory system might exist, then at least the LPD is located on the periplasmic side of the cytoplasmic membrane. Possibly, the low and hardly measurable PDC activity in broken *Synechocystis* cells could be explained by assuming that a partial dissociation of the PDC from the membrane occurs and that optimal activity is only obtained when a tight interaction of the PDC with the membrane is maintained. This aspect requires further investigation.

The essential nature of the LPD (which is probably a constituent of an unusual PDC) implies that under photoautotrophic growth conditions *Synechocystis* PCC 6803 has no functional pyruvate:ferredoxin (or flavodoxin) oxidoreductase, as described for several *Anabaena* species (Schmitz *et al.*, 1993; Bauer *et al.*, 1993), although comparative DNA sequence analysis revealed that a homologue DNA sequence to such an enzyme is present. Basepairs 3128396–3131985 of the *Synechocystis* PCC 6803 genome (database accession number sl10741) have 69% identity to the *nifJ* gene of *Anabaena* sp. 7120 (accession number L14925), which, however, is expressed during iron starvation only (Bauer *et al.*, 1993).

In conclusion, our results showing that LPD is an essential enzyme in *Synechocystis* PCC 6803, and that a reduced LPD activity leads to an acidification of the culture medium, suggest that the LPD is a constituent in a PDC. This interpretation is supported by the identification of the genes in *Synechocystis* PCC 6803 encoding E1 (pyruvate decarboxylase: α and β subunits) and E2 (dihydrolipoyl transacetylase) of the PDC (database accession numbers sl1712, slr1934 and sl1841: Genome Database of *Synechocystis* sp. strain PCC 6803). Thus,

all genes encoding the three enzymes of a PDC are present in *Synechocystis* PCC 6803. This makes the presence of a functional PDC in *Synechocystis* PCC 6803 highly likely. However, the cyanobacterial PDC is unusual in that the complex (or at least the LPD) is localized in the periplasmic space. For a final answer of the question whether the LPD in *Synechocystis* actually functions in a PDC, the localization of the E1 and E2 enzymes of a PDC has to be investigated.

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REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Arnold, W. & Pühler, A. (1988). A family of high-copy-number plasmid vectors with single end-label sites for rapid nucleotide sequencing. *Gene* **70**, 171–179.
- Bauer, C. C., Scappino, L. & Haselkorn, R. (1993). Growth of the cyanobacterium *Anabaena* on molecular nitrogen: NifJ is required when iron is limited. *Proc Natl Acad Sci USA* **90**, 8812–8816.
- Beaucage, S. L. & Caruthers, M. H. (1981). Desoxynucleoside phosphoramidites – a new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett* **22**, 1859–1862.
- Benen, J. A. E., Van Berkel, W. J. H., Van Dongen, W. M. A. M., Müller, F. & De Kok, A. (1989). Molecular cloning and sequence determination of the *lpd* gene encoding lipoamide dehydrogenase from *Pseudomonas fluorescens*. *J Gen Microbiol* **135**, 1787–1797.
- Block, M. A. & Grossman, A. R. (1988). Identification and purification of a derepressible alkaline phosphatase from *Anacystis nidulans* R2. *Plant Physiol* **86**, 1179–1184.
- Bothe, H. & Nolteernsting, U. (1975). Pyruvate dehydrogenase complex, pyruvate:ferredoxin oxidoreductase and lipoic acid content in microorganisms. *Arch Microbiol* **102**, 53–57.
- Bourguignon, J., Macherel, D., Neuburger, M. & Douce, R. (1992). Isolation, characterization, and sequence analysis of a cDNA clone encoding L-protein, the dihydrolipoamide dehydrogenase component of the glycine cleavage system from pea-leaf mitochondria. *Eur J Biochem* **204**, 865–873.
- Burnap, R., Koike, H., Sotiropoulou, G., Sherman, L. A. & Inoue, Y. (1989). Oxygen evolving membranes and particles from the transformable cyanobacterium *Synechocystis* sp. PCC6803. *Photosynth Res* **22**, 123–130.
- Camp, P. J. & Randall, D. D. (1985). Purification and characterization of the pea chloroplast pyruvate dehydrogenase complex. A source of acetyl-CoA and NADH for fatty acid synthesis. *Plant Physiol* **77**, 571–577.

