Production of the Secondary Metabolite Cyanide by Extracts of *Chromobacterium violaceum*

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The ability of cell-free extracts of *Chromobacterium violaceum* to form cyanide has been investigated. For maximal activity, extracts had to be prepared from bacteria harvested at the end of growth and stored under anaerobic conditions in the presence of dithiothreitol. Glycine was the substrate for cyanide formation by extracts and an electron acceptor such as phenazine methosulphate was required. Radiolabelling studies confirmed that cyanide was produced by decarboxylation of glycine. Formaldoxime, glyoxylic acid oxime, oxamic acid and *N*-hydroxyglycine, potential intermediates in the conversion of glycine to cyanide, were tested as substrates for cyanogenesis, but were inactive. However, *N*-hydroxyglycine was a non-competitive inhibitor of cyanogenesis from glycine. Cyanogenic activity was found principally in the particulate fraction, but a significant amount was also present in the supernatant fraction from high-speed centrifugation. The activity in the particulate fraction was solubilized with detergents.

INTRODUCTION

Cyanide production has recently been studied in growing cultures of *Chromobacterium violaceum* and in intact, harvested bacteria (Rodgers & Knowles, 1978; Collins *et al.*, 1980; Nazly *et al.*, 1981). Cyanide is formed by decarboxylation of glycine (Michaels *et al.*, 1965). Methionine stimulates cyanogenesis by growing cultures (Michaels & Corpe, 1965; Rodgers & Knowles, 1978). Cyanogenesis by *C. violaceum* has the typical properties of secondary metabolite formation (see Weinberg, 1971, 1977; Drew & Demain, 1977; Demain *et al.*, 1979); cyanide is produced only during a short period at the end of exponential growth and its level of synthesis depends on the iron and phosphate content of the medium (Rodgers & Knowles, 1978). Although differing in detail, similar data have been obtained for a *Pseudomonas* sp. (Wissing, 1974, 1975) and *Pseudomonas aeruginosa* (Castric, 1975, 1981). Harvested *C. violaceum* forms cyanide from glycine, but there is little stimulation by methionine (Nazly *et al.*, 1981). The ability of harvested bacteria to form cyanide depends on the time of harvesting, but is little affected by the growth conditions, though addition of glycine itself to the growth medium may cause a slight decrease in this capacity (Nazly *et al.*, 1981).

In order to study cyanogenesis by *C. violaceum* in more detail, a cell-free system was required. This paper reports a method for obtaining cyanide-producing extracts, how the activity of cyanogenesis varies in extracts derived from bacteria harvested at various stages of growth, and how the activities are affected by metabolites that could be involved in the catabolism of glycine to cyanide.

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Abbreviation: PMS, phenazine methosulphate.

METHODS

Organism and growth conditions. *Chromobacterium violaceum* strain NCIB 9131 was grown on a minimal salts medium containing 10 mM-glutamate as the sole source of carbon and nitrogen (Rodgers & Knowles, 1978). Where indicated, 2 mM-glycine and 0.5 mM-methionine were added to the medium.

Preparation of extracts. Bacteria were harvested by centrifugation at 20000 g for 5 min at 4 °C and washed twice in 100 mM-Tricine/NaOH buffer (pH 8.3) containing 1 mM-dithiothreitol. The bacteria were resuspended such that a 100-fold dilution had an A_{750} of 0.7 to 0.9. They were sonicated (4×20 s at 4 °C, MSE sonicator model 150W), with O₂-free N₂ passed over the surface of the sonication vessel. Whole cells and debris were removed by centrifugation at 16000 g for 30 min at 4 °C. The cell-free extract was separated into particulate and supernatant fractions by centrifugation at 150000 g for 90 min at 4 °C. The particulate fraction was suspended in 100 mM-Tricine/NaOH buffer (pH 8.3) containing 1 mM-dithiothreitol. Extracts were stored before assay by flushing storage vials with N₂, sealing and freezing at -15 °C.

