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PREPARATION, CRYSTALLIZATION AND PROPERTIES OF *CUCURBITA PEPO* PLASTOCYANIN AND FERREDOXIN

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Abstract—Homogeneous plastocyanin was obtained from *Cucurbita pepo* L. The MW was 11360 daltons based on 0·56% copper. Values of $S^{\circ}_{20w} = 1{\cdot}69\,S$ and $D^{\circ}_{20w} = 1{\cdot}46 \times 10^{-6}$ cm^2 sec^{-1} and $\bar{V} = 0{\cdot}73$ (amino acid analysis) or 0·74 (pycnometry) indicated 10300 to 10900 daltons. Gel chromatography on Sephadex G75 suggested 12200 daltons. Amino acid analyses of two cultivars indicated 10928 to 11000 daltons for 102 residues and one atom of copper, excluding 1·0% protein-bound carbohydrate. The single thiol group reacted slowly with 2-chloromercuri-4-nitrophenol but rapidly with mercuric acetate. The redox potential was +350 mV between pH 6·5 and 9·0 with a one-electron change and an ionizable group of pK 5·6. The isoelectric point was at pH 4·2. Light absorption maxima occurred at 253, 259, 264, 269, 278, 284, 460 (very weak), 597 and 775 nm. The best ratio E_{597}:E_{278} was 0·87. Extinction coefficient at 597 nm was $4{\cdot}75 \times 10^3$ l. mol^{-1} cm^{-1}. Copper was reversibly removed and 83% restored to apoprotein during successive treatments with mercuric acetate, Sephadex G25 and glutathione. The oxidized protein was crystallized from 58 or 60%-saturated ammonium sulphate containing 2% dioxan at pH 4·0 to 4·5. Oxidized crystals in ammonium sulphate were reduced by ascorbic acid or photochemically with glycerol and then dissolved. Homogeneous ferredoxin from the same source had a MW of 11400 daltons based on 0·98% iron and 11045 by amino acid analysis. Two atoms each of iron and labile sulphide were recorded. Methionine was present. Aggregation occurred during sedimentation which indicated 20500 daltons from $S^{\circ}_{20w} = 2{\cdot}44\,S$; $D^{\circ}_{20w} = 1{\cdot}0 \times 10^{-6}$ cm^2 sec^{-1} and $\bar{V} = 0{\cdot}71$. Gel chromatography indicated 12000 to 16000 daltons depending on media. Redox potential at 25° was −404 mV at pH 7·5. Light absorption maxima were at 277, 333, 422 and 462 nm. The best ratio E_{422}:E_{277} nm was 0·51. The extinction coefficient at 422 nm was $9{\cdot}8 \times 10^{-3}$ l. mol^{-1} cm^{-1}. The protein crystallized as red needles from 78% or 80%-saturated $(NH_4)_2SO_4$.

INTRODUCTION

Plastocyanins are blue copper-proteins present in chloroplasts of higher plants and algae. They have characteristic light-absorption spectra, fairly high redox potentials and are non-autoxidizable. Differences are in points of detail with respect to amino acid composition, critical spectral ratios, apparent state of aggregation and copper content. The plastocyanins may be useful in investigations of metallo-protein structures, especially the type of phylogenetic studies for which cytochrome *c* proteins have frequently been chosen [1, 2]. Plastocyanins first isolated from higher plants are from *Spinacea oleracea* [3], *Phaseolus vulgaris* [4], *Triticum vulgare* [5, 6] and *Chenopodium album* [7]. Following its discovery in *Chlorella ellipsoidea* [8],

Table 1. Amino acid analyses of plastocyanin from two cultivars of *Cucurbita pepo*

	Cultivar: (a) Suttons Green Bush			Cultivar: (b) Asmers F_1 hybrid			Integral numbers		
							Green bush	F_1 hybrid	
Residue	24 hr	72 hr	Best value	24 hr	72 hr	Best value	(by analysis)	(by analysis)	(from sequence)
Asx	10·64	11·96	11·30	13·71	13·16	13·43	11	13	12
Thr*	4·86	4·20	5·20	2·48	2·11	2·69	5	3	5
Ser*	7·54	5·79	8·60	7·14	6·03	7·76	9	8	7
Glx	11·80	12·80	12·30	11·70	11·75	11·73	12	12	9
Pro	5·40	5·59	5·49	5·20	5·36	5·28	5	5	5
Gly	10·56	9·61	10·08	10·45	10·53	10·49	10	10	13
Ala	8·53	7·90	8·21	7·36	7·72	7·54	8	8	8
Cys*	0·81	0·61	1·23	0·93	0·74	1·05	1	1	1
Val†	10·37	10·93	10·93	10·31	11·30	11·30	11	11	10
Met‡	1·93	1·98	1·95	2·16	1·91	2·03	2	2	2
Ile†	3·33	3·69	3·69	4·94	5·34	5·34	4	5	3
Leu	7·34	7·35	7·35	6·52	6·75	6·63	7	7	7
Tyr	3·08	2·98	3·03	2·99	3·29	3·14	3	3	3
Phe	6·59	6·44	6·51	6·78	6·96	6·87	7	7	6
His	2·12	2·24	2·18	2·16	2·24	2·20	2	2	2
Lys	4·81	5·80	5·31	5·02	4·78	4·90	5	5	7
Arg	0	0	0	0	0	0	0	0	0
Trp	0	0	0	0	0	0	0	0	0
Amide						6·18	6	6	6
Total residues							102	102	100
Total *N*			14·9%						

* Corrected for losses assuming first order kinetics [64].

