Sulfide Dehydrogenase Activity of the Monomeric Flavoprotein SoxF of *Paracoccus pantotrophus*[†,‡]

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ABSTRACT: Flavocytochrome *c*−sulfide dehydrogenases (FCSDs) are complexes of a flavoprotein with a *c*-type cytochrome performing hydrogen sulfide-dependent cytochrome *c* reduction *in vitro*. The amino acid sequence analysis revealed that the phylogenetic relationship of different flavoproteins reflected the relationship of sulfur-oxidizing bacteria. The flavoprotein SoxF of *Paracoccus pantotrophus* is 29−67% identical to the flavoprotein subunit of FCSD of phototrophic sulfur-oxidizing bacteria. Purification of SoxF yielded a homogeneous emerald-green monomeric protein of 42 797 Da. SoxF catalyzed sulfide-dependent horse heart cytochrome *c* reduction at the optimum pH of 6.0 with a k_{cat} of 3.9 s^{-1}, a K_m of 2.3 μM for sulfide, and a K_m of 116 μM for cytochrome *c*, as determined by nonlinear regression analysis. The yield of 1.9 mol of cytochrome *c* reduced per mole of sulfide suggests sulfur or polysulfide as the product. Sulfide dehydrogenase activity of SoxF was inhibited by sulfur (K_i = 1.3 μM) and inactivated by sulfite. Cyanide (1 mM) inhibited SoxF activity at pH 6.0 by 25% and at pH 8.0 by 92%. Redox titrations in the infrared spectral range from 1800 to 1200 cm^{-1} and in the visible spectral range from 400 to 700 nm both yielded a midpoint potential for SoxF of −555 ± 10 mV versus Ag/AgCl at pH 7.5 and −440 ± 20 mV versus Ag/AgCl at pH 6.0 (−232 mV versus SHE′) and a transfer of 1.9 electrons. Electrochemically induced FTIR difference spectra of SoxF as compared to those of free flavin in solution suggested a strong cofactor interaction with the apoprotein. Furthermore, an *activation*/*variation* of SoxF during the redox cycles is observed. This is the first report of a monomeric flavoprotein with sulfide dehydrogenase activity.

Hydrogen sulfide is oxidized by aerobic chemotrophic and anaerobic phototrophic sulfur-oxidizing bacteria, and the reaction is mediated by different enzymes (reviewed in refs *1*−*4*). The catalytic characteristic of flavocytochrome *c*−sulfide dehydrogenases (FCSDs)[1] is the transfer of two electrons from hydrogen sulfide to horse heart cytochrome *c in vitro* (*5*, *6*). The structural characteristic of FCSD is the formation of a complex of a flavoprotein with a *c*-type cytochrome. FCSD has first been identified from the phototrophic purple sulfur bacterium *Allochromatium vinosum* (*5*) [formerly *Chromatium vinosum* (*7*)] and later from other phototrophic purple and green sulfur bacteria (*1*−*4*, *8*−*10*) and from the chemotrophic sulfur-oxidizing bacterium *Thiobacillus* W5 (*11*). FCSD has been characterized from different sources with respect to its catalytic, spectroscopic, electrochemical, and structural (*12*) properties as well as its cellular location. The soluble FCSD of *A. vinosum* is composed of flavoprotein FccB and diheme cytochrome FccA of 21 kDa (*10*, *13*, *14*). FCSD of the green sulfur bacterium *Chlorobium limicola* is a soluble complex of flavoprotein SoxF1 with a monoheme cytochrome *c* of 11 kDa (*15*, *16*). FCSD of the phototrophic purple sulfur bacterium *Ectothiorhodospira vacuolata* is a complex with a monoheme cytochrome *c* of 9 kDa and is membrane-bound (*9*), as is FCSD of the chemotrophic bacterium *Thiobacillus* W5 (*11*). Genome data of *A. vinosum* (*4*, *14*) and *Chlorobium* species suggest FCSD is periplasmic (*15*). Thus, FCSDs from different sources differ in cellular location and characteristics of the cytochromes with which they are complexed (*17*, *18*; reviewed in refs *2* and *4*).

The sulfide dehydrogenase activity of FCSD *in vitro* was considered to reflect its function *in vivo*. However, inactivation of the cytochrome subunit of FCSD of *A. vinosum* does not affect phototrophic growth with hydrogen sulfide (*19*). Similarly, inactivation of SoxF of *Paracoccus pantotrophus* does not prevent strain GB*soxF*Δ from growing with or oxidizing thiosulfate (*17*), while mutations in other *sox* genes of photrophic and chemotrophic bacteria do (*20*−*22*).

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[1] Abbreviations: DGGE, density gradient gel electrophoresis; FCSD, flavocytochrome *c*−sulfide dehydrogenase; FTIR, Fourier transform infrared; Sox, sulfur oxidation.

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Although the involvement of these flavoproteins in sulfur oxidation is evident from their formation induced by thiosulfate or sulfide (*11*, *17*, *22*) and the location of the respective genes within the *sox* gene clusters in these bacteria (*22*, *23*), a key function in sulfur-dependent energy metabolism is not likely.

