Biochem. J. (1992) 288, 823–830 (Printed in Great Britain)

Purification, characterization and function of dihydrolipoamide dehydrogenase from the cyanobacterium *Anabaena* sp. strain P.C.C. 7119

Aurelio SERRANO
Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC y Universidad de Sevilla, Apdo. 1113, 41080-Sevilla, Spain

A dihydrolipoamide dehydrogenase (dihydrolipoamide:NAD^+ oxidoreductase, EC 1.8.1.4) (DLD) has been found in the soluble fraction of cells of both unicellular (*Synechococcus* sp. strain P.C.C. 6301) and filamentous (*Calothrix* sp. strain P.C.C. 7601 and *Anabaena* sp. strain P.C.C. 7119) cyanobacteria. DLD from *Anabaena* sp. was purified 3000-fold to electrophoretic homogeneity. The purified enzyme exhibited a specific activity of 190 units/mg and was characterized as a dimeric FAD-containing protein with a native molecular mass of 104 kDa, a Stokes' radius of 4.28 nm and a very acidic pI value of about 3.7. As is the case with the same enzyme from other sources, cyanobacterial DLD showed specificity for NADH and lipoamide, or lipoic acid, as substrates. Nevertheless, the strong acidic character of the *Anabaena* DLD is a distinctive feature with respect to the same enzyme from other organisms. The presence of essential thiol groups was suggested by the inactivation produced by thiol-group-reactive reagents and heavy-metal ions, with lipoamide, but not NAD^+, behaving as a protective agent. The function and physiological significance of *Anabaena* DLD are discussed in relation to the fact that 2-oxoacid dehydrogenase complexes have not been detected so far in filamentous cyanobacteria. Glycine decarboxylase activity, which might be involved in photorespiratory metabolism, has been found, however, in cell extracts of *Anabaena* sp. strain P.C.C. 7119 as the present study demonstrates.

INTRODUCTION

In eukaryotes and many aerobic eubacteria, dihydrolipoamide dehydrogenase (dihydrolipoamide:NAD^+ oxidoreductase, EC 1.8.1.4; DLD) catalyses the NAD^+-dependent oxidation of dihydrolipoamide, being an integral component of the 2-oxoacid (pyruvate and 2-oxoglutarate) dehydrogenase multienzyme complexes [1]. In addition to its extensively studied role in 2-oxo acid dehydrogenation, DLD functions in the glycine-cleavage system, namely glycine decarboxylase, a multienzyme complex which participates in glycine metabolism by animal mitochondria and micro-organisms [2]. In photosynthetic plant tissues the mitochondrial glycine dehydrogenase complex is involved in the metabolism of the glycine produced during photorespiration [3]. On the other hand, DLD could have cellular functions in addition to the aforementioned roles in multienzyme complexes, since the enzyme is present in both archaebacteria [4,5] and the parasite protozoan *Trypanosoma brucei* [6,7], in which the multienzyme complexes referred to above are absent. Lipoic acid and DLD have also been claimed to be involved in binding-protein-dependent and ubiquinone-mediated transport systems in *Escherichia coli* [8–10].

Cyanobacteria (blue–green algae) constitute a group of photosynthetic prokaryotes which perform oxygenic photosynthesis [11]. Lipoic acid has been demonstrated in *Anabaena*, although its cellular content is 10–50-fold lower than in *E. coli* and other micro-organisms [12]. DLD has, however, not been found to date in cyanobacteria. Acetate metabolism is severely restricted in these micro-organisms because the tricarboxylic acid cycle is blocked at the stage of 2-oxoglutarate oxidation [13]. The only report describing pyruvate dehydrogenase in the unicellular cyanobacterium *Anacystis nidulans* [12] has not been further corroborated. So far, neither pyruvate nor 2-oxoglutarate dehydrogenase activities has been detected in filamentous cyanobacteria [12,13]. Rather, pyruvate oxidation is driven by the less complex enzyme pyruvate:ferredoxin oxidoreductase, which has been extensively studied in several *Anabaena* species [14–16]. As stated above, DLD is involved in the photorespiratory carbon cycling in plants [3]. In the only complete report on photorespiratory metabolism in cyanobacteria [17], glycollate was found to be metabolized by *Anabaena cylindrica* mainly through the tartronic semialdehyde pathway, the glycine–serine pathway being of minor significance. To date, the glycine decarboxylase activity has not been demonstrated in any cyanobacterium.

We report here for the first time the presence of a DLD activity in cell extracts of both unicellular and filamentous cyanobacteria which lack 2-oxoacid dehydrogenase activities. DLD of the filamentous cyanobacterium *Anabaena* sp. strain P.C.C. [Pasteur Institute (Paris) Culture Collection] 7119 has been purified to homogeneity and characterized. The physiological role of DLD in the photorespiratory metabolism of *Anabaena* is discussed in the light of the finding of a glycine decarboxylase activity in cell-free extracts of this micro-organism.

MATERIALS AND METHODS

Chemicals

DL-Lipoamide (DL-6,8-thioctic acid amide), DL-lipoic acid (DL-6,8-thioctic acid), NAD^+, $NADP^+$, NADH, NADPH, GSSG, oxidized CoA, reduced CoA, FAD, 4-methyl-2-oxopentanoic acid, 3-methyl-2-oxobutanoic acid, L-glycine, L-lysine, piperazine, pyridoxal phosphate, Mops, Tris, quaternary aminoethyl (QAE)-Sephadex A-50, thiamin pyrophosphate, phenylmethanesulphonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), *p*-hydroxymercuribenzoate (*p*-HMB) and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDAC) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); DEAE-Sephacel, Polybuffer Exchanger 94, Polybuffer 74, Sephacryl S-300 (super-

Abbreviations used: EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; GR, GSSG reductase; *p*-HMB, *p*-hydroxymercuribenzoate; DLD, dihydrolipoamide dehydrogenase (dihydrolipoamide:NAD^+ oxidoreductase); NEM, *N*-ethylmaleimide; P.C.C., Pasteur Institute (Paris) Culture Collection; PMSF, phenylmethanesulphonyl fluoride; QAE, quaternary aminoethyl.

fine grade), CNBr-activated Sepharose 4B and protein standards for gel filtration and electrophoresis were from Pharmacia (Uppsala, Sweden); DEAE-cellulose DE-52 was from Whatman (Maidstone, Kent, U.K.); ingredients for PAGE were from Serva (Heidelberg, Germany). $NaH^{14}CO_3$ (55 mCi/mmol) was purchased from Amersham International (Bucks., U.K.). All other chemicals were of analytical grade and were acquired from Merck (Darmstadt, Germany).

