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MOLECULAR HETEROGENEITY OF FERREDOXIN:NADP$^+$ OXIDOREDUCTASE FROM THE CYANOBACTERIUM *ANABAENA CYLINDRICA*

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Summary

The enzyme ferredoxin:NADP$^+$ oxidoreductase (EC 1.18.1.2) from whole filaments of *Anabaena cylindrica* can be separated into four major fractions by chromatography on phosphocellulose; chromatography using ferredoxin-Sepharose 4B proved to be less satisfactory in separating the fractions. The purified fractions, designated 1, 2, 3 and 4, all showed diaphorase and ferredoxin-dependent cytochrome *c* reductase activity. The major fractions present were 2 and 3 which were each obtained in an electrophoretically homogeneous state (forms 2 and 3) and represented 30—37% and 30—42%, respectively, of the total enzyme activity. Each was a monomeric species with a molecular weight of approx. 33 000 as determined by gel filtration and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Evidence for the presence of a 70 000 molecular weight dimer was also obtained. Forms 2 and 3 had isoelectric points of 5.75 and 6.0, respectively, had similar kinetic properties and were flavoproteins. Extracts of isolated heterocysts showed no form 2 or 3 activity but contained a single form which closely resembled one of the species present in fraction 4; fraction 1 may have been a purification artifact because it was not detected in crude extracts of the cyanobacterium.

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Abbreviations: PMSF, phenylmethylsulphonyl fluoride; DCPIP, 2,6-dichlorophenolindophenol; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide.

Introduction

Ferredoxin:$NADP^+$ oxidoreductase (EC 1.18.1.2) is a component of the photosynthetic electron transport chain both in photosynthetic eukaryotes and cyanobacteria [1—4] and may function as a NADPH-specific diaphorase (NADPH:(acceptor)oxidoreductase, EC 1.6.99.1) [5,6], as a pyridine nucleotide transhydrogenase (NADPH:NAD^+ oxidoreductase, EC 1.6.1.1) [7,8] and as a NADPH-dependent cytochrome *f* reductase [9—11]. Enzyme activity has been detected in heterocysts and vegetative cells of the N_2-fixing cyanobacterium *Anabaena cylindrica* [12,13], although photoreduction of $NADP^+$ may not occur in heterocysts (see Ref. 12) and the enzyme may catalyse the NADPH-dependent reduction of ferredoxin there, using NADPH generated by reactions of the oxidative pentose phosphate pathway [13—17]. This could provide a supply of reduced ferredoxin for N_2 fixation in heterocysts [15] using carbon compounds transferred from vegetative cells [18,19].

Recently, it has been shown that multiple molecular forms of ferredoxin: $NADP^+$ oxidoreductase occur in chloroplasts and in whole filaments of cyanobacteria [20—30]. Two forms, isolated from *Spirulina platensis*, have been purified and characterised [29] and, in the case of *Nostoc* strain MAC, five forms have been distinguished on the basis of isoelectric focussing [30].

In this paper we present evidence that multiple molecular forms of ferredoxin:$NADP^+$ oxidoreductase occur in whole filament extracts of *A. cylindrica*, and provide preliminary evidence that the complement of enzyme forms in heterocysts is substantially different from that of vegetative cells.

Methods

Organism. A. cylindrica Lemm. (CU 1403/2a) was used in axenic culture.

Growth conditions. The cyanobacterium was grown in batch or continuous culture in BG-11 medium [31] in the absence of combined nitrogen at 28°C and 3000 lux.

