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CO oxidoreductase from *Streptomyces* strain G26 is a molybdenum hydroxylase

Judith M. BELL,*‡ John COLBY* and Edwin WILLIAMS†

*North East Biotechnology Centre, Biology Department, Sunderland Polytechnic, Sunderland SR1 3SD, U.K., and
†Microbial Technology Group, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

CO oxidoreductase was purified to 95% homogeneity from crude mycelial extracts of *Streptomyces* G26. The purified preparation has a specific activity of 25.7 units/mg, a 13-fold improvement on crude soluble mycelial extracts. The native enzyme (M_r 282000) is composed of non-identical subunits of M_r 110000 and 33000. It is a molybdenum hydroxylase containing 1.6 mol of FAD, 7.3 mol of Fe, 8.3 mol of acid-labile sulphide and 1.3 mol of Mo per mol of enzyme. Purified CO oxidoreductase catalyses the reduction of benzyl viologen, confirming the previously reported ability of this enzyme to interact with low-potential acceptors. Cytochrome *c* reduction cannot be accounted for entirely by non-enzymic reduction by superoxide radicals. NAD^+ and $NADP^+$ are not reduced, nor is clostridial ferredoxin.

INTRODUCTION

CO oxidoreductases (CO oxidase, CO dehydrogenase, CO:acceptor oxidoreductase) have been purified and characterized from several Gram-negative bacterial sources including the mesophiles *Pseudomonas carboxydovorans* (Meyer & Schlegel, 1979, 1980; Meyer, 1982; Meyer & Rajagopalan, 1984) and *Pseudomonas carboxydohydrogena* (Kim & Hegeman, 1981) and the moderate thermophile *Pseudomonas thermocarboxydovorans* (Turner *et al.*, 1984; Bell *et al.*, 1985). The results of M_r determinations, absorption spectrum measurements and cofactor analyses suggest that all these enzymes belong to the class of molybdenum hydroxylases (Williams *et al.*, 1986). CO oxidoreductases have also been found in crude extracts of some Gram-positive bacteria such as *Bacillus schlegelii* (Kruger & Meyer, 1984), *Arthrobacter* 11/x (Meyer & Rohde, 1984) and *Streptomyces* G26 (Bell *et al.*, 1985, 1987), but no studies with purified preparations have hitherto been published.

In previous papers we reported the finding that partially purified CO oxidoreductase from *Streptomyces* G26 catalysed the rapid CO-dependent reduction of viologen dyes (Bell *et al.*, 1985, 1987). These low-potential electron acceptors are not reduced by CO oxidoreductases purified from Gram-negative carboxydotrophs, but do interact with the nickel-containing CO dehydrogenases isolated from certain anaerobic bacteria (Ragsdale *et al.*, 1983*a*,*c*; Krzycki & Zeikus, 1984). It was therefore important to confirm that purified enzyme would still reduce these low-potential acceptors and to determine whether this CO oxidoreductase is a molybdenum hydroxylase or a nickel enzyme.

EXPERIMENTAL

Chemicals

Phenazine ethosulphate, 2,6-dichlorophenol-indophenol, Methylene Blue, benzyl viologen, Nitro Blue Tetrazolium, Coomassie Brilliant Blue G250 and R250, 2-mercaptoethanol, *NN'*-dimethyl-*p*-phenylenediamine, FAD, FMN, yeast NAD^+ and $NADP^+$, *Clostridium pasteurianum* ferredoxin, horse heart cytochrome *c*, *Aspergillus niger* glucose oxidase, bovine liver catalase, xanthine oxidase from buttermilk, superoxide dismutase from bovine erythrocytes, bovine serum albumin, bovine thyroglobulin, horse spleen ferritin, yeast alcohol dehydrogenase, yeast enolase, jack-bean urease, sweet-potato β-amylase and horse spleen apoferritin were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Acrylamide, Electran *NN'*-(1,2-dihydroxyethylene)bis-acrylamide, Bromophenol Blue, 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline), toluene-3,4-dithiol, mercaptoacetic acid, Na_2S and riboflavin were from BDH Chemicals, Poole, Dorset, U.K.

The pH values of buffer solutions were measured at room temperature.

Preparation of purified CO oxidoreductase

Streptomyces G26 was grown on CO in a 3-litre fermenter and washed mycelium was prepared as described by Bell *et al.* (1987).

Day 1. All procedures were done at 4 °C or on ice. About 12 g wet wt. of *Streptomyces* G26 mycelium was broken by sonication and a crude soluble extract prepared as described previously (Bell *et al.*, 1987). The crude extract was then loaded on to a DEAE-Sephacel (Pharmacia, Milton Keynes, Bucks., U.K.) anion-exchange column (35 cm × 2.5 cm) and eluted with a 300 ml gradient of 0–0.6 M-NaCl in 20 mM-Tris/HCl buffer, pH 7.0. The CO oxidoreductase-containing fractions, eluted at approx. 0.3 M-NaCl, were pooled, diluted 1:1 with 10 mM-sodium phosphate buffer, pH 7.0, and loaded on to a TSK DEAE-3SW column (75 mm × 7.5 mm). The CO oxidoreductase was eluted by h.p.l.c. with a 60 min gradient of 0–0.5 M-NaCl in 10 mM-sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. The fractions containing CO oxidoreductase activity were pooled and the CO oxidoreductase

‡ To whom correspondence should be addressed.

was precipitated from solution by the addition of $(NH_4)_2SO_4$ to 70% saturation. The precipitated protein was pelleted by centrifugation at 14300 *g* for 10 min, then resuspended in 0.5 ml of 50 mM-sodium phosphate buffer, pH 7.0. Ice-cold ethanediol (0.5 ml) was added and the preparation was stored at −20 °C until required.