Cyanobacterial strains and growth

Anabaena sp. strain P.C.C. 7119, *Synechococcus* sp. strain P.C.C. 6301 and *Calothrix* sp. strain P.C.C. 7601 [from the Pasteur Institute (Paris) Culture Collection] were grown photoautotrophically in liquid media with 20 mM-KNO_3 as nitrogen source under the conditions previously described [18]. The cells were harvested by centrifugation at 12000 *g* for 10 min, washed twice with 50 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mM-EDTA and 10 mM-2-mercaptoethanol (standard buffer), and stored at −70 °C before using. In the case of *Anabaena* sp., the yield of cells was about 3 g (wet wt.)/litre of culture medium.

Preparation of cell-free extracts

Unless otherwise specified, cyanobacterial cells were suspended in standard buffer containing 0.1 mM-PMSF at 0.1 g (wet wt.)/ml. The cells were disrupted in the cold with a Branson sonifier, model B-12, for 120 s at 70 W. Alternatively, cell lysis was achieved by gentle shaking of the cell suspension at 4 °C in the presence of both PMSF and 2% (v/v) Triton X-100. For glycine decarboxylase determinations, *Anabaena* sp. cells, previously resuspended in standard buffer containing 1 mM-PMSF and 2 mM-dithiothreitol at 2 g (wet wt.)/ml, were disrupted by using a pre-cooled French pressure cell at 110 MPa. Cell-free extracts were eventually obtained by centrifugation at 40000 *g* for 20 min at 4 °C.

Enzyme assays

The assay of DLD is based on measurement of the rate of NADH oxidation at 25 °C in the presence of a disulphide substrate. The reaction was monitored by the decrease of absorbance at 340 nm. Reaction mixtures contained, in a final volume of 1 ml: 100 μmol of Tris/HCl buffer, pH 7.0, 3 μmol of DL-lipoamide or, where stated, DL-lipoate [added in 30 μl of dimethyl sulphoxide/acetone (1:1, v/v)], 0.5 μmol of EDTA, 10 μmol of 2-mercaptoethanol, 0.2 μmol of NADH and an adequate amount of enzyme. For determination of optimal pH, Tris was replaced in the standard buffer by an equimolar mixture of Tris and piperazine (total concentration 0.1 M), and the pH value over the range 4.5–9.0 was adjusted by the addition of HCl. Glutathione reductase (EC 1.6.4.2) (GR) activity was assayed as described previously [19], by monitoring the GSSG-dependent oxidation of NADPH at 340 nm. Reactions were started by adding the enzyme, and the initial velocity was recorded. One unit of enzyme is defined as the amount which catalyses the oxidation of 1 μmol of NAD(P)H/min under the specified conditions. Studies on the effect of assay temperature on DLD activity were carried out in a Pye–Unicam SP8-150 recording spectrophotometer equipped with a thermostatically controlled cuvette holder. Temperature during the measurements was regulated with a Hetofrig refrigerated bath circulator provided with a calibrated thermocouple.

The overall activities of pyruvate, 2-oxoglutarate and branched-chain-2-oxoacid (4-methyl-2-oxopentanoic and 3-methyl-2-oxobutanoic) dehydrogenase complexes were assayed spectrophotometrically in crude extracts by monitoring the generation of NADH [20–22]. Glycine decarboxylase (EC 2.1.2.10) activity was measured in *Anabaena* sp. P.C.C. 7119 cell-free extracts by using the glycine–bicarbonate exchange reaction [3]. The 200 μl assay mixtures contained 25 mM-Mops/KOH, pH 7.0, 0.2 mM-pyridoxal phosphate, 20 mM-glycine, 2 mM-dithiothreitol, 20.5 mM-$NaH^{14}CO_3$ (100 mCi/mol) and crude enzyme extract. When indicated, sodium arsenite was added at a final concentration of 2 mM. Exchange reactions were terminated after 30 min at 30 °C by adding 25 μl of acetic acid. Dissolved $^{14}CO_2$ was driven off by shaking the samples under an i.r. lamp and the acid-stable [^{14}C]glycine was estimated by liquid-scintillation counting. The activity was expressed in nmol of CO_2 incorporated in the acid-stable fraction/h per mg of protein.

Gel electrophoresis

Non-denaturing PAGE was performed by the method of Jovin *et al.* [23] on tube gels (5 mm × 75 mm) containing 7.5% (w/v) acrylamide. Samples containing about 25 μg of protein were applied to the gels. SDS/PAGE was performed as described by Laemmli [24], using 7.5% (w/v) acrylamide slab gels. Proteins were stained with 1% (w/v) Coomassie Brilliant Blue in 7% (v/v) acetic acid for 4 h at room temperature. The NADH:lipoamide oxidoreductase activity was located in the gels by the disappearance of the green fluorescence exhibited by NADH under u.v. light (Sylvania BLB 15W). The gels were soaked for 20 min in the standard buffer plus 0.2 mM-NADH, washed with distilled water, and then placed in the standard buffer plus 0.3 mM-lipoamide for 10 min. In the presence of the disulphide substrate the green fluorescence faded as NADH was consumed by the enzyme. The DLD band was eventually located as a dark band when the gels were placed on a black background.

Coenzyme determination

The flavin coenzyme was quantified by measuring the fluorescence of an enzyme sample treated with 0.05 M-H_2SO_4 in a boiling-water bath, as described by Koziol [25]. The coenzyme released from DLD after trichloroacetic acid treatment was identified by t.l.c. on silica-gel plates by using purified flavin standards [26].

Physicochemical parameters

The molecular mass of native *Anabaena* sp. DLD was determined by gel filtration on a column (1.6 cm × 40 cm bed volume) of Sephacryl S-300 (superfine grade) calibrated with the following molecular-mass markers: horse spleen apoferritin, 440 kDa; bovine liver catalase, 232 kDa; yeast aldolase, 158 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; bovine pancreas chymotrypsinogen A, 25 kDa; horse heart myoglobin, 16.9 kDa; and bovine pancreas ribonuclease A, 13.7 kDa. The purified enzyme (50 μg in 0.5 ml of standard buffer plus 0.1 M-NaCl) was applied to the Sephacryl S-300 column equilibrated with the same buffer. Elution was carried out at a flow rate of 15 ml/h at 4 °C. Fractions (1 ml) were collected, checked for both absorbance at 280 nm and enzyme activity, and the $K_{av.}$ value calculated. The sedimentation coefficient, $s_{20,w}$, of *Anabaena* sp. DLD was determined by using 5–20%-(w/v)-sucrose gradients (total volume, 4.5 ml) in 5 ml Beckman cellulose nitrate tubes with catalase ($s_{20,w}$ = 11.25 S), aldolase (7.35 S), BSA (4.6 S) and chymotrypsinogen A (2.58 S) as protein standards. Sedimentation was for 12 h at 4 °C and 50000 rev./min (190000 *g* at $r_{av.}$ 6.51 cm) with an SW65 Ti rotor in a Beckman ultracentrifuge (model L2 65B). The Stokes' radius of the enzyme was estimated by gel filtration using the Sephacryl S-300 column described above. Subunit analysis was performed by SDS/PAGE using the following molecular-mass markers: rabbit muscle phosphorylase *b*, 97 kDa; BSA 67 kDa; ovalbumin, 43 kDa; and bovine erythrocyte carbonic anhydrase, 30 kDa.