Enzyme extraction and purification. Cultures (50—100 l, in the late exponential growth phase) were harvested by centrifugation, washed twice in 50 mM Tris-HCl buffer (pH 7.5) and resuspended in the same buffer. Cells were disrupted by passage through a French pressure cell at 110 MPa. $(NH_4)_2SO_4$ was added to 30% saturation for 30 min, followed by centrifugation at 35 000 × *g* for 30 min. The resulting supernatant was treated with $(NH_4)_2SO_4$ to 100% saturation for 30 min and recentrifuged at 35 000 × *g* for 30 min. The pellet was resuspended in a minimal volume of 50 mM Tris-HCl buffer (pH 7.5) and, after removal of $(NH_4)_2SO_4$ by dialysis, the resulting solution was chromatographed on DEAE-cellulose (30 × 3.2 cm column) and eluted with a gradient of 0—0.5 M KCl in 50 mM Tris-HCl (pH 7.5). Active fractions were combined, dialysed against 5 mM Tris-HCl (pH 7.5) and chromatographed on phosphocellulose (20 × 1.5 cm column). The enzyme was eluted with a gradient of 5—500 mM Tris-HCl (pH 7.5). Active fractions were combined and further purified as necessary; all forms were concentrated to less than 3 ml by vacuum dialysis and chromatographed on Sephadex G-150 (90 × 2.5 cm column), and forms 1, 2 and 3 (see below) were rechromatographed on phosphocellulose when necessary. All operations were carried out at 4°C.

Isolation of heterocysts and preparation of heterocyst cell-free extracts. Heterocysts were isolated from log phase cultures as before [32] and cell-free extracts were prepared from isolated heterocysts by passage through a French pressure cell at 110 MPa followed by centrifugation at 35 000 × *g* for 30 min. The 35 000 × *g* supernatant was used.

Purification of the enzyme by affinity chromatography using ferredoxin-Sepharose 4B. Ferrodoxin was purified from *A. cylindrica* by the method of Andrew et al. [33], purified preparations being electrophoretically homogeneous and having a 421 : 275 nm absorbance ratio of approx. 0.5.

Purified ferredoxin was coupled to Sepharose 4B as described by Ida et al. [34] (2 mg ferredoxin/g Sepharose 4B) and this was used to purify the enzyme by affinity chromatography. The enzyme was applied to the ferredoxin-Sepharose 4B column (5 × 1.5 cm) in 5 mM Tris-HCl (pH 7.5) and eluted with a gradient of 5—200 mM KCl in 5 mM Tris-HCl (pH 7.5).

Ferredoxin: $NADP^+$ oxidoreductase assays. Diaphorase activity. The reaction mixture contained, in addition to enzyme, in 1 ml: Tris-HCl buffer (pH 7.5), ionic strength 0.1/100 nmol DCPIP/60 nmol NADPH. The rate of reduction of DCPIP at 28°C was measured at 600 nm.

Ferrodoxin-dependent cytochrome *c* reductase activity. The reaction mixture contained, in addition to enzyme, in 1 ml: Tris-HCl buffer (pH 7.5), ionic strength 0.1/24 nmol horse heart cytochrome *c*/20 μg *A. cylindrica* ferredoxin/60 nmol NADPH. The rate of reduction of cytochrome *c* at 28°C was measured at 550 nm.

Determination of molecular weights. These were estimated by gel filtration on Sephadex G-150 (90 × 2.5 cm column). The molecular weight standards were alcohol dehydrogenase (150 000), bovine serum albumin (68 000), ovalbumin (46 000), chymotrypsinogen A (25 700) and cytochrome *c* (12 500).

Gel electrophoresis. Polyacrylamide gel electrophoresis was carried out according to Davis [35] on 7.5% (w/v) gel columns using Tris-glycine buffer (pH 8.8) and a current of 3 mA per gel tube. Bromophenol blue was used as the tracking dye. Gels were stained for protein with 0.2% (w/v) Coomassie brillant blue in acetic acid/methanol/water (10 : 45 : 45 by vol.) for 5—15 h at room temperature, destained with acetic acid/methanol/water (10 : 25 : 65 by vol.) and scanned at 600 nm using a Gilford 2410-S gel scanner. Enzyme activity in gels [3] was visualized by incubating in 50 mM Tris-HCl buffer (pH 8.0)/0.2 mM NADPH/1 mM MTT.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was carried out according to Weber and Osborn [36] using 7.5% (w/v) gel columns and a current of 6.5 mA per gel tube. The molecular weight standards were bovine serum albumin dimer (136 000) and monomer (68 000), γ-globulin-H-chain (50 000), aldolase (40 000), yeast alcohol dehydrogenase (37 000), chymotrypsinogen (25 700) and γ-globulin-L-chain (23 500). Proteins in 1% (w/v) SDS plus 1% (w/v) 2-mercaptoethanol in phosphate buffer (pH 7.2) were denatured by incubation at 100°C for 2 min. Gels were stained for protein as above.