Day 2. The stored CO oxidoreductase preparation was dialysed against 500 ml of 50 mM-sodium phosphate buffer, pH 7.0, for 2 h. The dialysis residue was loaded on to a TSK G3000SWG preparative gel-filtration column (300 mm × 21.5 mm) in 2 ml batches and eluted by h.p.l.c. with 50 mM-sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. The active fractions were combined to give the purified enzyme preparation. This rapid purification procedure decreased to a minimum the loss of CO oxidoreductase activity that occurred during the handling of the more highly purified preparations.

Determination of enzyme specific activity and purity

Standard aerobic assay. This was done as described previously (Bell *et al.*, 1987).

Anaerobic assay. The anaerobic assay used was based on that of Meyer & Schlegel (1980). A 3 ml reaction volume containing 0.15 mmol of sodium phosphate buffer, pH 7.15, 1 unit of bovine liver catalase (EC 1.11.1.6), 1 unit of *Aspergillus niger* glucose oxidase (EC 1.1.3.4), 0.3 μmol of glucose and 0.3 μmol of Methylene Blue, 3 μmol of benzyl viologen or 150 nmol of ferredoxin was placed in a 3 ml glass cuvette with a round ground-glass neck and the cuvette was sealed tightly with a Suba-Seal stopper. By using needle inserts through the stopper the contents of the cuvette were flushed with N_2 gas and then CO gas, each for 5 min. The cuvette was pre-warmed to 50 °C and the reaction was started by injecting N_2-flushed CO oxidoreductase preparation through the stopper. Methylene Blue reduction was monitored at 615 nm, benzyl viologen reduction at 550 nm and ferredoxin reduction at 390 nm.

Protein determination. The concentration of protein in CO oxidoreductase preparations was determined by the modified Lowry method of Kennedy & Fewson (1968), with bovine plasma albumin (fraction V) as standard.

Criteria for purity. The purification was monitored by observing the band pattern obtained by non-denaturing gel electrophoresis on 7.5% (w/v) polyacrylamide slab gels. Gels were stained for protein with methanolic Coomassie Brilliant Blue R250. CO oxidoreductase activity was detected on gels by using the method described previously (Turner *et al.*, 1984).

Holoenzyme and subunit M_r determination

Ferguson plot. The M_r of CO oxidoreductase was determined by the electrophoretic method of Hedrick & Smith (1968), which is based on the relationship between the M_r of a protein and its electrophoretic mobility in non-denaturing gels of a given acrylamide concentration. Rod gels covering the range 4–8% (w/v) polyacrylamide were used. Protein M_r markers employed were bovine thyroglobulin (M_r 669000), horse spleen ferritin (M_r 440000), bovine liver catalase (M_r 230000), yeast alcohol dehydrogenase (M_r 150000) and two preparations designed for use in M_r determinations by gel electrophoresis, bovine serum albumin (monomer M_r 66000, dimer M_r 132000) and jack-bean urease (dimer M_r 240000, tetramer M_r 480000). Samples of each marker protein and the CO oxidoreductase preparation were loaded on to separate gels covering the range of polyacrylamide concentrations. Gel loads contained, in running buffer, 20 μg of protein, 20% (w/v) sucrose and 0.2% Bromophenol Blue tracker dye. When electrophoresis was completed, the gels were stained with the $HClO_4$/Coomassie Brilliant Blue G250 stain (Reisner *et al.*, 1975). After staining was complete, the distances migrated by the tracker dye and the protein bands were measured and the R_F values were calculated. For each protein, the slope of the plot of $100 \times \log(R_F \times 100)$ against polyacrylamide concentration (%, w/v) was determined by linear regression.

Molecular exclusion chromatography. Standard proteins (M_r range 66000–443000) and purified CO oxidoreductase were loaded individually on to a TSK G3000SWG column (300 mm × 21.5 mm) as 10 mg/ml solutions by use of a 100 μl sample loop. The column was eluted with 50 mM-sodium phosphate buffer, pH 7.0 (1 ml/min), and the 280 nm absorbance of the eluate was monitored continuously. The standards were horse spleen apoferritin (M_r 443000), sweet-potato β-amylase (M_r 200000), yeast alcohol dehydrogenase (M_r 150000) and yeast enolase (M_r 66000). The void volume (V_0) of the column was 43.5 ml (elution volume of Dextran Blue 2000, M_r 2000000). The relative retention of each marker protein, measured as elution volume/void volume (V_e/V_0), was calculated and used to prepare the calibration curve.