Determination of cyanide production. Cyanide formation by extracts and whole cells was determined in a Wissing apparatus (Wissing, 1975, 1981). Bacteria or extract, plus any other required reagents, were added in 50 mM-Tricine/NaOH buffer (pH 8.3) to give a total volume of 1 ml. The reaction was initiated by addition of 0.1 ml 10 mM-glycine. Cyanide was removed by passing air over the surface of the stirred reaction mixture (800 ml min⁻¹). The incubation temperature was 30 °C. The cyanide was trapped in 1 ml 0.5 M-NaOH, taking samples at 5 min intervals. Samples were neutralized with 0.5 ml 1 M-HCl plus 0.5 ml 200 mM-KH₂PO₄/K₂HPO₄ buffer (pH 7.0) and assayed immediately by the method of Lambert (1975).

Other assays. The conversion of [1-¹⁴C]glycine and [2-¹⁴C]glycine to HCN and CO₂ was assayed, after the products had been trapped in 1 ml 0.5 M-NaOH by the method of Bunch & Knowles (1980). Samples of the reaction mixture were chromatographed in butan-1-ol/water/acetic acid (120:50:30, by vol.) on Eastman cellulose thin-layer chromatography plates. The radioactivity in each spot, located using amino acids as standards, was measured by scraping from the plate into phase combining system scintillation fluid and counting in a scintillation counter.

Protein was assayed by the Lowry method using bovine serum albumin as a standard. The O₂ content of O₂/N₂ mixtures was determined in an oxygen analyser (Taylor Servomix type OA 184).

Chemicals. [1-¹⁴C]Glycine (56 mCi mol⁻¹; 2.07 GBq mol⁻¹) and [2-¹⁴C]glycine (54 mCi mol⁻¹; 2.03 GBq mol⁻¹) were obtained from Amersham. Phase combining system was from Amersham Corp., Arlington Heights, Ill., U.S.A. *N*-Hydroxyglycine was kindly provided by Professor R. B. Beechey, Shell Research, Sittingbourne; TLC on Eastman cellulose plates, developed with butanone/*tert*-butyl alcohol/formic acid/H₂O (40:30:15:15, by vol.) yielded only one spot when detected with dimethylaminobenzaldehyde in acidic ethanol. The m.p. of *N*-hydroxyglycine was 130 °C, which agrees with previous data (Cooper & Griffith, 1979). Phenazine methosulphate (PMS) was obtained from Sigma. Whenever possible, all other reagents were of analytical grade; glass-distilled water was used throughout.

RESULTS

Maximum cyanide production by cell-free extracts of *C. violaceum* required the presence of dithiothreitol during preparation of the extracts; the optimum concentration was 1 mM. The presence of dithiothreitol during assay of the extracts was not necessary for maximal activity. Mercaptoethanol, cysteine and glutathione were less effective than dithiothreitol. Although strictly anaerobic conditions were not found to be essential during preparation of extracts, maximal cyanogenic activity was obtained when preparative steps were carried out, wherever possible, in the presence of O₂-free N₂.

The products of cyanogenesis (HCN and CO₂) are more oxidized than the substrate, glycine. Since only a low level of cyanogenesis was observed under aerobic assay conditions, the addition of an exogenous electron acceptor was required. Potential natural electron acceptors (NAD⁺, NADP⁺, FAD, FMN, FAD + FMN, mammalian cytochrome *c*, dihydrofolate) were ineffective. A range of redox dyes (PMS, phenazine ethosulphate, 2,6-dichlorophenolindophenol, tetrazolium blue, menadione, anthroquinone-2-sulphate, ubiquinone-9, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, 2-hydroxy-1,4-naphthoquinone, thionine, methyl viologen and potassium ferricyanide) were tested as electron acceptors. Of these, only PMS, phenazine ethosulphate and 2,6-dichlorophenolindophenol were effective. The activity of cyanide synthase in extracts was 0.25 ± 0.17 nmol cyanide min⁻¹ (mg protein)⁻¹ (mean $\pm$ S.D., 16 observations with separate extracts), using 1 mM-glycine and 100 μ M-PMS as substrates. The initial rate of cyanogenesis was proportional to the concentration of extract up to at least 20 mg protein ml⁻¹.