The isoelectric point of native purified *Anabaena* sp. DLD was estimated by both chromatofocusing and pH-dependent binding analysis studies. Column chromatofocusing was carried out over the pH range 6.0–3.0 with the pH gradient created by washing a Polybuffer Exchanger 94 column (1 cm × 18 cm bed vol.) with 15 bed vol. of 10-fold-diluted Polybuffer 74/HCl (pH 3.0) at a flow rate of 12 ml/h, according to the instructions of the manufacturer [27]. The pH-dependent binding analysis, also used to estimate the pI of *Anabaena* sp. DLD, was performed as described by Yang & Langer [28]. Briefly, the pH-dependent absorption on QAE-Sephadex A-50 of purified enzyme (~ 20 units/ml) was monitored over the pH range 5.0–3.0. The buffer used throughout the whole pH range was 0.1 M-piperazine adjusted by additions of HCl to different pH values. The midpoint of the transition from totally bound to the ion-exchanger (pH higher than 4.0) to unbound (pH lower than 3.5) was considered to be the pI of the protein.

Fluorescence measurements and absorbance spectra of purified *Anabaena* sp. DLD were made in an Aminco–Bowman spectrophotofluorimeter and an Aminco DW-2 spectrophotometer respectively.

Protein measurements

Protein in crude extracts was determined by a modification of the Lowry method [29]. In latter stages of the purification procedure protein was determined by the method of Bradford [30] or by u.v. absorption [31]. BSA was used as the standard.

Purification of *Anabaena* sp. strain P.C.C. 7119 DLD

Unless otherwise stated, all the operations were performed at 0–4 °C. Centrifugations were carried out at 40000 *g* for 20 min in a Sorvall RC-2B centrifuge with a SS-34 rotor.

***(a)* Preparation of cell extract.** *Anabaena* sp. cell paste (20 g wet wt.) was suspended in standard buffer in the presence of PMSF and treated with sonic energy as described above. The suspension of broken cells was centrifuged to remove insoluble debris. The resulting supernatant, which contained virtually all the DLD activity, was considered as the crude extract and used for the next step.

***(b)* DEAE-cellulose ion-exchange chromatography and $(NH_4)_2SO_4$ treatment.** The crude extract (about 224 ml) was applied to a DE-52 anion-exchanger bed (3 cm × 30 cm bed vol.) equilibrated with standard buffer. The column was then washed with 4 bed vol. of the same buffer, and the enzyme was eluted at a flow rate of 30 ml/h by using a linear gradient of NaCl (0–0.75 M; total volume, 1200 ml). A saturated aq. NH_3-neutralized $(NH_4)_2SO_4$ solution was added with stirring to the pooled active fractions (about 120 ml), up to 30% (w/v) saturation. After 30 min, the protein solution was centrifuged, and the pellet, which contained most of the chlorophyll pigments, was discarded. The supernatant was dialysed three times against 12 litres of 6 mM-Tris/HCl buffer, pH 7.5, containing 0.2 mM-EDTA and 10 mM-2-mercaptoethanol. The dialysed protein solution was then centrifuged and the resulting supernatant was used for the next step.

***(c)* DEAE-Sephacel ion-exchange chromatography.** The dialysed protein solution was applied to a DEAE-Sephacel column (1.6 cm × 28 cm bed vol.) equilibrated with standard buffer. The column was then washed with 2 bed vol. of 0.1 M-piperazine/HCl buffer, pH 5.0, to remove most adsorbed phycobiliproteins. The enzyme was eluted by using a linear gradient of NaCl (0–0.8 M-NaCl; total volume 200 ml) in standard buffer at a flow rate of 20 ml/h. The active fractions were collected and dialysed as described above.

***(d)* ε-Lysyl-lipoyl–Sepharose 4B affinity chromatography.** The dialysed protein solution (about 30 ml) was applied to an ε-lysyl-lipoyl–Sepharose 4B column (1 cm × 15 cm bed vol.) prepared by coupling lipoic acid to a CNBr-activated Sepharose matrix using lysine as the spacer arm and the water-soluble carbodi-imide EDAC as a cross-linking agent [32]. The column, which was equilibrated with 10 mM-Tris/HCl buffer, pH 7.5, containing 0.2 mM-EDTA and 5 mM-2-mercaptoethanol, was washed with 4 bed vol. of the same buffer. The enzyme activity remained tightly bound to the column, being eluted as a sharp peak at about half of the linear NaCl gradient used (0–0.5 M, in the buffer used for equilibration; total volume, 50 ml). The pooled active fractions were concentrated and equilibrated in standard buffer plus 0.1 M-NaCl by ultrafiltration on a Diaflo YM-30 Amicon membrane.

***(e)* Sephacryl S-300 gel filtration.** The concentrated DLD solution (2 ml) was applied to a Sephacryl S-300 column (1.6 cm × 40 cm bed vol.) equilibrated with standard buffer supplemented with 0.1 M-NaCl, and eluted at a flow rate of 20 ml/h. The active fractions were pooled, concentrated and equilibrated in standard buffer by ultrafiltration, and stored at −70 °C in the dark. This protein solution was used as the final enzyme preparation for the experiments described below.

RESULTS

Presence and cellular localization of dihydrolipoamide dehydrogenase in cyanobacteria

A DLD activity was detected in both unicellular (*Synechococcus* sp.) and filamentous (*Anabaena* sp. and *Calothrix* sp.) cyanobacteria. In the three strains the enzyme activity was found in the soluble protein fraction. Specific activities in crude extracts of sonicated cells were in the range 50–80 munits/mg of protein, and similar values were obtained when using other mechanical disruption methods (e.g. French press). Lower values (20–30 munits/mg of protein) were found, however, after cell solubilization with the non-ionic detergent Triton X-100. The cyanobacterial DLD is, therefore, probably located in the cytoplasm. The DLD activity of cell-free extracts of *Anabaena* sp. is absolutely specific for NADH, no lipoamide-dependent NADPH oxidation being detectable (see below). Moreover, no NADH oxidation was observed with cystine, GSSG or oxidized CoA, even at the millimolar concentration range. Under the assay conditions used, the rate of NADH oxidation was directly proportional to the amount of enzyme extract in the assay, and all the enzymic activity was destroyed on boiling the extract for 5 min.

The possibility was considered that the observed activity was a side reaction of another disulphide oxidoreductase enzyme, the most likely candidate being glutathione reductase (GR), which is closely related to eubacterial and eukaryotic DLD both in its catalytic mechanism and in its amino acid sequence around the catalytically active disulphide bridge [33,34]. This is unlikely, however, because the purified GR of *Anabaena* sp. strain 7119 is an NADPH-specific enzyme [19]. Moreover, the optimum pH values of the two activities are different. In fact, in cell-free extracts of this cyanobacterium, no lipoamide-dependent NADH oxidation was detectable at pH 9.0, which is the optimum for *Anabaena* GR [19]. Finally, cyanobacterial DLD and GR activities are clearly separable by ion-exchange chromatography (see below), thus demonstrating that they reside on different proteins.