Subunit M_r values. These were determined by polyacrylamide-gel electrophoresis in the presence of SDS, as described by Weber *et al.* (1972). Protein samples were run on slab gels prepared from 6.5% (w/v) acrylamide and 0.17% (w/v) bisacrylamide in 0.1 M-sodium phosphate buffer, pH 7.2, containing 0.1% (w/v) SDS. The running buffer contained 0.1% (w/v) SDS in 0.1 M-sodium phosphate buffer, pH 7.2. SDS/polyacrylamide gels were calibrated by using an Electran M_r calibration kit (M_r range 56000–280000) (BDH Chemicals). The contents of one vial of the M_r marker kit containing 2 mg of protein were dissolved in 1 ml of 10 mM-sodium phosphate buffer, pH 7.2, containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. The solution was then immersed in a boiling-water bath at 100 °C for 2 min. Purified CO oxidoreductase (0.45 mg of protein/ml) was treated similarly. Glycerol (4 drops) and 0.0125% Bromophenol Blue tracker dye were added to the incubation mixtures. Then 50 μl and 100 μl portions of the marker solution and enzyme solution were loaded on to the gel beneath the running buffer and the gels were electrophoresed at 60 V until the dye front had migrated about 10 cm down the gel. The dye front was marked with India ink before staining with methanolic Coomassie Brilliant Blue stain.

Analyses

Glassware used in the preparation of samples and standards for metal analysis was acid-washed by soaking in 2 M-HCl for 30 min and then rinsed copiously with de-ionized and glass-distilled water. Before use, dialysis tubing was boiled in ethanol, washed in glass-distilled

Table 1. Purification of CO oxidoreductase from *Streptomyces* G26

CO oxidoreductase was purified from crude soluble mycelial extracts by the procedure descrived in the Experimental section. CO oxidoreductase activities were determined by the standard spectrophotometric assay.

Fraction	Activity (units/ml)	Total activity (units)	Protein (mg/ml)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude sonicated extract	27.0	703	14.00	1.9	1.0	100
DEAE-Sephacel eluate	5.9	290	1.07	5.5	2.9	41
H.p.l.c. TSK DEAE-3SW eluate	26.6	253	2.37	11.2	5.8	36
H.p.l.c. TSK G3000SWG gel filtrate	18.8	90	0.73	25.7	13.3	13

water, boiled in 1 mM-Na_2EDTA and then finally washed with more distilled water.

Pretreatment of enzyme samples. Purified CO oxidoreductase was dialysed against 1 litre of 10 mM-Tris/HCl buffer, pH 7.0, containing 1 mM-Na_2EDTA for 12 h at 4 °C. The buffer was decanted and the dialysis vessel and the tubing were rinsed with glass-distilled water. The sample was then dialysed against 10 mM-Tris/HCl buffer, pH 7.0, for 3 h at 4 °C.

Determination of flavin. The flavin moiety was extracted by heating enzyme samples at 100 °C for 10 min, in the presence or in the absence of 1% (w/v) SDS, or by incubation in the presence of 5% (w/v) trichloroacetic acid at 4 °C for 20 min. In each case the precipitated apoenzyme was removed by centrifugation. The absorbance of the flavin extracts was measured at 450 nm and the flavin content of the enzyme calculated.

T.l.c. of extracted flavin. A sample of free flavin was prepared from purified CO oxidoreductase (5 mg/ml) by heating at 100 °C for 10 min. Samples (10 μl) of FAD, FMN and riboflavin (all 10 μM solutions in 50 mM-sodium phosphate buffer, pH 7.0) and a 20 μl sample of free flavin from CO oxidoreductase, having an absorbance of 0.383 at 450 nm, were spotted on to a 5 cm × 7.5 cm silica gel $60F_{254}$ pre-coated aluminium sheet (BDH Chemicals). The chromatogram was developed in solvent containing butanol/acetic acid/glass-distilled water (4:3:3, by vol.). After it had dried, the flavin standards were clearly visible as yellow spots on a white background.

Colorimetric determinations. Acid-labile sulphide in purified CO oxidoreductase was determined by the Methylene Blue method of Fogo & Popowski as modified by Brumby *et al.* (1965). Iron was determined by using the method of Johnson (1964) except that the assay was scaled down 1:20, to give a final assay volume of 1.25 ml. The amount of molybdenum in purified CO oxidoreductase was determined by the dithiol method of Cardenas & Mortenson (1974).

Atomic absorption spectroscopy. Molybdenum and nickel were determined with a model 157 atomic absorption spectrometer (Instrumentation Laboratories, Wilmington, MA, U.S.A.). Molybdenum was determined at 313.3 nm by use of an SJ Hollow cathode molybdenum lamp, supplied by Spectronic Services, Leeds, U.K. Nickel was determined by use of an SJ Hollow cathode nickel lamp. Enzyme samples were prepared by wet oxidation as described by Meyer (1982).

RESULTS

Purification of CO oxidoreductase

Partially purified CO oxidoreductase from *Streptomyces* G26 (Bell *et al.*, 1987) was further purified by ion-exchange and molecular exclusion chromatography by using h.p.l.c. techniques as described in the Experimental section. The final preparation had a specific CO oxidoreductase activity of 25.7 units/mg of protein, representing a 13-fold increase in specific activity over crude soluble extract, with a yield of 15.4% (Table 1). Non-denaturing polyacrylamide-gel electrophoresis gave one major band with two or three minor contaminating bands. The major protein band (comprising about 95% of the total protein on the gel) corresponded to the CO oxidoreductase activity band detected by the procedure described by Turner *et al.* (1984).

Enzyme stability during the purification process appeared to vary from batch to batch, as did the specific CO oxidoreductase activity in the crude extracts of *Streptomyces* G26. High specific activity in the crude soluble extract was associated with greater stability of the CO oxidoreductase during purification, whereas low CO oxidoreductase activity in crude extracts often meant that all activity would be lost before purification was complete. This activity could be lost during a purification step or simply by short-term storage (e.g. less than 12 h) at 4 °C. The reasons for this variation in enzyme stability are obscure at present, as every attempt was made to standardize the growth conditions under which mycelium was produced. Purified enzyme preparations were routinely stored at −20 °C in 50% (v/v) ethanediol, and under these conditions little loss of activity was observed over several weeks. At 4 °C purified preparations lost most of their activity on overnight storage.