The *sox* gene cluster of *P. pantotrophus* encodes four periplasmic proteins which reconstitute the sulfur-oxidizing (Sox) enzyme system. This enzyme system transfers electrons from hydrogen sulfide, sulfur, thiosulfate, and sulfite to reduce horse heart cytochrome *c in vitro*. SoxF is also located in the periplasm of the cell but is not part of this system, and its function *in vivo* has not been resolved (*17*).

The primary structure of SoxF of *P. pantotrophus* is 37% identical to that of FccB of *A. vinosum* (*17*) and 46% identical to the respective proteins from *C. limicola* and *Chlorobium tepidum* (*24*), and SoxF of *P. pantotrophus* has sequence features similar to those of the flavoprotein of FCSD from *E. vacuolata* (*9*). Although the primary structure of SoxF of *P. pantotrophus* is very similar to the structures of other flavoproteins, SoxF was reported to be monomeric according to native gradient gel analysis, and no activity was assigned to SoxF (*17*). Therefore, the structural and biochemical properties of SoxF were re-examined. We report here the hydrogen sulfide-dependent horse heart cytochrome *c* reduction of the flavoprotein SoxF of *P. pantotrophus*, and describe its monomeric nature and its catalytic and electrochemical characteristics. This is the first report of a sulfide dehydrogenase activity mediated by a flavoprotein not complexed with a *c*-type cytochrome.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. P. pantotrophus (*25*, *26*) and strains derived therefrom (*17*) were used in this study. *P. pantotrophus* was cultivated either aerobically at 30 °C with 20 mM thiosulfate or mixotrophically with succinate and thiosulfate (*27*). Chemotrophic mass cultivation of *P. pantotrophus* with thiosulfate was performed in a 300 L fermentor, and cells were harvested as described previously (*28*).

Purification of SoxF. Cell free extracts from cells grown mixotrophically with succinate and thiosulfate were obtained with a French press and subjected to differential centrifugation. Proteins were separated by ammonium sulfate fractionation and chromatography on Q-Sepharose (*17*) with some modifications. Instead of hydroxyapatite (*17*), hydrophobic interaction chromatography on Resource Q was applied. SoxF was identified from the column eluates by absorbance at 450 nm and by immunochemical analysis as detailed below.

Sulfide Dehydrogenase Assay. Sulfide-dependent horse heart cytochrome *c* reduction was assessed by following the absorbance at 550 nm with a Shimadzu UV160A UV−vis spectrophotometer. The assay (0.70 mL) contained, unless otherwise stated, 35 μmol of sodium potassium phosphate buffer (pH 6.0), 49 nmol of horse heart cytochrome *c*, 70 pmol of SoxF, and 7 nmol of disodium sulfide to start the reaction. Sodium sulfide solutions were freshly prepared and kept as 1 mM stock solutions under argon (*17*). Sulfur was dissolved in 1 M sodium hydroxide and added to the assays after dilution with water. The molar absorption coefficient ϵ of commercial horse heart cytochrome *c* was determined to be 19.8 cm^2 μmol^{-1} from the difference in absorption at 550 nm of the air-oxidized and dithionite-reduced sample. One unit of enzyme activity is defined as 1 μmol of cytochrome *c* reduced per minute at 30 °C.

Protein was quantified by the method of Bradford (*29*).

Electrochemistry. The ultra-thin layer spectroelectrochemical cell for the UV−vis and IR spectral range was previously described (*30*). Sufficient transmission in the 1800−1000 cm^{-1} range, even in the region of strong water absorbance around 1645 cm^{-1}, was achieved with the cell path length set to 6−8 μm. To avoid complexation of free flavin with the electrode or denaturation of the protein, the gold grid working electrode was found to be chemically modified conveniently by a 2 mM 1-mercaptohexanol solution. To accelerate the redox reaction, the mediators were used at a final concentration of 45 μM each as described previously (*31*). At the given concentrations, and with a path length of <10 μm, no spectral contributions from the mediators in the visible and IR range that was used were detected in control experiments with samples lacking the protein. Approximately 6−7 μL of the protein solution was sufficient to fill the spectroelectrochemical cell. Potentials quoted with the data refer to the Ag/AgCl/3 M KCl reference electrode; add 208 mV to the SHE′ electrode (pH 7).

Redox Titrations. The redox-dependent changes in absorbance were studied with electrochemical redox titrations in the visible and IR spectral range. The redox titrations were performed by setting, in a stepwise fashion, the potential steps and recording the spectrum after equilibration. Between each potential step, a full potential step was performed. Usually, data were recorded at steps of 20 mV between −0.6 and −0.4 V and an additional step to −0.2 V. All measurements were performed at 5 °C. The pH was adjusted in Tris buffer (pH 7.5). Data analysis was carried out with a program developed by S. Grzybek termed EHTIT (*30*, *32*). EHTIT allows one to obtain the midpoint potentials (E_m) and the number of transferred electrons (n) by adjusting a calculated Nernst curve to the measured absorbance change at a single wavelength by an interactive fit.

