Ferredoxins from Two Sulfonylurea Herbicide Monooxygenase Systems in *Streptomyces griseolus*†

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ABSTRACT: We have purified and characterized two ferredoxins, designated Fd-1 and Fd-2, from the soluble protein fraction of sulfonylurea herbicide induced *Streptomyces griseolus*. These cells have previously been shown to contain two inducible cytochromes P-450, P-450_{SU1} (CYP105A1) and P-450_{SU2} (CYP105B1), responsible for herbicide metabolism [O'Keefe, D. P., Romesser, J. A., & Leto, K. J. (1988) *Arch. Microbiol. 149*, 406–412]. Although Fd-2 is more effective, either ferredoxin can restore sulfonylurea monooxygenase activity to an aerobic mixture of NADPH, spinach ferredoxin:NADP oxidoreductase, purified cytochrome P-450_{SU1}, and herbicide substrate. The gene for Fd-1 is located in the genome just downstream of the gene for cytochrome P-450_{SU1}; the gene for Fd-2 follows the gene for P-450_{SU2}. The deduced amino acid sequences of the two ferredoxins show that, if monomeric, each has a molecular mass of ~7 kDa, and alignment of the two sequences demonstrates that they are ~52% positionally identical. The spectroscopic properties and iron and acid-labile sulfide contents of both ferredoxins suggest that, as isolated, each contains a single [3Fe-4S] cluster. The presence of only three cysteines in Fd-1 and comparisons with three [4Fe-4S] ferredoxins with high sequence similarity suggest that both Fd-1 and Fd-2 have an alanine in the position where these [4Fe-4S] proteins have a fourth cysteine ligand to the cluster. Transformation of *Streptomyces lividans*, a strain unable to metabolize sulfonylureas, with DNA encoding both P-450_{SU1} and Fd-1 results in cells capable of herbicide metabolism. *S. lividans* transformants encoding only cytochrome P-450_{SU1} do not metabolize herbicide. Taken together, our results suggest that the inducible herbicide monoxygenase systems of *S. griseolus* consists minimally of a cytochrome P-450 and its companion ferredoxin, but the inducibility and in vivo characteristics of reductase component(s) of the system remain poorly defined.

Cytochrome P-450 based monooxygenase systems typically consists of cytochrome P-450, which actually catalyzes substrate hydroxylation, and another protein or proteins which mediate electron transfer from reduced pyridine nucleotide. The camphor hydroxylase system from the bacterium *Pseudomonas putida* is a well-studied example; it uses a flavoprotein, putidaredoxin reductase, to transfer electrons from NADH to a [2Fe-2S] ferredoxin, putidaredoxin, which is in turn the direct reductant of the cytochrome P-450 (Gunsalus et al., 1974; Sligar & Murray, 1986). We have previously shown that *Streptomyces griseolus* ATCC 11796 has two inducible cytochrome P-450 monooxygenases capable of sulfonylurea herbicide metabolism (Romesser & O'Keefe, 1986; O'Keefe et al., 1988). The two cytochromes, P-450_{SU1} and P-450_{SU2} [CYP105A1 and CYP105B1 by the nomenclature of Nebert et al. (1987)], have been purified, and the genes encoding them, *suaC* and *subC*, respectively, have been cloned and sequenced (Omer et al., 1990). These cytochromes P-450 are enzymatically inactive alone. In previous studies, we have used either crude cell extracts from uninduced *S. griseolus* (O'Keefe et al., 1988) or ferredoxins and reductases from either *Ps. putida* or spinach to reconstitute activity (O'Keefe et al., 1987). While these results are consistent with the existence of an electron transfer system in *S. griseolus* similar to that in *Ps. putida*, we are interested in a complete biochemical description of the herbicide monooxygenase systems of *S. griseolus*. Using the optical absorption characteristics of a typical iron–sulfur protein as a guide, we therefore examined sulfonylurea-induced cell extracts for ferredoxin-like proteins. We found two major ferredoxins, which have been purified to homogeneity. These ferredoxins could be used to reconstitute herbicide monooxygenase activity in the presence of P-450_{SU1} and spinach ferredoxin:NADP oxidoreductase in vitro. We have also found that the coding region in the *S. griseolus* genome for each ferredoxin is shortly downstream from each of the two cytochrome P-450 coding regions. A *Streptomyces lividans* transformant containing genes for both cytochrome P-450_{SU1} and its associated ferredoxin metabolized herbicide, though another transformant containing only the P-450 gene did not. This suggests that the ferredoxin–P-450 pair also functions in vivo and that the *S. griseolus* ferredoxin can accept electrons from an *S. lividans* reductase.

Materials and Methods

Preparation and Analysis of Proteins. Cells of *Streptomyces griseolus* and preparations of cytochromes P-450_{SU1} and P-450_{SU2} were as previously described (Romesser & O'Keefe, 1986; O'Keefe et al., 1988; Omer et al., 1990) except that a 4.5 × 50 cm Sephacryl S-200 (Pharmacia) column was used for the first gel filtration step. Ferredoxins were found to copurify with the cytochromes P-450 through the first gel filtration step and subsequently were separated from the cytochromes by anion exchange HPLC on an LKB TSK-DEAE 5PW column (7.5 × 75 mm) and eluted at 0.75 mL/min with a 40-min linear gradient of 0–0.8 M sodium acetate in 20 mM

† The nucleic acid sequences in this paper have been submitted to GenBank under Accession Numbers M36480 (*suaB*) and M36481 (*subB*).

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Tris–acetate (ph 7.5). The ferredoxin fraction from the ion exchange column was concentrated over a 10-kDa cutoff membrane. Residual cytochrome P-450 and other impurities were removed by gel filtration through a 7.5 × 300 mm TSK G3000SW column (Bodman) in 0.1 M NaP_i, pH 7.0, at a flow rate of 0.75 mL/min. The ferredoxin peak was collected, reconcentrated, and applied to a 7.5 × 75 mm TSK-phenyl 5PW column (Pharmacia) in 20 mM Tris–SO_4, pH 7.5, containing 1 M $(NH_4)_2SO_4$. The ammonium sulfate concentration in the eluting buffer was decreased linearly to zero over 40 min at a flow rate of 1.0 mL/min. The separated ferredoxins were concentrated and stored at −80 °C.

Ferredoxin apoproteins were prepared for amino acid analysis and N-terminal sequencing by reverse-phase HPLC on a Vydac C-18 column (4.6 × 250 mm) with a 30-min linear gradient of 20%–80% acetonitrile in water (both the acetonitrile and water contained 0.1% TFA). To quantitate cysteine, samples were reduced with DTT, alkylated with iodoacetic acid, and rechromatographed (Shively, 1986). Amino acid analysis was performed on ~0.3 nmol of the apoferredoxins on a Beckman 6300 ion exchange/ninhydrin analyzer. N-Terminal sequence analysis on ~0.6 nmol of the apoferredoxins was performed by automated Edman degradation (Shively, 1986).

Protein concentrations of crude samples were determined with the Bio-Rad (Richmond, CA) protein assay with bovine serum albumin as a standard (Bradford, 1976). Purified ferredoxins were virtually undetectable with the Bio-Rad assay, and for these the Lowry method was used (Lowry et al., 1951). Cytochrome P-450 concentrations were determined by difference spectroscopy of ferrocytochrome–CO complex (Omura & Sato, 1964). Iron and acid-labile sulfide were quantitated as previously described (Kennedy et al., 1984; Beinert, 1983). Conversion of [3Fe-4S] to [4Fe-4S] clusters was attempted with a 1-h incubation of 50 μM Fd-1[1] or Fd-2 in the presence of 0.5 mM DTT and 5 mM $Fe(NH_4)_2(SO_4)_2$, followed by desalting over Sephadex G-25 (Kennedy et al., 1983; Kent et al., 1982). EPR spectra were recorded on a Bruker ER 200 spectrometer, equipped with an Air Products Heli-Tran LTR-3 liquid helium cryostat and Series 5500 temperature controller. Photoreduction of the samples was carried out with 5 μM deazaflavin sulfate and 5 mM potassium oxalate (Massey & Hemmerich, 1978). UV/vis spectra were recorded on a Johnson Foundation SDB-3A scanning dual-wavelength spectrophotometer or a HP Model 8450A diode array spectrophotometer.