† Maximum recovery adopted.

‡ Includes methionine sulphodixes.

Protein was "washed" on a Diaflo membrane and dialysed in 1 mM-ammonium carbonate; freeze-dried duplicate samples (50 μg approx.) of marrow protein were hydrolysed for 24 and 72 hr in 6 M-HCl at 110°C [64] as described in Methods. Values as residues/mol assuming MW of 10000 daltons. The integral numbers shown for sequence studies of F_1 hybrid are by M. D. Scawen and D. Boulter (unpublished work).

it was also found in *Chlamydomonas reinhardii* [9], *Phormidium lurum* [10] and *Anabaena variabilis* [11].

Ferredoxins are widely distributed and well-characterized [12–15]. In plants, in addition to reduction of NADP [16, 17] and methaemoglobin [18, 19] the same protein mediates electron-transfer in chloroplasts to nitrite reductase [20–22], to sulphite reductase [23] and for hydroxylamine reduction [24, 25].

Vegetable marrow was found to yield preparations of plastocyanin and ferredoxin of good quality by simple procedures [25]. This paper describes the isolation, crystallization and some properties of homogeneous plastocyanin and ferredoxin from field or greenhouse plants by a potentially large-scale method. A preliminary account relating to plastocyanin has been presented [26].

RESULTS AND DISCUSSION

Plastocyanin

Homogeneity, electrophoresis and sedimentation. Preparations obtained after the final Sephadex G100 chromatography (see Experimental) appeared homogeneous after electrophoresis on cellulose acetate (100 μg protein) at pH 4·5, 6·5, 7·7 and 8·6 and on acrylamide gel (200 μg protein) at pH 6·6 and 9·6. Impurities in the protein on acrylamide gel were less than 2% in terms of detectable protein stained with coomassie blue [27].

The protein sedimented as a single symmetrical component and showed no evidence for reversible aggregation or dimeric structure suggested for spinach [28]. Values of $S_{20w} = 1{\cdot}69$ S and $D^{\circ}_{20w} = 1{\cdot}46 \times 10^{-6}$ cm^2 sec^{-1} were obtained. The value of $\bar{V}$ was 0·73 ml/g from amino acid analysis and 0·74 ml/g by pycnometry from which MWs of 10300 and 10900 daltons were calculated.

The isoelectric point in a polyacrylamide gel was at pH 4·2. When the L.K.B. pH-focusing apparatus was used there was considerable precipitation and denaturation after 36 hr and the apparent isoelectric point was between pH 3·7 and 3·9.

Sephadex gel chromatography. For MW estimations Sephadex G75 was preferred to G100 which was used in the purification. In gels equilibrated with 0·1 M KCl in 0·05 M Tris–HCl (pH 7·6) a MW of 12200 ± 400 daltons was found. The elution profiles were symmetrical and there was no evidence of any higher-MW component. The higher value was possibly the result of hydration and occurred also with ferredoxin. The use of an acetate buffer system might have given lower values [28]. The value obtained by the same procedure for wheat plastocyanin (15000 daltons) [6] was notably high by comparison with our calculation from their amino acid analysis (10870 daltons).

Copper analysis. The copper content of the best preparations from F_1 hybrid material was 0·56 ± 0·06. Results were similar with either the acetic acid or nitric/perchloric acids methods. The MW based on one g atom copper/mol was about 13600 daltons. This was not significantly different (*t* test) from the value by gel chromatography.

Amino acid and Thiol-group analyses. The amino acid analyses for the two cultivars used are shown in Table 1. There were 102 integral residues in each and six amide groups corresponding to 10928 daltons (green bush) or 11000 daltons (F_1 hybrid) without the carbohydrate moiety.

Apparent differences between the two cultivars with respect to the proportions of some residues may not be real, although the substitution of lysine and serine at one position is established (M. D. Scawen and D. Boulter, unpublished work). Other homogeneous plant plastocyanins were reported to lack arginine and tryptophan [3, 6, 28]. The *C*-terminal group of the F_1 hybrid protein was isoleucine and the *N*-terminal asparagine [29]. Arabinose, glucose and a hexosamine are present up to 5% in spinach [3] and also in wheat [6]. The marrow protein contained only 1·0% anthrone-reactive carbohydrate, equivalent to one residue (approx.) per mol and no detectable hexosamine.

