Characterization of the Selenium-Substituted 2[4Fe–4Se] Ferredoxin from *Clostridium pasteurianum*†

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ABSTRACT: The sulfur atoms of the two [4Fe–4S] clusters present in the ferredoxin from *Clostridium pasteurianum* have been replaced by selenium. The substitution is readily carried out by incubating the apoferredoxin with excess amounts of Fe^{3+}, selenite, and dithiothreitol under anaerobic conditions. The UV–visible absorption spectrum of the Se-substituted ferredoxin, the core extrusion of its active sites, and analyses of its iron and selenium contents show that it contains two [4Fe–4Se] clusters. The Se-substituted ferredoxin is considerably less resistant to oxygen or to acidic and alkaline pH than the native ferredoxin: the half-lives of the former are 20–500 times shorter than those of the latter. The native ferredoxin and the Se-substituted ferredoxin display similar kinetic properties when used as electron donors to the hydrogenase from *C. pasteurianum*. It is of note, however, that the K_m and V_{max} values are lower for the 2[4Fe–4Se] ferredoxin than for the 2[4Fe–4S] ferredoxin. Reductive and oxidative titrations with dithionite and with thionine, respectively, show that both ferredoxins are two-electron carriers. The redox potentials of the ferredoxins have been measured by equilibrating them with the H_2/H^+ couple via hydrogenase: values of −423 and −417 mV have been found for the 2[4Fe–4S] ferredoxin and 2[4Fe–4Se] ferredoxin, respectively. Ferredoxins containing both chalcogenides in their [4Fe–4X] (X = S, Se) clusters have been prepared by reconstitution reactions involving mixtures of sulfide and selenide: the latter experiments show that sulfide and selenide are equally reactive in the incorporation of [4Fe–4X] (X = S, Se) sites into ferredoxin. The present report, together with former studies, establishes the general feasibility of the Se/S substitution in [2Fe–2S] and in [4Fe–4S] clusters of proteins and of synthetic analogues.

The prosthetic groups of ferredoxins contain two to four iron and inorganic sulfur atoms organized into $[Fe_2S_2(S\text{–}Cys)_4]$ (Tsukihara et al., 1981), $[Fe_3S_3(S\text{–}Cys)_5(O\text{–})]$ (Ghosh et al., 1981), or $[Fe_4S_4(S\text{–}Cys)_4]$ (Sweeney & Rabinowitz, 1980) clusters. Whereas the [3Fe–3S] unit has only recently been discovered, and still has incompletely defined properties, the [2Fe–2S] and [4Fe–4S] cores have been extensively studied in proteins as well as in synthetic analogues, where the cysteine residues of the protein are replaced by low molecular weight thiols (Holm & Ibers, 1977).

A number of studies have shown that inorganic sulfur can be substituted by inorganic selenium in iron–sulfur clusters. While maintaining the overall structure and physicochemical properties of the clusters, selenium introduces a variety of modifications that yield valuable information on the molecular structure of iron–sulfur complexes (Reynolds & Holm, 1980, and references cited therein). In addition, the substitution of sulfur by selenium in proteins may contribute to the understanding of why selenium has been selected over sulfur in some enzymes (Stadtman, 1980). To date sulfide has been replaced by selenide in binuclear (Reynolds & Holm, 1980) and tetranuclear (Bobrik et al., 1978; Christou et al., 1978) synthetic iron–sulfur clusters. In proteins, [2Fe–2Se] active sites have first been assembled in putidaredoxin from the corresponding apoprotein with iron and selenium reagents (Tsibris et al., 1968) and later in parsley ferredoxin (Fee & Palmer, 1971) and in adrenodoxin (Mukai et al., 1973). Incompletely characterized iron–selenium structures have recently been incorporated into bovine serum albumin (Arakawa & Kimura, 1979). The replacement of sulfur by selenium has little effect on the biological activity (Tsibris et al., 1968; Orme-Johnson et al., 1968; Fee & Palmer, 1971) and on the redox potentials of the [2Fe–2S] proteins (Fee et al., 1971; Wilson et al., 1973; Mukai et al., 1974). The UV–visible spectra display bathochromic shifts of ca. 25 nm (Tsibris et al., 1968; Fee & Palmer, 1971; Tang et al., 1973; Mukai et al., 1974), and some bands of the Raman resonance spectra are shifted to lower frequencies (Tang et al., 1973) when selenium is substituted for sulfur. Alterations probably associated with changes in the symmetry of the active site appear in the Mössbauer (Münck et al., 1972), EPR[1] (Tsibris et al., 1968; Fee & Palmer, 1971; Mukai et al., 1973, 1974), and ENDOR (Bowman et al., 1973) spectra of Se-substituted ferredoxins. Such studies were not extended to proteins containing [4Fe–4S] active sites, and it is only very recently that sulfide has been replaced by selenide in the two [4Fe–4S] clusters of the ferredoxin from *Clostridium pasteurianum* (Meyer & Moulis, 1981). Here, we describe more comprehensively the preparation, stability, biological activity, and redox properties of the Se-substituted ferredoxin from *C. pasteurianum*.