- Carothers, D. J., Pons, G. & Patel, M. S. (1989). Dihydrolipoamide dehydrogenase: functional similarities and divergent evolution of the pyridine-disulphide oxidoreductases. *Arch Biochem Biophys* 268, 409–425.
- Danson, M. J. (1988). Dihydrolipoamide dehydrogenase: a 'new' function for an old enzyme? *Biochem Soc Trans* 16, 87–89.
- Danson, M. J., McQuattie, A. & Stevenson, K. J. (1986). Dihydrolipoamide dehydrogenase from halophilic archaeobacteria: purification and properties of the enzyme from *Halobacterium halobium*. *Biochemistry* 25, 3880–3884.
- Danson, M. J., Conroy, K., McQuattie, A. & Stevenson, K. J. (1987). Dihydrolipoamide dehydrogenase from *Trypanosoma brucei*. Characterization and cellular location. *Biochem J* 243, 661–665.
- Douglas, S. E. (1994). Chloroplast origins and evolution. In *The Molecular Biology of Cyanobacteria*, pp. 91–118. Edited by D. A. Bryant. Dordrecht, Boston & London: Kluwer.
- Eisenberg, D., Schwarz, E., Komaromy, M. & Wall, R. (1984). Analysis of membrane and surface protein sequences with hydrophobic moment plot. *J Mol Biol* 179, 125–142.
- Else, A. J., Hough, D. W. & Danson, M. J. (1993). Cloning, sequencing, and expression of *Trypanosoma brucei* dihydrolipoamide dehydrogenase. *Eur J Biochem* 212, 423–429.
- Engels, D. H., Lott, A., Schmid, G. H. & Pistorius, E. K. (1994). Inactivation of the water-oxidizing enzyme in manganese stabilizing protein-free mutant cells of the cyanobacterium *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 during dark incubation and conditions leading to photoactivation. *Photosynth Res* 42, 227–244.
- Engels, A., Kahmann, U., Ruppel, H. G. & Pistorius, E. K. (1997). Isolation, partial characterization and localization of a dihydrolipoamide dehydrogenase from the cyanobacterium *Synechocystis* PCC 6803. *Biochim Biophys Acta* 1340, 33–44.
- Fox, B. & Walsh, C. T. (1982). Mercuric reductase: purification and characterization of a transposon-encoded flavoprotein containing an oxidation–reduction-active disulfide. *J Biol Chem* 257, 2498–2503.
- Ghassemian, M. & Straus, N. A. (1996). Fur regulates the expression of iron-stress genes in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Microbiology* 142, 1469–1476.
- Grant, S. G. N., Jessee, J., Bloom, F. G. & Hanahan, D. (1990). Differential plasmid rescue from transgenic mouse DNAs in *Escherichia coli* methylation-restriction mutants. *Proc Natl Acad Sci USA* 87, 4645–4649.
- Grimme, L. H. & Boardman, N. K. (1972). Photochemical activities of a particle fraction P₁ obtained from the green alga *Chlorella fusca*. *Biochem Biophys Res Commun* 49, 1617–1620.
- Hein, S. & Steinbüchel, A. (1994). Biochemical and molecular characterization of the *Alcaligenes eutrophus* pyruvate dehydrogenase complex and identification of a new type of dihydrolipoamide dehydrogenase. *J Bacteriol* 176, 4394–4408.
- Holmes, C. F. G. & Stevenson, K. J. (1986). The amino acid sequence encompassing the active-site histidine residue of lipoamide dehydrogenase from *Escherichia coli* labelled with a bifunctional arsenoxide. *Biochem Cell Biol* 64, 509–514.
- Jackman, S. A., Hough, D. W., Danson, M. J., Stevenson, K. J. & Oppendoes, F. R. (1990). Subcellular localisation of dihydrolipoamide dehydrogenase and detection of lipoic acid in blood-stream forms of *Trypanosoma brucei*. *Eur J Biochem* 193, 91–95.
