Journal of Bioenergetics and Biomembranes, Vol. 12, Nos. 3/4, 1980

Metronidazole and the Isolation of Temperature-Sensitive Photosynthetic Mutants in Cyanobacteria

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Received April 14, 1980

Abstract

A procedure has been developed for use of metronidazole (2-methyl-5-nitroimidazole-1-ethanol) as an enrichment agent during the isolation of temperature-sensitive, photosynthetic mutants in the cyanobacterium *Synechococcus cedrorum*. The protocol includes incubation with this drug following mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Incubation of photosynthetically active *S. cedrorum* cells with 1 mM metronidazole causes a light-dependent reduction of cell viability. Maximum reduction in cell viability occurred following 6 h of incubation. Cessation of electron transport reduced the impact of the drug by five orders of magnitude. Yet during the time of incubation, metronidazole did not influence the electron transport capacities of the *S. cedrorum* cells, suggesting that the thylakoid membrane was not the target of the toxic effects of this drug. In addition, this drug was found to be an effective electron acceptor to photosystem I although high concentrations were required to observe maximum rates of electron transfer. Metronidazole interacted in a noncompetitive manner with methyl viologen, which suggested that those two acceptors to photosystem I have unique reduction sites on the *S. cedrorum* thylakoid membrane. The temperature-sensitive strains that were isolated using the procedure presented here were assessed for photosynthetic electron transport and chlorophyll fluorescence (induction kinetics and low-temperature emission spectra) characteristics. Approximately one-half of the temperature-sensitive mutants isolated possessed abnormal photosynthetic properties when shifted to the restrictive temperature (40°C). A total of 31 strains have been characterized and initially classified, showing abnormalities throughout the photosynthetic electron-transport chain.

Introduction

An analysis of mutants defective in electron-transport activity provides an attractive approach to the study of photosynthetic membrane function. Such a genetic approach can offer information on the relationship of membrane

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structure and function as well as on the assembly and developmental control of the photosynthetic apparatus. Genetic strains of higher plants [1, 2] and of green algae [3–6] have been analyzed to study processes such as chlorophyll biosynthesis, chloroplast development, and thylakoid function. A majority of these studies have been accomplished using the green algae *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*. In many instances, genetic defects have been traced to deficiencies in lamellar protein structure and these deficiencies, in turn, correlated with abnormalities in photosynthetic function.

Until recently, no genetic strains of cyanobacteria have been recovered which show deficiencies in photosynthetic electron transport [7–9]. Owing to the relatively simple nature of the internal membrane structure and of the genetic apparatus of these prokaryotes, cyanobacteria would seem ideal for a genetic study of photosynthesis. In addition, the aerobic photosynthetic electron-transport system of these cells is similar to that of higher plants. Despite these advantages, two major problems have interfered with such a genetic study. First, the primarily phototrophic nature of the cyanobacteria would tend to make photosynthetically defective strains lethal. Most of the cyanobacterial species which have recently been shown capable of heterotrophic growth do so at only a fraction of their phototrophic growth rates [10, 11]. This is particularly true of the unicellular strains which are most suitable for genetic studies. Sherman and Cunningham [8, 9] have alleviated this problem by isolating temperature-sensitive (ts)[1] mutants. This ts technique allowed maintenance of the mutant strains at a permissive growth temperature while permitting the mutant phenotype to be analyzed following growth at a restrictive temperature.

A second problem is the isolation of photosynthetic mutants from cyanobacteria concerned a procedure to selectively enrich for mutants defective in electron transport. Several enrichment procedures have been developed for use with eukaryotic, photosynthetic organisms involving the treatment of these organisms with photosynthesis inhibitors, such as DCMU [12] and arsenate [12, 13], or with viologen dyes such as diquat [14]. However, attempts to adapt these procedures for use with cyanobacteria have proved unsuccessful [8]. Thus far, procedures to isolate cyanobacterial photosynthetic mutants have used rapid screening methods, such as visualization of highly fluorescent colonies.

In this communication, we present a mutant-enrichment protocol whereby metronidazole (2-methyl-5-nitroimidazole-1-ethanol) was used to

[1]Abbreviations used: DCMU: 3,4-dichlorophenyldimethylurea; F: magnitude of fluorescence. Subscripts *o*, *v*, and *m* denote original, variable, and maximal fluorescence. Superscript D denotes fluorescence in the presence of DCMU; PS I: photosystem I; PS II: photosystem II; ts: temperature sensitive.

select specifically for ts photosynthetic mutants of the cyanobacterium *Synechococcus cedrorum*. Metronidazole, a redox active drug used in the treatment of anaerobic infections [15, 16], was used by Schmidt et al. [17] to enrich for electron transport mutants in *Chlamydomonas reinhardtii*. Their studies suggested that metronidazole is toxic to photosynthetically active cells owing to its activity as an electron acceptor. We present here an assessment of the electron acceptor and toxic properties of metronidazole in cyanobacteria. These results demonstrate that metronidazole is an effective electron acceptor to PS I in these organisms and that the drug interacts in a noncompetitive manner with methyl viologen. The use of metronidazole in mutant isolation has yielded a large number of ts mutant strains which show aberrant photosynthetic electron-transport and fluorescence characteristics. The variety of characteristics obtained with these mutants attests to the utility of metronidazole as an enrichment agent.