The appearance of inactive forms of molybdenum hydroxylase enzymes during purification is a frequent problem and the addition of sodium salicylate and EDTA to buffers has been found useful in preventing the inactivation of samples during purification (Bray, 1975). In an experiment to test this, pelleted mycelium of *Streptomyces* G26 obtained from a single harvesting was divided into two equal parts. Each was broken by sonication, and crude extracts were prepared and then purified by the usual procedure except that one batch

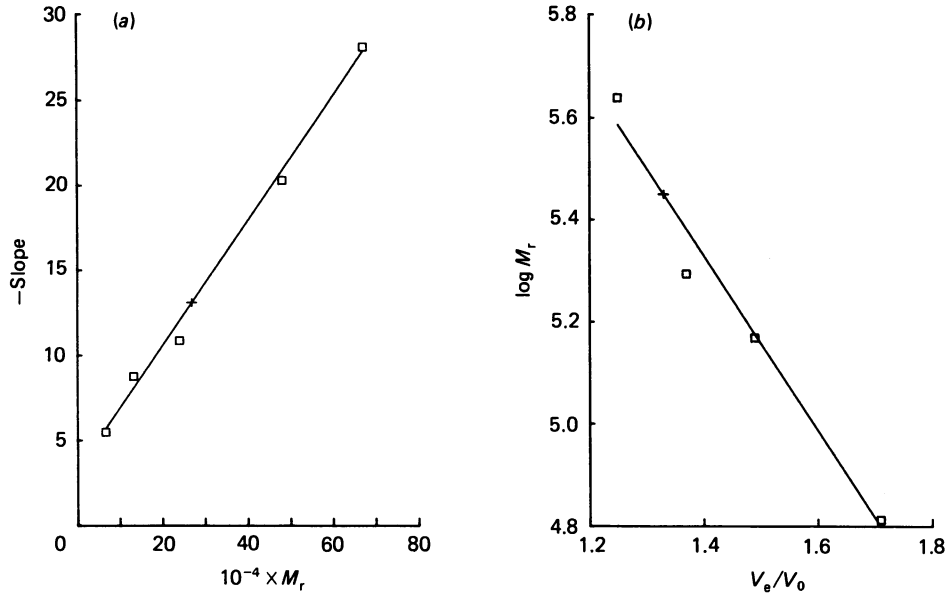

Fig. 1. Determination of the M_r of *Streptomyces* G26 CO oxidoreductase

(*a*) Ferguson plot. Samples of purified CO oxidoreductase, and of standard proteins of known M_r, were electrophoresed on a range of gels of 4% to 8% (w/v) polyacrylamide concentration. After protein staining, the R_F value for each protein was determined. The slope of the plots of polyacrylamide concentration (%, w/v) against $100 \times \log(R_F \times 100)$ for each standard (□) and for CO oxidoreductase (+) was calculated and used to plot the graph of the negative of the slope against M_r. (*b*) Molecular exclusion chromatography. Standard proteins (□) (M_r 30000–500000) and purified CO oxidoreductase (+) were loaded on to a TSK G3000SWG h.p.l.c. column (300 mm × 21.5 mm). The column was eluted with 50 mM-sodium phosphate buffer, pH 7.0 (1 ml/min); the 280 nm absorbance of the eluate was monitored continuously. The relative retention of each marker protein (V_e/V_0) was calculated and used to prepare the calibration curve.

was prepared with 1 mM-sodium salicylate and 1 mM-EDTA in all the buffers used. No significant improvement in yield or final specific activity was observed.

Holoenzyme and subunit M_r values

The M_r of the purified native enzyme was determined by two methods. The electrophoretic method of Hedrick & Smith (1968) yielded a value of 268000 ± 14000 (Fig. 1*a*), and molecular exclusion chromatography on TSK G3000SWG indicated an M_r of 282000 ± 9000 (Fig. 1*b*). On the h.p.l.c. sizing column, the elution time for CO oxidoreductase was 58 min compared with an elution time of 59 min for xanthine oxidase from buttermilk.

The subunit structure of CO oxidoreductase was determined by denaturing SDS/polyacrylamide-gel electrophoresis in 6.5% (w/v) polyacrylamide gels as described in the Experimental section. Two clear bands were always observed, and M_r values of 110000 ± 1100 and 33000 ± 1100 were calculated from their mobilities relative to the standards (Fig. 2). The smaller polypeptide (M_r 17000–21000) found on SDS treatment of the CO oxidoreductases from *Ps. thermocarboxydovorans* (Turner *et al.*, 1984; A. Halder & J. Colby, unpublished work) and from *Ps. carboxydovorans* (Meyer *et al.*, 1986) was not observed with this enzyme.

Cofactor composition

Absorption spectrum of CO oxidoreductase. The 600–300 nm absorption spectrum of purified CO oxidoreductase is shown in Fig. 3. The spectrum is typical of other molybdenum iron–sulphur flavoproteins such as xanthine oxidase and the CO oxidoreductases from Gram-negative bacteria (Meyer, 1982). It shows maxima at 430 and 345 nm, a shoulder at 465 nm and a pronounced trough at 395 nm. Treating the holoenzyme with 5% (w/v) trichloroacetic acid, or boiling, followed by centrifugation to remove the denatured protein, yielded a yellow solution with an absorption spectrum typical of free flavin (Fig. 3).