Fig. 1. HCN and CO₂ production from [1-¹⁴C]glycine and [2-¹⁴C]glycine by cell-free extracts. Extracts (6 mg protein ml⁻¹) were incubated in the presence of [1-¹⁴C]glycine (1 mM, 9.17 μCi μmol⁻¹) or [2-¹⁴C]glycine (1 mM, 8.3 μCi μmol⁻¹) and 100 μM-PMS in 50 mM-Tricine/NaOH (pH 8.3). Total radioactivity recovered in the NaOH trap (O), the H¹⁴CN from [2-¹⁴C]glycine (□) and the ¹⁴CO₂ from [1-¹⁴C]glycine (Δ) are recorded for a 30 min incubation.

Although phenazine ethosulphate gave about 20% greater activity than PMS, the latter was used routinely in further experiments since it gave more consistent results. The activity with 2,6-dichlorophenolindophenol was about 80% of that with PMS. There was no further stimulation of activity by adding FMN and/or FAD in the presence of PMS as the electron acceptor.

With 100 μM-PMS as the electron acceptor the initial rate of cyanogenesis and the total amount of cyanide produced under anaerobic conditions were the same as in the presence of air. When the concentration of O₂ was between 0.1% and 0.2% (v/v with N₂) the amount of cyanide produced was nearly doubled. Similar results were observed in the absence of PMS, although the amount of cyanide produced was very low.

The sensitivity of the cyanide synthase system to O₂ was further studied by exposing the extract to a stream of air in the assay vessel prior to initiating cyanogenesis by addition of PMS or glycine. After 3 min exposure to air in the presence of PMS, 45% activity was lost. Aeration in the presence of glycine, but not PMS, prevented loss of any activity for 11 min.

The apparent Michaelis constant for glycine was 300 μM in the presence of 100 μM-PMS. Higher concentrations of PMS resulted in less cyanide formation. Extracts prepared in 100 or 50 mM-Tris/HCl, 100 mM-KH₂PO₄/K₂HPO₄, 25 mM-HEPES/HCl or 25 mM-PIPES/HCl buffers (all at pH 8.3 and in the presence of 1 mM-dithiothreitol) had less than 50% of the activity of extracts prepared in 100 mM-Tricine/NaOH (pH 8.3) plus 1 mM-dithiothreitol. In each case there was a wide pH optimum between pH 7.5 and pH 8.3.

Dialysis of extracts overnight against buffer under anaerobic conditions resulted in no loss of activity. Nevertheless the requirement for cofactors was investigated. No stimulation of activity was found on addition, singly or in combination, of 10 mM-NADP⁺, 10 mM-NAD⁺, 5 mM-ATP, 5 mM-ADP, 10 mM-MgSO₄, 1 mM-pyridoxal phosphate or 1 mM-tetrahydrofolate. Methionine (0.5 mM), Fe³⁺ (30 μM) or K₂HPO₄ (10 mM), which maximally stimulated cyanogenesis by growing cultures (Rodgers & Knowles, 1978), had no effect on the amount of cyanide formed or the rate of cyanogenesis from glycine by cell-free extracts.