Spinach ferredoxin and NADPH:ferredoxin oxidoreductase were purified as described (Buchanan & Arnon, 1971; Zanetti & Curti, 1980). Cytochrome $P\text{-}450_{CAM}$, putidaredoxin, and putidaredoxin reductase were kindly provided by Dr. I. C. Gunsalus.

Assay Methods. Cell-free sulfonylurea herbicide metabolism was assayed by N-dealkylation of R7402 essentially as described previously (Romesser & O'Keefe, 1986). Assays were carried out in 25–50 μL of buffer at room temperature. For the experiments in Table I, crude P-450 was ammonium sulfate precipitated from the soluble protein fraction of chlorimuron ethyl induced cells (Romesser & O'Keefe, 1986) and used at a final concentration of 1 μM. Concentrations of other additions were as follows: $P\text{-}450_{CAM}$, 1.2 μM; putidaredoxin reductase, 0.5 μM; putidaredoxin, 40 μM; spinach FNR, 0.2 μM; spinach ferredoxin, 17 μM. Reactions were initiated by the addition of 0.4 mM NADPH or 0.4 mM NADH (when putidaredoxin reductase was present). The experiments in Table II were carried out in 0.1 M MOPS–NaOH (pH 7.0), 0.2 M NaCl, 0.2 mM R7402, 2 μM purified cytochrome $P\text{-}450_{SU1}$, spinach ferredoxin:NADP oxidoreductase (FNR), various ferredoxins as indicated, and an NADPH-regenerating system consisting of 5 mM glucose 6-phosphate and 2 units/mL *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Sigma). The reaction was initiated by the addition of NADPH to a final concentration of 0.03 mM. All of the in vitro metabolism reactions were terminated by the addition of a 10-fold excess of H_2O–acetonitrile–H_3PO_4 (80:19:1). After filtration of this mixture through a 0.2-μm filter, the amount of N-dealkylated R7402 (i.e., *N*-[[(4,6-dimethoxypyrimidin-2-yl)amino]carbonyl]-2,3-dihydro-1,2-benzoisothiazole-7-sulfonamide 1,1-dioxide) was analyzed by HPLC (Romesser & O'Keefe, 1986). By use of Zorbax ODS (3-μm particle size) column (6.2 × 80 mm; MacMod, Inc.) and a linear gradient from 20% to 75% acetonitrile over 10 min (both water and acetonitrile were supplemented with 1% H_3PO_4, flow = 1.5 mL/min), retention times of 8.9 and 7.0 min were found for R7402 and its dealkylated metabolite, respectively. No other metabolites of R7402 were observed under any of the conditions used here.

Measurements of electron transfer rates were carried out in CO-saturated buffers at room temperature. For the experiments in Figure 1, a crude preparation of *S. griseolus* soluble proteins was prepared by centrifugation and $(NH_4)_2SO_4$ precipitation of chlorimuron ethyl induced cell extracts (Romesser & O'Keefe, 1986). For experiments with the purified components, proteins were added to 0.1 M MOPS–NaOH (pH 7.0) containing 0.2 M NaCl and 2 mM chlorsulfuron. Protein concentrations were as follows: P-450, 1 μM; Fd, 1 μM; FNR, 2 μM. Reactions were initiated by addition of 0.1 mM NADPH, and spectra were recorded at 10-s intervals. The change in $\Delta A_{448-490}$ as a function of time was used to derive an initial rate. In most cases, cytochrome P-450 reduction rates were not affected by doubling the FNR concentration, indicating that Fd to P-450 electron transfer was rate limiting.

DNA Sequencing and Recombinant DNA Methodology. Plasmids pCAO302 (*suaC,B*) and pCAO304 (*subC,B*) (Omer et al., 1990) were sequenced by the dideoxy method of DNA sequencing, using 7-deaza-dGTP (Mizusawa et al., 1986; Sanger et al., 1977). The DNA sequences were analyzed with the University of Wisconsin DNA sequence analysis programs (Devereux et al., 1984). Recombinant DNA experiments in *Escherichia coli* were performed as previously described (Maniatis et al., 1982). The plasmid pCAO500 was made in *E. coli* by inserting the 2.4-kb *Bam*HI DNA fragment from pCAO302 (Omer et al., 1990) containing the *suaC,B* genes into the *Bam*HI site of pCAO200 (Omer et al., 1988). The plasmid pCAO400 was made similarly by inserting the 2.4-kb *Bam*HI DNA fragment from pCAO302 into the *Bam*HI site of pCAO170 (Omer et al., 1988). The plasmid pCAO501 was made in *E. coli* by inserting into the *Bam*HI site of pCAO200 a 1.9-kb *Bam*HI DNA fragment derived by exonuclease III digestion (Henikoff, 1984) from the 2.4-kb *suaC,B* DNA fragment of pCAO302. The 1.9-kb DNA fragment contains all of *suaC* and 6 bp downstream of the stop codon of *suaC* but has the entire *suaB* coding sequence deleted. Besides being

[1] Abbreviations: chlorimuron ethyl, *N*-[[(4-chloro-6-methoxypyrimidin-2-yl)amino]carbonyl]-2-(ethoxycarbonyl)benzenesulfonamide; chlorsulfuron, *N*-[[(4-methoxy-6-methyl-1,3,5-triazinyl)amino]carbonyl]-2-chlorobenzenesulfonamide; Fd, ferredoxin; FNR, spinach ferredoxin:NADP oxidoreductase; R7402, *N*-[[(4,6-dimethoxypyrimidin-2-yl)amino]carbonyl]-2,3-dihydro-2-(1-methylethyl)-1,2-benzoisothiazole-7-sulfonamide 1,1-dioxide.

Table I: Metabolism of the Sulfonylurea R7402 by Crude *S. griseolus* Cytochrome P-450 Fraction Supplemented with Ferredoxins and Reductases

additions	R7402 metabolism (% dealkylated in 30 min)[a]
crude P-450	7
complete camphor hydroxylase system[b]	<1
crude P-450 + putidaredoxin + putidaredoxin reductase	44
crude P-450 + spinach FNR	45
crude P-450 + spinach FNR + spinach ferredoxin	100

[a] Initial concentration of R7402 was 73 μM. [b] No *S. griseolus* proteins were added.

capable of replicating in *E. coli*, pCAO200 and pCAO170 can be used to integratively transform *Streptomyces lividans* (Omer et al., 1988). The plasmids pCAO500, pCAO400, and pCAO501 were transformed into *S. lividans* (Hopwood et al., 1985). Selection for thiostrepton resistance afforded strains *S. lividans* C500 (pCAO500), *S. lividans* C400 (pCAO400), and *S. lividans* C501 (pCAO501). *S. lividans* C200 (pCAO200) and another similar control strain, *S. lividans* C37, were made previously (Omer et al., 1988).

Growth of Cells for Analysis of Sulfonylurea Metabolism and Western Blot Analysis. *S. griseolus* cells were grown in sporulation broth at 30 °C (Omer et al., 1990), while *S. lividans* cells were grown in YEME broth (Hopwood et al., 1985) at 30 °C. If cells were to be induced, 125 mg/L chlorimuron ethyl was added to the cells 3–6 h before harvesting. Cells were harvested by centrifugation (6000–10000*g*, 10 min) washed in 0.05 M MOPS–NaOH (pH 7.0), and recentrifuged.

Resting Cell Metabolism of Sulfonylureas. Cells to be used for resting cell metabolism of sulfonylurea were resuspended in 5 cell volumes of 0.05 M MOPS–NaOH (pH 7.2) containing 0.2% glucose. One volume of the cell suspension was mixed with an equal volume of 0.05 M MOPS–NaOH (pH 7.2) and 0.6 mM chlorimuron ethyl and incubated at 30 °C with shaking. Aliquots were removed at 2-, 4-, and 6-h time points, and metabolites in the supernatants were identified by retention time on a 4.6 mm × 25 cm Zorbax ODS column (MacMod Inc.) as compared to known standards and by comparison of the UV spectra of the metabolites with those of authentic standards (Romesser & O'Keefe, 1986).