Materials and Methods

Cells of *Synechococcus cedrorum* UTEX 1191, obtained from the Indiana University culture collection (presently at the University of Texas), were grown axenically in liquid culture [18] as previously described [8]. The cultures were routinely monitored for bacterial contamination. Prior to electron-transport analysis, cells were concentrated to about 100 μg chlorophyll/ml [19] in Tricine (20 mM, pH 7.5), mannitol (0.5 M), $NaKPO_4$ (5mM), and $MgCl_2$ (5mM). Spheroplasts were prepared by addition of 20 mM EDTA and 10 mg lysosyme/ml and by incubation for 2 h at 37°C. Spheroplasts were broken by osmotic rupture when diluted into the reaction vessel. Cell viability was determined by plating serial dilutions of cell suspensions on agar plates (1.5% Difco agar in growth medium). Plates were incubated in constant-temperature rooms (± 1°C) for 5–7 days using cool white fluorescent illumination (4×10^4 ergs $\cdot$ cm^{-2} $\cdot$ sec^{-1}). Plating efficiency was over 90% at 30°C and over 80% at 40°C.

Photosynthetic electron flow was measured by assaying light-induced changes in oxygen concentration using a YSI Clark-type oxygen electrode. An illuminating beam of white light (10^6 ergs $\cdot$ cm^{-2} $\cdot$ sec^{-1} incident upon the reaction cuvette) was passed through a 5-cm heat filter of 0.5% $CuSO_4$. The 3-ml reaction vessel was regulated at 25°C and contained, as a basic mixture, Tricine (40 mM, pH 7.5), KCl (60 mM), and *S. cedrorum* material to a concentration of 5 μg chlorophyll/ml. PS II + I electron transport was measured as oxygen consumption in the presence of 2 mM KCN plus either 3.3 mM methyl viologen or 25 mM metronidazole. Likewise, PS I electron flow was assessed in the presence of DCMU (10^{-5} M), ascorbate (1 mM),

diaminodurene (1 mM), KCN (2 mM), and either methyl viologen (1.7 mM) or metronidazole (25 mM). Assays to assess PS II activity measured oxygen production in the presence of ferricyanide (5 mM) and a class III electron acceptor. The class III electron acceptor which yielded optimal rates was 2,6-dichloro-*p*-benzoquinone (400 μM). DCMU and 2,6-dichloro-*p*-benzoquinone were prepared as concentrated ethanolic solutions; the concentration of organic solvent in the reaction vessel never exceeded 2%. Exceptions to these reaction conditions were as noted in Results. Light intensity was varied using neutral density screens. Chlorophyll was measured by the procedure of Arnon [19].

Fluorescence Analyses

Fluorescence kinetics were determined using a kinetic fluorimeter as described earlier [20]. Cells, suspended in growth medium with a chlorophyll concentration of 1–2 μg chlorophyll · ml^{-1}, were dark adapted for 30 min and were subsequently illuminated at the intensity of 5.6×10^4 ergs · cm^{-2} · sec^{-1} through a Corning No. 9782 filter. The fluorescence emission was detected using a Corning No. 2030 red filter. DCMU (10^{-5} M) was added following a light–dark cycle and, after a 1-min dark interval, the cells were reilluminated. Representative fluorescence kinetic traces for wild-type *S. cedrorum* cells are given in Sherman and Cunningham [8].

Fluorescence spectra were obtained at 77°K using a modified Aminco-Bowman spectrofluorimeter as previously described [21]. Concentrated cells were suspended in 60% glycerol. Representative spectra for wild-type cells are given in Sherman [22].

Chemicals and Solutions

Diaminodurene (from Aldrich) and 2,6-dichloro-*p*-benzoquinone (from Eastman) were recrystallized prior to use. Methyl viologen was from Aldrich and lysosyme was obtained from Sigma. Metronidazole, obtained either through Ms. Elsie Rivera of Searle & Co. (lots XBO 756, and 637) or from Sigma, was freshly prepared as an aqueous (50 mM) solution prior to each experiment. The effects of this drug were consistent despite the variety of sources.

Results

Our goal in initiating this research was to isolate and characterize ts mutants of *S. cedrorum* which would show a variety of defects in the photosynthetic light reactions. To this end, we sought to find a compound that

would selectively enrich for conditional-lethal, photosynthetic mutants. Although several enrichment procedures have been effectively used with eukaryotic photosynthetic organisms, none have been found suitable for use with cyanobacteria. Metronidazole, a redox active nitroheterocyclic antibiotic ($E_o = -325$ mV at pH 6.9), was introduced by Schmidt et al. [17] for mutant enrichment in *C. reinhardtii*. Our initial findings on the toxic and electron-transport characteristics of this antibiotic with cells of *S. cedrorum* suggest that metronidazole is ideal for selectively enriching for ts electron transport mutants in this cyanobacterium.

Figure 1 shows the results of a 6-h incubation of *S. cedrorum* cells with metronidazole. Incubation in the presence of the drug causes a dramatic reduction in cell viability which is enhanced five orders of magnitude by

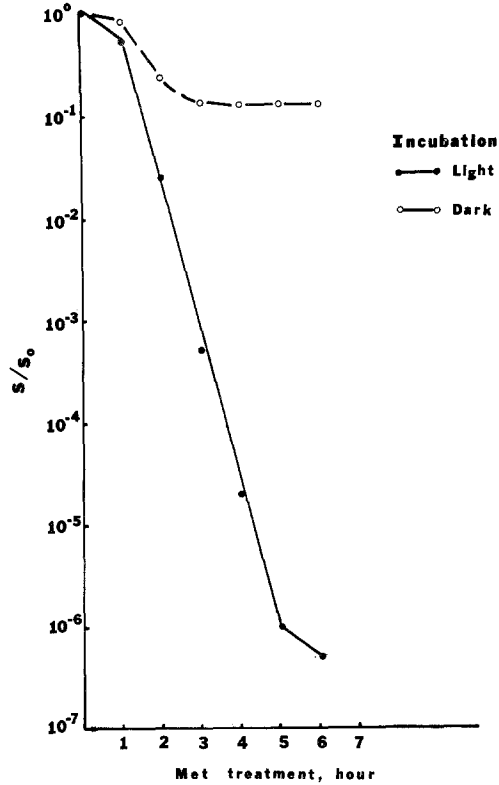

Fig. 1. Effect of metronidazole in cell viability of *S. cedrorum* cells. Cells (5×10^8 cells/ml) were incubated at 40°C with 1 mM metronidazole. Aliquots were centrifuged to remove the drug and were plated. S/S_o is the ratio of colonies recovered during the treatment to those recovered prior to incubation.