Isoelectric focussing. This was carried out on slab gels, using an LKB Multiphor and an LKB 2103 power pack, a suitable pH gradient being formed using

LKB ampholine ampholytes. Enzyme activity in the gels was visualized as described above, and the pH gradient was determined after extracting gel slices with water.

Chemicals. NADPH, horse heart cytochrome *c*, bovine serum albumin, chymotrypsinogen A, ovalbumin, γ-globulin, MTT, PMSF, DCPIP and cyanogen bromide-activated Sepharose 4B were obtained from Sigma London, Poole, U.K. Alcohol dehydrogenase and aldolase were obtained from Boehringer Ltd., Lewes, U.K. All other chemicals were from BDH Ltd., Poole, U.K.

Results

Purification and separation of multiple forms

Several procedures were tried to purify the enzyme. These included chromatography on phosphocellulose, on ferredoxin-Sepharose 4B and on Blue-Sepharose. The best separation was obtained using phosphocellulose and the purification protocol which proved most satisfactory is shown in Table I. Specific activity increased markedly after phosphocellulose chromatography when most of the protein which co-purified with the enzyme in previous steps was removed. The enzyme was separated into four fractions (Fig. 1) which showed both diaphorase and ferredoxin-dependent cytochrome *c* reductase activity. These fractions, designated 1, 2, 3 and 4, were invariably present and could be completely separated by rechromatography on phosphocellulose. A small fraction of diaphorase activity was associated with proteins eluted from the column before applying the Tris-HCl gradient; this fraction showed no ferredoxin-dependent cytochrome *c* reductase activity and was discarded. Fraction I varied from almost negligible (Fig. 1) to 25% of the total activity; fractions 2, 3 and 4 showed fairly constant activities which represented 30—37%, 30—42% and 8—20%, respectively, of the total activity.

TABLE I

PURIFICATION AND ACTIVITY OF FERREDOXIN.NADP$^+$ OXIDOREDUCTASE FROM *ANABAENA CYLINDRICA*

Activities are expressed as μmol DCPIP reduced/min^{-1}, and specific activities as μmol DCPIP reduced/min^{-1} · mg protein^{-1}.

Sample	Total protein (mg)	Specific activity	Total activity	Yield (%)
Crude extract	2520	0.17	438	100
30—100% $(NH_4)_2SO_4$ fraction	905	0.31	284	65
DEAE-cellulose eluate	241	0.63	151	35
Phosphocellulose eluate *	3.2		110	25
Fraction 1	0.67	9.40	6.3	
Fraction 2	0.80	39.40	31.5	
Fraction 3	0.96	53.90	51.7	
Fraction 4	0.72	28.00	20.2	

* The enzyme was eluted from phosphocellulose as four distinct peaks (fractions 1—4).