The reaction of the single thiol-group with 2-chloromercuri-4-nitrophenol (Fig. 1) was slow with native protein but rapid after denaturation by TCA or in 6 M urea [3], whereas stellacyanin remained inert in 6 M guanidinium chloride [30]. The equivalence point was obvious when TCA had been used but was less clear with guanidinium chloride (Fig. 1a). Equivalence was just detectable at pH 7·4 or above. The slow reaction between native plastocyanin and 2-chloromercuri-4-nitrophenol at pH 7·6, followed at 410 nm, was closely related to the change in extinction at 597 nm due to loss of copper (Fig. 1b). The denatured protein was bleached rapidly by mercuric acetate at pH 7·6 in 0·1 M Tris–HCl by reaction of the partially-protected thiol with this smaller mercurial reagent with loss of copper. The lower recoveries obtained with guanidinium chloride or TCA (0·79 to 0·86—SH/mol) compared with the native protein (0·96—SH/mol) probably reflects the formation of disulphide bonds by dimerization after denaturation.

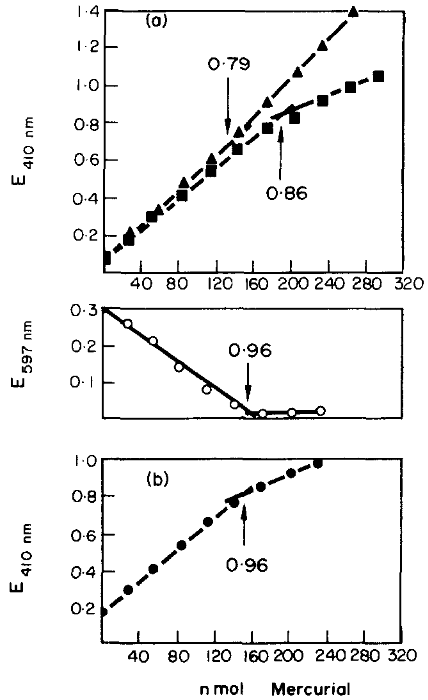

Fig. 1. Titration of free thiol group in marrow plastocyanin with 2-chloromercuri-4-nitrophenol. Plastocyanin (65–88 μm—as determined by E597 or by copper analysis) in 2·5 ml 0·1 M Tris–HCl buffer pH 7·4 and 0·1 mM EDTA was titrated with 2·88 mM mercurial in 0·1 M NaOH and changes in light absorption were recorded at wavelengths shown, using a Unicam SP500 spectrophotometer. Protein denatured by trichloroacetic acid or in the presence of 4 M guanidinium hydrochloride reacted immediately (1a), but native plastocyanin (1b) required an interval of several hours between each point for completion of the reaction. Equivalence points and estimated molar ratios (-SH/mol) are indicated by the vertical arrows. —▲—, Guanidine-denatured plastocyanin (169 nmol); —■—, TCA-denatured plastocyanin (220 nmol); —○—, native plastocyanin (162 nmol); —●—, native plastocyanin (162 nmol).

Redox potential. This was estimated with F_1-hybrid plastocyanin to be +350 mV with unit slope for a single-electron reaction and, depending on E_0 adopted for the ferri-/ferrocyanide couple, was probably lower than +370 mV recorded for spinach [3] and *Chlamydomonas reinhardii* [9] or +390 mV for *Chlorella ellipsoidea* [8] but well above that of umecyanin (+280 mV) from horseradish root [31]. The value was constant between pH 6·8 and 9·5, and increased progressively between pH 6·5 and 4·8 with a slope approaching 58 mV/pH unit and an intercept which indicated a single ionising-group of p*K* 5·6, compared with p*K* 5·4 in spinach [3].

Absorption spectrum. Light absorption maxima (above 220 nm) occurred at 775, 597, 460, 284, 278, 269, 259 and 253 nm. The fine structure in the UV is characteristic for other plant plastocyanins [3, 5, 28] and for *Phormidium lurum* [10]. It is masked or weak below 278 nm in *Chlorella*, *Chlamydomonas* and *Anabaena* [8, 9, 11]. The maximum at 460 nm was very weak; it is pronounced in spinach, beans and *Chlamydomonas* but weak in the blue-green algae and in *Chlorella*. The far-red maximum at 775 nm was also weak in marrow compared with spinach, French bean, wheat and *Chenopodium album*. The best ratio $E_{597}:E_{278}$ was 0·87 in homogeneous protein. This was similar to that for beans (0·93), which also has three tyrosine residues [28] but notably less than that for spinach (1·12) which has only two tyrosine residues per 10000 daltons [3]. The algal proteins cited above all appear to have notably lower $E_{597}:E_{278}$ ratios between 0·5 and 0·7, which could result from one tryptophan residue which has four times the molar extinction of tyrosine [32]. The molar extinction coefficient at 597 nm was 4·75 (±0·31) × 10^3 l. mol^{-1} cm^{-1} per g atom copper for the marrow protein and was similar to other plant plastocyanins summarized elsewhere [29], except for the unusual finding of two copper atoms per mol (11 500 daltons) from *Chenopodium album* [7] giving a nominal value of 2·35 × 10^3 l. mol^{-1} cm^{-1} per g atom copper. E_{597} was also higher than for other single-copper plant proteins, namely umecyanin (3·4 × 10^3) [31] or stellacyanin (4·08 × 10^3) which also has a marked maximum at 450 nm [30]. The bands between 269 and 253 nm due to phenylalanine and that at 284 due to tyrosine were lost in the presence of 5 M guanidinium chloride which bleached the protein at 597 nm with loss of copper. Bleaching by reduction with ascorbate did not have this effect. Bleaching with mercuric acetate, which removed copper, was associated with large changes around 250 nm, but the fine structure due to aromatic residues was still discernible.