Spectroscopy. FTIR and vis difference spectra were obtained as a function of the applied potential from a modified IFS 25 instrument (Bruker) for the 2000−1000 cm^{-1} range, combining an IR beam from the interferometer and a dispersive spectrometer for the 400−900 nm range as reported previously (*33*). First, the protein was equilibrated with an initial potential at the electrode, and single-beam spectra in the visible and IR range were recorded. Then a potential step toward the final potential was applied, and single-beam spectra of this state were again recorded after equilibration. Difference spectra were calculated from the two single-beam spectra with the initial single-beam spectrum taken to be a reference. No smoothing or deconvolution procedures were applied. The equilibration process for each applied potential was followed by monitoring the electrode current and by successively recording spectra in the visible range until no further changes were observed. The equilibration generally took less than 3 min for the full potential step from −0.65 to −0.3 V. Typically, 128 interferograms at 4 cm^{-1} resolution were coadded for each single-beam IR spectrum and Fourier transformed using triangular apodiza-

Table 1: Identities of Flavoproteins of FCSD of Different Sources with SoxF of *P. pantotrophus*

organism	gene designation	M_w (Da)	% identity with Pp SoxF[a]	accession no. or reference
P. pantotrophus	*soxF*	42 006		X79242
Aquifex aeolicus	*dhsU*	45 978	30.3 (402)	AE000679
A. aeolicus	*fccB*	42 297	28.8 (325)	AE000679
C. tepidum	*fccB-1*	42 935	41.2 (396)	NC_002932
C. tepidum	*fccB-2*	45 343	47.6 (361)	NC_002932
Rhodopseudomonas palustris	Rpal0917	42 346	58.3 (393)	ZP_00009243
R. palustris	Rpal0916	47 230	48.9 (395)	ZP_00049242
Rhodovulum sulfidophilum	*soxF*	41 892	66.6 (395)	AY005800
Ralstonia solanacearum	*soxF*	42 368	39.2 (400)	AL646074
C. limicola	*soxF1*	42 308	45.6 (397)	AY074396
C. limicola	*soxF2*	42 749	41.8 (397)	AY074395
Allochromatium vinosum	*fccB*	42 784	44.5 (400)	AF031149
E. vacuolata	*fcsD*	43 457	49.2 (400)	*9*

[a] Accession numbers refer to GenBank except that for *R. palustris* (Joint Genome Institute).

tion. Approximately 20−30 difference spectra have been averaged.

Analytical Procedures. The molecular masses of denatured proteins were determined by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE) according to Laemmli (*34*) and of native proteins by nondenaturing density gradient gel electrophoresis (DGGE) with a linear gradient from 5 to 27.5% polyacrylamide (*35*). Proteins were stained with Coomassie blue (*36*). The molecular mass of SoxF was determined by nano-electrospray mass spectrometry with a Finnigan LCQ mass spectrometer at the facilities of the Max-Planck-Institut für molekulare Physiologie (Dortmund, Germany).

Immunoblots (*37*) were performed according to the "semidry" procedure using the multiphor electrophoresis system (Pharmacia, Freiburg, Germany). Antibodies against the internal oligopeptide of SoxE, MRDHSDRPQDIPEAE (OP-E), and SoxF, LDPKDKFSKQALFE (OP-F), were raised in rabbits by Eurogentec (Seraing, Belgium).

Hemochromes from protein preparations were analyzed as described previously (*27*, *38*).

Oxidized flavin was quantified spectrometrically using a molar extinction coefficient (ϵ_{450}) of 11 300 cm^2 M^{-1}.

Kinetic constants were obtained by nonlinear regression analysis using Scientist for Windows version 2.01 (Micro-Math Research, St. Louis, MO).

RESULTS AND DISCUSSION

Genome Analysis for Flavoproteins. The primary structure of SoxF of *P. pantotrophus* as deduced from the *soxF* gene has been verified by Edman degradation of internal peptides of SoxF (*17*). Amino acid sequence comparison revealed that the primary structure of flavoproteins from FCSD of various sources is 29−67% identical to that of SoxF (Table 1). The SoxF sequence shared essential features with respect to the proposed FAD-binding site (*9*, *18*), and sequence identities were distributed over the entire polypeptide (data not shown).

The flavoproteins are also phylogenetically closely related, which is evident from the neighbor-joining tree (Figure 1) which also reflected the relationship of the bacteria. The flavoproteins of the phototrophic green sulfur bacteria (*Chlorobia*) form one group as do the proteins of the purple sulfur bacteria (*γ-Proteobacteria*) and the photo- and chemotrophic *α-Proteobacteria* (Figure 1).

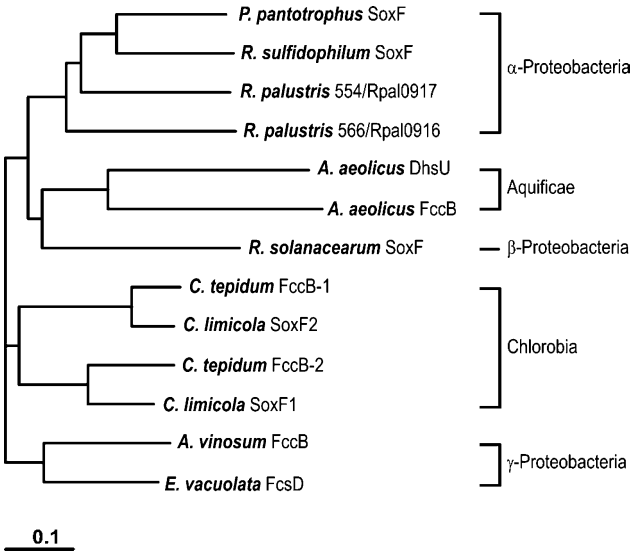

FIGURE 1: Phylogenetic relationships of SoxF homologous proteins from different bacteria. The bars indicate the estimated distance of accepted point mutations per 100 amino acids.

