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Oxidation of Diaminobenzidine in the Heterocysts of *Anabaena cylindrica*

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Abstract. Hemoproteins were localized in the cyanobacterium *Anabaena cylindrica* with diaminobenzidine (DAB). Incubation of whole cells in the light with DAB resulted in deposition of oxidized DAB on the lamellae of the vegetative cells and central heterocyst region. This reaction was greatest at pH 7.5, light-dependent, insensitive to 3-(3,4-dichlorophenyl)-1, 1-dimethyl urea, and abolished by glutaraldehyde fixation. A light-independent oxidation of DAB was also observed with light and electron microscopy in the "honeycomb" region and periphery of heterocysts. This reaction was greatest at pH 7.5, enhanced by H_2O_2, and active in glutaraldehyde-fixed frozen sections. Inhibitors such as sodium cyanide, sulfide, and hydroxylamine severely reduced DAB oxidation and nitrogenase activity under aerobic but not anaerobic conditions. These results indicate that the heme proteins, localized in heterocysts by light-independent DAB oxidation, are involved in the oxygen-protection mechanism of the O_2-labile nitrogenase.

3,3′-Diaminobenzidine (DAB) has been used extensively as a cytochemical reagent for the localization of hemoproteins in a variety of cells and tissues. Hemoproteins containing an iron-porphyrin prosthetic group appear to be the principal, if not the only, class of proteins which oxidize DAB [5,19,24]. DAB oxidation has been reported to occur on the thylakoids of chloroplasts and cyanobacteria (blue-green algae) [4,13,15,16]. The reaction is light-dependent and is thought to involve one or more components of the photosynthetic electron transport chain. In the present study, we report a light-independent oxidation of DAB in glutaraldehyde-fixed and frozen sections and in intact, unfixed cells that is located in the periphery and "honeycomb" regions of the heterocysts of *Anabaena cylindrica*. The hemoproteins localized may be involved in the O_2-protection mechanism of nitrogenase.

Materials and Methods

Cells. Axenic nitrogen-fixing batch cultures of *Anabaena cylindrica* 629 were grown aerobically in Allen and Arnon [1] media as described [14]. Cells were harvested in exponential phase and resuspended to a density of 0.3 mg (dry weight)/ml in 0.05 M Tris buffer adjusted to the desired pH. 3,3′-Diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, Missouri,) was prepared just before use in 0.05 M Tris buffer, filtered through No. 1 Whatman filter paper, and adjusted to the desired pH. The DAB solution was added to the algal suspension to a final concentration of 0.5 mg/ml. Hydrogen peroxide (Sigma) was added to a final concentration of 3.4 mM. The suspensions were incubated in 100-ml Erlenmeyer flasks on a reciprocal shaker for 30 min at 30°C in the dark (covered with aluminum foil) or at a light intensity of 1×10^4 ergs · cm^{-2} · sec^{-1}.

Microscopy. Wet mounts were prepared for immediate observation (or the cells were kept on ice and viewed within 8 h) using bright field illumination. For electron microscopy, the cells were harvested and washed three times in 0.1 M cacodylate buffer (pH 7.2). The cells were fixed according to the procedure of Lang and Fay [12] in 4% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer for 4 h on ice. After rinsing twice in cold buffer, the pelleted cells were resuspended in 1% OsO_4 in cacodylate buffer. The secondary fixation was ended after 12 h of incubation at 0°C by three rinses in cold buffer.

The cells were dehydrated in a graded ethanol series followed by two changes in propylene oxide and then embedded in Araldite (Polysciences). The resin was polymerized for 48 h at 60°C. Sectioning was carried out on an LKB MT-1 ultramicrotome with either glass knives or a Westfall-Healy diamond knife. Sections were collected on carbon-stabilized, Formvar-coated, 200-mesh copper grids and viewed either with a Zeiss 9S microscope at an accelerating voltage of 60 kV or under a Siemens Elmiskop 1A at 80 kV.