Determination of flavin. Molybdenum hydroxylases such as xanthine oxidase and aldehyde oxidase contain 2 mol of FAD/mol of enzyme (Rajagopalan & Handler, 1964). CO oxidoreductase from *Ps. carboxydovorans* also contains this ratio of flavin to enzyme (Meyer, 1982). The amount of flavin released from a known amount of CO oxidoreductase by heat treatment, trichloroacetic acid precipitation or SDS and heat treatment was calculated after measuring the absorbance at 450 nm of the resultant preparation. Values were calculated by using a molar absorption coefficient for air-oxidized FAD of 11.3×10^3 litre·g^{-1}·cm^{-1} at 450 nm (Colby & Dalton, 1979) or by assuming a molar absorption coefficient at 450 nm of 10.5×10^3 M^{-1}·cm^{-1} for oxidized minus reduced flavin (Meyer, 1982); in practice both methods gave very similar values. Flavin/CO oxidoreductase ratios were calculated by assuming an M_r of 282000 for CO oxidoreductase. The results obtained with five different enzyme preparations are summarized in Table 2, giving an average molar ratio of 1.55 mol of FAD/mol of CO oxidoreductase.

The flavin released from CO oxidoreductase by boiling was run against flavin standards by t.l.c. on silica gel $60F_{254}$. The R_F values obtained were 0.29 for FAD, 0.66

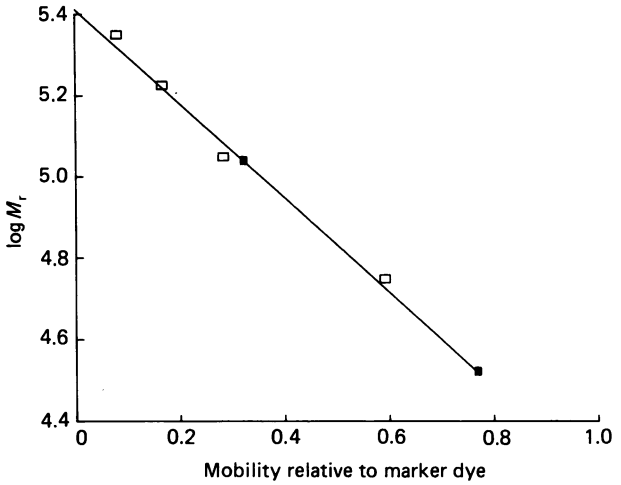

Fig. 2. Determination of the subunit M_r of *Streptomyces* G26 oxidoreductase

SDS/polyacrylamide gels were calibrated by using a Sigma Electran M_r marker kit (M_r range 56000–280000). Gels were loaded with 50 μl and 100 μl portions of a solution containing M_r marker (2 mg/ml) or CO oxidoreductase (0.45 mg/ml) that had been incubated with 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol at 100 °C for 2 min. After electrophoresis the gel was stained and R_F values for the M_r markers (□) and CO oxidase bands (+) were calculated.

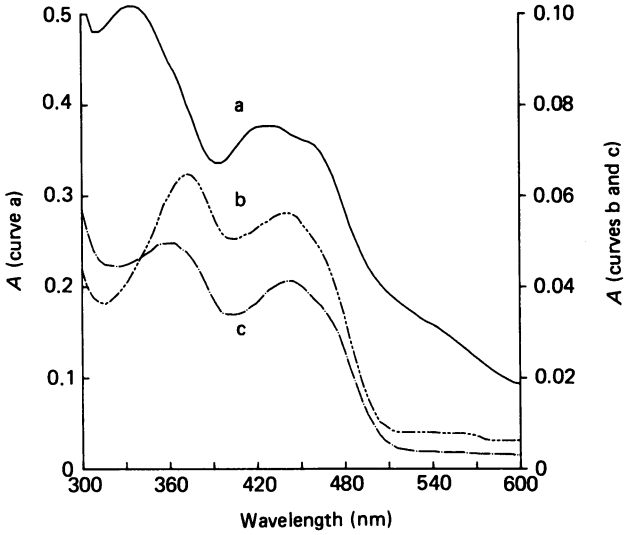

Fig. 3. Absorption spectra of *Streptomyces* G26 CO oxidoreductase and of the flavin released by heat or trichloroacetic acid treatment

Flavin preparations were obtained by treating purified CO oxidoreductase (4.2 mg/ml; curve a) by heating at 100 °C for 10 min (curve b) or by treatment with 5% (w/v) trichloroacetic acid (curve c). Denatured protein was removed from treated samples by centrifugation before the spectra were recorded.

for riboflavin, 0.69 for FMN and 0.29 for the flavin from CO oxidoreductase. The CO oxidoreductase flavin was therefore assumed to be FAD.

Acid-labile sulphide content. The acid-labile sulphide contents of five CO oxidoreductase preparations were determined, giving values of 13.24, 9.3, 8.1, 5.3 and 5.4 mol/mol of CO oxidoreductase (single determinations on different preparations). The mean value of 8.27 is close to the molar ratio of 8 found with other molybdenum hydroxylases.