illumination. Maximum losses in cell viability occur after about 5 h. *S. cedrorum* cells are apparently more sensitive to the effects of metronidazole than are *C. reinhardtii,* since the effective concentration for killing was tenfold less in the case of *S. cedrorum* [17]. In addition, incubation of cells in darkness produced an 80% loss in cell viability with *S. cedrorum,* whereas no such loss was reported for *C. reinhardtii.*

Table I demonstrates that photosynthetic electron transport is required to obtain the maximum inhibitory effect by metronidazole. A cessation of electron flow, induced by darkness, by DCMU, or by a mutation blocking electron transport, decreased by five orders of magnitude the inhibitory effects of metronidazole. In addition, we have found that the loss of cell viability observed in the dark can be attenuated by incubating cells in the dark for 24 h prior to metronidazole treatment (unpublished observations).

Since the mode of killing by metronidazole is not well understood, and since the mechanism of metronidazole action can determine the types of electron-transport mutants which would be recovered, it became necessary to study the mechanism by which metronidazole kills photosynthetically active cells. Our experiments were designed to provide information on the following four aspects of metronidazole cytocidal activity during enrichment: (1) the electron-acceptor activity of metronidazole and the site at which metronidazole is reduced on the thylakoid membrane; (2) the mode of action whereby reduced metronidazole produces toxic products; (3) the target cell processes influenced by the toxic products; and (4) the ensuing loss of cell survivability caused by metronidazole treatment. This report focuses on the first three aspects of metronidazole activity.

Metronidazole is an effective Hill electron acceptor when exogeneously added to whole cells and to thylakoid membrane preparations of cyanobacteria. Addition of metronidazole causes a light-dependent increase in oxygen uptake, which is evidence of the participation of this drug in a Mehler

Table I. Effect of Photosynthesis on Metronidazole Killing[a]

Strain	Incubation	% Survival
Wild type	Light	6.7×10^{-5}
Wild type	Dark	16
Wild type	Light + 10^{-5} DCMU	12
Wild type	Dark + 10^{-5} DCMU	15
ts-53	Light	19
ts-53	Dark	22

[a]Cells were incubated with 1 mM metronidazole (in BG-11) for 6 h at 40°C, and then plated as detailed in Materials and Methods. Dark cultures were obtained by wrapping culture flasks with two layers of aluminum foil. Strain ts-53 is a temperature-sensitive mutant isolated by the procedure of Sherman and Cunningham [8] that does not grow at 40°C. It was incubated overnight at 40°C before metronidazole treatment.

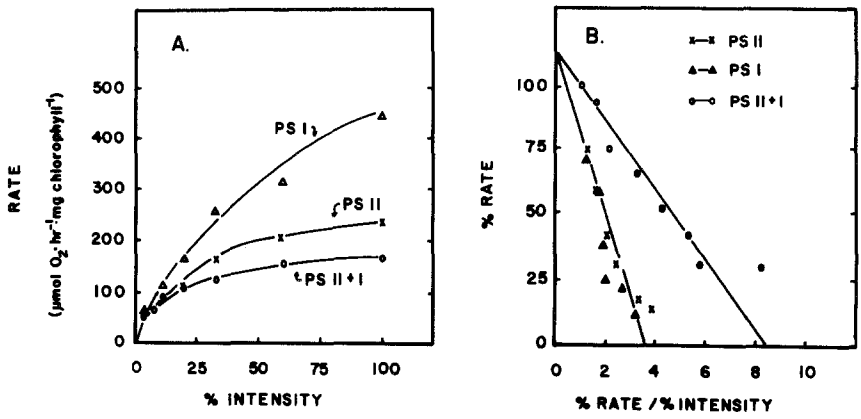

Fig. 2. Effects of light intensity on electron-transport reactions in cells of *S. cedrorum*. (A) Light saturation curves for reactions involving PS II ($H_2O \rightarrow$ 2,6-dichloro-*p*-benzoquinone), PS I (ascorbate/diaminodurene $\rightarrow$ metronidazole), and PS II + I ($H_2O \rightarrow$ metronidazole). (B) Woolfe–Augustinsson–Hofstee plot of the data shown in (A).

reaction with dioxygen [23]. Figure 2 shows the light-saturation curves of three electron-transport reactions in whole cells, two of which involve metronidazole. The first reaction utilizes water as an electron source and involves both PS II and I, while the second, using an ascorbate/diaminodurene electron couple as an electron source in the presence of DCMU, involves only PS I. These two reactions are compared with a 2,6-dichloro-*p*-benzoquinone mediated electron-transport reaction which assesses the capacity of PS II. Figure 2B shows that the intensities of light required to saturate the PS I and the PS II reactions are similar and are greater than that required to saturate a reaction assessing PS II + I. These findings are similar to the light intensities required to saturate electron-transport reactions with isolated thylakoid preparations of higher plants [24].