Reduction of copper by hydrogen and palladized asbestos (used to avoid introduction of soluble reagents) or by potassium borohydride, ascorbate or granular zinc induced only slight changes in the fine-structure absorption in the UV. This appeared to be less pronounced, notably for the region around 284 nm and increased the extinction 15–30% between 280 and 253 nm. Re-oxidation, immediately by ferricyanide or slowly by hydrogen peroxide, mainly restored the original spectrum in this region as well as in the visible. All of these experiments were complicated by interference in the accurate recording of changes in extinction between 253 nm and 285 nm in spite of various control procedures. Palladized asbestos washed before and after reduction was possibly the most satifactory for the purpose (T. A. Dransfield and E. J. Hewitt, unpublished work). Reduction of umecyanin induced significant reversible changes in circular dichroism in the same UV region, indicating that the aromatic residues probably participate in the ligand field of the copper atom [33]. Carbon monoxide produced no spectral change between 200 and 800 nm with either oxidized or reduced plastocyanin. The absence of tryptophan from plant plastocyanins contradicts the hypothesis [30] that tryptophan chelation of the copper atom is an essential feature of the blue copper-proteins.

Electron paramagnetic resonance. This was observed with a Varian E3 spectrometer at 77°K in a 1 mM solution of oxidized protein free of ferricyanide. The spectrum was axial with only weak hyperfine coupling typical of type-1 Cu^{2+} proteins [34]. Parameters obtained were $g_{\perp}$ 2·05; $g_{\parallel}$ 2·23; $A_{\parallel}$ 60 gauss, 0·0059 cm^{-1}. These were identical with values for spinach plastocyanin [35] and similar to those for the axial spectrum of umecyanin ($g_{\perp}$ 2·05; $g_{\parallel}$ 2·317; $A_{\parallel}$ 35 gauss, 0·0035 cm^{-1}) [36].

Reversible removal and restoration of copper. Plastocyanin (940 nmol by copper analysis) was treated with 3 μmol mercuric acetate at pH 7·6 in 0·1 M Tris–HCl buffer, resulting in bleaching. The mixture was passed through a Sephadex G25 column (12 cm × 1·5 cm), eqilibrated with 0·1 M Tris–HCl (pH 7·6), and collected in 4 ml fractions. The excluded protein was monitored by extinction at 280 nm and not rendered blue with ferricyanide. The two sharply eluted fractions were found to have substantially decreased copper content, 80% of the copper recovered being in the retarded fractions. The main two exluded fractions were bulked and 3 ml of the original 8 ml supplemented with glutatione (2 mM) and copper sulphate (0·2 mM). This was stored at 4° for 18 hr before chromatography on a second similar Sephadex G25 column. The excluded colourless protein eluted sharply in three 4 ml fractions. These were rendered blue with ferricyanide and were analysed for copper and extinction at 597 nm. Total copper recovered in the excluded fractions and in the retarded fraction was 820 n-atoms. Copper in the three excluded fractions was 711 n-atoms, and plastocyanin calculated from E_{597} was 694 nmol. The recovery of plastocyanin after reconstitution was therefore 86% of the apo- and holo-protein represented by the total of the exluded and retarded fractions and the reconstituted apoprotein (calculated as 540 nmol for E_{597} and copper analysis) after correction for residual copper-containing protein was equivalent to 83% of the retarded copper.

The reconstituted blue protein was reduced by ascorbate. The fine structure between 253 and 269 nm was normal after restoration of copper and removal of mercurial and other reagents. Treatment with 5 M guanidinium chloride followed by passage through Chelex-100 resin removed 90% of the protein-bound copper but also caused irreversible denaturation after which copper could not be restored. Treatments of the protein with citrate buffer at pH 2 and 50%-saturated ammonium sulphate [37] with additional 1 mM EDTA [4] or with dithizone in carbon tetrachloride all caused bleaching and were presumed to remove copper. However, no restoration of the blue colour could be achieved by adding copper sulphate after the removal of these reagents from the protein.

Apoplastocyanin was not detectable in extracts of copper-deficient marrow or spinach plants processed as described (Experimental) and examined by absorption spectra or when estimated [38] after incubation with copper. Copper deficiency caused 80% reduction in plastocyanin content of spinach, barley and wheat on both fr. wt and total chlorophyll bases. Plastocyanin concentrations were restored to nearly normal only 140 hr after giving copper to the plants; increases were negligible up to 60 hr.