A DLD activity is present, therefore, in several cyanobacterial strains representative of this group of photosynthetic prokaryotes, many of which lack the NAD^+-linked 2-oxoacid dehydrogenase multienzyme complexes [13]. In fact, in agreement with results obtained with other *Anabaena* strains [13–15], attempts to detect pyruvate dehydrogenase, 2-oxoglutarate

Table 1. Purification of DLD from *Anabaena* sp. strain P.C.C. 7119

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification factor (fold)	Yield (%)
Crude extract	224.0	2054.8	127.4	0.062	1	100
30% $(NH_4)_2SO_4$ supernatant of DE-52 eluate	157.5	85.3	97.2	1.14	18	76
DEAE-Sephacel eluate	26.4	3.1	73.9	23.27	387	58
ε-Lysyl-lipoyl-Sepharose-4B eluate	7.1	0.43	70.1	163.40	2635	55
Sephacryl S-300 eluate	8.5	0.32	61.2	190.10	3066	48

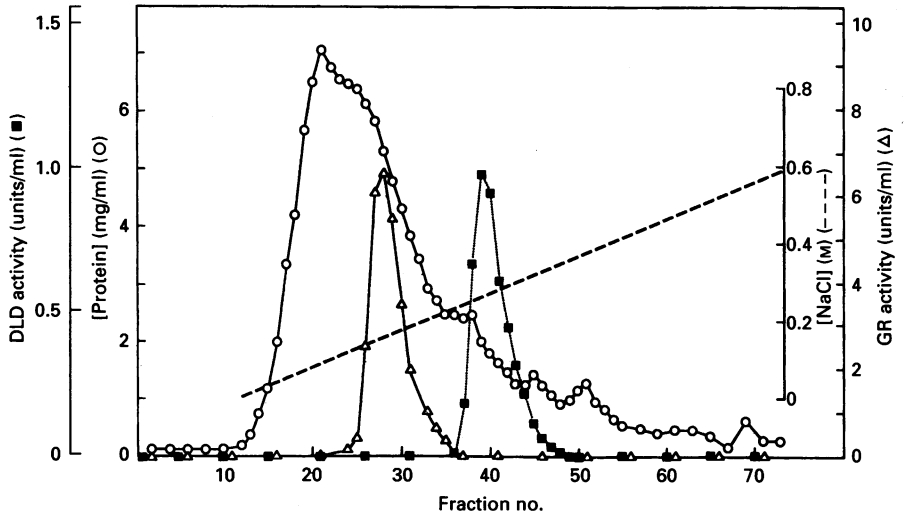

Fig. 1. Ion-exchange chromatography of a crude extract of *Anabaena* sp. strain P.C.C. 7119 on DEAE-cellulose

The enzyme preparation was applied to the DE-52 cellulose bed (3 cm × 30 cm bed volume), and adsorbed protein was eluted with a linear NaCl gradient as described in the Materials and methods section. Fractions (15 ml each) were collected. Note that a clear separation of DLD and GR activities was achieved.

dehydrogenase and branched-chain-2-oxoacid dehydrogenase activities in cell-free extracts of *Anabaena* sp. strain P.C.C. 7119 and *Synechococcus* sp. strain P.C.C. 6301 were unsuccessful.

DLD is also involved in the oxidative glycine decarboxylation of the photorespiratory cycle [3]. Therefore we looked for the activity of the glycine decarboxylase complex in *Anabaena* sp. strain P.C.C. 7119. The properties of this complex allow estimation of its activity by measuring the exchange between the carboxy carbon of glycine and bicarbonate by the use of ^{14}C. Crude extracts of *Anabaena* catalyse the ^{14}C exchange reaction with a specific activity level of about 20 nmol/h per mg of protein. The exchange activity was inhibited about 60% by 2 mM-arsenite, a glycine decarboxylase inhibitor [35]. Moreover, all the enzymic activity was destroyed on boiling the extract for 5 min. Therefore we can suggest that a glycine decarboxylase complex is present in the cyanobacterium *Anabaena* sp. strain P.C.C. 7119.

Enzyme purification

The purification protocol of *Anabaena* DLD, summarized in Table 1, yielded electrophoretically pure enzyme with a specific activity of about 190 units/mg of protein and a recovery rate of 48%. Ion-exchange-chromatography steps represented by far the largest contribution to the overall purification (purification factor ~ 400), because during these steps most of the chlorophyll–protein complexes and water-soluble phycobiliproteins, which constitute the bulk of proteins in crude extracts of cyanobacteria, were removed from the enzyme preparation. As Fig. 1 shows, by using a linear salt gradient, the DLD activity could be eluted from the DEAE-cellulose column as a single peak, being clearly separable from GR, the other outstanding NAD(P)H-dependent disulphide reductase present in cyanobacterial cells [19], which was eluted first. The behaviour of *Anabaena* DLD in ion-exchange chromatography, namely its remarkable affinity for the DEAE-cellulose matrix, indicated that it should be a very acidic protein, even more than GR [19], as was actually confirmed with the purified enzyme (see below).

Since chromatography on columns of lipoyl-bound Sepharose 4B has been used with success for the purification of DLD from mammalian tissues [32], we have used this matrix-bound ligand in the case of *Anabaena* DLD. At this stage, the enzyme preparation still contained, as checked by SDS/PAGE, a few contaminating proteins which were eventually separated from DLD by gel filtration on Sephacryl S-300. The enzyme activity was eluted from the Sephacryl S-300 column as a symmetrical peak with constant specific activity, a fact which speaks in favour of only one protein in all these fractions.

Homogeneity of the resulting enzyme preparation was checked

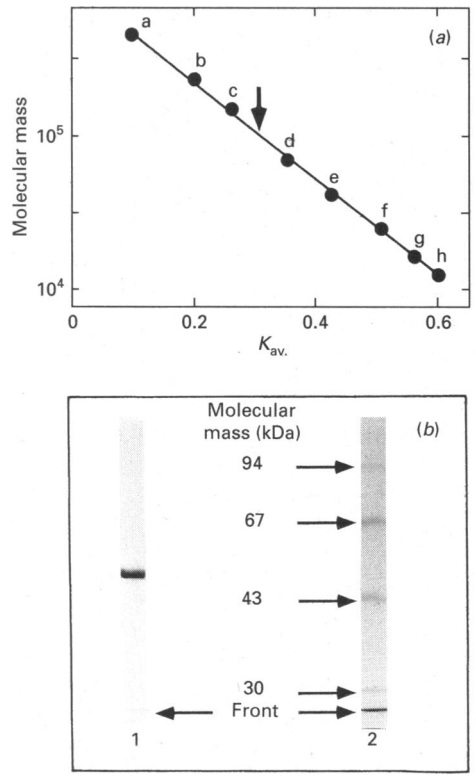

Fig. 2. Determination of the molecular mass of native (*a*) and denatured (*b*) DLD from *Anabaena* sp. strain P.C.C. 7119