Schmidt et al. [17] showed that optimal rates of metronidazole reduction by PS I particles were obtained only when the electron-transport system was reconstituted by an addition of ferredoxin (also Guikema and Sherman, unpublished observations). In contrast, methyl viologen reduction required no ferredoxin addition. Since these two electron acceptors respond differently to ferredoxin in a reconstituted system, it is likely that they respond differently with thylakoid membranes *in vivo* as well. Figures 3A and B show the results of an experiment designed to confirm this in whole cells. The efficiency of methyl viologen as a PS II + I electron acceptor was assessed in the presence of differing, subsaturating concentrations of metronidazole. As shown in Figure 3A, titration curves for methyl viologen were obtained using three experimental conditions: in the presence of 0, 3.3, and 6.7 mM metronidazole. For each titration curve, the reaction rate obtained in the absence of methyl

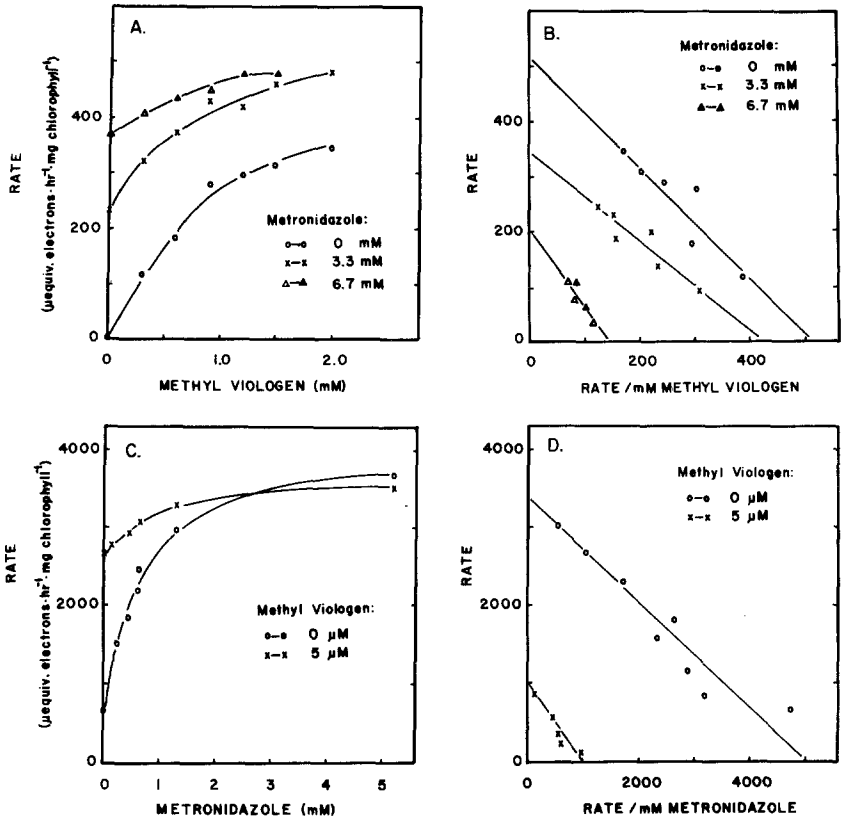

Fig. 3. Interaction of methyl viologen and metronidazole as electron acceptors in cells (A and B) or broken spheroplasts (C and D) of *S. cedrorum*. (A) Titration curves for methyl viologen in a light-induced PS II + I reaction in the presence of increasing metronidazole concentrations. (B) Woolfe–Augustinsson–Hofstee plot of the data in (A) following a correction for basal rates of electron transport as described in the text. (C) Titration curves for metronidazole in a light-induced PS I reaction in the presence of increasing methyl viologen concentrations. (D) Woolfe–Augustinsson–Hofstee plot of the data in (C) following correction for basal electron flow.

viologen was subtracted from the remaining points and the data, corrected in this manner, was replotted as in Figure 3B. The results of Figure 3 show that a noncompetitive relationship exists between methyl viologen and metronidazole since the "K_m" for methyl viologen remains stable despite increasing concentrations of metronidazole whereas the maximum velocity changes drastically. In addition, the "$K_{i\ \text{apparent}}$" for metronidazole was between 4.8 and 6.1 mM, equal to the "K_m" values obtained for this compound in other rate-saturation experiments (data not shown).

Figures 3A and B show that methyl viologen and metronidazole have

different rate-limiting factors governing their interactions with the thylakoid membrane. It seemed possible that the plasma membrane could provide such a rate limitation for reactions involving either metronidazole or methyl viologen by providing a barrier to efficient entry of these compounds into the cell. It is known, for example, that high concentrations and extended incubation times are needed to saturate methyl viologen-supported electron-transfer reactions in assays involving intact cells [25]. An experiment performed to dismiss this possibility is shown in Figs. 3C and D where the efficiency of metronidazole was assessed using broken spheroplasts in the presence or absence of methyl viologen. The data of Fig. 3C were corrected in the manner described for Fig. 3A and are replotted in Fig. 3D. Even under conditions which diminish the effects of cell integrity on methyl viologen, metronidazole and methyl viologen have a noncompetitive relationship. The findings of Fig. 3 suggest that these two electron acceptors interact with *S. cedrorum* thylakoids at different acceptor sites [26]. This conclusion is supported by the differences in E_o between the two compounds [27].

These results, in concert with those of Schmidt et al. [17], indicate that reduced metronidazole, generated by reducing equivalents from a site(s) in the electron-transport chain distinct from site(s) at which methyl viologen is reduced, is responsible for the toxic effects of this compound observed in photosynthetically active cells. Yet little information is available concerning the mechanism by which the reduced drug imposes its inhibitory influence. Schmidt et al. [17] propose two alternative mechanisms. First, reduced metronidazole, owing to its reactivity with oxygen [27, 23], generates superoxide radical and peroxide. These compounds would be toxic [28] if produced in excess or in microenvironments not protected by catalase and superoxide dismutase. In the second mechanism, the reduction of metronidazole produces an unstable intermediate [29] which, if not quenched by autooxidation, could degrade rapidly to form toxic breakdown products [30] or to covalently bind to DNA and protein [31]. Furthermore, it is not known which cellular processes, such as photosynthesis, DNA replication, or protein synthesis, are targets of these mechanisms to cause metronidazole-induced cell death.