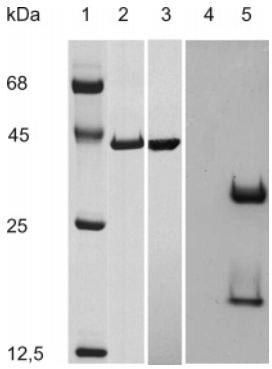

FIGURE 2: SDS−PAGE of SoxF as isolated. Samples of SoxF (2.0 μg) were prepared as described previously (20) and applied to each well: lane 1, marker proteins; lane 2, Coomassie stain; lane 3, immunoblot using OP-F antibodies; lane 4, heme stain SoxF; and lane 5, heme stain cytochrome SoxXA (2.0 μg). Samples in lanes 4 and 5 were prepared without boiling.

Characterization of SoxF. SoxF was purified as described in Materials and Methods and identified from the various purification steps by antibodies to oligopeptide OP-F which detected a specific internal immunogenic epitope of SoxF. SoxF eluted from the 0.05 M sodium chloride fraction of the Q-Sepharose column (data not shown). The purification procedure yielded a SoxF preparation of 8.0 mg/mL with an intense emerald-green color. The preparation was homogeneous as judged from SDS−PAGE by Coomassie stain and immunoblot analysis using OP-F antibodies, and was free of cytochromes as judged from heme staining (sample preparation without boiling) (Figure 2). Immunoblots using OP-E antibodies did not detect traces of cytochrome SoxE (data not shown).

The electronic absorption spectrum of the air-oxidized SoxF (100 μM) as monitored at pH 6.0 exhibited maxima at 650, 473, 451, and 377 nm, of which the peaks at 451 and 377 nm were characteristic of flavin (Figure 3). The

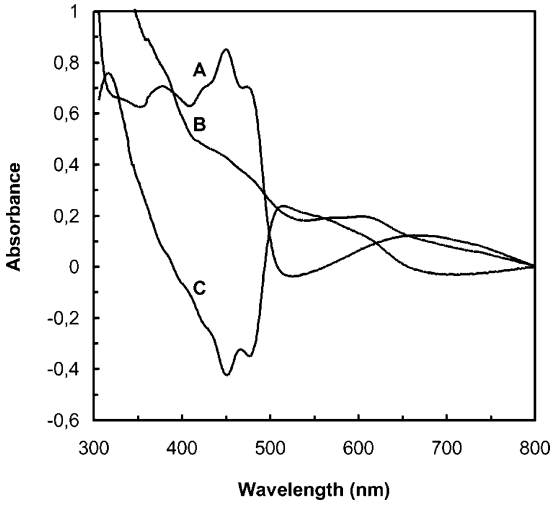

FIGURE 3: Electronic absorption spectrum of SoxF. SoxF (100 μM) in 25 mM sodium potassium phosphate buffer (pH 6.5) with a light path of 1 cm: (A) air-oxidized SoxF, (B) dithionite-reduced SoxF, and (C) reduced minus oxidized difference spectrum.

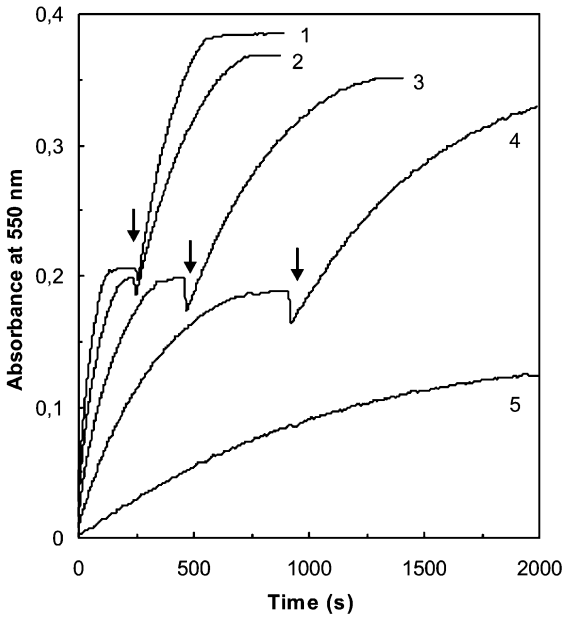

FIGURE 4: Time course of hydrogen sulfide cytochrome *c* reduction by SoxF. The reaction was started with disodium sulfide; arrows indicate further addition of 7 nmol of disodium sulfide. The concentrations of SoxF were 0.5 (1), 0.25 (2), 0.1 (3), and 0.05 μM (4). Trace 5 shows an assay without SoxF.

broad absorption around 650 nm is diagnostic for the semiquinone form. The oxidized form of flavin is yellow and the semiquinone blue, giving together the observed emerald color of the "as isolated" preparation. The absorbance at 450 nm indicated a content of 0.85 ± 0.03 mol of flavin per mole of SoxF in the oxidized form, and the absorption at 650 nm suggested 0.15 mol of flavin in the semiquinone form.