Ferredoxin

Homogeneity and sedimentation. Crystalline marrow (F_1 hybrid) ferredoxin appeared homogeneous during electrophoresis in acrylamide gel at pH 6·6 and 9·6 when gels were loaded with 200 μg protein. Purity was probably greater than 98% [27]. A single component sedimented with S°_{20W} = 2·44 S and $D^{\circ}_{20W} = 1 \times 10^{-6}$ cm^2 sec^{-1}. From amino acid analysis (Table 2) $\overline{V}$ was 0·71 ml/g, indicating a MW of 20 500 daltons. This value was high by a factor approaching two by all criteria and probably resulted from aggregation, as found for alfalfa ferredoxin [39].

Sephadex gel chromatography. In G100 gel equilibrated with 0·1 M KCl in 0·01 M Tris–HCl (pH 7·6) a MW of 12 370 daltons was obtained, whereas with NaCl the value was 15 800 daltons for the same sample. This was probably due to hydrated Na^+ ions in the protein environment. In both salt-systems the elution profiles were symmetrical and there was no evidence of aggregation. On several occasions during preparation, when Sephadex G25 was used to desalt an extract after protamine precipitation and prior to adsorption on DEAE cellulose, two clearly separate bands with similar ferredoxin spectra were eluted from the gel. The retarded form eluted at 12 500 daltons from G75 and at 12 100 daltons from G100. The retarded fraction could not be resolved from the normal fraction on DEAE-cellulose, whereas two similar ferredoxin fractions were obtained from DEAE-cellulose for *B. polymyxa* which could not be resolved on further purification [40], possibly for analogous reasons.

Iron and labile sulphide content. The iron content was 0·98% giving a minimum MW of 5700 daltons from which two iron atoms/mol were inferred for a MW of 11 400 daltons. The labile sulphide content was 2·02 ± 0·05 atoms/mol based on extinction at 420 nm.

Amino acid and thiol-group analysis. The amino acid content of the F_1 hybrid protein is given in Table 2. As is usual for ferredoxins, there is an excess of acidic residues but it is less usual in having one residue of methionine, otherwise observed only in three other angiosperms, namely cotton [41], pigweed [42] and nutsedge [43]. Total reac-

Table 2. Amino acid analysis of ferredoxin from *Cucurbita pepo* (Asmers F_1 hybrid)

Residue	Hydrolysis 24 hr	Period 72 hr	Best value	Nearest integer
Asp	13·12	11·36	12·24	12
Thr*	5·75	5·52	5·90	6
Ser*	8·28	6·80	9·15	9
Glx	14·52	15·19	14·85	15
Pro	4·26	4·25	4·26	4
Gly	7·48	7·52	7·50	8
Ala	7·87	8·88	8·37	8
Cys†	4·07		4·07	4
Val‡	6·88	7·51	7·51	8
Met	1·09	1·08	1·08	1
Ile‡	4·39	4·88	4·88	5
Leu	7·03	7·40	7·22	7
Tyr	3·33	3·42	3·37	3
Phe	1·69	1·76	1·72	2
His	0·88	0·97	0·93	1
Lys	5·36	5·87	5·62	6
Arg	1·50	1·37	1·45	1
Trp§	—	—	1·01	1
Total				101
Fe				2
Labile S				2

* Corrected for losses assuming first order kinetics [64].

† After separate performate oxidation.

‡ Maximum recovery adopted.

§ Determined spectrophotometrically relative to tyrosine [32].

The procedure was that described for Table 1. Values are as residues/mol calculated for a MW of 11 000 daltons.

tive sulphur, as indicated with 2-chloromercuri-4-nitrophenol, required 8 mol of mercurial/mol ferredoxin in accordance with the presence of two atoms of sulphide and four half-cysteine residues found after separate performate oxidation and hydrolysis. Thus, in eight out of ten angiosperms [15] the sum of methionine and cysteine is five, but is four for maize [44] and six for pigweed [42]. Pteridophyta and algae show wider variations [15]. As in other angiosperm ferredoxins [15], marrow had one tryptophan. Reported absence from parsley based on spectrophotometric evidence [45] may be mistaken, since its presence in nutsedge was not apparent from the absorption spectrum [43]. In other respects it was consistent with other angiosperm ferredoxins already reported [15]. The MW based on 101 amino acid residues (Table 2), two iron atoms and two labile sulphides was 11 045 daltons, in good agreement with the value of 11 400 daltons based on iron analysis. Values obtained by gel chromatography were appreciably high, possibly because of hydration of the protein, and sensitivity to the ionic environment as for plastocyanin [28].

Redox potential. The value at 25°, pH 7·7 was −404 mV, as found after reduction by dithionite in the presence of methyl viologen [46, 47] and stepwise enzymic reoxidation by nitrite. The slope was 59 mV/decade, indicating a one-electron process.