Table II shows the results of experiments designed to test the effects of metronidazole treatment on the process of photosynthetic electron transport. Whereas the light-induced loss of cell viability was maximal at 6 h (Table II and Fig. 1), electron transport was not diminished until much later. After a 24-h incubation with metronidazole in the light, the capacity of the membranes to perform electron transport was completely lost. However, when metronidazole was incubated with cells in the absence of light, only cell viability was influenced after 24 h. In addition, no detectable loss of metronidazole occurred in these experiments, as assayed by absorbance at

Table II. Effects of Extended Incubation with Metronidazole on Cell Viability and Photosynthetic Activities in *Synechococcus Cedrorum* Cells[a]

Parameter	% Activity of zero incubation control		
	6-hr incubation, light	24-hr incubation, light	24-hr incubation, dark
Cell viability (plating efficiency)	6.0×10^{-4}%	4.8×10^{-4}%	10%
PS II electron transport ($H_2O \rightarrow$ dichlorobenzoquinone)	92%	0%	89%
PS II + I electron transport ($H_2O \rightarrow$ methyl viologen)	116%	0%	102%
A_{320}	101%	102%	99%

[a]Cells ($1–5 \times 10^8$ cells/ml) were incubated with 1.0 mM metronidazole at 40°C. A_{320} measurements (determining the concentration of metronidazole remaining) were performed following removal of cells via centrifugation. Photosystem assays and viable cell counts were measured as described in Materials and Methods. A_{320} measurement is expressed as the % of absorbance at 320 obtained immediately after metronidazole addition.

320 nm. Thus, the amount of metronidazole lost to degradation or to binding with cell material was below detectable levels.

It would appear from these experiments and from the data of Fig. 1 and Table I that there are two modes of action for metronidazole killing: (1) light-dependent, which occurs within the first 6 h of incubation; (2) light-independent, which takes place within the first few hours of treatment. Neither mode significantly consumes metronidazole, and it appears that neither mode of metronidazole killing affects the photosynthetic membrane as the primary target. It is the light-dependent mode of metronidazole-induced loss of cell viability upon which the use of this drug in mutant isolation is based.

Thus, the rationale for the use of metronidazole for selectively enriching for ts electron transport mutants in *S. cedrorum* is as follows. During photosynthetic electron transport, metronidazole is reduced with the concomitant formation of toxic products. Cells which are photosynthetically competent will be killed by the drug whereas cells defective in electron transport will not be able to reduce the drug nor form the toxic products. When a cell population is mutagenized and then raised to the nonpermissive temperature, mutants defective in electron transport will be unaffected by metronidazole. In this way, most of the competent cells will be destroyed, enriching the survivors for photosynthetic mutants.

The use of metronidazole, coupled with a ts mutant-isolation procedure, would be expected to yield four classes of ts mutants: (1) ts mutants which prohibit metronidazole entry into the cell; (2) ts mutants which can actively detoxify the inhibitory compounds produced by the interaction of metronida-

zole and the electron-transport chain; (3) ts mutants in which the targets of the toxic compounds are altered to reduce the impact of this drug; and (4) ts mutants deficient in electron transport which are unable to reduced metronidazole. Occurrence of the first class of mutants is unlikely owing to the lipophilic nature of the drug [30]. There is almost no confirmed evidence of resistance of anaerobic trichomonads to metronidazole [15], presumably for the same reason. Occurrence of mutants in the second and third classes is difficult to assess since the precise effects of metronidazole during enrichment are still unknown. However, the fourth class of ts mutant can be easily detected using fluorescence and electron-transport measurements.

The protocol for mutagenesis and for the isolation of ts mutants using metronidazole enrichment is summarized in Fig. 4. The mutagen, nitrosoguanidine, was previously found to be the most effective chemical mutagenic agent in cyanobacteria [8]. Under the conditions listed in Fig. 4, 50% of the cells survived the nitrosoguanidine treatment. The cells were then grown for

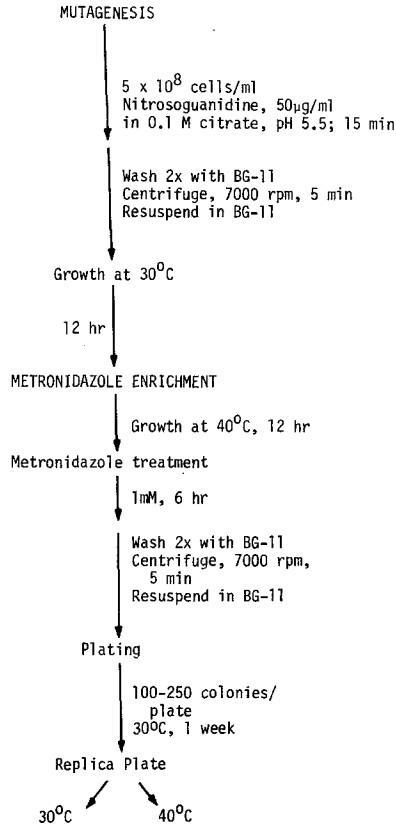

Fig. 4. Protocol for the isolation of ts mutants of *S. cedrorum* using metronidazole enrichment.

one to two generations at the permissive temperature to allow expression of the mutation and were subsequently shifted to the restrictive temperature to halt electron flow in the presumptive mutants. Cells were incubated in the light with 1 mM metronidazole, and the survivors plated at 30°C. In five separate experiments, a total of over 6,000 surviving colonies were replica plated, of which approximately 70 appeared to be temperature-sensitive. Colonies from the 30°C replicate were then grown in liquid culture and replated at 30 and 40°C. Sixty colonies showed significant differences in plating efficiency between the two temperatures (greater than 10,000-fold) and were chosen for further characterization. Of these, 31 separate strains have shown some abnormalities in photosynthetic properties. This finding of at least 50% photosynthetic mutants among the total number of ts strains shows the effectiveness of the metronidazole enrichment procedure.