Protein Gel Electrophoresis and Western Blot Analysis. Cells to be disrupted to obtain protein extracts were resuspended in 1–2 cell volumes of 0.05 M MOPS–NaOH (pH 7.2) and broken in a French pressure cell (20 000 psi). Cellular debris was removed by centrifugation at 10000*g* for 10 min. Protein extracts of cells were subjected to SDS–polyacrylamide (7.5% T, 2.7% C) gel electrophoresis as previously described (Laemmli, 1970). Proteins in the gels were transferred to nitrocellulose filters (Schleicher & Schuell, BA85), and cytochrome P-450$_{SU1}$ was detected with specific antiserum as described (Omer et al., 1990).

Results

Ferredoxin-Dependent Herbicide Monooxygenase Activity in Crude S. griseolus Preparations. Previous experiments had shown that the sulfonylurea monooxygenase activity of *S. griseolus* could be found in the soluble protein fraction and that over one-third of this activity was still present in the 40–60% ammonium sulfate pellet from induced cells (Romesser & O'Keefe, 1986; O'Keefe et al., 1988). As Table I shows, the activity is stimulated >6 fold by the addition of ferredoxin:NADP oxidoreductase (FNR) from spinach chloroplasts and stimulated >14fold by the additional presence of spinach chloroplast ferredoxin. The putidaredoxin–putidaredoxin reductase system was about as effective as FNR supplementation. A similar ferredoxin requirement was also suggested by the results of an electron transfer assay; spinach ferredoxin stimulated single equivalent reduction (as monitored by absorbance increase at 450 nm) in a CO-saturated crude cytochrome P-450 preparation that had also been supplemented with FNR (see Figure 1). These results suggest that while a complete herbicide monooxygenase system is present in the soluble protein fraction from *S. griseolus*, it is limited by the ability of the endogenous reductase system to deliver reducing equivalents from NAD(P)H in vitro (Romesser & O'Keefe, 1986). The ability of FNR to stimulate activity in the absence of added ferredoxin is probably due to the presence of endogenous ferredoxin(s) in this preparation and not to the direct reduction of P-450 by FNR (see below).

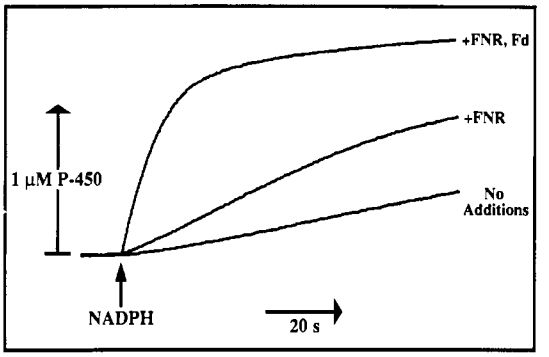

FIGURE 1: Enhancement of cytochrome P-450 reduction by addition of spinach ferredoxin (Fd) and spinach ferredoxin:NADP oxidoreductase (FNR). To continuously stirred CO-saturated buffer containing 20 mM MOPS–NaOH (pH 7.0) and 2 mM chlorsulfuron, under an atmosphere of CO, were added crude protein extracts from chlorimuron ethyl induced *S. griseolus* to a final concentration of 1.8 μM P-450. Where indicated, 10 μM Fd and/or 60 nM FNR was also present. The reduction was initiated by the addition of NADPH to a final concentration of 200 μM, and the reduction of cytochrome P-450 was monitored by $\Delta A_{450-475nm}$.

Purification of S. griseolus Ferredoxins. Search for ferredoxin in the soluble extract from *S. griseolus* was based on the characteristics of the visible absorption spectrum of [Fe–S] proteins. Iron–sulfur proteins have broad absorbance bands in the 400–500-nm region ($A_{460} \geq 0.9A_{420}$ for [2Fe-2S] proteins), whereas heme proteins like low-spin ferricytochromes P-450 have relatively sharp absorbance bands centered at about 418 nm ($A_{460} \leq 0.2A_{420}$) (Palmer, 1973; Beinert & Thomson, 1963; O'Keefe et al., 1988). Analysis at 460 nm of *S. griseolus* total soluble protein fraction (not shown, but see Figure 2) revealed a major peak at slightly longer retention times than that of cytochrome P-450$_{SU1}$; this peak was only a minor component when analyzed by 420-nm absorbance. The appearance of this peak during anion exchange HPLC is the basis of an assay for putative ferredoxins. This peak is absent in extracts from untreated cells [see O'Keefe et al. (1988)], suggesting that it also represents herbicide-inducible protein(s). It was found that >78% of the putative ferredoxin present in the broken cell soluble fraction copurified with the cytochrome P-450 fraction through ammonium sulfate precipitation, gel filtration chromatography, and concentration over a 30-kDa cutoff membrane. Upon preparative anion exchange HPLC of the material after these three steps, the major activity peak in Figure 2 at 30–32 min was collected. After gel filtration HPLC, two distinct ferredoxins in this fraction could be separated in their native form (retaining chromophore) by hydrophobic interaction chromatography to

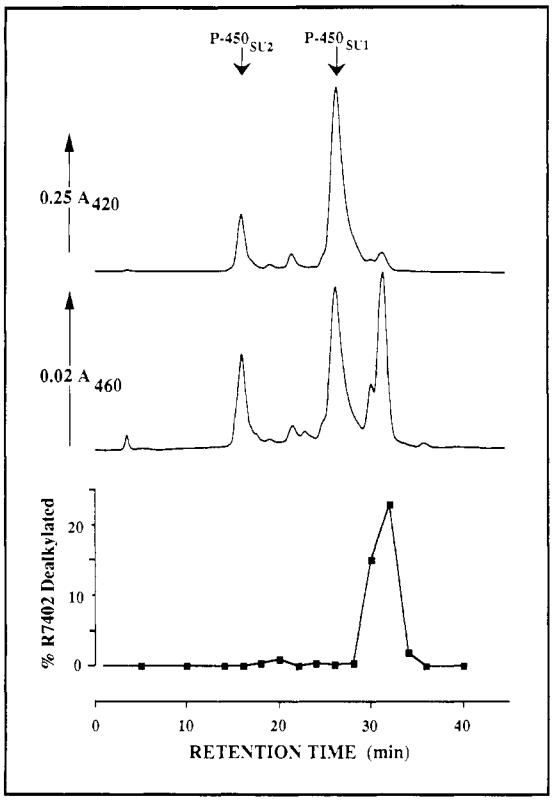

FIGURE 2: Anion exchange HPLC of *S. griseolus* ferredoxins. Subsequent to ammonium sulfate precipitation, gel filtration chromatography, and concentration by ultrafiltration, ~10 mg of protein from chlorimuron ethyl induced *S. griseolus* was applied and eluted from the column as described under Materials and Methods. Fractions collected manually over the time intervals as indicated were assayed for R7402 dealkylase activity by addition of 50 μL of the fraction to 100 μL of a solution containing 0.1 M MOPS–NaOH (pH 7.0), 0.5 μM purified cytochrome $P\text{-}450_{SU1}$, 250nM FNR, and 75 μM R7402. Reactions were terminated after 30 min and analyzed as described under Materials and Methods. The 30- and 32-min fractions were pooled and carried through subsequent procedures.