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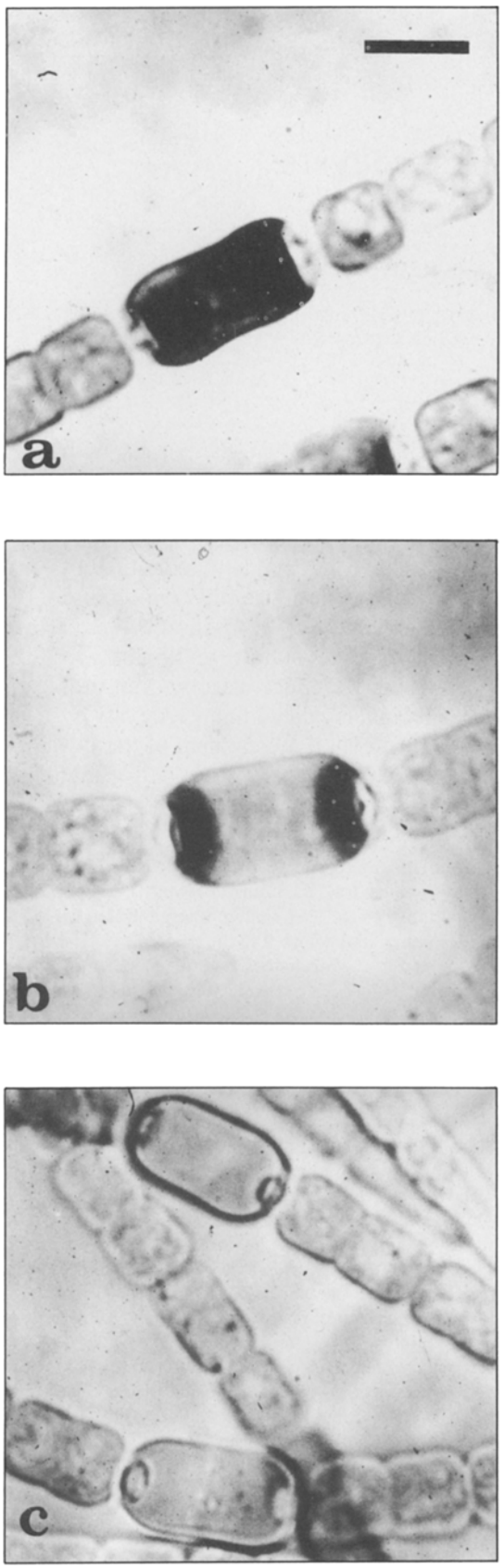

Frozen sections. Exponential-phase aerobic cultures of *A. cylindrica* were fixed in a 0.05% glutaraldehyde in cold (4°C) 0.05 M Tris (pH 7.5) buffer for 30 min. After 3 washes in cold buffer, the cells were infused with a 2 M sucrose plus 1.5% (wt/vol) gelatin mixture for 1 h and frozen in liquid nitrogen–cooled Freon. Frozen sections (0.5 μm thick) were obtained using a Sorvall-Blum MT-2 ultramicrotome equipped with a Sorvall cryokit model FTS (DuPont Co., New Town, Connecticut). The sections were retrieved with an eyelash and transferred to agar-coated (1%) glass slides. Immediately after the sections were transferred, the slides were placed in a large volume of 0.05 M Tris buffer (pH 7.5) to prevent the samples from drying and to dilute the sucrose. The samples were incubated in the same DAB reaction medium as used for whole cells for 30 min at 22°C in magnetically stirred 100-ml beakers. The slides were washed in buffer for 10 min and subjected to a final wash in distilled water.

Acetylene reduction. The effect of inhibitors on nitrogenase activity was measured using in vivo acetylene reduction assays as described previously [14]. Air-grown cultures were sparged with N_2/CO_2 for 5 min to remove most of the dissolved O_2, and two-ml aliquots were assayed for 30 min in serum-stoppered 6-ml Fernbach flasks under an atmosphere of argon or argon plus 20% O_2. The cells were incubated at 30°C on a reciprocal shaker in saturating light (6×10^4 ergs · cm^{-2} · sec^{-1}). Inhibitors were dissolved in 0.05 M Tris, pH 7.5, and added just before the start of the assay.

Results

Localization of the DAB reaction product. Incubation of whole cells of *Anabaena cylindrica* in diaminobenzidine (DAB) media (pH 7.6) resulted in deposition of the brown oxidation product in 3 distinct locations within the filament (Fig. 1). In the light, the thylakoid membranes of the photosynthetic vegetative cells are outlined with the reaction product (Fig. 1a). A distinct reaction is also apparent in the polar or "honeycomb" region and throughout the central portion of the heterocyst in the light. Time-course experiments showed that the polar reaction was visible within 5 min, while the reaction in the photosynthetic lamellae and central region of the heterocyst required 20 to 35 min to become obvious (data not shown); 2×10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) did not inhibit any of the reactions.