A preliminary characterization of the 31 ts strains isolated by the procedures of Fig. 4 was accomplished as follows: (1) cells of wild type and the ts strains were incubated in the light for 3–4 days at both the permissive (30°C) and restrictive (40°C) temperature, and (2) the characteristics of the ts strains were compared to those of wild type (which had been identically treated) using three types of assays performed on intact cells. Those assays were: electron-transport assays, fluorescence induction kinetics, and fluorescence emission spectra at liquid-nitrogen temperatures. We used a 3–4 day incubation period prior to assay in this initial characterization to allow complete expression of the ts phenotype in each strain. During this incubation time, the growth characteristics of these strains were observed. All strains

Table III. Summary of Temperature-Sensitive Mutations Obtained from Metronidazole Enrichment

Class I: Low PS I activity
- Ia. High F_v and F_{max}
  - 78-91, 78-114 (high F696), 109, 79-8
- Ib. High F_v
  - 78-92, 101, 110
- Ic. Low V_v and F_{max}; high F716
  - 78-102, 103
- Id. High F_o and F_{max}; low F_{max}^{D}/F_{max}
  - 79-5, 6, 13

Class II: Low PS II and PS I activity
- High F_o and F_{max}; low F_{max}^{D}/F_{max}
  - 79-4, 79-18, 14, 19, 17

Class III: Higher photosystem activities and High F_v
- 78-93, 97

Class IV: Photosystem activities near normal
- IVa. High F_v and F_{max}
  - 78-87, 78-104, 94, 115, 96
- IVb. High F_v
  - 79-1, 79-9, 3, 10
- IVc. High F_v, low F_o, and low F_{max}; high F716
  - 79-98, 111
- IVd. Low F_v and F_{max}; high F696
  - 78-107

showed similar growth characteristics at 30°C (doubling time about 14 h; see Sherman and Cunningham [9]). However, variable growth patterns were observed at 40°C. Certain ts strains continued to increase in cell number for several generations followed by a cessation in growth (such as TsMet 79-4, 5, 14, and 19). Other strains showed continued growth at rates below those observed for wild type at 40°C (such as TsMet 78-91, 79-8, 10, 13, and 18). Cells of the former category, when shifted to the permissive temperature following an extended (5–7 day) incubation at the restrictive temperature, resumed growth and again assumed a phenotype typical of that of cells growing at 30°C.

An initial characterization of the 31 ts strains is given in Table III. Photosynthetic electron-transport capacity was the first parameter used to distinguish ts mutants in the classification scheme of Table III. Two reactions were employed to assess the transport capacities of *S. cedrorum* cells: $H_2O \rightarrow$ 2,6-dichloro-*p*-benzoquinone (PS II) and ascorbated/diaminodurene $\rightarrow$ methyl viologen (PS I). The transport capacities of these cells were compared with those of wild-type cells treated in an identical manner. Table IV shows that representative rates of electron flow obtained with wild-type cells possessed an enhanced PS II activity and a decreased PS I activity when grown at 40°C. Interestingly, the enhanced PS II activity observed with cells grown at 40°C is not seen when PS II activity is assessed by the reduction of 2,6-dichlorophenolindophenol [22]. This second PS II assay yields rates which are lower than those obtained with the quinone and shows decreased activity in cells grown at 40°C.

As shown in Table IV, the electron-transport activities of wild-type and ts strains were virtually identical when the cells were grown at 30°C. However, significant differences were observed when cells incubated at 40°C were compared. Nearly half of the 31 ts mutant strains showed low PS I activity following 40°C incubation (see also Table III); activities ranged from 20–50% of the rates obtained with 30°C-grown cells. A few strains, those of

Table IV. Electron Transport Rates of *S. cedrorum* Wild-Type and Selected ts Mutants

	Rates of electron transfer[a]			
	PS II		PS I	
Strain	30°C cells	40°C cells	30°C cells	40°C cells
Wild type	270	330	1200	930
TsMet 78-114	288	345	1380	570
79-5	230	261	1525	513
79-17	230	130	1025	340
79-19	290	107	1480	330

[a]Rates expressed as μmoles O_2 evolved (PS II) or consumed (PS I) per hour per milligram chlorophyll.

Class II, showed low PS II activity (40%–60% of the rates obtained with 30°C-grown cells) and showed a concomitant ts loss of PS I activity as well.

Within the framework of photosynthetic activity, the ts mutants were further categorized according to their fluorescence characteristics. Most of the ts strains in Table III showed higher than normal fluorescence for 40°C-cells and were kinetically very distinct. Figure 5 presents the fluorescence induction kinetics obtained for two mutant strains and illustrates the differences which were observed. TsMet 78-91 (Fig. 5A) was typical of strains in Classes Ia, Ib, and IVa–c in that incubation at 40°C resulted in an enhanced F_v. In addition, this ts strain showed complex, multiphasic induction kinetics with F_m being very sensitive to 10^{-5} M DCMU (F_m^D/F_m = 1.5–2.0). In contrast, TsMet 79-17 (Fig. 5B) is an example of a strain showing an enhanced F_o, a depressed F_v, and little sensitivity to DCMU (F_m^D/F_m = 1.0–1.1). In these strains (Classes Id and II), the F_v component of fluorescence, when present, shows a fast rise. In contrast with ts mutants showing elevated fluorescence levels, only a few strains showed lowered fluorescence (Classes Ic and IVd). Interestingly, all of the strains showing abnormally low fluorescence emission also showed an abnormal low-temperature emission spectra, with a high F_{716} peak for Class Ic and a high F_{696} peak for IVd.