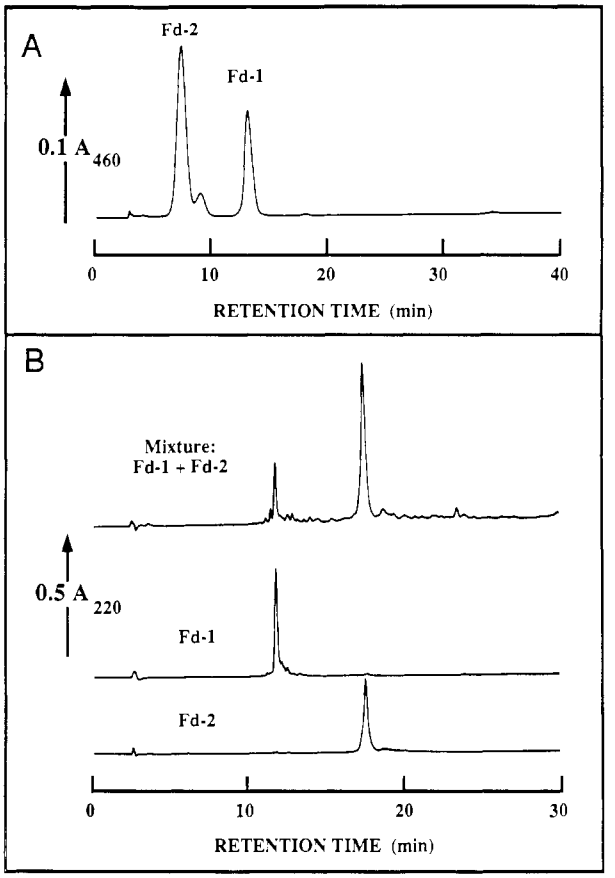

FIGURE 3: Hydrophobic interaction and reverse-phase chromatography of *S. griseolus* ferredoxins. Pooled fractions from Figure 2, subjected to gel filtration HPLC as described under Materials and Methods, were further separated. (A) Separation of native ferredoxins by hydrophobic interaction chromatography. About 35 nmol of the combined ferredoxins was injected. (B) Separation of ferredoxin apoproteins by reverse-phase HPLC. The mixture is the same as in (A) except that only ~4 nmol was injected. Fd-1 (~4.4 nmol) and Fd-2 (~4.0 nmol), collected from the hydrophobic interaction column in (A), were injected individually in the lower two traces.

yield essentially pure proteins (Figure 3A). These proteins have been designated ferredoxin 1 (Fd-1) and ferredoxin 2 (Fd-2). The yield of ferredoxins from this procedure is uncertain since our only accurate measure of ferredoxin content in the initial soluble protein fraction is based on analysis by anion exchange HPLC, a technique which clearly yields poor recovery (~10%) of the *S. griseolus* cytochromes P-450 (O'Keefe et al., 1988). Prior to the anion exchange step recovery of the combined ferredoxins was 78%, and of the ferredoxins collected from the anion exchange column, 72% were ultimately recovered as pure native Fd-1 + Fd-2. The total recovery of material from the purification was ~35 nmol of each ferredoxin from ~200 mL of cell paste.

Both Fd-1 and Fd-2 exhibit M_R ~ 23 kDa by gel filtration chromatography; however, both spinach ferredoxin (M_R = 10.5 kDa) and putidaredoxin (M_R = 12.5 kDa) appear to have abnormally high molecular masses (30 and 24 kDa, respectively) by this particular procedure. It is also noteworthy that only after separation from the bulk cytochromes P-450 by anion exchange do the ferredoxins readily pass through a 30-kDa ultrafiltration membrane and become resolvable from residual P-450 upon gel filtration chromatography. Denaturing lithium dodecyl sulfate gel electrophoresis (LDS–PAGE) gave faint, diffuse bands near the tracking dye front (M_R < 20 kDa). However, the ferredoxins were well-separated and could be quantitatively recovered from a C-18 reverse-phase HPLC column under denaturing conditions (Figure 3B). Other (C-3 and C-4) reverse-phase supports worked equally well. This technique has proven to be the superior analytical tool for the detection of these polypeptides in partially purified preparations. It is noteworthy that the apoproteins are almost invisible to a detector at 280 nm, due to the total absence of Tyr and Trp in the polypeptides (see below).

Properties of S. griseolus Ferredoxin 1 and Ferredoxin 2. The data strongly suggest that, as isolated, Fd-1 and Fd-2 each contain a [3Fe-4S] cluster. The iron content of the two proteins assuming M_R = 7 kDa for both proteins (see below) is 2.6 and 2.8 Fe/polypeptide for Fd-1 and Fd-2, respectively. Determination of the acid-labile sulfide contents of the two proteins yielded an Fe/S ratio of 0.80 ± 0.06 for Fd-1 and of 0.93 ± 0.05 for Fd-2. The Fe/S ratio for Fd-2 is high enough to suggest that it could contain a [4Fe-4S] cluster. However, EPR spectra of the two proteins (Figure 4B) revealed a single strong resonance at g ~ 2.0 for each protein; these resonances contained >89% of the predicted spin content for a [3Fe-4S] cluster. Intensity of the EPR signal is nearly undetectable at temperatures of ≥30K, which is characteristic for the g = 2.0 signal from an oxidized [3Fe-4S] cluster (Beinert & Thomson, 1983). These spectra were obtained with the proteins as isolated; treatment with potassium ferricyanide does not alter the UV/vis spectra (after the ferricyanide is removed), suggesting that up to E_h ~ 400 mV the purified proteins cannot be further oxidized. Photochemical reduction

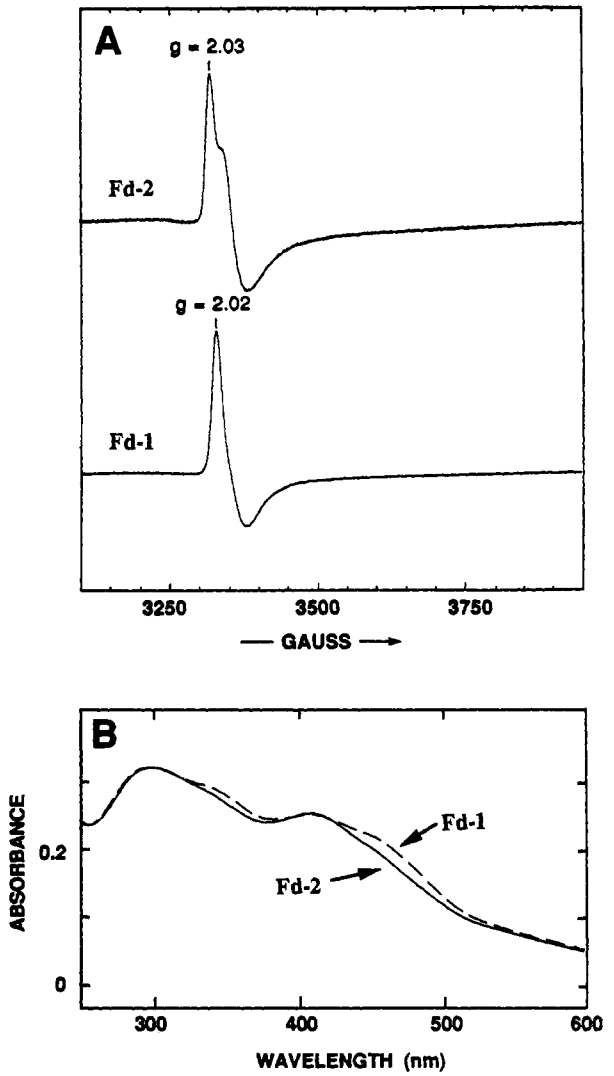

FIGURE 4: EPR and UV/visible spectra of *S. griseolus* ferredoxins. (A) EPR spectra of the ferredoxins as isolated (temperature, 9.8 K, power, 1 mW; modulation amplitude, 0.8 mT; microwave frequency, 9.45 GHz). (B) UV/visible spectra of the two ferredoxins at ~11 μM, normalized so the peaks at ~297 nm are equal.

of the proteins (Massey & Hemmerich, 1978) led to complete loss of the $g = 2.02$ resonance in Fd-1; in Fd-2, it was replaced by a less intense signal that could reflect a low (~10%) [4Fe-4S] cluster content. The optical spectra of the proteins were also consistent with their identification as [3Fe-4S] proteins (Bruschi et al., 1976; Emptage et al., 1983; Beinert & Thomson, 1983). Although the absorption bands are broad, the electronic spectra of the two ferredoxins are distinct because that of Fd-1 has shoulders at 340 and at 460 nm that are missing from the spectrum of Fd-2. Assuming the presence of 4S per cluster, the sulfide content was used to determine an extinction coefficient at 410 nm of 17.0 mM^{-1} cm^{-1} for Fd-1 and of 20.1 mM^{-1} cm^{-1} for Fd-2.