Materials and Methods

Chemicals. Bathophenanthroline disulfonate, benzenethiol, thionine, and sodium selenite were purchased from Aldrich. Benzenethiol was distilled and stored under argon. Me_2SO was distilled on barium oxide under reduced pressure and stored under argon. $Na_2{}^{75}SeO_3$ was from the Radiochemical Center, Amersham, U.K. Elemental selenium (99%) was purchased from Prolabo, France. High-purity gases were from l'Air Liquide, Grenoble, France. Traces of oxygen in argon were removed by passage through a tower of BASF R3-11 catalyst heated to 120 °C.

Ferredoxin and Hydrogenase. *C. pasteurianum* W5 (ATCC 6013) cells were grown as described by Rabinowitz (1972). The cells (600 g wet weight) were suspended in 1200

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[1] Abbreviations: EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol; Fd, ferredoxin; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ox, oxidized; red, reduced.

0006-2960/82/0421-4762$01.25/0

mL of Tris-HCl, 0.1 M, pH 8.0, broken by sonication with a W185D sonifier (Heat Systems Ultrasonics, Inc., Plainview, NY), and centrifuged (20000*g*, 30 min). The supernatant was heated to 55 °C for 15 min and centrifuged (20000*g*, 30 min) to eliminate precipitated material. The supernatant was adjusted to pH 7.4 by adding solid Tris–base, diluted 2-fold with distilled water, and loaded on a 5 × 15 cm DE-52 (Whatman) column equilibrated with Tris-HCl, 0.02 M, pH 7.4, and NaCl, 0.1 M. The column was washed with 3 bed volumes of the same buffer, after which hydrogenase was eluted with 0.15 M NaCl. Ferredoxin was subsequently eluted with 0.35 M NaCl, diluted 3-fold with distilled water, and loaded on a second DE-52 column (3.5 × 10 cm) equilibrated with Tris-HCl, 0.02 M, pH 7.4, and NaCl, 0.1 M. The column was washed with NaCl, 0.2 M, in order to elute rubredoxin, which moved down as a reddish band in front of the dark brown ferredoxin band. Ferredoxin was subsequently eluted with 0.3 M NaCl, and the fractions having an A_{390}/A_{280} ratio higher than 0.80 were pooled and precipitated by adding solid ammonium sulfate to 90% saturation. The precipitate was sedimented (20000*g*, 20 min), redissolved in 10 mL of Tris-HCl, 0.05 M, pH 8.0, and loaded on a 3 × 80 cm column of Ultrogel AcA 202 (LKB) equilibrated with the same buffer. After this step the yield was 30 mg of ferredoxin with an A_{390}/A_{280} ratio of 0.83, 50 mg with an A_{390}/A_{280} ratio of 0.82, and 20 mg with an A_{390}/A_{280} ratio of 0.81. All steps, including cell disruption, were carried out in the absence of oxygen, all flasks, buffers, and columns being flushed with argon.

The hydrogenase from the first DE-52 column was further purified as described by Chen & Mortenson (1974), except that the hydroxylapatite chromatography was omitted. The purified proteins were stored in liquid nitrogen.

Apoferredoxin. Apoferredoxin was prepared similarly to procedure II of Rabinowitz (1972). The ferredoxin solution (20–30 mg in 15–20 mL of Tris-HCl, 0.05 M, pH 8.0) was made 8% in trichloroacetic acid, stirred 30 min at 0 °C under a flow of argon, and centrifuged (20000*g*, 10 min). The precipitate was dissolved in 10 mL of Tris-HCl, 0.1 M, pH 8.0, and the whole procedure was repeated. The pellet of the second centrifugation was taken up in 3 mL of Tris-HCl, 0.5 M, pH 8.5, and passed over a Sephadex G-25 column (2 × 50 cm) equilibrated with Tris-HCl, 0.02 M, pH 8.5. After this step the apoprotein was usually colorless and showed no absorbance at 390 nm. Occasionally the apoprotein retained a pale yellow color: In this case the precipitations and chromatography were repeated. The apoferredoxin was obtained with an 80–100% yield and contained less than 0.5% of its initial iron and sulfur content (Meyer & Moulis, 1981).