The spectral features of the dithionite-reduced minus air-oxidized difference spectrum of SoxF did not indicate absorption of a heme (Figure 3). Hemochrome analysis (*27*, *38*) of SoxF as isolated did not indicate the presence of even traces of heme (data not shown), again demonstrating that SoxF was not complexed with a cytochrome. These data were also in accordance with the result of the heme stain of blots of SoxF from SDS−PAGE.

Electrospray mass spectrometry of SoxF as isolated yielded masses of 42 797 and 42 832 Da (data not shown). These masses were close to that of the mature polypeptide with protonated FAD (821 Da) as a prosthetic group. The predicted signal peptide cleavage site was between G^{26} and Q^{27}, and since the N-terminus was blocked, cycloglutamate was likely as the N-terminus to give a mature SoxF polypeptide of 41 989 Da. The mass difference of 35 Da may have resulted from a post-translational modification created by addition of one sulfur atom or two oxygen atoms to SoxF.

The data of the electronic absorption spectrum, hemochrome analysis, SDS−PAGE and DGGE, and electrospray mass spectrometry were in accordance and demonstrated the monomeric nature of SoxF.

Catalytic Properties of SoxF. SoxF catalyzed hydrogen sulfide-dependent horse heart cytochrome *c* reduction. The rate of cytochrome *c* reduction was not linear and decreased with time, and reduction ceased completely due to exhaustion of sulfide from the assay as demonstrated from an additional supply of sodium sulfide to the assay which led to the recovery of the reaction rate and increased the final level of conversion of sulfide (Figure 4). The catalytic activity of SoxF was determined from the initial rate as extrapolated from the reaction rate versus concentration of reduced cytochrome *c* course to initial substrate concentrations by nonlinear regression analysis. After repeated addition of sodium sulfide, the initial rate decreased significantly. From the final degree of sodium sulfide-dependent reduction of horse heart cytochrome *c*, a yield of 1.9 ± 0.3 mol of electrons per mole of hydrogen sulfide was determined. This yield suggested sulfur or polysulfide as a product of the reaction. The identical yield of electrons has been determined, and the same products of the reaction have been suggested for FCSD of *C. limicola* (*1*) and *A. vinosum* (*6*). FCSD activity of *A. vinosum* very similarly decreases with time (*1*, *6*), while the isolated flavoprotein subunit of FCSD of *A. vinosum* does not have sulfide dehydrogenase activity (*6*).

The pH optimum of SoxF activity was determined to be pH 6.0 and used for the standard assay. At this pH, the spontaneous sulfide-dependent cytochrome *c* reduction rate was negligible as the blank rate increased with an increase in pH (Figure 5). The pH dependence of the SoxF activity was expressed as the total reaction rate minus the rate without SoxF. From the net rate, two pK_a values of 4.8 and 7.6 were calculated according to the general titration equation. The only other chemotrophic bacterium from which FCSD was isolated was *Thiobacillus* W5; its pH optimum is around pH 8.6 (*11*), as is that of FCSD of *A. vinosum* (*6*).

The initial sulfide-dependent cytochrome *c* reduction rate of SoxF depended on the sulfide concentration and followed a random bireactant Michaelis−Menten type of kinetics with independent substrate dissociation constants (*43*). The kinetic constants were obtained by nonlinear regression analysis and are summarized in Table 2. The rate constant K_m for sodium sulfide was 2.3 ± 0.1 μM and for horse heart cytochrome *c* 116 ± 9 μM.

Competitive product inhibition of the binding of the respective substates was assessed by a fit of the final kinetic

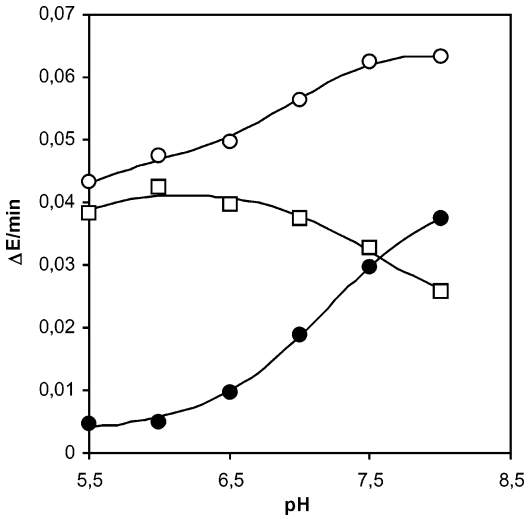

FIGURE 5: pH optimum of sulfide-dependent cytochrome *c* reduction. The assay contained 70 μM cytochrome *c*, 50 mM potassium phosphate buffer, and 10 μM disodium sulfide (start). The pH was determined after completion of the reaction: (●) assay without SoxF, (○) assay with 70 pmol of SoxF, and (□) SoxF net activity.