Fig. 1. Diaminobenzidine (DAB) oxidation in intact, unfixed filaments of *Anabaena cylindrica*. Filaments were incubated for 30 min in DAB solution without H_2O_2 at pH 7.6. Line scale = 5 μm. (a) Filaments were incubated in light. DAB deposition can be seen along the photosynthetic lamellae of the vegetative cells. An intense and uniform deposition of reaction product is seen in the heterocyst. (b) Filaments were incubated in the dark. A DAB reaction is seen only in the polar region of the heterocyst. (c) Control culture without DAB.

Table 1. Effect of pH on the light-independent diaminobenzidine (DAB) reaction in the heterocysts of *Anabaena cylindrica*. Whole, unfixed filaments were incubated in the dark for 30 min with DAB (0.5 mg/ l) and 3.4 mM H_2O_2.

pH	Reaction intensity[a]
6.1	−−
7.5	++++
9	++
10	+

[a] Symbols: − = no reaction; + = slight reaction; ++ = moderate reaction; +++ = strong reaction; ++++ = intense reaction.

Figure 1b shows that only the polar region of the heterocysts reacted with DAB in the dark. The reaction was greatest at pH 7.5 but discernible at pH 10 (Table 1). This reaction was also resistant to glutaraldehyde fixation (Fig. 2), which abolished DAB oxidation in the central portion of the heterocyst and in the vegetative cells. The reaction in glutaraldehyde-fixed sections was discernible within 5 min and essentially complete within 30 min. This reaction was enhanced with 3.4 mM H_2O_2.

Electron microscopic examination of filaments treated with DAB in the dark confirmed that DAB oxidation was confined to the heterocysts. Figure 3 shows the electron-dense osmiophilic reaction product in the "honeycomb" region of the heterocyst. DAB deposition can also be seen in the peripheral region of this heterocyst, although the reaction is weaker.

Effect of hemoprotein inhibitors on DAB oxidation and nitrogenase activity. Table 2 summarizes the effects of several hemoprotein inhibitors on the intensity of the light-independent DAB reaction in whole, unfixed heterocysts and on aerobic and anaerobic nitrogen fixation rates in the light. Aminotriazole, an inhibitor of catalase, had no effect on either DAB oxidation or nitrogenase activity. Sulfide and hydroxylamine nearly abolished DAB oxidation in the polar region and substantially inhibited aerobic but not anaerobic nitrogenase activity; 20 mM sodium azide was somewhat less inhibitory to both processes but abolished nitrogenase in the dark under 20% O_2 (data not shown). The inhibitory effect of azide on aerobic nitrogenase activity could not be reversed by removing O_2. Filaments preincubated with 20 mM azide under 20% O_2 for 30 min were sparged with N_2/CO_2 to remove O_2 and assayed for acetylene reduction. Activity was reduced to 37% of the anaerobic, azide-treated control. Cyanide strongly inhibited DAB oxidation and nitrogenase activity under both aerobic and anaerobic conditions.

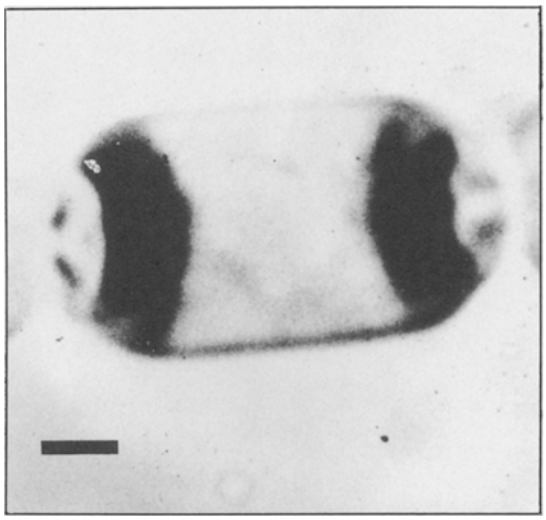

Fig. 2. Light micrograph of diaminobenzidine (DAB) oxidation in a glutaraldehyde-fixed cryosection of a heterocyst of *Anabaena cylindrica*. Incubated in dim light. Line scale = 1.5 μm.