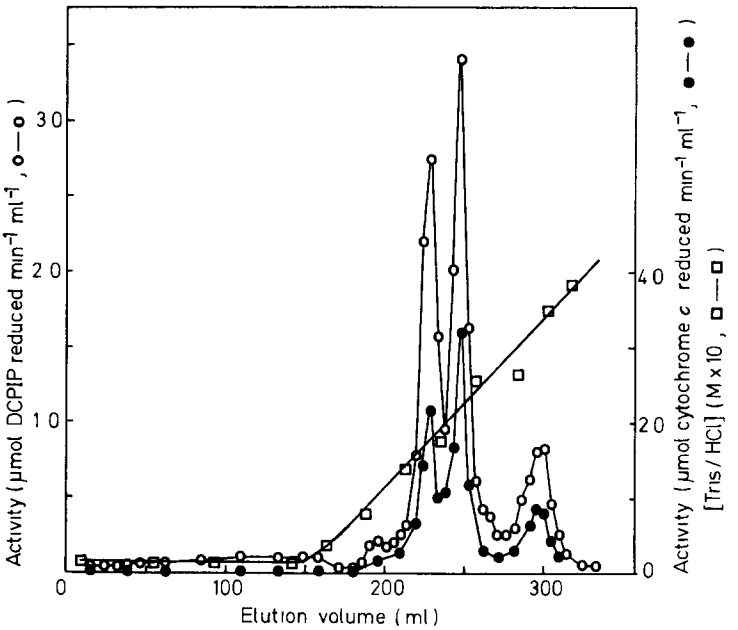

Fig. 1. Elution profile of *A. cylindrica* ferredoxin:$NADP^+$ oxidoreductase from a phosphocellulose column (20 × 1.5 cm). Enzyme (110 ml) was applied in 0.01 M Tris-HCl, pH 7.5 and eluted with a gradient of 0.01—0.5 M Tris-HCl, pH 7.5.

On polyacrylamide gel electrophoresis of the fractions, fraction 1 gave 3—4 electrophoretically distinct protein bands but only two could catalyse the NADPH-dependent reduction of MTT; those which did not were removed by chromatography on Sephadex G-150 and the specific activity of the remaining fraction increased to 34.1 μmol DCPIP reduced/min^{-1} · mg $protein^{-1}$ (Figs. 2 and 3). Fractions 2 and 3 each gave one band on gel electrophoresis; these bands were indistinguishable. Electrophoresis of fractions 1, 2 and 3 separately and together confirmed that both protein species in fraction 1 were electrophoretically distinct from those of fractions 2 and 3 (Fig. 2). The protein bands of all three fractions corresponded with bands of catalytic activity (MTT reduction) in duplicate gels. Fraction 4 showed several bands of protein and catalytic activity on electrophoresis; these were not characterised further here.

Isoelectric focussing of crude extracts was then carried out to determine whether the four fractions could be recognized prior to purification. Using whole filament extracts, bands corresponding to forms 2 and 3 were clearly seen, but no band corresponding to fraction 1 was observed; fraction 4 was not characterised further because of its heterogeneity. These results suggest that while fractions 2 and 3 probably occur in vivo, fraction 1 may not and may be a purification artifact, due possibly to proteolytic activity although 5 mM PMSF had no effect on any of the enzyme forms indicating that a serine protease was not involved. It did not seem to result from thiol oxidation [8] since treatment of fractions 1, 2 and 3 with dithiothreitol or 2-mercaptoethanol did not affect their electrophoretic properties. On electrophoresis of