Table 2. Determination of flavin content of *Streptomyces* G26 CO oxidoreductase

Flavin concentrations were calculated by assuming a molar absorption coefficient for FAD at 450 nm of 11.3×10^3 M^{-1}·cm^{-1} (Colby & Dalton, 1979) or, for the sample marked with an asterisk (*), by using a molar absorption coefficient for oxidized minus reduced FAD at 450 nm of 10.5×10^3 M^{-1}·cm^{-1} (Meyer, 1982). The molar content of FAD in CO oxidoreductase was determined by assuming an M_r of 282000 for the holoenzyme. The A_{450}/A_{550} ratio is calculated from the respective absorbances at 450 nm and 550 nm of the enzyme sample. For molybdenum hydroxylases an A_{450}/A_{550} ratio close to 3 indicates an iron–sulphur/flavin ratio of 4:1 (Rajagopalan & Handler, 1964). Abbreviations: TCA, trichloroacetic acid; N.D., not determined.

A_{450}/A_{550} ratio	Method of flavin release	FAD content (mol/mol of CO oxidoreductase)
2.48	100 °C+1% SDS; 10 min	2.4
N.D.	4 °C+5% TCA; 20 min	1.3
	4 °C+5% TCA; 20 min	1.4*
2.42	4 °C+5% TCA; 40 min	1.3
	100 °C; 10 min	1.4
2.74	4 °C+5% TCA; 40 min	1.3
	100 °C; 10 min	1.6
2.72	100 °C; 10 min	1.7

Metal content. The iron content of the CO oxidoreductase from *Streptomyces* G26 was determined by the colorimetric method of Johnson (1964). A mean value of 7.3 mol of Fe/mol of CO oxidoreductase was obtained from five separate determinations (7.1, 8.4, 8.2, 6.2 and 6.5) by assuming an M_r of 282000 for the enzyme. A commercial preparation of xanthine oxidase from buttermilk, used as a control, gave a value of 8.6 mol of Fe/mol of enzyme, calculated by assuming an M_r of 283000 (Bray, 1975). The molybdenum content of CO oxidoreductase was determined by the dithiol method described by Cardenas & Mortenson (1974) and a value of 1.33 mol/mol of enzyme was calculated.

The molybdenum and nickel contents of purified CO oxidoreductase were determined by atomic absorption spectroscopy, and xanthine oxidase from buttermilk was again used as a control sample. CO oxidoreductase was found to contain 1.16 mol of Mo/mol of enzyme, and the xanthine oxidase preparation contained 0.69 mol of Mo/mol of enzyme. No nickel was found in either enzyme. The low value for xanthine oxidase may reflect the presence of a high proportion of the demolybdo form of the enzyme in the commercial preparation.

Kinetic properties of the purified enzyme

Effect of temperature on CO oxidoreductase activity. The relationship between temperature and enzyme activity is given in Fig. 4(*a*). The highest measurable

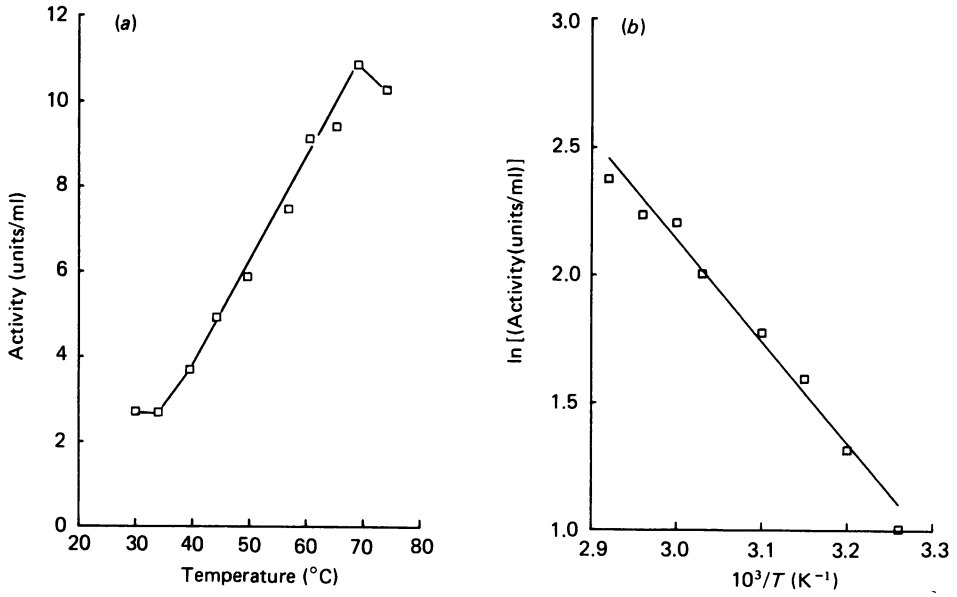

Fig. 4. Temperature–activity (*a*) and Arrhenius plots (*b*) for purified *Streptomyces* G26 oxidoreductase

The activity of CO oxidoreductase over the temperature range 30–74 °C was determined by using the standard spectrophotometric assay.

Table 3. Reduction of cytochrome *c* by *Streptomyces* G26 CO oxidoreductase

Enzyme activities with cytochrome *c* as electron acceptor were measured by using a spectrophotometric assay medium (3 ml) containing 0.15 mmol of sodium phosphate buffer, pH 7.0, 0.25 μmol of cytochrome *c*, 0.5 ml of CO-saturated water and 0.6 unit of purified CO oxidoreductase. Reactions were started by adding enzyme solution. Anaerobic assays were bubbled with N_2 for 5 min before starting the reaction. Some assays contained 10 units of superoxide dismutase (SOD).