Light absorption spectrum. The absorption spectrum resembles those of chloroplast ferredoxins. Maxima (relative extinctions to E_{277} nm shown in parentheses) were at 462 (0·45), 422 (0·51), 333 (0·67) and 277 (1·0) nm. The ratio of $E_{260}:E_{277}$ was 0·81. Extinction coefficient at 420 nm was $9·8 \times 10^3$ litre mol^{-1} cm^{-1} based on iron analysis. The best ratio of $E_{420}:E_{277}$ was 0·51, and was obtained from crystalline material. This compares favourably with 0·49 for spinach [48, 49], 0·48 for alfalfa [39] and 0·43 for taro [50]. Highly-purified non-crystalline protein had a maximum ratio of 0·48. A shoulder at 290 nm is indicative of the presence of tryptophan [51], as determined by analysis, but not all ferredoxins show this feature [43, 45].

EXPERIMENTAL

Vegetable marrow, cvs Asmers Gourmet (F_1 hybrid) and Sutton's Green Bush, were grown in the plantation between

June and Sept or in sand cultures supplied with Long Ashton nutrient solution [52]. Leaves were washed thoroughly with H_2O, drained and stored for several weeks at −18° or for 18 months at −30° without deterioration.

Copper was estimated with bathocuproine in AcOH without pre-digestion [53], thereby avoiding loss with perchloric acid [54]. Iron was determined using *o*-phenanthroline [39]. Estimation of thiol groups was carried out by spectrophotometric titration at 410 nm with 2-chloromercuri-4-nitrophenol [55] either over a period of several hours in the native state or after denaturation by 5 M guanidinium chloride at pH 7·0 or by 10% w/v TCA and re-soln in 0·1 M Tris–HCl (pH 7·6). Labile sulphide was determined as methylene blue in acid soln [56].

The redox potential of plastocyanin was determined with the ferri-/ferrocyanide system [57]. Ratios of oxidized: reduced protein relative to extremes produced with ferricyanide or ascorbate were determined spectrophotometrically at 597 nm in citrate (pH 4·9–6·0), phosphate (pH 6·0–7·8) and glycine (pH 8·5–9·5) all at 50 mM concn. The redox potential (E_0 at pH 7·0) for the 10 mM ferri-/ferrocyanide couple with a total K^+ concn of 60 mM was found to be +411 mV at 25° in the phosphate buffer. This value accords more closely with those of Kolthoff and Tomsicek [58], Reilly and Rae [59] and O'Reilly [60] (400–418 mV) than with the older and more often adopted value of Clark *et al.* [61]. The redox potential of ferredoxin at pH 7·7 was estimated using methyl viologen and dithionite [46, 47], but using nitrite and nitrite reductase for step-wise reoxidation instead of air (Hewitt and Dransfield, unpublished) to avoid destruction by autoxidation).

Plastocyanin concentrations in leaves and crude preparations were determined by rate of photo-oxidation of ferrocytochrome *c* [38] using a dual wavelength spectrophotometer, calibrated at 550 and 541 nm with pure plastocyanin, and chloroplasts [62] obtained from greenhouse-grown spinach. Comparatively pure plastocyanin was estimated spectrophotometrically after oxidation by potassium ferricyanide, using an E.C. of $4{\cdot}75 \times 10^3$ l. mol^{-1} cm^{-1} at 597 nm. Ferredoxin estimations were done using an E.C. of $9{\cdot}8 \times 10^3$ l. mol^{-1} cm^{-1} at 422 nm.

Amino acids were analysed in salt-free protein, obtained by repeated washing against an Amicon UM2 membrane with 1 mM NH_4HCO_3, pH 7 followed by dialysis against the same and freeze-drying. Approximately 50 μg protein was dissolved in 0·5 ml 6 M HCl, frozen, evacuated and thawed under vacuum. The de-gassed soln was sealed in evacuated tubes and hydrolysed for 24 or 72 hr at 110°. When cool, the HCl was removed under vacuum over NaOH and the residue dissolved in 1 ml of 0·2 M citrate buffer (pH 2·2) containing dithioglycol [63, 64].

Tryptophan was estimated by ninhydrin and glycyltryptophan as standard [65] or spectrophotometrically [32]. Cysteine was estimated in a separate 24 hr hydrolysate after performate oxidation [66]. Amide nitrogen was determined on duplicate samples after hydrolysis for 2–4 hr at 100° in 2 M HCl followed by distillation and estimation of ammonia [67]. A micro-Kjeldahl procedure [67, 68] was used for total-nitrogen determinations. Protein-bound carbohydrate was estimated using anthrone [69], and hexosamine by a modification [70] of the method of Elson and Morgan [71], after 4 hr hydrolysis at 100° in 4 M HCl [72] and prior to removal of neutral sugars on Dowex 50 (H^+ form) [73].