Discussion

Diaminobenzidine (DAB) is oxidized in whole, unfixed filaments of *Anabaena cylindrica* by at least two different mechanisms. A light-dependent reaction was observed in the thylakoids of the central region of heterocysts and in vegetative cells. The light-dependent reaction did not require exogenous hydrogen peroxide and, in fact, was mildly inhibited by 3.4 mM H_2O_2. However, photosynthetic production of hydrogen peroxide has been reported in the cyanobacterium *Anacystis* [18,23], so this reaction may not necessarily be independent of peroxide. This reaction was independent of photosystem II activity since DCMU had no inhibitory effect. Thus, the light-dependent oxidation of DAB appears to be effected by photosystem I activity, as was shown in chloroplasts [4,15]. Since the heterocysts lose photosystem II but not photosystem I activity during differentiation [22], the light-dependent DAB deposition in the central region of the heterocyst must be mediated by photosystem I.

We could not demonstrate the light-dependent reaction in glutaraldehyde-fixed whole cells or frozen sections. In contrast, Lauritis and co-workers [13] observed uniform staining of photosynthetic lamellae of several glutaraldehyde-fixed cyanobacteria after incubation at 37°C for 1 h. Aldehyde-fixed chloroplasts can perform photosynthetic light

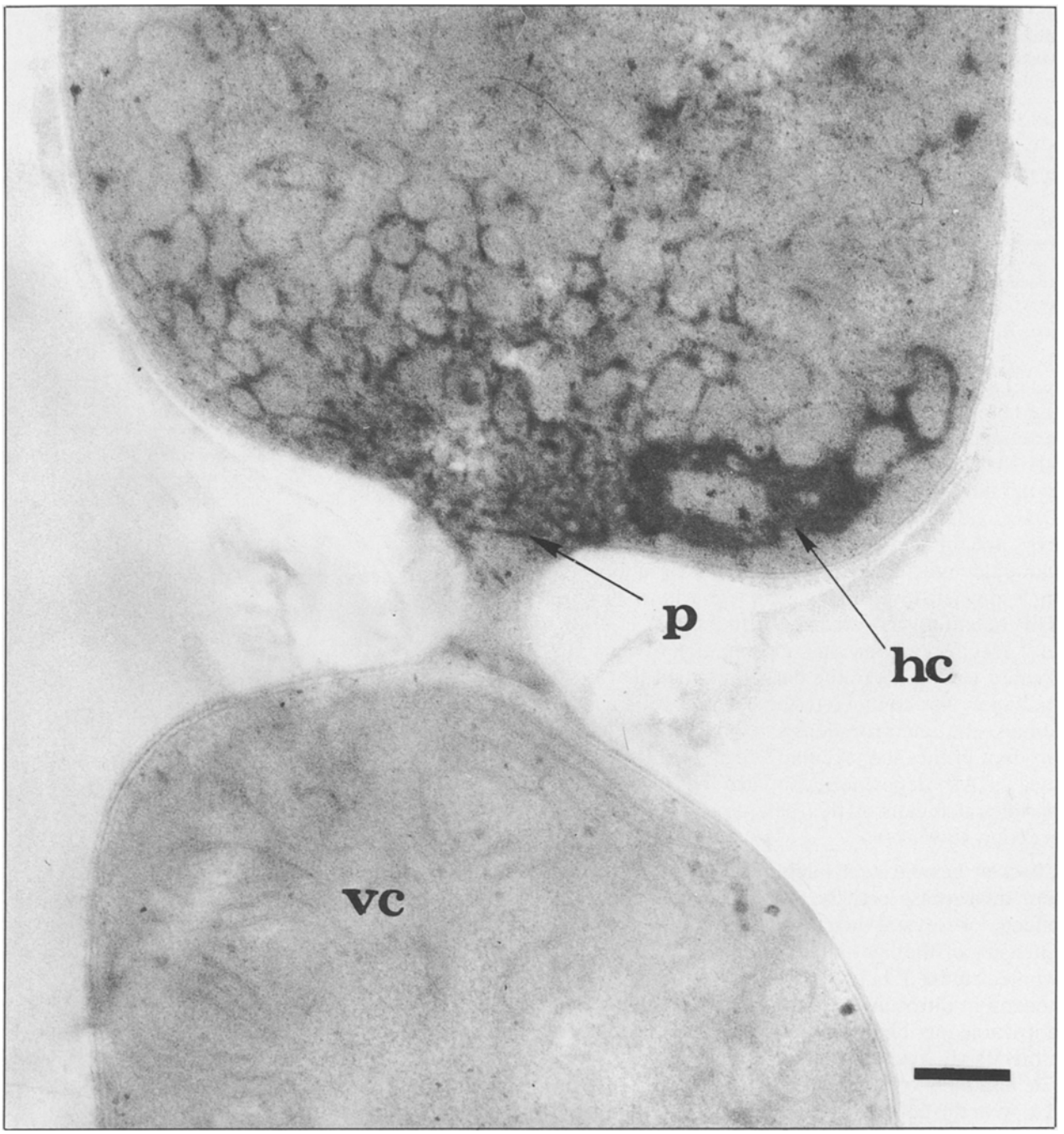

Fig. 3. Electron micrograph of the heterocyst–vegetative cell junction of *Anabaena cylindrica* incubated with diaminobenzidine (DAB) in the dark. Dense osmiophilic DAB deposition is seen in the polar (P), "honeycomb" (HC), and peripheral regions of the heterocyst but not in the vegetative cells (VC). Line scale = 0.5 μm.

reactions at low rates [9]. A longer incubation period may have revealed DAB oxidation in the thylakoids of fixed material.

The light-independent oxidation of DAB in the heterocyst is clearly correlated with what Lang and Fay [12] term the "honeycomb network". This reaction also extends around the periphery of the heterocyst but is less dense (Fig. 3). Several characteristics of the light-independent DAB reaction, namely its aldehyde stability, neutral pH optimum, and stimulation by H_2O_2, suggest that this reaction is due to a non-enzyme hemoprotein [see reference

Table 2. Effect of inhibitors on the light-independent diaminobenzidine (DAB) reaction of the heterocyst and nitrogenase activity of *Anabaena cylindrica*.

Inhibitor	DAB reaction (relative intensity)[a]	Acetylene reduction (% control)[b]	
		Argon	Argon + 20% O_2
None	++++	100	100
Aminotriazole 10 mM	++++	96	95
Na sulfide 10 mM	+	100	48