Table 2: Kinetic Constants of the Sulfide-Dependent Cytochrome *c* Reduction of SoxF

k_{cat} (s^{-1})	3.9 ± 0.2
$K_{m-Sulfide}$ (μM)	2.3 ± 0.1
$K_{m-Cytochrome\ c\ oxidized}$ (μM)	116 ± 9
$K_{i-Sulfur}$ (μM)	1.3 ± 0.2
$K_{i-Cytochrome\ c\ reduced}$ (μM)	7.2 ± 1.1

model (eq 1) to the full set of experimental data in the range of 1−20 μM sulfide and 17.5−105 μM cytochrome *c*.

$$\frac{v}{E} = (k_{cat} C_{Sulfide} C_{Cytochrome\ c}) / \left\{\left[C_{Sulfide} + K_{m-Sulfide}\left(1 + \frac{C_{Sulfur}}{K_{i-Sulfur}}\right)\right]\left[C_{Cytochrome\ c} + K_{m-Cytochrome\ c}\left(1 + \frac{C_{Cytochrome\ c\ reduced}}{K_{i-Cytochrome\ c\ reduced}}\right)\right]\right\} \quad (1)$$

From these analyses, a k_{cat} of 3.9 ± 0.2 s^{-1} was calculated, resulting in a specificity constant of 1.7×10^6 s^{-1} M^{-1}. This second-order rate constant was in the same order of magnitude as that reported for FCSD of *A. vinosum* (7.6×10^6 s^{-1} M^{-1}) and *C. limicola* (5.5×10^6 s^{-1} M^{-1}) (*10*).

Amazingly, similar kinetic data were reported for FCSD from *Thiobacillus* W5 with respect to k_{cat} (1.8 s^{-1}) and K_m for sulfide (1.7 μM). The K_m for cytochrome *c* of SoxF of *P. pantotrophus* was much more than 1 order of magnitude higher than that of FCSD of *Thiobacillus* W5 (3.8 μM) (*11*) and almost 1 order of magnitude lower than that of FCSD of *C. limicola* (0.60 mM) (*10*, *42*). The similar kinetic features suggested the activity of SoxF is not due to contaminating traces of other proteins or a cytochrome, as the latter was beyond the limits of detection of different methods. Each of these results and their combination confirm the monomeric nature of SoxF as isolated.

The sulfide-dependent cytochrome *c* reduction rate decreased with the duration of the reaction (Figure 4). From the kinetic data given above, the decrease in rate reflected the changing concentrations of the substrate and product during the reaction. The decreasing rates were in accordance with the inversely changing concentrations of the substrate and product and close to the K_m and K_i values. Thus, the kinetics were in accordance with the classical product inhibition and did not indicate inactivation of SoxF during the reaction (*43*), which is also evident from the repetitive sulfide addition (Figure 4). In that respect, it is worthy to note that the K_i of sulfur (1.3 μM) is lower than the K_m of hydrogen sulfide (2.3 μM), preferring the reverse reaction of sulfide dehydrogenase.

SoxF did not catalyze thiosulfate- or sulfite-dependent cytochrome *c* reduction. SoxE, purified partially by chromatography on Q-Sepharose and added to the assay, did not enhance sulfide dehydrogenase activity of SoxF of *P. pantotrophus* (data not shown).

Sulfite and cyanide are potent inhibitors of FCSDs from various sources (*11*, *44*−*46*). The initial sulfide dehydrogenase activity of SoxF of *P. pantotrophus* was inhibited by 1 mM sodium sulfite by 59.3%. Preincubation of SoxF with 1 mM sodium sulfite for 20 min inactivated SoxF, and a residual sulfide dehydrogenase activity of 9.0% was determined (data not shown). Sulfite has been previously reported to be a potent modulator of FCSD of *A. vinosum* due to formation of a stable N-5 adduct of sulfite to flavin at pH 5 (*45*). In analogy, the inactivation suggested adduct formation of sulfite to SoxF. This property, however, could not be examined spectroscopically as SoxF precipitated at pH 5 (data not shown).

Potassium cyanide (1 mM) inhibited sulfide dehydrogenase activity of SoxF of *P. pantotrophus* by 25.2% when assayed at pH 6.0. However, at pH 8, the sulfide dehydrogenase activity of SoxF was inhibited by cyanide by 92%. At this pH, the net activity of SoxF ia ∼50% of that at pH 6.0 (Figure 5). FCSD of *C. limicola* is inhibited by 1 μM cyanide by 80% (*1*), and 0.67 mM cyanide inhibits FCSD of *C. vinosum* completely (*6*). The inhibition of SoxF of *P. pantotrophus* at high pH is in accordance with previous results of FCSD of *A. vinosum*, where the active form of cyanide is the anion (*17*). At pH 6, the absorption difference spectrum of SoxF of *P. pantotrophus* with 0.10 mM potassium cyanide minus air-oxidized SoxF did not indicate formation of an adduct of cyanide with SoxF (data not shown), and the spectral features differed from those obtained for FCSD of *Thiobacillus* W5 (*11*) and *A. vinosum* (*46*).