Because [3Fe-4S] clusters have been shown in other systems to result from degradation of, or interconversion with, [4Fe-4S] clusters (Beinert, 1990; Kennedy et al., 1983; Kent et al., 1982), Fd-1 and Fd-2 were treated with Fe(II) and DTT (Kennedy et al., 1983) to see if a [4Fe-4S] cluster could be reconstituted. The optical spectra of the Fe(II) + DTT treated proteins were very similar to those of the photoreduced [3Fe-4S] proteins, suggesting that the ferredoxins were simply reduced by this procedure and that no cluster interconversion took place. Some EPR-detectable [4Fe-4S] cluster (~10%) was evident after this procedure upon anaerobic photoreduction of Fd-1, but there was no apparent increase in the [4Fe-4S] cluster content of Fd-2.

Table II: Metabolism of the Sulfonylurea R7402 by Aerobic Mixtures of Cytochrome P-450_{SU1}, Spinach Ferredoxin:NADP Oxidoreductase (FNR), and Various Ferredoxins[a]

Fd addition	FNR concn (μM)	rate (min^{-1})[b]
none	0.2	<0.03
none	2.0	0.06
4 μM Fd-1	0.2	0.25
4 μM Fd-1 (Fe, DTT treated)[c]	0.2	0.17
4 μM Fd-1	2.0	0.27
20 μM Fd-1	2.0	0.36
4 μM Fd-2	0.2	1.03
4 μM Fd-2 (Fe, DTT treated)[c]	0.2	0.82
4 μM Fd-2	2.0	2.63
20 μM Fd-2	2.0	5.08
4 μM spinach Fd	0.2	0.18
4 μM spinach Fd	2.0	0.18
20 μM spinach Fd	2.0	0.36
4 μM Fd-1 + 4 μM Fd-2	2.0	1.74
4 μM Fd-1 + 4 μM spinach Fd	2.0	3.08
4 μM Fd-2 + 4 μM spinach Fd	2.0	4.27

[a] Further details under Materials and Methods. [b] Samples were incubated for 15 min at room temperature and are reported as P-450 turnover. A turnover of 7 min^{-1} corresponds to total consumption of the substrate during this time. [c] *S. griseolus* ferredoxins were subjected to a treatment for conversion of [3Fe-4S] clusters to [4Fe-4S] clusters. See Materials and Methods.

Activity of Purified Fd-1 and Fd-2. The activity of the purified ferredoxins was assayed by measuring herbicide (R7402) dealkylation in a reconstituted system consisting of spinach FNR, ferredoxin (Fd-1 or Fd-2 and/or spinach ferredoxin), and cytochrome P-450_{SU1}. R7402 is one of the best P-450_{SU1} sulfonylurea substrates available, although at pH 7 it is not soluble above ~400 μM and the dealkylation rate is a nearly linear function of concentration to that point. The results of a number of assays are shown in Table II. It is apparent that any of the three ferredoxins afforded significant activity at 4 μM, though none was saturating, since the rate of dealkylation increased at higher ferredoxin concentrations (20 μM). It is also clear that Fd-2 gave more activity than Fd-1. FNR may have been partially rate limiting in the experiments with Fd-2 but not with Fd-1 or spinach Fd. Treatment intended to restore a [4Fe-4S] cluster did not enhance the function of either Fd-1 or Fd-2; it is possible that any [4Fe-4S] cluster formed was lost during the vigorous shaking in air that is part of the assay, or in light of the EPR experiments reported above, it is possible that the treatment largely failed. It is also possible that [3Fe-4S] and [4Fe-4S] forms of the ferredoxin have similar activity. Surprisingly, spinach ferredoxin had a synergistic effect on activity when added with either Fd-1 or Fd-2; with Fd-1, a 10-fold rate enhancement was observed. Since FNR is apparently not rate limiting when Fd-1 or spinach ferredoxin is the only ferredoxin added, the rate enhancement when both are present suggests that a subsequent limiting step in the formation of product has been accelerated. The activity with the two ferredoxins in a P-450_{SU2}-based monooxygenase assay has not been specifically addressed because P-450_{SU2}-mediated dealkylation of R7402 is virtually undetectable (Harder et al., in preparation).

Fd-1 and Fd-2 have been compared in electron transfer assays similar to that in Figure 1, but with FNR and the purified cytochromes P-450_{SU1} and P-450_{SU2}. The initial rates of P-450_{SU1} reduction were 90 nM/min for Fd-1 and 60 nM/min for Fd-2. Substantially higher rates were observed for the initial reduction of P-450_{SU2}: 410 nM/min for Fd-1 and 1810 nM/min for Fd-2 (a minimum estimate for Fd-2,

A

***suaB* Sequence (Ferredoxin-1)**

```
-39   ACTCCCGGTCACCTGGTGACGGGGGAGAGGGGCAAGGACATGACCATGCGGGTGAGTGCG   21
      -LeuProValThrTrpEnd                    MetThrMetArgValSerAla

22    GATCGGACGGTCTGCGTCGGTGCCGGGCTGTGTGCGCTGACGGCGCCGGGCGTCTTCGAC   61
      AspArgThrValCysValGlyAlaGlyLeuCysAlaLeuThrAlaProGlyValPheAsp

62    CAGGACGACGACGGGATCGTCACGGTGCTGACGGCCGAACCCGCCGCCGACGACGACCGG   121
      GlnAspAspAspGlyIleValThrValLeuThrAlaGluProAlaAlaAspAspAspArg

122   CGCACCGCGCGCGAGGCCGGCCATCTCTGTCCGTCCGGTGCGGTCCGCGTCGTCGAGGAC   181
      ArgThrAlaArgGluAlaGlyHisLeuCysProSerGlyAlaValArgValValGluAsp

182   ACGGAATAGGGTCAAGGACACGGAACAGGCGAGCGGGGATTCCGGCCGTCGGCCGGGGCG   241
      ThrGluEnd
```

B

***subB* Sequence (Ferredoxin-2)**

```
-63   CTGCCGATCGCCTGGTAGCCCGGGCGCCCCCACCACCGACCACCACGCACCCTTGGGAGC   -4
     -LeuProIleAlaTrpEnd

-3    ACCATGCGCATCCACGTCGACCAGGACAAGTGCTGCGGCGCCGGCAGTTGCGTCCTCGCC   57
        MetArgIleHisValAspGlnAspLysCysCysGlyAlaGlySerCysValLeuAla

58    GCGCCCGACGTCTTCGACCAGCGGGAGGAGGACGGCATCGTGGTCCTCCTCGACACCGCG   117
      AlaProAspValPheAspGlnArgGluGluAspGlyIleValValLeuLeuAspThrAla

118   CCGCCCGCCGCGCTGCACGACGCGGTCCGTGAGGCGGCGACCATCTGCCCCGCCGCCGCG   177
      ProProAlaAlaLeuHisAspAlaValArgGluAlaAlaThrIleCysProAlaAlaAla

178   ATCACGGTGACCGACTGAGCCACCGGCCGCCCCGCCCGCCCGCGCCCCGGTCCCCGCATC   237
      IleThrValThrAspEnd
```