- Jorgensen, R. A., Rothstein, S. J. & Reznikoff, W. S. (1979). A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. *Mol Gen Genet* 177, 65–72.
- Karplus, P. A. & Schulz, G. H. (1987). Refined structure of glutathione reductase at 1.54 Å resolution. *J Mol Biol* 195, 701–729.
- Kikuchi, H. & Hiraga, K. (1982). The mitochondrial glycine cleavage system. Unique features of the glycine decarboxylation. *Mol Cell Biochem* 45, 137–149.
- Krüger, N., Oppermann, F. B., Lorenz, H. & Steinbüchel, A. (1994). Biochemical and molecular characterization of the *Clostridium magnum* acetoin dehydrogenase enzyme system. *J Bacteriol* 176, 3614–3630.
- Kubo, A., Sano, T., Saji, H., Hikaru, S., Kunisuke, T., Kondo, N. & Tanaka, K. (1993). Primary structure and properties of glutathione reductase from *Arabidopsis thaliana*. *Plant Cell Physiol* 34, 1259–1266.
- Kyte, J. & Doolittle, R.-F. (1992). A simple method for displaying the hydrophobic character of a protein. *J Mol Biol* 157, 105–132.
- Laddaga, R. A., Chu, L., Misra, T. K. & Silver, S. (1987). Nucleotide sequence and expression of the mercurial-resistance operon from *Staphylococcus aureus* plasmid p1258. *Proc Natl Acad Sci USA* 84, 5106–5110.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Luinenburg, I. & Coleman, J. R. (1990). A requirement for phosphoenolpyruvate carboxylase in the cyanobacterium *Synechococcus* PCC 7942. *Arch Microbiol* 154, 471–474.
- Matteucci, M. D. & Caruthers, M. H. (1981). Synthesis of deoxy-oligonucleotides on a polymer support. *J Am Chem Soc* 103, 3185–3191.
- Mattevi, A., Schierbeek, A. J. & Hol, W. G. J. (1991). The refined crystal structure of *Azotobacter vinelandii* lipoamide dehydrogenase at 2.2 Å resolution. A comparison with the structure of glutathione reductase. *J Mol Biol* 220, 974–995.
- Mattevi, A., Obmolova, G., Sokatch, J. R., Betzel, C. & Hol, W. G. J. (1992). The refined crystal structure of *Pseudomonas putida* lipoamide dehydrogenase complexed with NAD⁺ at 2.45 Å resolution. *Proteins* 13, 336–351.
- Mattevi, A., Obmolova, G., Kalk, K. H., van Berkel, W. J. H. & Hol, W. G. J. (1993). Three-dimensional structure of lipoamide dehydrogenase from *Pseudomonas fluorescens* at 2.8 Å resolution. Analysis of redox and thermostability properties. *J Mol Biol* 230, 1200–1215.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Myers, E. W. & Miller, W. (1988). Optimal alignment in linear space. *Comput Appl Biosci* 4, 11–18.
- Otulakowski, G. & Robinson, B. H. (1987). Isolation and sequence determination of cDNA clones for porcine and human lipoamide dehydrogenase. Homology to other disulfide oxidoreductases. *J Biol Chem* 262, 17313–17318.
- Patel, M. S. & Roche, T. E. (1990). Molecular biology and biochemistry of pyruvate dehydrogenase complexes. *FASEB J* 4, 3224–3233.
- Patel, M. S., Naik, S., Wexler, I. D. & Kerr, D. S. (1995). Gene regulation and genetic defects in the pyruvate dehydrogenase complex. *J Nutr* 125, 1735S–1757S.
- Rao, M.-J.-K. & Argos, P. (1986). A conformational preference parameter to predict helices to integral membrane proteins. *Biochim Biophys Acta* 869, 197–214.
- Richarme, G. & Heine, H.-G. (1986). Galactose- and maltose-