Assay conditions	CO oxidoreductase activity (units/ml)
Aerobic	6.33
Aerobic + SOD	2.68
Anaerobic	2.41
Anaerobic + SOD	2.38

activity was recorded at 69 °C. The value for activity at 74 °C represents that measured over the first 5 s of reaction time. CO oxidoreductase was rapidly inactivated at this temperature, as witnessed by a dramatic decrease in activity with incubation time. Fig. 4(*b*) shows an Arrhenius plot of the data, from which the activation energy was calculated to be 33.14 kJ/mol.

Electron-acceptor specificity. The activity of purified *Streptomyces* G26 CO oxidoreductase with either Methylene Blue or benzyl viologen as electron acceptor was determined by using the standard anaerobic spectrophotometric assay. The CO:Methylene Blue oxidoreductase activity was 38.21 units/mg, assuming a molar absorption coefficient for Methylene Blue at 615 nm of 37.1×10^3 $M^{-1} \cdot cm^{-1}$ (Ragsdale *et al.*, 1983*a*), and the CO:benzyl viologen oxidoreductase activity was 4.33 units/mg, assuming a molar absorption coefficient of 12.0×10^3 $M^{-1} \cdot cm^{-1}$ at 550 nm (Ragsdale *et al.*, 1983*a*). NAD^+ and $NADP^+$ were not reduced. Reduction of *Clostridium pasteurianum* ferredoxin was monitored by using a modification of the standard spectrophotometric assay as described in the Experimental section. No CO-dependent reduction of ferredoxin was observed.

A simple spectrophotometric assay was used to determine the activity of *Streptomyces* G26 CO oxidoreductase with cytochrome *c* under aerobic and anaerobic conditions. Superoxide dismutase (10 units) from bovine erythrocytes (EC 1.15.1.1) was added to both aerobic and anaerobic assay mixtures, to assess the role of superoxide radicals ($O_2^{\cdot -}$) in cytochrome *c* reduction. The results are given in Table 3.

DISCUSSION

CO oxidoreductase from the moderate thermophile *Ps. thermocarboxydovorans* was purified to 95% homogeneity in our laboratory by using conventional column-chromatographic methods (Turner *et al.*, 1984) and subsequently purified to homogeneity and crystallized (A. Halder & J. Colby, unpublished work). This methodology was not found successful for the less stable CO oxidoreductase from *Streptomyces* G26 (Bell *et al.*, 1987). This enzyme was, however, amenable to purification by h.p.l.c. techniques, although purification to homogeneity was not achieved in the present study. Two slight contaminating bands, corresponding to proteins of much higher M_r, were present in non-denaturing gels. These bands may have been due to CO oxidoreductase aggregates, although no CO oxidoreductase activity within the gel was associated with them.

The M_r of native *Streptomyces* G26 CO oxidoreductase was determined by two different methods, yielding values of 262000 ± 14000 and 282000 ± 9000. The latter is in good agreement with determinations for other molyb-

Table 4. Properties of some CO oxidoreductases and CO dehydrogenases

Abbreviations: ALS, acid-labile sulphide; N.D., not determined.

Species	M_r	Subunit M_r	Analytical data (mol/mol of holoenzyme) Mo	Ni	Fe	ALS	FAD	Reference
CO oxidoreductases								
Pseudomonas carboxydovorans	310000	86000, 34000, 17000	0.7	N.D.	7.3	8.2	2.0	Meyer & Rajagopalan (1984); Meyer (1985)
Pseudomonas thermocarboxydovorans	300000	87000, 34000, 21000	0.9	< 0.2	6.9	6.9	1.8	Turner *et al.* (1984); A. Halder & J. Colby, unpublished work
Streptomyces G26	282000	110000, 33000	1.25	0.0	7.2	8.3	1.7	Present paper
CO dehydrogenases								
Acetobacterium woodii	480000	80000, 68000	0.0	5.0	27	36	0.0	Ragsdale *et al.* (1983*a*)
Clostridium thermoaceticum	440000	77000, 71000	< 0.3	6.0	32	42	0.0	Ragsdale *et al.* (1983*c*)
Methanosarcina barkeri	232000	91000, 18000	N.D.	N.D.	N.D.	N.D.	N.D.	Krzycki & Zeikus (1984)

denum hydroxylases at 283000±18000 (Bray, 1975) and with determinations for CO oxidoreductases from mesophilic carboxydotrophic bacteria, at 300000 (Meyer & Rajagopalan, 1984). Quite different M_r values for CO oxidoreductases have been obtained by using sucrose-density-gradient centrifugation. Values of about 230000 were obtained for the enzymes from *Ps. carboxydovorans* (Meyer & Schlegel, 1980) and *Ps. thermocarboxydovorans* (Turner *et al.*, 1984). This may be due to low protein density resulting from high hydrophobic amino acid contents within the enzyme structure: it is noticeable that the CO oxidoreductases from *Streptomyces* G26 and *Ps. thermocarboxydovorans* bind very strongly to hydrophobic matrices such as phenyl-Sepharose.

