Isolation and characterization of a novel haemoprotein *b*-559 from Bengal gram (*Cicer arietinum*)

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A haemoprotein was purified to apparent homogeneity from Bengal-gram seeds. The purified protein exhibited an absorption maximum at 412 nm (Soret band) that upon reduction with dithionite gave rise to a shift in the Soret band to 426 nm with concomitant appearance of an α -band at 559 nm and a β -band at 530 nm. In the reduced state the Bengal-gram haemoprotein showed reactivity towards CO, nitrite and hydroxylamine. SDS/polyacrylamide-slab-gel electrophoresis showed that the haemoprotein has M_r 78 000. Gel-filtration and ultracentrifugal analyses suggest that the Bengal-gram haemoprotein is oligomeric in nature. Since it differs from photosynthetic membrane cytochrome *b*-559 in solubility in buffer, in reactivity towards CO and in molecular size, it appears to be a novel haemoprotein *b*-559.

INTRODUCTION

In plants, haemoproteins are involved in a wide variety of functions, such as catalyst in oxidation–reduction reactions [1], as mediators of electron-transfer reactions in both respiratory and photosynthetic processes [2] and as O_2 carrier for N_2 -fixing nodules in legumes [3]. A number of enzymes having haem as the prosthetic group, such as catalase, peroxidase, nitrate reductase and nitrite reductase, have been well characterized. The haemoproteins involved in respiratory and photosynthetic electron-transfer systems have also been the subject of extensive investigation [4].

During the course of isolation of lipxygenase from the seeds of Bengal gram, we noticed the presence of a coloured component. Analysis of this led to the identification of a novel haemoprotein *b*-559. Although the spectral properties of this haemoprotein were essentially the same as that of the cytochrome *b*-559 component of the photosynthetic membranes, it appeared to be quite different in solubility in buffer, in reactivity towards CO and in apparent molecular size. Here we report on the purification and partial characterization of this haemoprotein.

MATERIALS AND METHODS

Materials

Bengal-gram seeds (variety Annegeri-I) were purchased from the local Seed Corporation of India, Mysore, India.

Sephacryl S-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Peroxidase, catalase, cytochrome *c*, NADPH, NADH, tryptophan and phenylmethane-sulphonyl fluoride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. NaF, hydroxylamine, ascorbic acid, NaCN, $NaNO_2$ and $NaNO_3$ were obtained from BDH Chemicals, Bombay, India. $Na_2S_2O_4$ and $NaNO_3$ were purchased from Riedel–DeHaen A.G.,

Hannover, Germany. Hydroxyapatite was prepared according to the method described by Bernardi [5]. All other reagents used were of analytical grade.

Enzyme assays

Catalase and peroxidase were assayed spectrophotometrically as described by Chance & Maehly [6]. NADH:nitrate reductase and methyl viologen:nitrate reductase activities were measured as previously described [7,8]. Nitrate reductase was assayed by the method of Ramirez *et al.* [9], and NADPH:cytochrome *c* reductase by the method of Masters *et al.* [10]. Tryptophan oxygenase activity was determined as described by Yamamoto & Hayaishi [11]. Some of these enzymic assays were standardized with commercially available enzymes.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out with the Laemmli system [12] with 7.5% gel. The gel thickness was 1.5 mm and a constant current of 25 mA was applied.

Ultracentrifugal studies

Velocity-sedimentation experiments were performed in a Beckman model E ultracentrifuge equipped with an RTIC unit. A standard 12 mm Kel-F cell was used for the experiment. The $s_{20,w}$ values were calculated by using standard procedures [13].

Determination of haem

The haem content of the enzyme was determined from the pyridine haemochrome spectrum measured at 25 °C in 25% (v/v) pyridine in 0.1 M-NaOH after reduction with $Na_2S_2O_4$ [14]. The millimolar absorption coefficients $\epsilon_{557} = 30.2 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{557} - \epsilon_{540} = 20.8 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ determined for haemin under the same conditions were used for calculating the haem content in the enzyme.

Abbreviation used: BGHP, Bengal-gram haemoprotein.