C

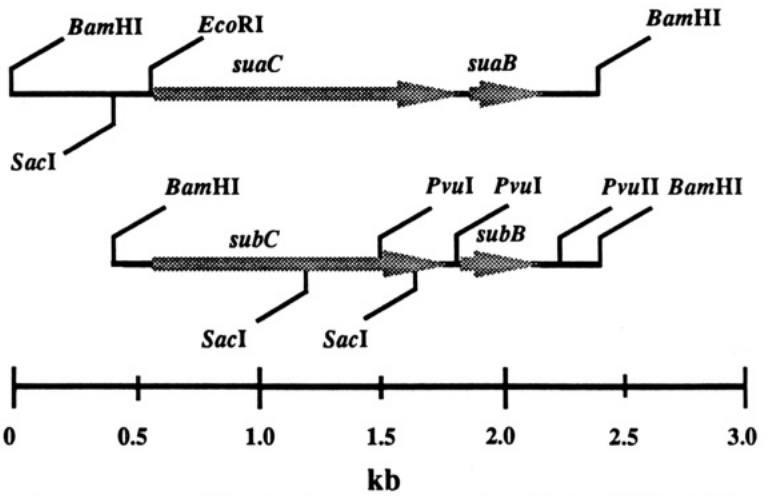

FIGURE 5: Sequences and restriction endonuclease map of the *S. griseolus* genes encoding Fd-1 and Fd-2. DNA sequences shown are the *suaB* (A) and *subB* (B) genes. Numbering starts at the ATG initiation codons, and the sequences determined by amino acid sequencing of the ferredoxin apoproteins are underlined. Note the C-terminal ends of the two cytochrome P-450 genes [−39 to −24 is from the *suaC* gene in (A), and −63 to −49 is from the *subC* gene in (b) (Omer et al., 1990)]. (C) Restriction endonuclease map of the 2.4- and 2.0-kb *Bam*H1 DNA fragments showing the locations of the *suaC,B* and *subC,B* genes, respectively.

since it could not be confirmed that FNR was not rate limiting). In the absence of ferredoxin there was not detectable (<3 nM/min) reduction of either P-450. These results demonstrate that either ferredoxin can transfer electrons to either P-450 but that each ferredoxin works at least somewhat faster with its cognate P-450.

Gene-Derived Sequences of the S. griseolus Ferredoxin 1 and Ferredoxin 2. Amino-terminal sequencing of the carboxymethyl derivatives of the Fd-1 and Fd-2 polypeptides yielded amino acid sequences for the first 43 residues of both proteins. Further sequence analysis of the 2.4-kb *Bam*H1 DNA fragment of pCAO302 containing the *suaC* gene (encoding $P\text{-}450_{SU1}$) described previously (Omer et al., 1990) revealed another open reading frame only 21 bases from the end of the $P\text{-}450_{SU1}$ coding region that, excluding the initiation methionine, encodes a 68 amino acid polypeptide (Figure 5A). With the exception of the initiation Met, the first 44 residues of this open reading frame are identical with those found by protein sequencing for Fd-1, and the amino acid compositions measured from Fd-1 and predicted from the sequence are in close agreement (Table III). Similarly, the 2.0-kb *Bam*H1 DNA fragment from pCAO304 containing the *subC* gene ($P\text{-}450_{SU2}$) contains an open reading frame, starting 46 bases after the termination of $P\text{-}450_{SU2}$, that encodes a 64-residue polypeptide. This polypeptide (Figure 5B) is identical in its first 43 residues to the N-terminal sequence of Fd-2 and also exhibits a close correlation with the measured amino acid composition of Fd-2 (Table III). We conclude that the shorter open reading frames in the 2.4- and 2.0-kb *Bam*H1 fragments encode Fd-1 and Fd-2, respectively. Consistent with the no-

```
                                10        20        30
                                 '*     *  '        '
S. griseolus Fd-1               tmrvsaDrtvCvgaGlCaltAPgVFDq.DdDGIvtVl......ta  38
S. griseolus Fd-2               .mrihVDQDkCcgaGsCvlaAPdVFDqreEDGIv.VllD....ta  39
C. thermoaceticum Fd            ..kvtVDQDlCIACGtCidlcPsVFD.wDdeGlshVivD......  36
D. africanus FdI                arkfyVDQDeCIACesCveiAPgaFamdpEiekayVk.Dve....  40
B. stearothermophilus Fd        pkytiVDketCIACGaCgaaAPdiyDy.DEDGIayVtlDdnqgiv  44

                                40        50        60
                                '         '     *   '
S. griseolus Fd-1               EpaadddrrtA.REaghlCPsgAvrVvedte                68
S. griseolus Fd-2               ppaalhd...AvREaatiCPaaAItVtd                   64
C. thermoaceticum Fd            EvpegaedscA.REsvneCPteAIkev                    62
D. africanus FdI                gasqeeveeamdt.....CPvqsIee                     61
B. stearothermophilus Fd        Evpdiliddmmdafe..gCPtesIkVadepfdgdpnkfd        81
```

FIGURE 6: Comparison of the *S. griseolus* Fd-1 and Fd-2 amino acid sequences with the three most similar ferredoxin sequences in the NBRF data base. Numbering of the *S. griseolus* ferredoxins is based on the observed polypeptide N-terminal residues. The sequences were aligned by pairwise comparison with the GAP program (Devereux et al., 1984) and taking the resulting gapped sequences and rerunning the GAP program until no more gaps appeared. A gap weight of 2.0 and length weight of 0.1 were used. The aligned sequences have capital letters for those residues found in at least three of the five sequences at a particular position. The three positions at which cysteines (C) appear in all five proteins are indicated (*).

Table III: Comparison of the Amino Acid Compositions of *S. griseolus* Ferredoxins As Measured from the Purified Apoproteins (Observed) and Deduced from the DNA Sequence (Predicted)

	ferredoxin 1 (*suaB*)			ferredoxin 2 (*subB*)		
residue	observed mol %	predicted mol %	predicted residue/mol[a]	observed mol %	predicted mol %	predicted residue/mol[b]
Asx	13.2	13.2	9	12.5	12.5	8
Thr	9.8	10.3	7	6.0	6.3	4
Ser	2.9	3.0	2	1.6	1.6	1
Glx	7.9	7.4	5	8.1	7.8	5
Pro	4.3	4.4	3	6.5	6.3	4
Gly	9.0	8.8	6	5.0	4.7	3
Ala	14.7	14.7	10	18.9	18.8	12
Val	12.3	13.2	9	10.8	11.0	7
Met	1.2	1.5	1	1.2	1.6	1
Ile	1.2	1.5	1	5.7	6.3	4
Leu	6.2	5.9	4	6.7	6.3	4
Tyr	0	0	0	0	0	0
Phe	1.5	1.5	1	1.6	1.6	1
His	1.4	1.5	1	3.0	3.1	2
Trp	nd[d]	0	0	nd[d]	0	0
Lys	0.3	0	0	1.7	1.6	1
Arg	11.0	8.8	6	6.1	4.7	3
Cys[c]	3.4	4.4	3	4.8	6.3	4

[a] Using molecular weight = 7044. The N-terminal Met in the open reading frame of *suaB* is excluded from this calculation. [b] Using molecular weight = 6647. [c] Measured as (carboxymethyl)cysteine. [d] Not determined.

menclature of the *Ps. putida* camphor monooxygenase system (Unger et al., 1986) and previous assignment of the *S. griseolus* genes (Omer et al., 1990), these genes are named *suaB* and *subB*, and are located in the two *Bam*H1 DNA fragments as shown in Figure 5C.