Na cyanide 5 mM	+	23	3
Na azide 20 mM	++	100	25
Hydroxylamine 10 mM	+	97	33

[a] See footnote [a] Table 1 for symbols.

[b] Acetylene reduction activity of the control culture was 10.6 nm $C_2H_4 \cdot min^{-1} \cdot mg$ (dry wt)$^{-1}$ under argon and 9.65 nm $C_2H_4 \cdot min^{-1} \cdot mg$ (dry wt)$^{-1}$ under 20% O_2. Assays were conducted in saturating light for 30 min.

5], perhaps one or more of the cytochromes or cytochrome-*c* peroxidase [3]. Since this reaction is not inactivated by glutaraldehyde and exogenous H_2O_2 is not required, it is probably not due to a true peroxidase. Nevertheless, peroxidase activity is reported to increase dramatically during differentiation of heterocysts [25], so it is possible that DAB oxidation in unfixed heterocysts is at least partially due to peroxidase activity. Catalase was evidently not involved in either the light-dependent or-independent reactions, since DAB oxidation was substantially inhibited at pH 10, the optimum for catalase oxidation of DAB [20,21]. Furthermore, aminotriazole, an effective inhibitor of catalase-effected DAB oxidation [6,8,10,17], had no effect on DAB deposition.

The components responsible for the light-independent oxidation of DAB may be involved in the O_2 protection mechanism of nitrogenase. The heterocysts are known to have high rates of O_2 uptake [7,19], which may scavenge O_2 from the site of nitrogen fixation. Both the light-independent DAB reaction and aerobic (but not anaerobic) nitrogenase activity were inhibited by sodium azide, sulfide, and hydroxylamine. These inhibitors are thought to act on cytochrome oxidase [11]; however, they are relatively nonspecific and interact to some degree with all hemoproteins [5]. The cytochromes of the photosynthetic electron transport chain of the heterocysts are not inhibited by these compounds at the levels used, since no inhibition of nitrogenase activity in the light was observed without O_2, which, under these conditions, is dependent on cyclic photophosphorylation for ATP [2]. However, dark nitrogenase activity, which requires oxidative phosphorylation for ATP [26], was abolished by azide.

Although we are unable to identify the component(s) involved in light-independent DAB oxidation, the location of this reaction indicates that the components involved may constitute an important means by which O_2 diffusing through the heterocyst wall and from the junction with the photosynthetically competent vegetative cells can be scavenged and thus prevent inactivation of the O_2-labile nitrogenase.

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