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RESULTS

Preparation of extract and $(\text{NH}_4)_2\text{SO}_4$ fractionation

Bengal-gram flour (50 g) was extracted with 250 ml of 50 mM-sodium borate buffer, pH 8.0, containing 5% (v/v) glycerol, 1 mM-EDTA and 1 mM-phenylmethanesulphonyl fluoride. The extract was centrifuged at 16000 *g* for 15 min. The supernatant (214 ml) was adjusted to 30% saturation by addition of 38 g of $(\text{NH}_4)_2\text{SO}_4$. The precipitate formed was removed by centrifugation, and to the supernatant (227 ml) was added 45 g of $(\text{NH}_4)_2\text{SO}_4$ to give 60% saturation. After standing for 1 h in an ice bath, the precipitated protein was spun down at 16000 *g* for 15 min. The sediment was enriched in lipoxygenase. The supernatant (240 ml) was adjusted to 80% saturation by the addition of 34.5 g of $(\text{NH}_4)_2\text{SO}_4$ and left overnight at 0–4 °C. After centrifugation at 16000 *g* for 15 min, the sediment was taken up in 50 mM-sodium phosphate buffer, pH 6.8 (buffer A), and dialysed against 1 litre of the same buffer overnight with two changes. The dialysed sample was briefly centrifuged to remove insoluble material.

Hydroxyapatite chromatography

A 3.5 cm × 3.0 cm column of hydroxyapatite previously equilibrated with buffer A was loaded with the dialysed protein sample. The column was washed with buffer A until all non-adsorbed proteins were eluted and then developed with 200 ml linear gradient of 50–250 mM-sodium phosphate buffer, pH 6.8. This was followed by elution with 250 ml of 250 mM-sodium phosphate buffer, pH 6.8 (buffer B). The fractions were monitored for absorbances at 280 and 410 nm. The elution profile of Bengal-gram haemoprotein (BGHP) from hydroxyapatite is given in Fig. 1. Some 410 nm-absorbing material was eluted in the buffer wash itself. However,

Fig. 1. Elution profile of BGHP from hydroxyapatite

The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was applied; 5 ml fractions were collected. ○, A_{280} ; ●, A_{410} . For details see the text.

Fig. 2. Elution profile of BGHP from Sephacryl S-200

A constant flow rate of 12 ml/h was maintained by a peristaltic pump; 2 ml fractions were collected. ○, A_{280} ; ●, A_{410} . For details see the text.

this was not a haemoprotein, as judged from its spectral properties. The haemoprotein was eluted with buffer B. Fractions 66–70 were pooled and the protein was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation). After centrifugation at 16000 *g* for 10 min, the orange-red precipitate was taken up in a small volume of 50 mM-sodium phosphate buffer, pH 6.5 (buffer C). The preparation obtained from the hydroxyapatite column had an A_{280}/A_{410} ratio of 1.44:1.

Sephacryl S-200 chromatography

An 88 cm × 1.45 cm column of Sephacryl S-200 previously equilibrated with buffer C was loaded with the sample obtained from the hydroxyapatite column. The column was calibrated with catalase (M_r 240000), lactate dehydrogenase (M_r 140000), haemoglobin (M_r 64000), pepsin (M_r 35000) and myoglobin (M_r 17000). The peak fractions (fractions 33–36) were pooled and concentrated by the use of Aquacide (Calbiochem, La Jolla, CA, U.S.A.). The elution profile of BGHP from this column is given in Fig. 2. The preparation gave an A_{280}/A_{410} ratio of 1.17:1. Details of the purification data are summarized in Table 1. The M_r of the native BGHP calculated from the molecular-sieving data appeared to be larger than that of catalase, which has M_r 240000 [15].

SDS/polyacrylamide-gel-electrophoretic analysis

At this stage of the purification, the protein was analysed by SDS/polyacrylamide-slab-gel electrophoresis as indicated in the Materials and methods section. The protein appeared to be more than 95% pure, with very minor contaminants of both higher- M_r and lower- M_r species (Fig. 3). The M_r of the protein as determined from electrophoretic data was 78000. Since M_r of the native BGHP was greater than 250000, it appears that the protein is composed of at least three or four subunits. Although there are no data to support the

Table 1. Summary of the purification of haemoprotein *b*-559 from *C. arietinum*

For experimental details see the text.

Fraction	Total volume (ml)	Concn. of protein (mg/ml)	Total A_{280}	Total A_{410}	A_{280}/A_{410} ratio
Crude extract	214.0	15.3	6258.0	672.0	9.31
(NH ₄) ₂ SO ₄ fraction (60–80% -satn.), dialysed	19.5	23.0	372.5	50.7	7.34
Hydroxyapatite chromatography	2.3	9.7	15.75	10.9	1.44
Sephacryl S-200 chromatography	1.3	3.8	7.6	6.5	1.17

Fig. 3. SDS/polyacrylamide-slab-gel electrophoresis of BGHP

Electrophoresis of BGHP in the presence of SDS was carried out as described in the Materials and methods section. Lane A, marker proteins [β -galactosidase (M_r 116000), phosphorylase *b* (M_r 97400), bovine serum albumin (M_r 66000), egg albumin (M_r 45000) and carbonic anhydrase (M_r 29000)]; lane B, 30 μ g of BGHP; lane C, 60 μ g of BGHP.

view that the subunits are identical, the protein is at least composed of subunits of the same size as each other.