(*a*) The purified enzyme (50 μg in 0.5 ml of standard buffer plus 0.1 M-NaCl) was applied to a column (1.6 cm × 40 cm bed vol.) of Sephacryl S-300 equilibrated with the same buffer. Elution was carried out at a flow rate of 15 ml/h at 4 °C. The column was calibrated with the following molecular-mass markers: ferritin (a), catalase (b), aldolase (c), BSA (d), ovalbumin (e), chymotrypsinogen A (f), myoglobin (g) and RNAase A (h). The arrow indicates the $K_{av.}$ value obtained for *Anabaena* DLD. (*b*) Purified enzyme (20 μg) was applied to an SDS/PAGE slab gel. Electrophoresis was conducted overnight at 4 °C by applying a constant current of 6 mA. On completion, the gel was stained with Coomassie Brilliant Blue to locate proteins; lane 1, DLD; lane 2, standards.

by PAGE and ultracentrifugation. The gel-filtration eluate exhibited on 7.5% polyacrylamide gels a single protein band which gave positive reaction for DLD activity *in situ* (results not shown). Moreover, the final enzyme preparation was also homogeneous when subjected to SDS/PAGE (Fig. 2). Ultracentrifugation on sucrose gradients revealed only one protein peak containing the enzyme activity. The purified enzyme preparation had an A_{280}/A_{456} ratio of about 5.6; this characteristic index of purity for flavoproteins is similar to those previously obtained for the enzyme from other sources [1].

Protein structure and cofactor identity

Molecular-mass determination of the native DLD from *Anabaena* sp. was performed by gel filtration on Sephacryl S-300 and yielded a value of 104 kDa (Fig. 2*a*). SDS/PAGE of the purified enzyme showed a single stained protein band indicating a molecular mass of 53 kDa for the DLD subunit (Fig. 2*b*). Thus the *Anabaena* sp. enzyme should have a homodimeric structure like that of other DLDs [1,33]. The sedimentation coefficient, $s_{20,w}$, of the pure enzyme was estimated to be 6.02 S by sucrose-density-gradient ultracentrifugation, and the Stokes' radius was determined to be 4.28 nm by Sephacryl S-300 gel filtration. From these data a native molecular mass of 106 kDa, in fairly good agreement with the above-mentioned value, and a frictional quotient, f/f_0, of 1.38 were calculated [36,37]. The strong acidic character of *Anabaena* DLD was shown by both column chromatofocusing (Fig. 3) and pH-dependent binding analysis experiments (results not shown), values of pI in the range 3.6–3.8 being estimated for different purified enzyme preparations. This result is in agreement with the behaviour in ion-exchange chromatography of *Anabaena* DLD shown above (since this enzyme exhibits higher affinity for the DEAE-cellulose matrix than GR, an acidic flavoprotein with a pI of 4.0 [19]). Moreover, in accordance with previous reports on bacterial DLD [38], only one peak, suggesting the existence of only one isoelectric form of the protein, was observed in chromatofocusing (Fig. 3).

The purified *Anabaena* DLD displayed a typical flavoprotein absorption spectrum with peaks at 274, 365 and 456 nm (see Fig. 4). The absorption coefficient (1 mM; 1 cm path length) at 456 nm is virtually the same to that of free FAD at 448 nm and was calculated to be 11.28 on the basis of a subunit molecular mass of 54 kDa. The flavin cofactor was released from the enzyme and identified as FAD by t.l.c.; its content was calculated to be 1.88 mol/mol of native enzyme. Therefore, the enzyme appears to contain two identical subunits, each one with one molecule of FAD. The native *Anabaena* sp. DLD also shows an intense flavin fluorescence at 540 nm when excited at 360 nm, thus indicating no quenching effect by the apoprotein.

Catalytic properties and substrate specificity

As it was the case for the enzyme in crude extracts, the purified *Anabaena* sp. DLD was specific for NADH as the electron donor. No detectable activity was observed when NADH was replaced by NADPH, even at alkaline values, at which the related disulphide-reducing flavoprotein GR of *Anabaena* sp. exhibits its maximum NADPH-dependent activity [19]. Lipoamide and, to a lesser extent (relative rate ~ 50%), lipoic acid, were disulphide substrates of the enzyme. The effect of pH on the activity of cyanobacterial DLD was also studied. Whereas the greatest activity was achieved over the pH range 6.0–7.0 with lipoamide as electron acceptor, a clear optimum pH value of about 6.5 was obtained using lipoate. Moreover, in contrast with the enzyme of eukaryotes [39,40] and many eubacteria [38], NAD^+ is not required for the catalytic oxidation of NADH by the cyanobacterial DLD. On the contrary, in the presence of the oxidized nucleotide even a slight inhibition (up to 10% of control rates) was observed, specially at neutral and alkaline pH values. The *Anabaena* DLD was also specific for the disulphide substrate, no detectable NADH oxidation being observed when L-cystine, GSSG or oxidized CoA were used, even at the millimolar concentration range, at different pH values. DLD activity is strongly affected by the ionic strength. By using 20 mM-buffer in the assays the optimum rate was found in the presence of 0.1 M-NaCl. The activity sharply decreased as the NaCl concentration increased over the range 0.15–0.3 M, a 90% inhibition being eventually obtained. Therefore the concentration of buffer in the reaction mixture was adjusted to give the maximum reaction rate. From reciprocal plots of reaction rates in the presence of a saturating concentration of the other substrate, apparent K_m values for lipoamide and NADH were estimated to be 1.6 mM and 21 μM respectively. Optimum temperature for enzyme activity was 51 °C, and an activation energy (E_a) of about 25 kJ/mol could be calculated from the Arrhenius plot in the range of temperatures between 10 and 50 °C. The *Anabaena* DLD is quite resistant to thermal inactivation. No significant enzyme inactivation (less than 5%) was observed after incubation for 5 min in standard buffer at different temperature values up to 60 °C.

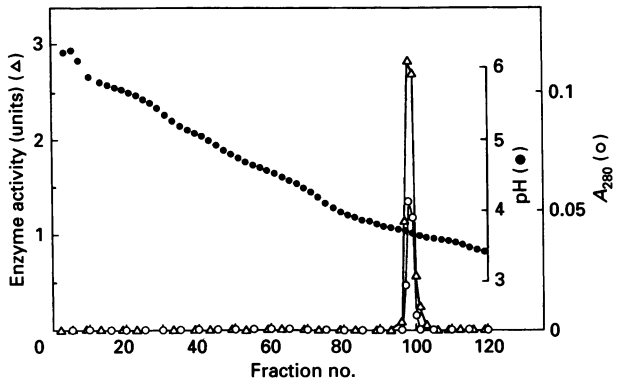

Fig. 3. Analytical chromatofocusing on Polybuffer Exchanger 94 of DLD purified from *Anabaena* sp. strain P.C.C. 7119

A sample containing about 0.1 mg of native protein was applied to a Polybuffer Exchanger 94 column (1 cm × 18 cm bed vol.) equilibrated with 25 mM-piperazine/HCl buffer, pH 6.4, containing 0.5 mM-EDTA and 10 mM-2-mercaptoethanol. The enzyme was eluted by using a pH gradient generated by 15 bed vol. of Polybuffer 74/HCl, pH 3.0 (7.5 μmol/pH unit per ml). Fractions (1.5 ml) were collected.