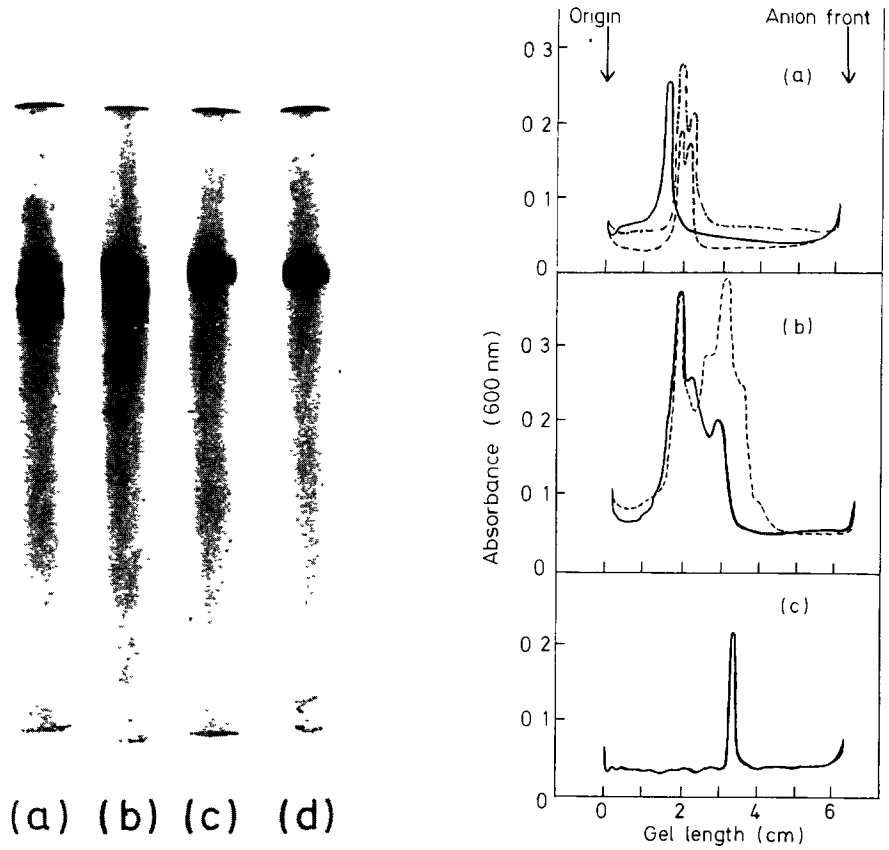

Fig. 2. Polyacrylamide gel electrophoresis of fractions 1, 2 and 3 of *A. cylindrica* ferredoxin:$NADP^+$ oxidoreductase. (a) 10 μg fraction 1; (b) 5 μg fraction 1 plus 5 μg fraction 2; (c) 10 μg fraction 2; (d) 7 μg fraction 3 on 7.5% gels. The preparation of fraction 1 shown here contained a trace amount of fraction 2. The origin is at the upper end of each gel.

Fig. 3. Polyacrylamide gel electrophoresis of fractions 1, 2 and 4 of *A. cylindrica* ferre-doxin: $NADP^+$ oxidoreductase. (a) 7.5% gels were loaded with 9 μg fraction 1 and stained for protein (— — —) or for enzyme activity (· — · — ·), or with 6 μg fraction 2 and stained for protein (———). (b) 7.5% gels were loaded with 11 μg fraction 4 and stained for protein (———) or for activity (— — —). (c) 7.5% gel loaded with heterocyst (———) extract (about 5—10 μg protein) and stained for activity.

isolated heterocyst extracts a single band which corresponded to one of the bands of fraction 4 was clearly detected.

When DEAE-cellulose chromatography followed by gel filtration was used to purify the enzyme, it was generally eluted from the column of Sephadex G-150 as a single peak (spec. act. approx. 20 μmol DCPIP reduced/min^{-1} · mg $protein^{-1}$), with a shoulder, corresponding to a higher molecular weight species (Fig. 4) occurring sometimes; its possible significance is considered later. Further purification of the main peak, and shoulder when present, using ferredoxin-Sepharose 4B gave the data presented in Fig. 5. There was no separation into the 4 distinct fractions obtained on phosphocellulose chromato-

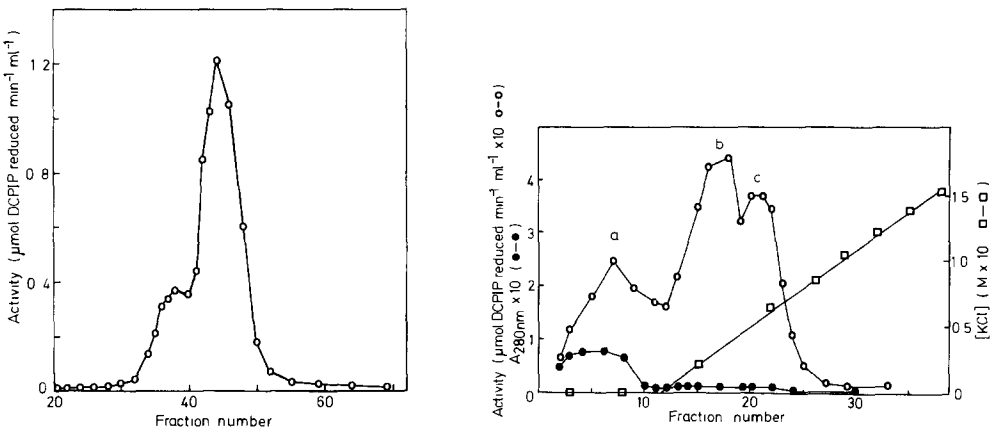

Fig. 4. Chromatography of partially purified ferredoxin:$NADP^+$ oxidoreductase on Sephadex G-150.