Analysis of the deduced sequences (Figure 5) and amino acid compositions (Table III) reveals that the Fd-1 polypeptide only has three total cysteines. Fd-1 is therefore incapable of forming a monomer with protein-derived four-cysteine coordination of a [4Fe-4S] cluster. This is consistent with our interpretation of the spectroscopic and biochemical evidence suggesting that Fd-1 contains a [3Fe-4S] cluster. We have searched the National Biomedical Research Foundation (NBRF) protein data base for other iron–sulfur proteins with sequence similarity to the *S. griseolus* ferredoxins 1 and 2. Of 125 ferredoxins and ferredoxin-like proteins in the data base, three showed significantly more similarity (≥50%) to either Fd-1 or Fd-2. The three proteins found were the [4Fe-4S] ferredoxins from *Bacillus stearothermophilus* (Hase et al., 1976), *Clostridium thermoaceticum* (Elliot et al., 1982), and *Desulfovibrio africanus* (Bruschi & Hatchikian, 1982), and sequence comparisons are shown in Figure 6. A fourth ferredoxin from *Bacillus thermoproteolyticus*, nearly identical with the *B. stearothermophilus* ferredoxin but not present in the NBRF data base, is very significant for this comparison since its three-dimensional structure has been determined (Fukuyama et al., 1988). The *B. stearothermophilus* ferredoxin differs from *B. thermoproteolyticus* ferredoxin simply by the transposition of the *B. stearothermophilus* Glu-64 and Asp-81. In contrast to the *S. griseolus* ferredoxins, these similar proteins all contain [4Fe-4S] clusters, and cysteines involved in attachment of the *B. thermoproteolyticus* [Fe-S] cluster align with Cys-11, Cys-17, Cys-56, and, notably, Ala-14 of Fd-1 (corresponding to Cys-10, Cys-16, Cys-55, and Ala-13 of Fd-2; see Figure 6). The location of this alanine is identical with that of an acid residue in some [3Fe-4S] ferredoxins, thought to stabilize a three-iron center (Bovier-Lapierre et al., 1987). Although Fd-2 does contain a fourth Cys (Cys-11), it does not align with the proposed coordination sites of the [4Fe-4S] proteins but is immediately adjacent to Cys-10, one of the proposed ligands of the cluster, in such a position as to make its involvement in [Fe-S] coordination unlikely (Fukuyama et al., 1988).

Expression of suaB and suaC in S. lividans. Expression of the *suaB* and *suaC* genes in *S. lividans* was obtained by cloning the 2.4-kb *Bam*HI DNA fragment from pCAO302 (Omer et al., 1990) into two chromosomally integrating vectors, pCAO170 or pCAO200 (Omer et al., 1988), creating pCAO400 and pCAO500, respectively. Expression of the *suaC* gene only was accomplished by cloning a 1.9-kb *Bam*HI fragment that contains the *suaC* gene and 6 bp beyond the end of the gene (i.e., no *suaB* gene) into pCAO200, creating pCAO501. This 1.9-kb DNA fragment was made from an exonuclease III deletion (used in DNA sequencing) to which a *Bam*HI linker was added to replace the deleted *Bam*HI site. Transformed *S. lividans* expressed cytochrome P-450$_{SU1}$ (~0.2% and ~0.8% of total soluble protein for *S. lividans* C501 and *S. lividans* C500, respectively) when analyzed by Western blot (see Figure 7). No reproducible differences in the levels of P-450$_{SU1}$ were evident in transformed *S. lividans*

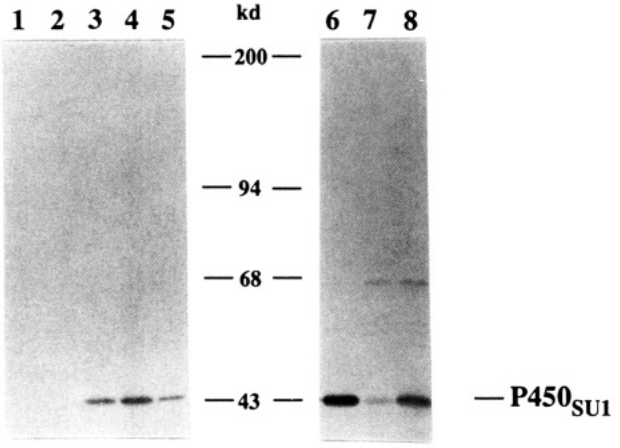

FIGURE 7: Western blot analysis of expression of cytochrome P-450$_{SU1}$ in transformed *S. lividans*. Samples loaded were 30 μg of either soluble protein from broken cells grown with or without 125 mg/L chlorimuron ethyl or purified cytochrome P-450$_{SU1}$. Lanes contained the following: (1) *S. lividans* C37 (control); (2) *S. lividans* C37 + chlorimuron ethyl; (3) *S. lividans* C400 (transformed with the *suaB* and *suaC* genes); (4) *S. lividans* C400 + chlorimuron ethyl; (5) P-450$_{SU1}$ (0.1 μg); (6) P-450$_{SU1}$ (0.2 μg); (7) *S. lividans* C501 (transformed with the *suaC* gene); (8) *S. lividans* C500 (transformed with the *suaB* and *suaC* genes).

grown in the presence or absence of chlorimuron ethyl (compare lanes 3 and 4, Figure 7). By contrast, P-450$_{SU1}$ was detectable in *S. griseolus* only after induction with sulfonylureas (O'Keefe et al., 1988; Omer et al., 1990).

S. lividans expressing both P-450$_{SU1}$ and Fd-1 was capable of metabolizing a number of sulfonylurea herbicides in liquid culture, and the results of incubation in the presence of chlorimuron ethyl are shown in Table IV. Transformed *S. lividans* C500 (containing both P-450$_{SU1}$ and Fd-1) metabolized chlorimuron ethyl faster than induced or uninduced *S. griseolus* (Table IV); the rate and pattern of metabolite formation was unaltered by pretreatment of *S. lividans* C500 with sulfonylureas known to be P-450 inducers in *S. griseolus* (data not shown). Cells expressing only cytochrome P-450$_{SU1}$ (*S. lividans* C501) appear to be nearly incapable of chlorimuron ethyl metabolism. These results suggest that *S. lividans* does not have an electron transfer system capable of transferring reducing equivalents directly to cytochrome P-450$_{SU1}$ but that it does have a component that effectively reduces ferredoxin 1, which can in turn reduce cytochrome P-450$_{SU1}$, resulting in sulfonylurea monooxygenase activity.

Discussion

The data presented here demonstrate that there are at least two forms of ferredoxin present in herbicide-induced *S. griseolus*, which we have previously shown to contain two inducible cytochromes P-450, P-450$_{SU1}$ and P-450$_{SU2}$ (O'Keefe et al., 1988; Omer et al., 1990). Their inducibility and the position of the *suaB* and *subB* open reading frames in the *S. griseolus* genome 21 and 46 bases, respectively, from the stop codon of the *suaC* and *subC* genes suggest that these ferredoxins may be cotranscribed and coordinately regulated with the adjacent cytochrome P-450 protein genes. In further sequence analysis of DNA downstream of *suaB* and *subB*, the next open reading frames found were over 250 bp away (data not shown). Whether or not these downstream open reading frames are involved in cytochrome P-450 mediated metabolism of sulfonylureas is unclear at this time.

The two ferredoxins are of similar molecular weight as determined by gel filtration and by LDS–PAGE even though we do not presently know how to evaluate these estimates of

Table IV: Metabolism of the Sulfonylurea Chlorimuron Ethyl by Cultures of *S. griseolus* and Transformed *S. lividans*

cells	time (h)	chlorimuron ethyl (mol %)	metabolite I (mol %)[a]	metabolite II (mol %)[b]
S. griseolus 11796 (uninduced)	2	87.8	7.2	5.0
	4	64.7	20.9	14.4
	6	36.7	36.7	26.6
S. griseolus 11796 (induced)	2	52.0	30.0	18.0
	4	9.8	55.3	34.9
	6	0.8	60.0	39.2
S. lividans C500 (uninduced)	2	12.7	48.0	39.3
	4	0	55.0	45.0
	6	0	54.4	45.6
S. lividans C501 (uninduced)	2	98.6	0.7	0.7
	4	97.2	1.4	1.4
	6	96.2	2.1	1.7
S. lividans C200 (uninduced)	2	99.0	0.5	0.5
	4	99.4	0.3	0.3
	6	98.8	0.6	0.6

[a] O-Demethylated metabolite of chlorimuron ethyl; *N*-[[(4-chloro-6-hydroxypyrimidin-2-yl)amino]carbonyl]-2-(ethoxycarbonyl)benzenesulfonamide (O'Keefe et al., 1987). [b] Deesterified metabolite of chlorimuron ethyl; *N*-[[(4-chloro-6-methoxypyrimidin-2-yl)amino]carbonyl]-2-(carboxycarbonyl)benzenesulfonamide (O'Keefe et al., 1987).