The subunit structure of CO oxidoreductases is a subject for much debate. *Streptomyces* G26 CO oxidoreductase was found to be composed of two subunits of M_r 110000 (L) and 33000 (S). This can be compared with values of 86000, 34000 and 17000 for the L, M and S subunits of *Ps. carboxydovorans* CO oxidoreductase, determined by using similar methods (Meyer *et al.*, 1986). Those authors suggested a subunit structure of $L_2M_2S_2$ or $(LMS)_2$, the latter dimer structure being similar to the configuration of other molybdenum hydroxylases. CO oxidoreductase from *Ps. thermocarboxydovorans* also yields three subunits, of M_r 87000, 29000 and 21000 (A. Halder & J. Colby, unpublished work). On the basis of an M_r of 283000, a subunit structure of L_2S_2 or $(LS)_2$ is indicated for *Streptomyces* G26 CO oxidoreductase.

The CO oxidoreductase from *Streptomyces* G26 is clearly a molybdenum hydroxylase. The results of cofactor analyses (mol/mol of native enzyme) were 1.25 Mo:7.3 Fe:8.3 acid-labile sulphide:1.7 FAD, assuming an M_r of 282000. These cofactor contents are in agreement with those believed to be present in fully functional molybdenum hydroxylases, with values of 2 Mo:8 Fe:8 acid-labile sulphide:2 FAD (Rajagopalan & Handler, 1964; Hart *et al.*, 1970). No nickel was found in the enzyme preparation, indicating that *Streptomyces* G26 CO oxidoreductase does not resemble the CO dehydrogenases from anaerobic bacteria, which contain 5–6 mol of Ni/mol of enzyme (Ragsdale *et al.*, 1983*a*). These latter enzymes also have a much higher M_r, much higher Fe and acid-labile sulphide contents, e.g. *Clostridium thermoaceticum* CO dehydrogenase at M_r 440000 and 32 and 42 mol/mol respectively (Ragsdale *et al.*, 1983*b*), and [4Fe-4S] centres rather than the [2Fe-2S] centres found in CO oxidoreductases from aerobic bacteria (Ragsdale *et al.*, 1982; Bray *et al.*, 1983). The molecular properties of CO oxidoreductases and CO dehydrogenases from various aerobic and anaerobic bacterial sources are summarized in Table 4.

CO oxidoreductases from Gram-negative bacteria do not interact with low-potential electron acceptors. Low activity with viologen dyes was observed with crude extracts of CO-grown *B. schlegelii* (Kruger & Meyer, 1984), but work with purified CO oxidoreductase from this source has not been published. Purified CO oxidoreductase from *Streptomyces* G26 interacted with phenazine ethosulphate ($E_0' = +80$ mV), Methylene Blue ($E_0' = +11$ mV) and benzyl viologen ($E_0' = -359$ mV) in spectrophotometric assays, confirming the earlier observations with impure enzyme preparations (Bell *et al.*, 1987). The enzyme thus appears to have a uniquely low specificity towards its oxidizing substrate.

The E_0' of the CO/CO_2 couple (-540 mV) is sufficiently negative to allow direct reduction of $NAD(P)^+$ [$E_0' = -320$ (-324) mV]. However, in *Ps. carboxydovorans* and *Ps. carboxydohydrogena* electrons from CO oxidoreductase are believed to enter the electron-transport chain at the level of *b*-type cytochromes; NAD(P)H would be formed via reversed electron flow (Meyer & Schlegel, 1983). The ability of *Streptomyces* G26 CO oxidoreductase to catalyse electron transfer from CO to viologen dyes ($E_0' = -440$ mV

to −359 mV) suggests that this enzyme might be able to interact with natural redox-active compounds of low redox potential such as ferredoxin, rubredoxin or flavodoxin, which might be involved in direct reduction of nicotinamide nucleotides *in vivo*. The finding of a natural low-potential electron acceptor able to interact with CO oxidoreductase would represent an important advance in carboxydotroph physiology. However, it must be borne in mind that CO oxidoreductases, like other molybdenum hydroxylases, have very low specificity for oxidizing substrate, owing to the multiplicity of redox centres carried by the enzyme. Cleere & Coughlan (1974) identified seven possible routes for electron egress from liver xanthine oxidase. Assuming that electrons transfer by similar routes, interaction of *Streptomyces* G26 CO oxidoreductase with benzyl viologen may be a function of a slightly different redox-centre configuration at any one of the three sites of electron egress from the enzyme (eg. the presence of [4Fe-4S] centres rather than [2Fe-2S] centres) and have no physiological significance.

Purified *Streptomyces* G26 CO oxidoreductase reduces cytochrome *c* at a high rate under aerobic conditions (Bell *et al.*, 1987), but under these conditions cytochrome *c* can be non-enzymically reduced by superoxide radicals generated by the oxidation of reduced flavin with molecular O_2. The results in Table 3 show that about 60% of the apparent cytochrome *c* reduction rate by CO oxidoreductase under aerobic conditions is via the non-enzymic route, but that 40% of the activity is still observed in the absence of O_2. Molybdenum hydroxylases differ in their ability to reduce cytochrome *c*. With *Veilonella alcalescens* xanthine dehydrogenase the anaerobic reduction of cytochrome *c* proceeded at 75–100% of the aerobic rate, depending on the cytochrome *c* concentration (Smith *et al.*, 1967). In contrast, O_2 is required for cytochrome *c* reduction by milk xanthine oxidase, milk aldehyde oxidase and dihydroorotate dehydrogenase (Smith *et al.*, 1967) and by *Ps. carboxydovorans* CO oxidoreductase (Meyer, 1982).

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