Chromatography and electrophoresis. DEAE cellulose was equilibrated with 0·01 M Tris–HCl (pH 7·6) and used as columns (12 cm × 2·5 cm). The columns were fed by Marriotte bottle and the flow regulated to approx. 1 ml/min. Chromatography [74] on Sephadex G75 and G100 was performed at 4° in columns (90 × 3 cm), upward flow being maintained at 40 ml/hr by peristaltic pump. Columns were equilibrated with blue dextran, glucagon and 6 proteins in two mixtures [75]. Fractions (2–4 ml) were collected and monitored at 280 nm. Columns and applied solns were routinely equilibrated with 0·1 M KCl in 0·05 M Tris–HCl (pH 7·6) for both fractionation and MW determinations.

Electrophoresis on cellulose acetate strips (5 × 10 cm) was carried out at 4° at a current of 2 mA per strip in buffers [76] of $I = 0{\cdot}1$ at pH values of 4·5 (acetate), 6·5 and 7·7 (phosphate) and 8·6 (veronal). Protein was revealed with 0·001% nigrosine in 2% w/v HOAc overnight [77]. Electrophoresis on polyacrylamide gel columns was carried out without spacer- or sample-gels [78], using the Tris–glycine (pH 9·4) [79] and 2,6-lutidine-glycine (pH 6·6) [80] buffers. Protein was stained with coomassie blue [27]. The isoelectric point of plastocyanin was determined in polyacrylamide gel using a pH 3 to 6 ampholine gradient at room temperature [81].

Sedimentation. Plastocyanin or ferredoxin solutions (10 mg/ml or serially diluted) were examined in the Beckmann Model E analytical ultracentrifuge and photographed using red or blue filters. The synthetic boundary cell was used with 0·1 M NaCl in 0·01 M phosphate (pH 7·0).

Purification and crystallization of plastocyanin. Frozen leaf (18) (10–15 kg) was minced in a Hobart E 4522 chopper using coarse plates. The frozen mince in batches of 1·5 kg was mixed thoroughly with 45 g NaCl and 90 ml 1 M Tris–HCl buffer (pH 7·3) and allowed to thaw overnight at 4°, the temp. used for all subsequent stages. The juice was expressed through muslin and 0·5 vol. redistilled Me_2CO at −18° was added slowly with stirring and after 15 min the ppt. was removed by centrifugation for 15 min at 2000 *g*. To the supernatant was added 2 vol. Me_2CO and after 1 hr standing the supernatant was removed by gentle suction. The ppt. was redissolved in minimum 0·05 M Tris–HCl (pH 7·6), adding 2–5 g of polycaprolactam powder per kg leaf when necessary to aid dispersion. The dispersed slurry was centrifuged for 5 min at 5000 *g*. The pellet was twice re-extracted with minimum buffer and the combined extracts (about 500 ml) passed through a 2·5 l. column of Sephadex G25 equilibrated with 0·01 M Tris–HCl (pH 7·6). The first excluded fraction was collected and applied to a DEAE-cellulose column (see above). The cellulose was washed with 0·2 M NaCl in 0·05 M Tris–HCl (pH 7·6) until the extinction of the eluate was less than 0·1. Ferredoxin remained on the column.

The 0·2 M NaCl eluate was brought to 60% satn of $(NH_4)_2SO_4$. After 30 min the ppt. was removed by centrifugation for 30 min at 5000 *g*. The supernatant was brought to satn with $(NH_4)_2SO_4$, 10–50 mg potassium ferricyanide added and left to stand overnight. The grey–blue protein was collected by centrifugation, redissolved in 50–80 ml 0·01 M Tris–HCl (pH 7·6) and either dialysed overnight against 5 l. Tris–HCl or passed through a 5-vol. column of Sephadex G25 and applied to a second column of DEAE-cellulose. The column was washed with 0·07 M NaCl in 0·01 M Tris–HCl (pH 7·6) until the extinction of the effluent at 280 nm fell to 0·02. Plastocyanin was then slowly eluted with 0·12 M NaCl in 0·01 M Tris–HCl (pH 7·6). The eluted plastocyanin was brought to the maximally oxidized state with minimum ferricyanide, diluted with 2 vol. Tris–HCl and readsorbed on a fresh DEAE column. Again it was eluted with 0·12 M NaCl until about to leave the column and was then eluted sharply with 50 ml of 0·25 M NaCl in 0·01 M Tris–HCl (pH 7·6). Excess of ferricyanide added to prevent reduction during elution from DEAE-cellulose [3] caused losses of the marrow protein, apparently by removal of copper (E. J. Hewitt and P. Sims, unpublished work). The eluted plastocyanin was just fully-oxidized, concentrated to about 15 ml on a "Diaflo" UM2 membrane using N_2 at 2·5 kg/cm^2 and applied to a column of Sephadex G100. The blue plastocyanin fractions were collected and bulked when the ratio of $E_{597}:E_{278}$ was between 0·56 and 0·71. This represented approx. the "half-band-width" of the elution peak at 597 nm and about 80% of the plastocyanin applied to the column. The soln was concn. to between 10 and 15 ml on the membrane. For the amino acid and copper analyses, measurements of molar extinction coefficients, extinction ratios, sedimentation and electrophoresis, the G75 chromatography and concn procedures were repeated. Conc. solns were *ca* 1 mM plastocyanin with a yield of 80–120 mg pure protein from 10–15 kg leaf material. The yields, based on enzymic assays [38], were about 20% of the initial leaf content. This indicates about 5 μM (55 mg/kg) in *C. pepo* leaves. Estimates for spinach, based on chlorophyll content (as 2 mM) and EPR signals or on enzymic estimation, indicate 5 μM [35], 4·5 μM [82] or 3 to 8 μM [83]. Initial drying of the chloroplast sludge [3] or use of surface active compounds [49, 83] to solubilize were not necessary. Presumably the lipoprotein complex is more readily dissociated in marrow than in spinach.