Oxidized minus Reduced FTIR Difference Spectra of SoxF. The monomeric structure of SoxF allowed the biophysical characterization of the protein without interference from another redox-active protein such as a cytochrome. The oxidized minus reduced FTIR difference spectra were examined of free FAD in solution (Figure 6A), of the SoxF protein during the first redox cycles (Figure 6B), and of the SoxF protein as found after several redox cycles (Figure 6C) for a potential step from −0.65 to −0.3 V versus Ag/AgCl (−0.442 to −0.092 V versus SHE′).

The redox-induced FTIR difference spectra of flavins are dominated by the strong positive signal of the ν(C═C) mode of the neutral flavin at 1548 cm^{-1} (*31*). The signals between 1714 and 1634 cm^{-1} are attributed to ν(C═O) modes, and the signals at 1604 and 1582 cm^{-1} may be assigned to the ν(C═N) modes. Ring modes of the isoalloxazine system contribute in the spectral range between 1460 and 1200 cm^{-1}. These assignments are consistent with resonance Raman data

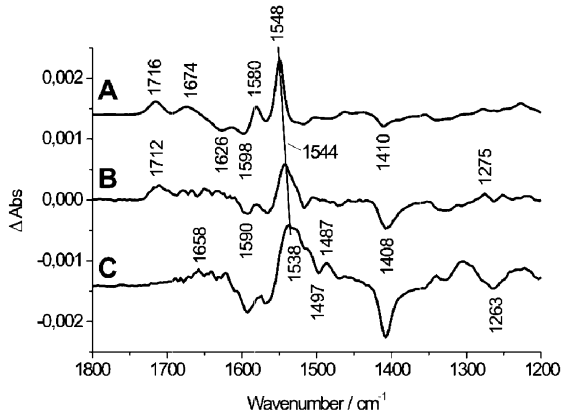

FIGURE 6: Oxidized minus reduced FTIR difference spectra of free FAD in solution. (A) The SoxF protein during the first redox cycles (B) and the SoxF protein as found after several redox cycles (C) for a potential step from −0.65 to −0.3 V versus Ag/AgCl (−0.442 to −0.092 V versus SHE′).

(*48*). As demonstrated recently, the spectra of FAD and FMN are very similar, revealing that the reorganizations of the flavin upon electron and proton transfer are essentially limited to the isoalloxazine ring and both flavin types are essentially indistinguishable in the mid-infrared region (*31*).

The redox behavior of flavins is highly complex (*49*). Monomeric and dimeric species are involved in the equilibrium reactions of the oxidized and reduced forms from FMN and FAD, $FMNH_2$ and $FADH_2$, and free radicals $FMNH^{\bullet}$ and $FADH^{\bullet}$, respectively. Therefore, the infrared spectra of the model compound could include contributions of semiquinoid and dimeric species (*50*). Spectral contributions of various flavin adducts are reflected in the variation of the amplitude of the difference signals at 1515 and 1530 cm^{-1} and between 1714 and 1408 cm^{-1} at high flavin concentrations.

Redox-induced FTIR difference spectra of SoxF were recorded for a potential step from −0.65 to −0.3 V (−0.442 to −0.092 V versus SHE′) (Figure 6B,C). Interestingly, the observed spectral features change during the initial redox cycles and are stable then. The difference spectrum obtained from the as prepared sample exhibits strong similarities to free flavin in solution (Figure 6B). The mode at 1712 cm^{-1} can be tentatively assigned to the ν(C═O) mode of the isoalloxazine ring. The ring mode at 1548 cm^{-1} is downshifted to 1544 cm^{-1}. The shoulder at 1538 cm^{-1} can be attributed to the Tris buffer. From the protein side, typically seen between 1710 and 1640 cm^{-1} (amide I range), only small contributions can be detected.

However, from the redox-induced FTIR difference spectrum observed after several redox cycles, the mode at 1712 cm^{-1} is missing, and the mode at 1544 cm^{-1} is broadened and downshifted to 1538−1520 cm^{-1}, indicating a strong change at the flavin cofactor. Clear modes in the so-called amide I region, where contributions from the ν(C═O) mode of the polypeptide backbone are present (Figure 6C), in addition to several strong signals appear below 1500 cm^{-1}.

Two possibilities may account for the redox-dependent behavior. (i) The flavin cofactor is not stable toward the applied potential, or (ii) the redox reaction induces a variation, not present in the as prepared form of the enzyme. Since the spectra strongly differ from those of free flavin and are fully reversible and stable and the redox potential is shifted in comparison to free flavin (see below), the first alternative seems to be less likely, inasmuch as the variation of the flavin during redox cycles being catalytically relevant has to be shown in the future.