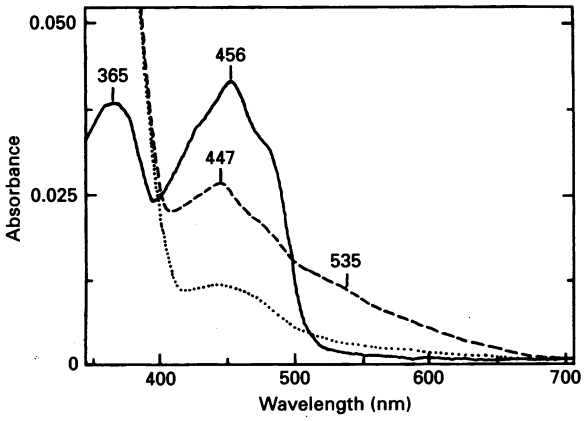

Fig. 4. Absorption spectra of DLD purified from *Anabaena* sp. strain P.C.C. 7119

Spectra were taken under an N_2 atmosphere at 15 °C in 1 ml anaerobic cuvettes containing 1.7 μM purified DLD in standard buffer. The oxidized protein (——) was reduced by addition of 1.5 μmol of NADH (----) and further addition of 0.1 μmol of *p*-HMB (·····). NADH and *p*-HMB were added in 10 μl aliquots by using a gas-tight syringe.

Table 2. Inhibition of *Anabaena* sp. strain P.C.C. 7119 DLD by thiol reagents and heavy-metal ions

The preincubation mixtures (1 min, 25 °C) included, in a final volume of 0.1 ml, 100 μmol of Tris/HCl buffer, pH 7.0, the above indicated additions and an adequate amount of purified enzyme. An aliquot of 0.02 ml of these mixtures was added to 0.98 ml of the assay mixture.

Additions	Relative activity (%)
None	100*
DL-Lipoamide (2 mM)	100
NADH (0.5 mM)	97
p-HMB (20 μM)	19
+DL-Lipoamide (2 mM)	82
+NADH (0.5 mM)	0
Sodium arsenite (0.5 mM)	40
+DL-Lipoamide (2 mM)	85
+NADH (0.5 mM)	0
$ZnCl_2$ (0.5 mM)	0
+DL-Lipoamide (2 mM)	56
$HgCl_2$ (0.5 mM)	0
$CuSO_4$ (0.5 mM)	0

* An activity of 100% corresponded to 176 munits.

The cyanobacterial DLD showed a marked sensitivity to thiol reagents (NEM and *p*-HMB), *vicinal* dithiol reagents (sodium arsenite) and heavy-metal ions (Table 2). The sensitivity of the enzyme to thiol reagents was increased if NADH was present in the preincubation mixture; in contrast, lipoamide, but not $NAD(P)^+$, behaved as a protective agent. When incubated in the presence of Zn^{2+}, Cu^{2+} or Hg^{2+}, which are known to form chelates with dithiol groups [41], the enzyme activity was completely inhibited, lipoamide behaving again as it did with thiol-group-reactive reagents. However, EDTA, even at the millimolar concentration range, did not reverse heavy-metal inhibition. The increased inhibition by incubation with NADH is due to the generation of the reduced form of the enzyme (see Fig. 4) in which the catalytic dithiol group become reactive [1]. Cyanobacterial DLD is not, therefore, different from DLD from other sources in that it has a disulphide group reducible by NADH in the catalytic centre for lipoamide [1,33].

Several spectral changes were observed when the DLD of *Anabaena* was incubated in the presence of an excess of NADH (Fig. 4); namely, a decrease of absorbance in the range 400–500 nm, the 456 nm peak of the oxidized enzyme being shifted to 447 nm, as well as the appearance of new absorbance at longer wavelengths, up to 670–680 nm. Similar changes in DLD from other sources have been ascribed to the formation of an e.p.r.-silent charge-transfer complex when the enzyme was reduced by the nicotinamide nucleotide [1]. As Fig. 4 shows, subsequent treatment with a thiol-group-reactive reagent, such as *p*-HMB, produced a total reduction of the enzyme-bound flavin, as suggested by the dramatic decrease in absorbance over the 400–500 nm range and the simultaneous disappearance of the long-wavelength absorption. These results are consistent with the participation of thiol groups, probably the catalytic dithiol, in the stabilization of the spectral changes observed.

DISCUSSION

The above results represent the first report of a cyanobacterial DLD, the purification procedure described herein being an effective way to obtain the enzyme of *Anabaena* sp. strain P.C.C. 7119 in a homogeneous state, with a specific activity which is similar to those reported for DLDs purified from other sources.

The cyanobacterial DLD is a homodimeric FAD-containing protein with a molecular mass of about 100 kDa, which is specific for NAD(H) as coenzyme. It is noteworthy that the gross structures of DLDs isolated from different sources appear to be very similar, all of them being homodimeric flavoproteins (Table 3). However, in recent reports on anaerobic glycine-utilizing bacteria [42,43] several new types of DLDs have been defined which differ from the classical enzyme in molecular mass and nicotinamide-nucleotide specificity, although the enzyme from *Eubacterium acidaminophilum* is actually a thioredoxin reductase-like flavoprotein exhibiting DLD activity ([44]; see Table 3). Structural studies described here show that the *Anabaena* DLD

Table 3. Comparison of some structural and functional properties of DLDs from different sources

Source (reference)	Molecular mass (kDa)		pI	Coenzyme		Metabolic role
	Native	Subunit*		NAD(H)	NADP(H)	
Eukaryotes						
Mammalian tissues, yeast [1,33,59,60]	100–115	50–55	6–7	+	–	Mitochondrial 2-oxo acid oxidation
Trypanosoma brucei [6]	ND†	ND	ND	+	–	Function not known, membrane-bound enzyme
Trypanosoma cruzi [61]	100	55	ND	+	–	Probably 2-oxo acid oxidation
Spinach leaves [40]	102	ND	ND	+	–	Mitochondrial 2-oxo acid and glycine oxidation, but cf. [49]
Spinach chloroplasts [49]	ND	ND	ND	+	–	Function not clearly established (rhodanese cofactor?)
Prokaryotes						
Aerobic eubacteria [1,33]	100–110	50–55	ND	+	–	2-Oxo acid oxidation, but cf. [54] for a third DLD of *P. putida* without known function
Archaebacteria [5,47,62]	115–122	58–61	ND	+	–	Function not known
Cyanobacteria‡ (*Anabaena* sp.)	104	53	3.7	+	–	Probably photorespiratory glycine oxidation
Anaerobic, glycine-utilizing bacteria						
Clostridium cylindrosporum [42]	105	52	ND	+	+	Uric acid and glycine utilization
Clostridium sporogenes [42]	100	52	ND	–	+	Function not known
Eubacterium acidaminophilum [26]	68	34.5	ND	(+)§	+	Glycine utilization