Figure 1 shows total radioactivity, H¹⁴CN and ¹⁴CO₂ trapped during assay of extracts using either [1-¹⁴C]glycine or [2-¹⁴C]glycine as substrate. The amount of ¹⁴CO₂ produced from [2-¹⁴C]glycine and H¹⁴CN from [1-¹⁴C]glycine are not recorded as they accounted for less than 5% and 7%, respectively, of the total radioactivity recovered. The carbon of HCN was derived from the C-2 group of glycine and the carbon of CO₂ from the carboxyl group of glycine. During the course of the assay the rate of trapping of the total radioactivity, H¹⁴CN and ¹⁴CO₂ was

Fig. 2. Effect of growth phase on cyanogenic capacity and activity of cyanide synthase by extracts. *Chromobacterium violaceum* was grown on the 10 mM glutamate/minimal salts medium containing 2 mM-glycine and 0.5 mM-methionine. Cyanide formation by harvested bacteria and cell-free extracts was measured as described in Methods, with 10 mM-glycine as the substrate and, in the extracts, 100 μ M-PMS as the electron acceptor. Δ , Growth; $\circ$, cyanide content of the medium; $\square$, cyanogenic capacity of harvested bacteria; $\bullet$, cyanide synthase activity of cell-free extracts.

constant for 9 min and then declined. This decrease in activity with time could not be prevented by the addition of more glycine, PMS or dithiothreitol, or any of the compounds given above. In addition, the protease inhibitor phenylmethylsulphonyl fluoride, catalase, superoxide dismutase or the use of O_2 -free N_2 instead of air did not prevent this time-dependent loss of activity. The slower apparent rate of release of CO_2 than HCN from glycine was due to the lower rate of scavenging of CO_2 from the Wissing apparatus (Wissing, 1975, 1981) rather than the efficiency of trapping.

The cyanide synthase system was found primarily in the particulate fraction, suggesting that it is membrane bound. However, a significant amount of activity (up to 40% of the total) was also recovered in the high-speed supernatant fraction, and this was not precipitated by further centrifugation. Total recovery of activity following high-speed centrifugation was variable, from 50 to 100% of the applied activity. The reasons for this are not clear, but it is probably due to oxygen lability of the cyanide synthase. Density-gradient centrifugation of the supernatant fraction did not reveal any discrete bands of activity in the supernatant fraction. Treatment of the particulate fraction with detergents solubilized cyanogenic activity. The recovery of activity in the soluble fraction after treatment of 4.7 mg protein (ml particulate fraction) $^{-1}$ with 0.5% (w/v) Lubrol PX, Nonidet P-40, Renex 690, SDS and Triton X-100 was 92%, 80%, 89%, 51% and 60%, respectively.

Several potential inhibitors of cyanide formation from glycine by the extract were tested. EDTA (1 mM) caused a slight stimulation, whereas relatively high concentrations of monovalent and divalent metals (7 mM- $CaCl_2$, 50 mM-KCl, 50 mM-NaCl, 50 mM-RbCl, 50 mM- $MgSO_4$, 50 mM- $MgCl_2$) inhibited activity by about a third to a half; $(NH_4)_2SO_4$ (100 mM) had little

effect. The respiratory inhibitor antimycin A caused only slight inhibition, as did the ionophore valinomycin. Up to 50% inhibition was caused by carbonyl cyanide *m*-chlorophenylhydrazone, which is an inhibitor of oxidative phosphorylation. Because of the possible relationship of cyanide synthase to amino acid oxidases (Wissing, 1975; Knowles, 1976), the effect of benzoate, which inhibits the latter enzymes, was tested. At a high concentration (30 mM) it caused 50% inhibition of cyanide synthesis.

Several intermediates of the pathway of conversion of glycine to cyanide have been proposed (Michaels *et al.*, 1965; Conn, 1973, 1979, 1981; Wissing, 1974; Knowles, 1976). The stable intermediates, oxamic acid, formaldoxime and glyoxylic acid oxime (all at 10 mM) did not act as substrates for cyanogenesis nor did they inhibit the conversion of glycine (10 mM) to cyanide. The addition of methionine (0.5 mM) with these compounds had no effect. Betaine, *N,N*-dimethylglycine, sarcosine, glycineamide and the methyl or ethyl esters of glycine (all at 10 mM) did not inhibit conversion of glycine (10 mM) to cyanide. Although *N*-hydroxyglycine (10 mM) did not act as a substrate for cyanogenesis, it was a non-competitive inhibitor for the conversion of glycine to cyanide, with 50% inhibition of activity at a 6 to 7 mM concentration in the presence of 10 mM-glycine.