stimulated lipoamide dehydrogenase activities related to the binding-protein-dependent transport of galactose and maltose in toluenized cells of *Escherichia coli*. *Eur J Biochem* **156**, 399–405.

Robinson, B. H., Taylor, J. & Sherwood, G. (1977). Deficiency of dihydrolipoyl dehydrogenase (a component of the pyruvate and α -ketoglutarate dehydrogenase complexes): a cause of congenital chronic lactic acidosis in infancy. *Pediatr Res* **11**, 1198–1202.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schirmer, R. H. & Schulz, G. E. (1987). Pyridine nucleotide-linked sulfur metabolism. In *Pyridine Nucleotide Coenzymes: Chemical, Biochemical and Medical Aspects*, vol. B, pp. 333–379. Edited by D. Dolphin, R. Poulson & O. Avramovic. New York: Wiley.

Schmitz, O., Kentemich, T., Zimmer, W., Hundeshagen, B. & Bothe, H. (1993). Identification of the *nifJ* gene coding for pyruvate:ferredoxin oxidoreductase in dinitrogen-fixing cyanobacteria. *Arch Microbiol* **160**, 62–67.

Schulz, G. E., Schirmer, R. H., Sachsenheimer, W. & Pai, E. F. (1978). The structure of the flavoenzyme glutathione reductase. *Nature* **273**, 120–124.

Serrano, A. (1992). Purification, characterization and function of dihydrolipoamide dehydrogenase from the cyanobacterium *Anabaena* sp. strain PCC 7119. *Biochem J* **288**, 823–830.

Shames, S. L., Fairlamb, A. H., Cerami, A. & Walsh, C. T. (1986). Purification and characterization of trypanothione reductase from *Crithidia fasciculata*, a newly discovered member of the family of disulphide-containing flavoprotein reductases. *Biochemistry* **25**, 3519–3526.

Smith, A. J. (1973). Synthesis of metabolic intermediates. In *The Biology of Blue-green Algae*, pp. 1–38. Edited by N. G. Carr & B. A. Whitton. Oxford: Blackwell Scientific Publications.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goecke, N. M., Olson, B.

J. & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**, 76–85.

Staden, R. (1986). The current status and portability of our sequence handling software. *Nucleic Acids Res* **14**, 217–232.

Sumegi, B. & Srere, P. A. (1984). Complex I binds mitochondrial NAD-coupled dehydrogenases. *J Biol Chem* **259**, 15040–15045.

Taylor, A. E., Cogdell, R. J. & Lindsay, J. G. (1992). Immunological comparison of the pyruvate dehydrogenase complexes from pea mitochondria and chloroplasts. *Planta* **188**, 225–231.

Thieme, R., Pai, E. F., Schirmer, R. H. & Schulz, G. E. (1981). Three-dimensional structure of glutathione reductase at 2 Å resolution. *J Mol Biol* **152**, 763–782.

Turner, S. R., Ireland, R. & Rawsthorne, S. (1992). Purification and primary amino acid sequence of the L subunit of glycine decarboxylase. Evidence for a single lipoamide dehydrogenase in plant mitochondria. *J Biol Chem* **267**, 7745–7750.

Williams, C. H., Jr (1976). Flavin-containing dehydrogenases. In *The Enzymes*, vol. 13, 3rd edn, pp. 89–172. Edited by P. D. Boyer. New York: Academic Press.

Williams, C. H., Jr (1991). Lipoamide dehydrogenase, glutathione reductase, thioredoxin reductase, and mercuric ion reductase – a family of flavoenzyme transhydrogenases. In *Chemistry and Biochemistry of Flavoenzymes*, vol. III, pp. 121–211. Edited by F. Müller. Boca Raton, FL: CRC Press.

Williams, J. G. K. (1988). Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. *Methods Enzymol* **167**, 766–778.

Yeaman, S. L. (1986). The mammalian 2-oxoacid dehydrogenases: a complex family. *Trends Biochem Sci* **11**, 293–296.

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