Ultracentrifugal analysis

The sedimentation-velocity experiment on BGHP gave a major symmetrical peak and a small very-slow-moving broader peak. The apparent $s_{20,w}$ value calculated for the major peak was 9.1 ± 0.3 S (Fig. 4). The slow-moving component in all likelihood was the dissociated subunit of BGHP, as in the SDS/polyacrylamide-gel electrophoresis there was only one major protein band seen. Thus the ultracentrifugation experiments also confirm the oligomeric nature of BGHP.

Haem content

The haem content of the BGHP was determined from the pyridine haemochrome spectrum to be 8.87 nmol/mg

Fig. 4. Ultracentrifugal analysis of BGHP

The sedimentation-velocity experiment on BGHP was carried out at 25 °C. The protein concentration was 6.5 mg/ml in 50 mM-sodium phosphate buffer, pH 6.5. The picture was taken at 39.5 min after reaching the maximum speed of 59 750 rev./min. The bar angle was 60°.

of protein. The protein concentration was determined by measuring the nitrogen content of the sample by the method of Jaenicke [16]. A haem value of 0.7 mol/mol of subunit was obtained on the basis of the subunit M_r of 78000. The somewhat lower haem content perhaps reflects the inaccuracy in the M_r determination by the SDS/polyacrylamide-gel-electrophoretic method. It is also possible that some loss of the prosthetic group occurs during purification as a result of partial denaturation.

Absorption spectra of BGHP

The absorption spectrum of purified BGHP is given in Fig. 5(a). The protein as isolated exhibited an absorption maximum at 412 nm besides the usual absorbance due to the protein at 278 nm. Upon reduction of the protein with dithionite there was a shift in the Soret band to 426 nm with concomitant appearance of an α -band at 559 nm and a β -band at 530 nm (Figs. 5b and 5c).

Several agents, such as azide, cyanide, CO, fluoride, nitrite and hydroxylamine, are known to perturb the haem spectrum by their binding to iron. These compounds were tested to check their effect on BGHP.

Fig. 5. Absorption spectra of BGHP

(a) Absorption spectrum of BGHP (0.26 mg/ml) dissolved in 50 mM-sodium phosphate buffer, pH 6.5. (b) Absorption spectra of oxidized BGHP (spectrum A) and dithionite-reduced BGHP (0.20 mg/ml) in 50 mM-sodium phosphate buffer, pH 6.5. (c) Reduced (dithionite)-minus-oxidized (ferricyanide) difference spectrum of BGHP. The spectra were recorded on a Beckman DU-7 spectrophotometer that had been calibrated with a holmium oxide filter.

Fig. 6. Absorption spectra of oxidized BGHP (spectrum A), dithionite-reduced BGHP (spectrum B) and CO-treated BGHP (spectrum C)

Fig. 7. Absorption spectra of oxidized BGHP (spectrum A), dithionite-reduced BGHP (spectrum B) and 2 mM-nitrite-treated BGHP (spectrum C)

The bubbling of CO through a solution of dithionite-reduced BGHP for 2 min resulted in the disappearance of the α -band (559 nm) and β -band (529–530 nm) and the concomitant appearance of broader bands at 570 nm and 540 nm respectively. The Soret band at 426 nm showed a 6 nm blue-shift with an increase in absorbance (Fig. 6). Thus there is formation of a definite complex between CO and the haemoprotein. Nitrite at a concentration of 2 mM markedly decreased the intensity of the Soret band and shifted the peak to 422 nm. The α -band and β -band intensities were very much diminished (Fig. 7). Hydroxylamine at a concentration of 5 mM oxidized BGHP (Fig. 8). NaF at a concentration of 1 mM also produced an effect similar to that of nitrite (results not shown). Besides these, cyanide, nitrate, ascorbic acid

and sulphite at a concentration of 1 mM were tested and found not to perturb the spectrum of reduced BGHP.