The ability of harvested whole cells to produce cyanide from glycine, termed the cyanogenic capacity by Castric *et al.* (1979), was measured at various stages of growth on bacteria grown under conditions of high cyanide formation (Fig. 2). Cyanogenic capacity increased during growth to a maximum about 8.5 h after inoculation. Cyanogenic capacity was rapidly lost between 9 and 11 h after inoculation. The appearance and disappearance of cyanogenic capacity correlated with the activity of cyanide synthase in extracts. This suggests that the loss of activity observed in cultures was due to degradation of the enzyme rather than regulation of its activity or removal of cyanide from the medium by β -cyanoalanine synthase (Rodgers & Knowles, 1978).

The cyanogenic capacity of whole cells or the activity of cyanide synthase in extracts was unaffected by the presence or absence of glycine and methionine in the growth medium, but if glycine alone was added a slight decrease in activity was observed.

DISCUSSION

Cyanide synthase of *C. violaceum* is relatively sensitive to oxygen. Partial protection is provided by glycine, which also protects cyanide synthase of *P. aeruginosa* (Castric *et al.*, 1981). The enzyme from *C. violaceum* is also stabilized by dithiothreitol, suggesting that there is a thiol group at the active site. Wissing & Andersen (1981) have shown a requirement for dithiothreitol during preparation of cyanide synthase from *Pseudomonas* strain C.

The distribution of the cyanide synthase of *C. violaceum* between the particulate and supernatant fractions following high-speed centrifugation suggests that the enzyme is membrane bound but relatively easily removed from the membrane. Wissing (1975) has also suggested that cyanide synthase of *Pseudomonas* strain C is membrane bound. Like the enzyme in *C. violaceum* it can also be solubilized by detergents (Wissing & Andersen, 1981).

The cyanide synthase of *Pseudomonas* strain C and *P. aeruginosa* (Wissing, 1975; Castric *et al.*, 1979), as well as *C. violaceum* can use PMS as an electron acceptor. A natural electron acceptor has yet to be found, but the location of the enzyme in the membrane suggests that it may be coupled to the respiratory system. An inhibitor of electron transport, an inhibitor of oxidative phosphorylation and an ionophore did not greatly affect cyanogenesis by extracts of *C. violaceum*. In contrast, the enzyme is sensitive to some monovalent and divalent ions. This is not due simply to an increase in ionic strength, since 100 mM-(NH₄)₂SO₄ did not inhibit the enzyme. Collins *et al.* (1980) noted a decrease in cyanogenesis by cultures in which glycine was the sole source of nitrogen compared to those containing both ammonia and glycine.

No evidence has been gained about intermediates involved in the conversion of glycine to cyanide. Although oxamic acid, formaldoxime, glyoxylic acid oxime and *N*-hydroxyglycine did not act as substrates, this does not rule them out as intermediates. For example, substrate channelling might occur as happens in plant cyanogenesis (Conn, 1979), or an electron/hydro-

gen acceptor or donor might be required which is recycled when glycine is the substrate. The inhibition of cyanogenesis from glycine by *N*-hydroxyglycine possibly suggests that it is acting as a substrate analogue, though if this were the case it might be expected to be a competitive rather than a non-competitive inhibitor.

The appearance and disappearance of cyanide synthase activity during growth of *C. violaceum* corresponds well to the activities of the enzyme predicted from earlier indirect studies with growing cultures (Rodgers & Knowles, 1978; Nazly *et al.*, 1981). Similar data have been obtained with *P. aeruginosa* (Castric *et al.*, 1979). This pattern of enzyme formation is typical of enzyme synthesis seen with other secondary metabolites, for example gramicidin (Matteo *et al.*, 1975).

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