The data in Table III are interesting in that there is no direct correlation between fluorescence characteristics and electron-transport properties when comparing specific classes of mutants. Certain fluorescence characteristics, such as a high F_v and F_m, were observed in both the presence and absence of a

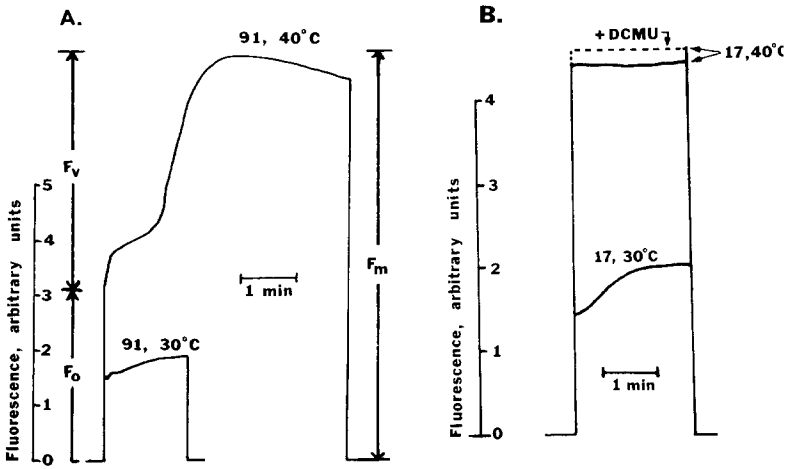

Fig. 5. Fluorescence induction kinetics of (A) TsMet 78-91 and (B) TsMet 79-17 cells grown at 30 and 40°C. The results were normalized to chlorophyll concentration and are given as arbitrary units.

concomitant inhibition of PS II. These mutants thus provide an excellent system with which to study the effect of membrane structure on fluorescence.

Classes III and IV as described in Table III might seem at variance with the metronidazole-enrichment techniques in that cells with normal or enhanced electron-transport reactions should be eliminated by this technique. However, since we were interested initially in assessing the capacities of PS I and PS II we employed partial electron-transport reactions which specifically analyzed portions of the electron-transport chain very near the photosystems. The acceptor 2,6-dichloro-*p*-benzoquinone is presumed to be reduced very near the primary acceptor to PS II [26]; the same is true for methyl viologen and PS I [17]. Thus, a significant portion of the electron-transport chain was not assayed and may well be loci for additional electron-transport lesions in Classes III and IV. We plan to study this possibility using a H_2O-to-metronidazole assay which will allow us to probe the entire chain between the water oxidizing site and ferredoxin.

Since the ts strains TsMet 78-91 and 79-17 represent mutant classes which possess vastly different fluorescence induction kinetics (Table I and Fig. 2), they were selected for further characterization. Cells of these strains and of wild type were grown at the permissive temperature of 30°C. Following growth, cells were briefly dark adapted (30 min) at either room temperature or at the restrictive temperature of 40°C and, subsequent to adaptation, were assessed for fluorescence induction kinetics at the appropriate temperature. As shown in Fig. 6, the strains grown at 30°C and assessed at room temperature had similar fluorescence induction kinetics. However, when adapted and assayed at 40°C, differences occurred. TsMet 79-17 showed a faster rise to F_m from F_o while TsMet 78-91 showed a slightly biphasic rise in F_v. Thus, it is evident that, at least in these two ts strains, a ts phenotype is expressed following brief (30 min) exposures to the restrictive temperature.

Discussion

In this communication we have shown that the antibiotic metronidazole can be used as a highly effective enrichment agent for the isolation of temperature-sensitive photosynthetic mutants in a cyanobacterium. The entire protocol is straightforward and allows for mutant isolation throughout the electron-transport chain. The basis for the use of metronidazole in this protocol is the reduction of the antibiotic by photosynthetically competent cells with a resulting loss of cell viability. Metronidazole apparently interacts with thylakoid membranes at a point on the reducing side of PS I and at a site