Fig. 5. Elution of *A. cylindrica* ferredoxin:$NADP^+$ oxidoreductase from a column of ferredoxin-Sepharose 4B (5 × 1.5 cm). The enzyme preparation was applied to the column in 0.005 M Tris-HCl, pH 7.5 and eluted with a gradient of 0—0.2 M KCl in 0.005 M Tris-HCl, pH 7.5. The ratios of ferredoxin-dependent cytochrome *c* reductase/diaphorase activities for peaks a, b and c were 0.25, 0.76 and 0.86, respectively. Peak b was enriched with an enzyme form which, on the basis of electrophoretic mobility, was similar to fraction 1 and peak c with enzyme corresponding to fractions 2 and 3 (see Fig. 1). Enzyme corresponding to 25—30% of the diaphorase activity did not bind to the column, and this fraction (peak a) showed only low ferredoxin-dependent cytochrome *c* reductase activity.

graphy; instead activity was eluted as two peaks which, on electrophoresis, gave bands characteristic of fractions 1, 2 and 3 (see legend to Fig. 5 for further details).

Molecular weights

The apparent molecular weights of fractions 1—4, estimated by gel filtration, were 37 000, 33 600, 33 500 and 33 200, respectively. SDS-polyacrylamide gel electrophoresis gave values of 33 300, 31 000 and 32 000 for fractions 1—3,

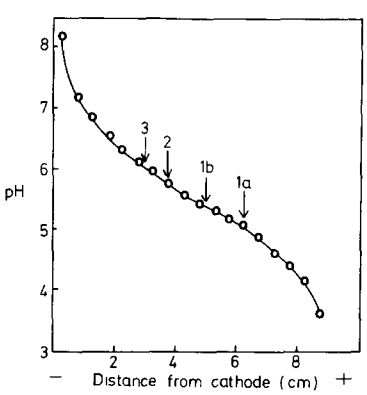

Fig. 6. Isoelectric focussing pattern of fractions 1, 2 and 3 of ferredoxin:$NADP^+$ oxidoreductase of *A. cylindrica*. Gels were stained for enzyme activity using MTT.

TABLE II

SOME PROPERTIES OF FRACTIONS 1, 2 AND 3 OF FERREDOXIN:$NADP^+$ OXIDOREDUCTASE FROM *ANABAENA CYLINDRICA*

n.d. not determined. (1) Potassium phosphate, Tris-HCl, glycine-NaOH and Na_2CO_3-$NaHCO_3$ buffer systems (all 0.1 ionic strength) were used. (2) Michaelis constants were calculated from Lineweaver-Burk plots of the reaction velocities vs. substrate concentration at various fixed concentrations of the other substrate; all plots gave a series of parallel straight lines.

Enzyme fraction	Diaphorase activity			Ferredoxin-dependent cytochrome *c* reductase activity		
	pH (1) optimum	K_m NADPH (2) (μM)	K_m DCPIP (2) (mM)	pH (1) optimum	K_m NADPH (2) (μM)	K_m Ferredoxin (2) (μM)
1	n.d.	33	0.16	7.4	11	4.5
2	10.1	40	0.11	7.8	26	4.6
3	10.1	62	0.10	7.8	35	4.5

respectively. These purified fractions are probably monomeric species. A minor species (the shoulder on Fig. 4) had a molecular weight of approx. 70 000 and may have been a dimer.

Isoelectric points

These were estimated as pH 5.0 and 5.4 for the minor and major species of fraction 1, 5.75 for fraction 2 and 6.0 for fraction 3 (Fig. 6).