Testing for enzyme activities

Several haemoproteins show enzyme activities, and it was therefore decided to check whether BGHP has any enzymic function. It is known that dormant Bengal-gram seeds contain catalase and peroxidase activities that increase several-thousand-fold during germination [17]. Although the spectral properties of catalase and peroxidase are quite different from those of BGHP, the haem protein was tested and found to possess neither of these enzyme activities. There was striking similarity between the spectra of BGHP and the nitrate reductase of *Chlorella vulgaris* [18]. The *Chlorella* enzyme had the

Fig. 8. Absorption spectra of oxidized BGHP (spectrum A), dithionite-reduced BGHP (spectrum B) and 5 mM-hydroxylamine-treated BGHP (spectrum C)

Soret band at 410 nm, which upon reduction shifted to 423 nm with the appearance of α - and β -bands at 557 nm and 525 nm respectively. Nitrate reductase activity, however, could not be detected in BGHP with either NADPH or NADH as electron donor. Partial activities usually associated with nitrate reductase such as methyl viologen:nitrate reductase and NADPH/NADH:cytochrome *c* reductase were also not detected. The sulphite/ferricyanide oxidase activity was also not detected with BGHP. It may be mentioned here that the addition of NADPH or NADH to BGHP did not generate the spectrum of reduced BGHP. Also, there was no binding of BGHP to Cibacron Blue–Sephacel when it was applied in 10 mM-sodium phosphate buffer, pH 6.8. These results suggest that there is probably no nucleotide-binding site on BGHP.

As mentioned above, the spectrum of BGHP was perturbed by both hydroxylamine (5 mM) and nitrite (2 mM). Since the reduced protein was completely oxidized with hydroxylamine, the possibility of its turning over with hydroxylamine as the electron acceptor was checked with reduced methyl viologen as electron donor. There was no turnover of the enzyme with nitrite or hydroxylamine, which suggests that it is neither a hydroxylamine reductase nor a nitrite reductase. Tryptophan oxygenase, which is also a haem protein, has been shown to be induced in germinating Bengal gram [19]. This activity was also not detected with BGHP. The absence of some of the above-mentioned enzyme activities certainly does not rule out the possibility that BGHP is an enzyme, however.

DISCUSSION

The occurrence of cytochrome *b*-559 in chloroplasts is well documented. It has been postulated that cytochrome *b*-559 functions either as an electron acceptor in the

water-splitting reaction or in the electron-transfer reaction between Photosystems II and I or even in a bypass chain reaction around Photosystem II [4]. The presence of cytochrome *b*-559 in microsomal membranes has also been reported in *Vicia faba* leaves [20]. The spinach protein exhibits α -, β - and γ -bands at 559, 530 and 429 nm respectively in the reduced state and the Soret band at 415 nm in the oxidized form [21]. Although the general features of BGHP, in particular the α -band at 559 nm, are similar to those reported for cytochrome *b*-559, the difference lies in their reactivity towards CO and ascorbate. The photosynthetic as well as the microsomal cytochrome *b*-559 are reduced by ascorbate and their ferrocycytochrome *b*-559 does not combine with CO [20,21]. In contrast, as discussed above, the BGHP is not reduced by ascorbate but in the dithionite-reduced form reacts with CO. The cytochromes *b*-559, depending upon their reducibility with hydroquinone, ascorbate or dithionite, are termed high-potential, middle-potential or low-potential form respectively [4]. Judged by such criteria with regard to the reducibility, BGHP appears to be in the low-potential form.

Another important difference between BGHP and the photosynthetic cytochromes *b*-559 is in the solubility. Photosynthetic cytochrome *b*-559 is a membrane-bound protein and, as such, requires a combination of detergent and protein denaturant (2% Triton X-100 in 4 M-urea) for solubilization and purification [22]. BGHP has been isolated from mature seeds as a soluble protein. The requirement for a high concentration of $(\text{NH}_4)_2\text{SO}_4$ for precipitation attests to its high solubility.

BGHP, which has been isolated from a non-photosynthetic tissue, may represent a precursor form that is eventually processed during germination to be incorporated into the plastid membrane compartment. Such a precursor-product relationship for the synthesis of spinach cytochrome *b*-559 was initially suggested on the basis of the isolation of its mRNA and its translation *in vitro* in a rabbit reticulocyte lysate system [23]. However, recent evidence on the localization of the gene for apo-cytochrome *b*-559 on the plastid chromosome of spinach as well as its nucleotide sequence data [24,25] rule out such a relationship, at least in spinach.

It may also be noted that the M_r values of the cytochromes *b*-559 isolated from spinach and *Vicia faba* are around 10000 and 28000 respectively [20,22]. The BGHP is certainly a much larger protein. Further, the oligomeric nature of this protein is yet another interesting feature. The cytochrome *b*-559 of spinach is thought to exist as a dimer in the membrane [26]. The BGHP appears to consist of more than two subunits each of M_r 78000. Thus BGHP, for which no enzyme activity has been found so far, appears to be a novel haemoprotein *b*-559. The functional significance of this protein either in seed development or in the germination process merits further investigation.

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