* Values obtained by SDS/PAGE. A consensus value of ~ 50 kDa, calculated from the amino acid sequences deduced from the nucleotide sequences, has been recently reported for the mammalian and eubacterial DLD subunits [33].
† ND, not determined.
‡ The present work.
§ NADP(H) was the preferred cofactor; this enzyme is actually a thioredoxin reductase-like flavoprotein exhibiting some DLD activity [44].

is a typical DLD enzyme with essential thiol groups, but it is characterized by having a very acidic pI in comparison with the mammalian DLD (see Table 3). Interestingly this characteristic is shared with two other outstanding flavoproteins of *Anabaena* sp. strain P.C.C. 7119 which have pI values over the range of 4.0–4.5, namely the closely related disulphide reductase GR [19], and ferredoxin:$NADP^+$ oxidoreductase [45], a component of the photosynthetic electron-transport chain. Although pI values of bacterial DLDs have not been reported, studies on electrophoretic mobility and amino acid composition, performed respectively with the enzymes of *E. coli* [46] and *Halobacterium halobium* [47], indicate that they should be more acidic than mammalian DLDs, which are almost electroneutral proteins (see Table 3). It is noteworthy, however, that an electroneutral pI of 6.8 has been determined for asparagus mitochondrial DLD [48], being the only pI value reported for a higher-plant enzyme.

The presence of a DLD activity in three cyanobacterial species, one unicellular and two filamentous, has been clearly established in the present work. Lipoic acid has been unequivocally demonstrated in the cyanobacteria *Anacystis nidulans* (unicellular) and *Anabaena cylindrica* (filamentous) [12]. The disulphide substrate for DLD is present, therefore, in these microorganisms, although its cellular content varies notably, depending on the cyanobacterial species examined [12]. Cyanobacteria are so far the only group of photosynthetic prokaryotes in which the presence of a DLD has been investigated. The enzyme has been found also in leaves [40] and isolated chloroplasts [49] from spinach (Table 3). Indeed, lipoic acid is a known chloroplast constituent, although its physiological function in these organelles is not yet clear [41]. Although plant mitochondria and proplastids have a pyruvate dehydrogenase activity, the presence of such multienzyme complex in mature chloroplasts has not been demonstrated [50], and these organelles have been reported to import acetate from other cellular compartments [51].

The presence of a true DLD in filamentous cyanobacteria raises the question of its function *in vivo*. It is well known that DLD is a component of two outstanding multienzyme complexes, namely 2-oxoacid dehydrogenase and glycine decarboxylase. However, most cyanobacteria so far examined do not have the 2-oxoacid dehydrogenase multienzyme complexes [12–15]. Pyruvate is presumably metabolized by these organisms via pyruvate:ferredoxin oxidoreductase, which has been demonstrated in both filamentous [14,15] and unicellular [52] cyanobacterial species. In agreement with these data, no 2-oxoacid dehydrogenase activities have been detected in the strains of *Anabaena* and *Synechococcus* used in the present work. Therefore the DLD found in these organisms do not seem to be involved in 2-oxoacid oxidation. Lipoic acid and DLD have also been found in the absence of 2-oxoacid dehydrogenase complexes in archaebacteria [4,5,53], which possess both pyruvate and 2-oxoglutarate ferredoxin-dependent oxidoreductases [5], and in the endoparasite *T. brucei* [7]. Furthermore, a third DLD, which does not appear to be involved in 2-oxoacid oxidation, has been found recently in *Pseudomonas putida* [54].

As stated above, it is well known that DLD is involved in photorespiratory glycine decarboxylation in plant tissues. Although the photorespiratory glycine–serine pathway seems to be a non-major route in filamentous cyanobacteria [17], the presence of a glycine decarboxylase complex in crude extracts of *Anabaena* sp. strain P.C.C. 7119 has actually been shown in the present work. Therefore the cyanobacterial DLD might be active in the glycine decarboxylase system and the photorespiration. The high enrichment factor obtained for *Anabaena* sp. DLD also agrees with an involvement of this enzyme in photorespiratory

glycine metabolism. To my knowledge this is the first time that a function in photorespiratory metabolism which has been so far demonstrated only in green tissues of plants has been proposed for a bacterial DLD.

Apart from these specific metabolic roles, DLD may also have a general function in reducing intracellular thiol groups. In fact, it has been reported that reduced lipoic acid dramatically enhances glyceraldehyde-3-phosphate dehydrogenase activity in both heterocysts and vegetative cells of several *Anabaena* species [55]. Furthermore, the highly hydrophobic character of lipoic acid and its derivate lipoamide allows them to permeate biological membranes at high rates. These compounds should therefore be particularly efficient in the maintenance of thiol groups of membrane proteins in the proper redox state [56]. In this respect it is noteworthy that recent work [57] supports the contention that dihydrolipoic acid has an important role in mitochondrial ATP synthesis, improving the energetic state of cells, i.e. increases ATP synthase activity and prevents ATPase activation. Finally, the possible involvement of cyanobacterial DLD in binding-protein-dependent transport systems, as it has been claimed in *E. coli* [9,10,58] and the eukaryotic parasite *T. brucei*, in which DLD seems to be associated with the cytoplasmic membrane [7], appears to be rather unlikely, as no evidence has been found in the present study for a localization of cyanobacterial DLD in the cytoplasmic membrane.

I thank Dr. E. Flores for critically reading the manuscript before its submission and his helpful advice during the performance of ^{14}C experiments. The help of F. Valverde is also acknowledged. This work was supported in part by grant BT45/85 from CAICYT and grants PB 87/401 and PB 90/99 from DGICYT (Ministerio de Educación y Ciencia, Spain).