Kinetic properties

Differences in kinetic properties of fractions 1, 2 and 3 are small (Table II), the greatest differences being in the K_m value for NADPH in the ferredoxin-dependent cytochrome *c* reduction assay. Fraction 1 showed a substantially lower K_m value in this assay than did the other two fractions, but the interpretation of this finding is difficult because this fraction contained two distinct species (see Figs. 2 and 3).

Absorption spectra

Fractions 1, 2 and 3 showed typical flavoprotein absorption spectra, with 275 nm : 460 nm absorbance ratios of 8.5, 10.3 and 9.3, respectively.

Discussion

We have demonstrated, using several methods, that multiple forms of the enzyme ferredoxin:$NADP^+$ oxidoreductase occur in the photosynthetic prokaryote *A. cylindrica*. Chromatography on phosphocellulose (Table I) and Blue-Sepharose (our unpublished results) gave the most satisfactory separation of the enzyme fractions; chromatography on ferredoxin-Sepharose 4B was less satisfactory, giving only a partial separation of the above fractions.

The occurrence of multiple forms in *A. cylindrica* is in accord with recent

findings for the enzyme from *Spirulina platensis* [29], *Nostoc* strain MAC [30] and chloroplasts [20—28]. The two major fractions of the *A. cylindrica* enzyme (forms 2 and 3) have almost identical molecular weights (approx. 33 000) and kinetic properties (Table II) and, as they can be detected in cell-free extracts prior to purification, appear to occur in vivo. Fraction 1, on the other hand, may be a purification artifact being undetectable prior to purification. It is possible that one or more of the enzyme forms exists as a dimer of molecular weight 70 000 (Fig. 4) which dissociates during purification. This interpretation, however, must be treated cautiously because we have been unable to obtain any of the purified fractions in a dimeric form. The enzymes of higher plant chloroplasts [23,25,28] and algae [26] have also been reported to occur in dimeric form. The conditions which we used to release the enzyme from the cells were similar to those used by Zanetti and Arosio [28]. However, unlike the latter workers we did not find the proportion of the enzyme present as the dimeric form to increase on re-extraction of the 30% $(NH_4)_2SO_4$ pellet (Table I).

Fraction 4 appears to be a mixture of several enzyme forms, one of which is similar to that extracted from heterocysts. We have been unable to further purify and characterise this fraction.

The cyanobacterial enzyme is present in vegetative cells as a component of the photosynthetic electron transport chain and functions in vivo in the photoreduction of $NADP^+$, and in whole filament extracts the enzyme forms detected are probably mainly of vegetative cell origin since heterocysts represent only about 5% of the total cells present. It is not known whether any of the enzyme forms detected in *A. cylindrica* have an alternative function, whether they are interconvertible, or whether they have any regulatory significance. There is evidence for the interconversion of forms of the spinach chloroplast enzyme by oxidation/reduction [27] and for the involvement of a sulphydryl group in a pH-dependent transition between two forms of this enzyme [37]. The physiological regulation of the enzyme of *Nostoc muscorum* by oxidation/reduction has also been suggested [38] and it has recently been shown that the membrane-bound ferredoxin:$NADP^+$ oxidoreductase of chloroplasts undergoes a light-induced conformational change [39]. The mechanism of any interconversion of enzyme forms remains to be determined but it will be interesting to know whether, for example, the light-effect-mediator [40] or thioredoxin [41] systems are involved.

Our data, showing that only one form of the enzyme could be detected in heterocyst extracts is of particular note since heterocysts are also deficient in other components, which are characteristic of vegetative cells such as ribulose-1,5-bisphosphate carboxylase [42] and components of photosystem II (see Ref. 43). If it is confirmed that there is only one form of ferredoxin:$NADP^+$ oxidoreductase in heterocysts and that the others have not simply been lost during heterocyst isolation or are not readily extracted, it will be of interest to determine the role of the form present. In this connection it may be noted that the reduction of ferredoxin, an electron donor to nitrogenase, can involve ferredoxin:$NADP^+$ oxidoreductase [13] with reversed electron transport possibly being driven by a proton motive force [44].

Acknowledgements

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