Plastocyanin from F_1 hybrid was crystallized in two ways. Originally [26], a 1 mM solution of the protein was dialysed against 62%-saturated $(NH_4)_2SO_4$ at about pH 4·5, which produced a faint turbidity. On standing for 10–15 weeks at 4°, fan-shaped or stellate clusters of deep-blue needles, apparently octagonal with 4 major and 4 minor faces, were produced. Similar clusters formed still more slowly in 58%-saturated $(NH_4)_2SO_4$. Crystals were subsequently obtained readily after 3 weeks at room temp. in 60%-saturated $(NH_4)_2SO_4$ containing 2% dioxan at pH 4·0–4·5 and more slowly in 56 to 58%-saturated $(NH_4)_2SO_4$ with 2% dioxan. These crystals formed in paired or tetrad fan-shaped clusters or in collateral bundles and accumulated most of the protein from the soln. Separate crystals appeared to have 4 major and 4 minor faces. Oxidized crystals embedded in Canada balsam or suspended in $(NH_4)_2SO_4$ + 2% dioxan remained blue during repeated intense microscopic illumination, but bleaching and disappearance of the crystals occured after a few minutes' illumination when anhydrous glycerol had been used to seal the cover-slip, apparently as a photo-reduction effect. Addition of a minute amount of solid ascorbic acid to crystals resulted in slow bleaching followed by erosion of the surface and dissoln which could be arrested by further addition of $K_3Fe(CN)_6$ (E. J. Hewitt and P. Sims, unpublished work). Plastocyanin in phosphate buffer containing EDTA or redissolved after freeze-drying was bleached in light [4]. After freeze-drying, preparations lost EPR signals which could not be restored by ferricyanide, indicating probable loss of protein-bound copper. Repeated freezing and thawing bleached spinach plastocyanin [82].

Purification and crystallization of ferredoxin. Ferredoxin was eluted from the first DEAE-cellulose column with 0·5 M NaCl, diluted to 0·02 M NaCl, re-adsorbed on a fresh DEAE column and washed with 0·25 M NaCl in 0·01 M Tris–HCl (pH 7·6) until the extinction of the eluate at 280 nm was 0·1. The ferredoxin was then eluted as a dark-brown band with 0·8 M NaCl in 0·01 M Tris–HCl (pH 7·6) and desalted by passing through a 5 vol. column of Sephadex G25. Further purification was achieved by fractionation with protamine sulphate [16]. The crude ferredoxin was adjusted to pH 6·5 with 0·5 M acetate (pH 4·5) and protamine sulphate (10 mg/ml, adjusted to pH 6·5) was added to give a final concn of 1 mg protamine/15 mg protein. After 20 min the ppt. was removed by centrifugation at *ca.* 10000 *g* for 10 min and discarded. More protamine was added to the supernatant to give a concn of 1 mg protamine/7 mg protein. The ppt. was collected by centrifugation, and the colourless or pale-yellow supernatant discarded. The pellet was extracted with successive 5 ml portions of 0·5 M Tris–HCl (pH 8·5) with addition of about 1 g of polycaprolactam powder to aid the extraction of ferredoxin. The extract was diluted with 5 vol. of H_2O and adsorbed on a small (5 cm × 1·5 cm) DEAE-cellulose column which was then washed with 0·2 M NaCl until the extinction at 280 nm of the eluate was 0·02. Ferredoxin was then eluted with minimum 0·8 M NaCl and dialysed overnight against 0·01 M Tris–HCl (pH 7·6). At this stage the ratio $E_{420}:E_{277}$ was 0·44–0·47. Preparations for analytical studies were purified further by gel filtration on Sephadex G75. Fractions with ratio $E_{420}:E_{277} > 0·46$ were bulked, conc. on the Diaflo membrane to 10 mg/ml and about 10 ml applied to the G75 column. About 80% recovered in the half-band-width of the elutate was reconcentrated on the Diaflo membrane.

Marrow ferredoxin was crystallized either by dialysis of a conc. solution (10–12 mg/ml) against 80%-saturated $(NH_4)_2SO_4$ (pH 4·8) or by addition of solid $(NH_4)_2SO_4$ until a faint turbidity appeared (about 70%-satn). In either case, red-brown needles appeared after one or two days at 4°. The crystals were washed with 80%-saturated $(NH_4)_2SO_4$ and redissolved in 0·01 M Tris–HCl (pH 7·6) for further study.

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