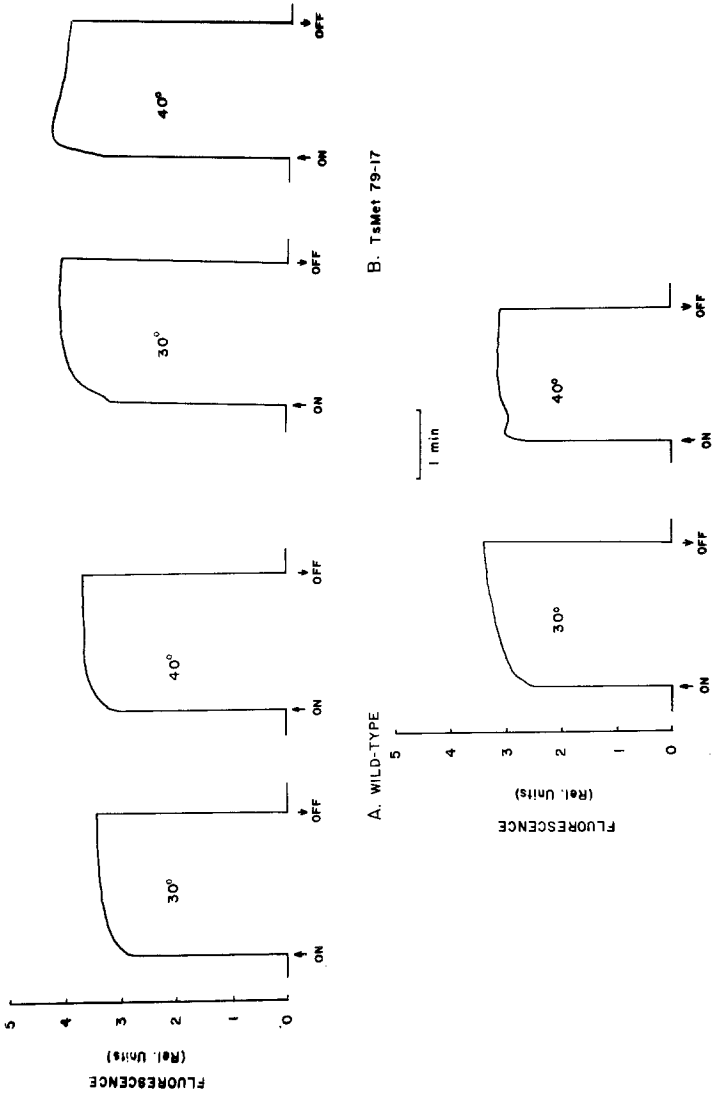

Fig. 6. Fluorescence induction kinetics of (A) wild-type, (B) TsMet 79-17, and (C) TsMet 78-91 cells grown at 30°C. The dark incubation (30 min) and kinetic assay were performed at either 30°C or at the restrictive temperature of 40°C. The results were normalized to chlorophyll concentration and expressed as arbitrary units.

which is distinct from the site of methyl viologen reduction. Antibiotic reduction is thought to be the cause of metronidazole inhibition of CO_2 fixation [32] and N_2 fixation [33] and is certainly the cause for cell death observed here.

Despite our findings and those of Schmidt et al. [17], the precise mechanism of metronidazole enrichment remains unknown. Anaerobic reduction of the drug causes covalent binding to DNA [29], destabilization of the DNA helix and strand breakage [34], and an inhibition of nucleic acid synthesis in bacteria [35]. Although metronidazole killing of bacterial cells has been accompanied by a disappearance or consumption of the drug [36], no such result has been observed with *S. cedrorum*. Thus, major topics for future study include the identity and target of the toxic products formed upon the interaction of metronidazole with cyanobacteria.

Using the protocol which we have presented here, 31 strains showing ts aberrations in photosynthesis have been isolated. These have been briefly characterized according to defects in electron transport and fluorescence. A variety of characteristics were observed, indicating both the usefulness of metronidazole in mutant isolation and the potential which the study of these cells has in the study of photosynthesis. The mutants appear to be genetically stable and have a number of properties that should make them useful in photosynthetic research.

We would expect that the isolation protocol would allow us to obtain two types of ts photosynthetic mutants: (1) those in which the lesion is in a gene coding for a "structural" membrane protein, and (2) those in which the lesion is in a gene that codes for enzymes involved in membrane synthesis and development. It would appear that both types have been isolated. As shown in Fig. 6, mutants such as TsMet 78-91 and TsMet 79-17 respond almost immediately upon shift to the nonpermissive temperature. They first show abnormal fluorescence kinetics which is followed by a decline in photosystem activity. Thus, it is likely that these mutations are in genes that code for membrane proteins. In addition, mutants in Class Id and the remainder of Class II appear to be developmental mutants. When these strains are shifted to 40°C, characteristic, temporal changes in photosynthetic parameters take place. In these mutants, the loss of photosynthetic function and the appearance of aberrant fluorescence properties develop over a number of generations at 40°C. These mutants should be of great value in the study of membrane assembly and development of the photosynthetic apparatus. Analyses of protein composition by one- and two-dimensional acrylamide electrophoresis indicate that membrane protein composition is altered in many of these strains (unpublished observations).

However, routine characterization of these ts strains is complicated by the interesting changes observed in wild-type cells grown at the permissive

and restrictive temperatures. Previous work [9, 22] has defined many of these structural and functional differences owing to differences in growth temperature. Aside from the insertion of different proteins into the thylakoid membrane [9], growth at 40°C results in a fatty acid composition of the thylakoids with a higher ratio of saturated to unsaturated fatty acids and longer-chain unsaturated fatty acids [22], observations consistent with those using *Anacystis nidulans* [37, 38]. Thus, an analysis of the ts characteristics of mutant cells must consider temperature-dependent changes which occur in wild type. Study of the structural gene mutations will not suffer from this complication.

The ability to readily isolate photosynthetic mutants in the cyanobacteria can have profound consequences for the genetic study of photosynthesis. This is particularly true now that techniques for the transfer of genetic information have been obtained. Recent work has demonstrated that *S. cedrorum* and related species of cyanobacteria contain plasmid DNA [39, 40]. Work is now in progress to add bacterial antibiotic markers to this plasmid DNA and thus construct a cloning vector [41]. Genes from wild-type DNA could then be cloned on the plasmid and inserted into a mutant cell. This would allow a direct identification of gene, gene product, and function. Unfortunately, it is a related strain, *Anacystis nidulans* R2 [42] and not *S. cedrorum* which gives the best transformation frequencies ([41]; van den Hondel and Sherman, unpublished observations). Thus, it is likely that *A. nidulans* R2 would be the ideal organism with which to pursue the genetic control of photosynthesis. The metronidazole enrichment technique can then be used to isolate mutants in strain R2 as a prelude to these genetic studies.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Ms. Jill Cunningham. This research has been supported by N.I.H. grant GM21827.

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