~18–23 kDa given that the sequence data suggest molecular masses on the order of 7 kDa. It is possible that the native proteins are not monomeric; it is also possible that as small, unusually acidic proteins they simply do not behave like the "typical" ones used to calibrate the column, as we have shown for spinach ferredoxin and putidaredoxin. They may behave anomalously upon LDS–PAGE for the same reasons. The two proteins exhibit 52.4% sequence identity (Figure 6), so it is reasonable to suppose that they are structurally as well as functionally similar; some structural similarity is demonstrated by the finding that, as isolated, each contains a (somewhat unusual) [3Fe-4S] cluster. Their functional similarity is borne out by the observation that either Fd-1 or Fd-2 can mediate electron transfer to either P-450$_{SU1}$ or P-450$_{SU2}$. Furthermore, either ferredoxin will support herbicide metabolism by P-450$_{SU1}$ (Table II). The apparent discrepancy between the reduction data, which show faster rates for the genetically linked protein pairs (P-450$_{SU1}$–Fd-1 and P-450$_{SU2}$–Fd-2), and the metabolism experiments, in which Fd-2 was more active than Fd-1 with P-450$_{SU1}$, suggest that the overall rate of turnover (metabolism) was not limited by the rate of transfer of the first electron to the P-450. It has been shown that the overall rate of metabolism of camphor by cytochrome P-450$_{CAM}$ is limited by the rate of delivery of the second reducing equivalent to the P-450 (Gunsalus et al., 1974; Sligar & Murray, 1986). In the case of liver microsome P-450$_{LM}$, the reaction rate appears to be controlled at or after the second reduction step (Coon & White, 1980).

The experiments with reconstituted systems included spinach ferredoxin:NADP oxidoreductase in addition to the purified ferredoxins and cytochromes P-450. It is apparent that *S. griseolus* must contain NAD(P)H:ferredoxin reductase(s) of its own, and we have in fact been able to assay such an activity in extracts, though we have yet to find out how many there are or how to purify them and to determine their specificities. However, the experiments with *S. lividans* suggest that it may have a constitutive reductase capable of donating reducing equivalents to various ferredoxins, since there was P-450 activity in cells that had been transformed with ferredoxin and P-450, but not reductase, genes. The finding that uninduced *S. griseolus* extracts, which contain only an herbicide metabolism incompetent cytochrome P-450$_{CON}$, also confer ac-

tivity on purified P-450_{SU1} (O'Keefe et al., 1988) suggests that it also may have a constitutive ferredoxin reductase of broad specificity, as well as ferredoxins other than Fd-1 and Fd-2. It is also notable that, in contrast to *S. griseolus*, P-450 synthesis is constitutive in transformed *S. lividans*, which suggests that the *suaB* and *suaC* genes are negatively regulated in *S. griseolus*.

The activity of spinach ferredoxin in reconstitution of P-450_{SU1}-catalyzed metabolism closely parallels the activity of the "natural" partner of P-450_{SU1}, Fd-1 (see Table II). This occurs despite the low redox midpoint potential of spinach ferredoxin which results in only about 10% steady-state reduction of this ferredoxin in the presence of FNR and NADPH under conditions similar to those used here (O'Keefe, 1983). The practical utility of a cytochrome P-450 which is capable of herbicide metabolism and detoxification and the finding that ferredoxin, a common point of reducing equivalent distribution in the higher plant chloroplast, can function as electron transfer partners in vitro suggest that such a heterologous combination could be functional in vivo. This would require that plants could be transformed with the *suaC* gene in such a way that the mature bacterial-derived cytochrome P-450 ended up in the chloroplast stromal space of the plant, to intercept reducing equivalents from photoreduced ferredoxin. This possibility is currently the subject of further investigation.

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References

Beinert, H. (1983) *Anal. Biochem. 131*, 373–378.
Beinert, H. (1990) *FASEB J. 4*, 2483–2491.
Beinert, H., & Thomson, A. J. (1983) *Arch. Biochem. Biophys. 222*, 333–361.
Bovier-Lapierre, G., Bruschi, M., Bonicel, J., & Hatchikian, E. C. (1987) *Biochim. Biophys. Acta 913*, 20–26.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Bruschi, M., & Hatchikian, E. C. (1982) *Biochimie 64*, 503–507.
Bruschi, M., Hatchikian, E. C., LeGall, J., Moura, J. J. G., & Xavier, A. V. (1976) *Biochim. Biophys. Acta 449*, 275–284.
Buchanan, B. B., & Arnon, D. I. (1971) *Methods Enzymol. 23*, 413–440.
Coon, M. J., & White, R. E. (1980) in *Metal Ion Activation of Oxygen* (Spiro, T. G., Ed.) pp 73–124, Wiley, New York.
Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res. 12*, 387–395.
Elliot, J. I., Yang, S.-S., Ljungdahl, L. G., Travis, J., & Reilly, C. F. (1982) *Biochemistry 21*, 3294–3298.
Emptage, M. H., Dreyer, J.-L., Kennedy, M. C., & Beinert, H. (1983) *J. Biol. Chem. 258*, 11106–11111.
Fukuyama, K., Nagahara, Y., Tsukihara, T., Katsube, Y., Hase, T., & Matsubara, H. (1988) *J. Mol. Biol. 199*, 183–193.
Gunsalus, I. C., Meeks, J. R., Lipscomb, J. D., Debrunner, P., & Münck, E. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) pp 559–613, Academic Press, New York.
Hase, T., Omiya, N., Matsubara, H., Mullinger, R. N., Rao, K. K., & Hall, D. O. (1982) *Biochem. J. 159*, 55–63.
Henikoff, S. (1984) *Gene 28*, 351–359.
Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., & Schrempf, H. (1985) *Genetic Manipulation of Streptomyces: a Laboratory Manual*, John Innes Foundation, Norwich, England.
Kennedy, M. C., Emptage, M. H., Dreyer, J.-L., & Beinert, H. (1983) *J. Biol. Chem. 258*, 11098–11105.
Kennedy, M. C., Kent, T. A., Emptage, M. H., Merkle, H., Beinert, H., & Münck, E. (1984) *J. Biol. Chem. 259*, 14463–14471.
Kent, T. A., Moura, I., Moura, J. J. G., Lipscomb, J. D., Huynh, B. H., LeGall, J., Xavier, A. V., & Münck, E. (1982) *FEBS Lett. 138*, 55–58.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Massey, V., & Hemmerich, P. (1978) *Biochemistry 17*, 9–17.
Mizusawa, S., Nishimura, S., & Seela, F. (1986) *Nucleic Acids Res. 14*, 1319–1324.
Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R., & Waterman, M. R. (1987) *DNA 6*, 1–11.
O'Keefe, D. P. (1983) *FEBS Lett. 162*, 349–354.
O'Keefe, D. P., Romesser, J. A., & Leto, K. J. (1987) *Recent Adv. Phytochem. 21*, 151–173.
O'Keefe, D. P., Romesser, J. A., & Leto, K. J. (1988) *Arch. Microbiol. 149*, 406–412.
Omer, C. A., Stein, D., & Cohen, S. N. (1988) *J. Bacteriol. 170*, 2174–2184.
Omer, C. A., Lenstra, R., Litle, P. J., Dean, C., Tepperman, J. M., Leto, K. J., Romesser, J. A., & O'Keefe, D. P. (1990) *J. Bacteriol. 172*, 3335–3345.
Omura, R., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.
Palmer, G. (1973) in *Iron Sulfur Proteins* (Lovenberg, W., Ed.) Vol. 2, pp 285–325, Academic Press, Orlando, FL.
Romesser, J. A., & O'Keefe, D. P. (1986) *Biochem. Biophys. Res. Commun. 140*, 650–659.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Shively, J. E. (1986) in *Methods of Protein Microcharacterization* (Shively, J. E., Ed.) pp 41–87, Humana Press, Clifton, NJ.
Sligar, S. G., & Murray, R. I. (1986) in *Cytochrome P-450 Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 429–503, Plenum Press, New York.
Unger, B. P., Gunsalus, I. C., & Sligar, S. G. (1986) *J. Biol. Chem. 261*, 1158–1163.
Zanetti, G., & Curti, B. (1980) *Methods Enzymol. 69*, 250–255.