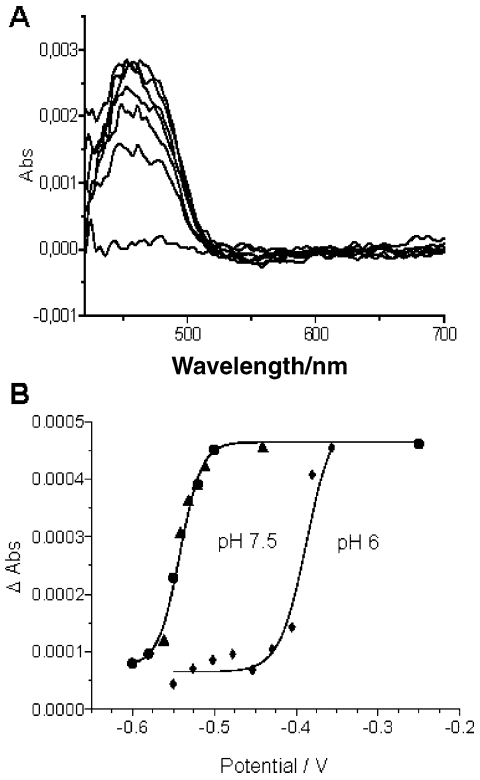

FIGURE 7: Redox-dependent behavior of SoxF as followed in the visible spectral range (A). The midpoint potentials were determined in the IR and visible spectral range for a pH of 7.5 (● and ▲) and a pH of 6 (♦) on the basis of a calculated Nernst fit (full line). Values of −555 ± 10 mV versus Ag/AgCl at pH 7.5 (−340 mV versus SHE′) and −410 ± 20 mV (−202 mV versus SHE′) at pH 6 with an *n* value of 1.9 for the transferred electrons were found.

Redox Titration of SoxF. The reduction of SoxF in the visible spectral range from 420 to 700 nm is shown in Figure 7A. Between 640 and 660 nm, no strong signal can be detected, indicating a full two-electron−two-proton reaction of the flavin under the given experimental conditions. The redox-dependent behavior of SoxF was monitored in the visible spectral range at 460 nm [Figure 7B (▲)] and at 1304 cm^{-1} in the infrared spectral range [Figure 7B (●)]. The calculated Nernst fit is shown as a solid line (Figure 7B). Three titrations in the infrared and in the visible spectra range, each, and the potential dependent development of the modes at 1304, 1408, 1488, 1506, and 1592 cm^{-1} have been evaluated and averaged, revealing a midpoint of −555 ± 10 mV versus Ag/AgCl at pH 7.5 and of −410 ± 20 mV versus Ag/AgCl at pH 6.0 (−202 mV versus SHE′) with an *n* value of 1.9 for the transferred electrons. Free flavin in solution has a midpoint potential of −427 mV versus Ag/AgCl at pH 7 (−219 mV versus SHE′).

We note that the small broad signal at 650 nm in Figure 3 and the emerald-green color observed for the oxidized enzyme indicate the presence of a small fragment of the blue semiquinone radical. Since it cannot be stabilized electrochemically for the chosen pH conditions and, however, the color changes with acidification, we suggest that the fraction

of radical is stabilized at lower pH values. The role of this radical form for the mechanism will be studied in the future.

CONCLUSIONS

This is the first report of a monomeric flavoprotein to perform hydrogen sulfide-dependent cytochrome *c* reduction. FCSDs of all other sulfur-oxidizing bacteria comprise a complex of a flavoprotein and a mono- or diheme *c*-type cytochrome. However, the catalytic characteristics of SoxF of *P. pantotrophus* were very similar to those of FCSD reported from another chemotrophic bacterium, *Thiobacillus* W5 (*11*), and were similar to those of FCSD of the purple sulfur bacterium *A. vinosum*, the best studied example of this family of proteins (reviewed in refs *8*, *10*, *11*, *19*, and *45*).

Analysis of the genomic data uncovered a close relationship of the flavoproteins. Therefore, the monomeric nature of SoxF *in vitro* may not reflect the actual situation *in vivo*, and SoxE may be the temporary partner of SoxF, although SoxE does not enhance the sulfide dehydrogenase activity of SoxF. This view considers the transfer of electrons from SoxF to horse heart cytochrome *c* not to be specific for the diheme cytochrome *c* SoxE, as horse heart cytochrome *c* is commonly used as final electron acceptor for all FCSDs. Moreover, in *Paracoccus*, the sulfur-oxidizing enzyme system is reconstituted of four proteins and the coordinated reaction of the enzyme system requires temporary and thus loose complexes with the different proteins that are involved (*3*, *23*).

Paracoccus strains oxidize hydrogen sulfide, thiosulfate, and sulfur (*47*), and *P. pantotrophus* harbors a periplasmic sulfur-oxidizing (Sox) enzyme system which *in vitro* performs hydrogen sulfide-dependent cytochrome *c* reduction at a high rate. The reconstituted system yields 4 mol of electrons per mole of hydrogen sulfide (*17*) as compared to two electrons of SoxF. In-frame deletion in *soxF* of *P. pantotrophus* does not eliminate growth with thiosulfate or sulfur oxidation of the respective homogenote strain, while inactivation of other *sox* genes does (*20*, *39*, *40*). Therefore, the function of SoxF *in vivo* is considered to be different from the sulfide dehydrogenase reaction observed *in vitro*. This conclusion is in accordance with the genetic evidence from disruption of *fccA* of *A. vinosum* (*19*). Since, however, the formation of SoxF is induced by thiosulfate and the *soxF* gene is located within the *sox* gene cluster of *P. pantotrophus*, we propose its close link to the sulfur-oxidizing enzyme system.

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