REFERENCES

1. Williams, C. H., Jr. (1976) Enzymes 3rd Ed. **13**, pp. 89–173
2. Kikuchi, G. (1973) Mol. Cell. Biochem. **1**, 169–187
3. Blackwell, R. D., Murray, A. J. S. & Lea, P. J. (1990) Methods Plant Biochem. **3**, 129–144
4. Danson, M. J. (1988) Biochem. Soc. Trans. **16**, 87–89
5. Danson, M. J. (1988) Adv. Microb. Physiol. **29**, 165–231
6. Danson, M. J., Conroy, K., McQuattie, A. & Stevenson, K. J. (1987) Biochem. J. **243**, 661–665
7. Jackman, S. A., Hough, D. W., Danson, M. D., Stevenson, K. J. & Opperdoes, F. R. (1990) Eur. J. Biochem. **193**, 91–95
8. Owen, P., Kaback, H. R. & Graeme-Cook, K. A. (1980) FEMS Microbiol. Lett. **7**, 345–348
9. Richarme, G. (1985) J. Bacteriol. **162**, 286–293
10. Richarme, G. & Heine, H. H. (1986) Eur. J. Biochem. **156**, 399–405
11. Carr, N. G. & Whitton, B. A. (eds.) (1982) The Biology of Cyanobacteria, Blackwell Scientific Publications, Oxford
12. Bothe, H. & Nolteernsting, U. (1975) Arch. Microbiol. **102**, 53–57
13. Smith, A. J. (1973) in The Biology of Blue–Green Algae (Carr, N. G. & Whitton, B. A., eds.), pp. 1–38, Blackwell Scientific Publications, Oxford
14. Bothe, H., Falkenberg, B. & Nolteernsting, U. (1974) Arch. Microbiol. **96**, 291–304
15. Leach, C. K. & Carr, N. G. (1971) Biochim. Biophys. Acta **245**, 165–174
16. Neuer, G. & Bothe, H. (1982) Biochim. Biophys. Acta **716**, 358–365
17. Codd, G. A. & Stewart, W. D. P. (1973) Arch. Mikrobiol. **94**, 11–28
18. Serrano, A. & Losada, M. (1988) Plant Physiol. **86**, 1116–1119
19. Serrano, A., Rivas, J. & Losada, M. (1984) J. Bacteriol. **158**, 317–324
20. Pettit, F. H., Yeaman, S. J. & Reed, L. J. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 4881–4885
21. Reed, L. J. & Mukherjee, B. B. (1969) Methods Enzymol. **13**, 55–61
22. Schwartz, E. R. & Reed, L. J. (1970) Biochemistry **9**, 1434–1439
23. Jovin, T., Charamback, A. & Naughton, M. A. (1964) Anal. Biochem. **9**, 351–364
24. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
25. Koziol, J. (1971) Methods Enzymol. **18B**, 253–285
26. Fazekas, A. G. & Kokai, K. (1971) Methods Enzymol. **18B**, 385–398
27. Pharmacia Fine Chemicals (1980) Chromatofocusing, Pharmacia Fine Chemicals, Uppsala, Sweden
28. Yang, V. C. & Langer, R. (1985) Anal. Biochem. **147**, 148–155
29. Yocum, R. R., Blumberg, P. M. & Strominger, J. L. (1974) J. Biol. Chem. **249**, 4863–4871
30. Bradford, M. (1976) Anal. Biochem. **72**, 248–254
31. Layne, E. (1957) Methods Enzymol. **3**, 447–454
32. Visser, J. & Strating, M. (1975) Biochim. Biophys. Acta **384**, 69–80
33. Carothers, D. J., Pons, G. & Patel, M. S. (1989) Arch. Biochem. Biophys. **268**, 409–425
34. Rice, D. W., Schulz, G. E. & Guest, J. R. (1984) J. Mol. Biol. **174**, 483–496
35. Walker, G. H., Oliver, D. J. & Sarojini, G. (1982) Plant Physiol. **70**, 1465–1469
36. Phelps, C. F. (1974) in Chemistry of Macromolecules (Biochemistry, series 1, vol. 1) (Gutfreund, H., ed.), pp. 325–374, Butterworths, London
37. Siegel, L. M. & Monty, K. J. (1966) Biochim. Biophys. Acta **112**, 346–362
38. Scouten, W. H. & McManus, I. R. (1971) Biochim. Biophys. Acta **227**, 248–263
39. Massey, V. (1966) Methods Enzymol. **9**, 272–278
40. Matthews, J. & Reed, L. J. (1963) J. Biol. Chem. **238**, 1869–1876
41. Jocelyn, P. C. (1972) The Biochemistry of the SH Group, Academic Press, London
42. Dietrichs, D. & Andreesen, J. R. (1990) J. Bacteriol. **172**, 243–251
43. Freudenberg, W., Dietrichs, D., Lebertz, H. & Andreesen, J. R. (1989) J. Bacteriol. **171**, 1346–1354
44. Dietrichs, D., Meyer, M., Schmidt, B. & Andreesen, J. R. (1990) J. Bacteriol. **172**, 2088–2095
45. Serrano, A. (1986) Anal. Biochem. **154**, 441–448
46. Cohn, M. L., Wang, L., Scouten, W. & McManus, I. R. (1968) Biochim. Biophys. Acta **159**, 182–185
47. Danson, M. J., McQuattie, A. & Stevenson, K. J. (1986) Biochemistry **25**, 3880–3884
48. Yanagawa, H. & Egami, F. (1976) J. Biol. Chem. **251**, 3637–3644
49. Jacobi, G. & Öhlers, U. (1968) Z. Pflanzenphysiol. **58**, 193–206
50. Stumpf, P. K. (1980) in The Biochemistry of Plants, vol. 4 (Stumpf, P. K., ed.), pp. 177–204, Academic Press, New York
51. Leech, R. M. & Murphy, D. J. (1976) in The Intact Chloroplast (Barber, J., ed.), pp. 365–401, Elsevier/North-Holland Biomedical Press, Amsterdam
52. Bothe, H., Neuer, G., Kalbe, I. & Eisbrenner, G. (1980) in Nitrogen Fixation (Stewart, W. D. P. & Gallon, J. R., eds.), pp. 83–112, Academic Press, London
53. Pratt, K. J., Carles, C., Carne, T. J., Danson, M. J. & Stevenson, K. J. (1989) Biochem. J. **258**, 749–754
54. Burns, G., Sykes, P. J., Hatter, K. & Sokatch, J. R. (1989) J. Bacteriol. **171**, 665–668
55. Papen, H., Neuer, G., Sauer, A. & Bothe, H. (1986) FEMS Microbiol. Lett. **36**, 201–206
56. Malbon, C. C., George, S. T. & Moxham, C. P. (1987) Trends Biochem. Sci. **12**, 172–175
57. Zimmer, G., Mainka, L. & Krüger, E. (1991) Arch. Biochem. Biophys. **288**, 609–613
58. Richarme, G. (1989) J. Bacteriol. **171**, 6580–6585
59. Kenney, W. C., Zakim, D., Hogue, P. K. & Singer, T. P. (1972) Eur. J. Biochem. **28**, 253–260
60. Wilson, J. E. (1971) Arch. Biochem. Biophys. **144**, 216–223
61. Lohrer, H. & Krauth-Siegel, R. L. (1990) Eur. J. Biochem. **194**, 863–869
62. Sundquist, A. R. & Fahey, R. C. (1988) J. Bacteriol. **170**, 3459–3467

Received 12 May 1992/23 June 1992; accepted 26 June 1992