Hydrogenase Assay. Hydrogenase activity was assayed at 30 °C in 8-mL flasks stoppered with rubber septa and flushed with argon. The assay mixture contained 100 mM Tris-HCl, 15 mM dithionite, hydrogenase, and ferredoxin in a final volume of 1 mL. The pH values and the amounts of ferredoxin and hydrogenase are to be found in the legend of Figure 5 and Table III. The reaction was started by the injection of hydrogenase and stopped after 8 min by injecting 0.1 mL of 50% trichloroacetic acid. Hydrogen was assayed in the gas phase as described by Meyer (1981).

Redox Titrations of Ferredoxin with Dithionite and Thionine. Anaerobic titrations were performed under argon in 2-mm path length round-necked quartz cells fitted with rubber septa. After the absorbance of the ferredoxin solution (in Tris-HCl, 0.05 M, pH 8.0) was read at 425 nm, aliquots of a standardized dithionite solution in Tris-HCl, 0.05 M, pH 8.0 [ϵ_M = 8000 M^{-1} cm^{-1} at 315 nm (Dixon, 1971)], were added, and the decreasing values of A_{425} were read after equilibrium had been reached. When the ferredoxin was completely reduced, it was reoxidized stepwise by adding aliquots of a thionine solution (in Tris-HCl, 0.05 M, pH 8.0) that had previously been calibrated with a standard dithionite solution. After correction for dilution, the absorbance at 425 nm was plotted as a function of the amount of added reductant or oxidant. Additional corrections were made for the excess of oxidized thionine present at the end of the titrations (ϵ_{425} = 2000 M^{-1} cm^{-1} was calculated from the UV–visible spectrum of oxidized thionine). These experiments allowed the determination of the number of electrons accepted or donated by the ferredoxins, as well as the absorbance ratios of fully reduced to fully oxidized ferredoxins. The latter ratios were used for the redox potential determinations (see below).

Redox Potential Measurements. The redox potentials of both ferredoxins (S and Se) were measured by allowing them to equilibrate with the H_2/H^+ couple at 25 °C in the presence of hydrogenase (Tagawa & Arnon, 1968). The hydrogen pressure was maintained constant by equilibration with atmospheric pressure through a water bubbler, and the redox states of the ferredoxins were measured spectrophotometrically as a function of pH (Lode et al., 1976b). Pools of either ferredoxin were divided into the number of measurements to be made. These fractions (ca. 0.5 mg each) were filtered through Sephadex G-10 columns (25 × 1 cm) equilibrated with argon-flushed Tris–Mes (0.025 M of each) buffers adjusted at the required pH. The pH ranges were 5.5–8.5 and 6.5–8.5 for the native ferredoxin and the selenium-substituted ferredoxin, respectively. After gel filtration, 0.4-mL samples (ca. 0.25 mg) of ferredoxin were injected into 2-mm path length cells stoppered with rubber septa and flushed with hydrogen. Hydrogen was passed through the ferredoxin solutions for a few minutes, whereafter a UV–visible (250–450 nm) spectrum was recorded to assess the quality of the sample (those with an A_{390}/A_{280} ratio of less than 0.81 were rejected) and to measure A_{425}. Hydrogenase (0.5 μL, 2.4 μg) was then injected, and the mixture was shaken for a few seconds and allowed to equilibrate. When the absorbance at 425 nm was stable (after 5–10 min), another spectrum was recorded. The ratio of reduced to oxidized ferredoxin was calculated from the absorbance data (Stombaugh et al., 1976). The partial pressure of hydrogen was equal to the atmospheric pressure corrected for the water pressure in the bubbler (29 mmH_2O = 2.13 torr) and for the water vapor pressure (23.76 torr at 25 °C). These data were used in the Nernst equation for the hydrogen and ferredoxin couples in equilibrium (Stombaugh et al., 1976), which allowed the calculation of the redox potential at each pH value. It should be kept in mind that the Nernst equation as written by Stombaugh et al. (1976) only holds if the two redox sites of the ferredoxin are reduced independently at similar potentials. It will be shown below (Figure 9) that the latter requirement is met by both the native and the Se-substituted ferredoxins.

Iron Assay. The protein samples (containing 50–150 nmol of iron) were mixed with 0.3 volume of HCl, 12 N, in Eppendorff plastic centrifuge tubes (1.5 mL). The tubes were stoppered and heated to 100 °C for 15 min. Distilled water (0.4 mL) was then added to each tube. The precipitated material was removed by centrifuging 4 min in an Eppendorff 3200 microcentrifuge. Aliquots from the supernatants (0.05–0.2 mL) were transferred to disposable plastic tubes and diluted to 1.5 mL with Tris-HCl, 0.5 M, pH 8.5. Sodium ascorbate (0.1 mL, 5% in water) and 0.4 mL of bathophenanthrolinedisulfonate (0.1% in water) were subsequently

added, and the absorbance at 535 nm was measured after 1 h against a blank containing the buffer and the reagents. The molar extinction coefficient of the iron–bathophenanthroline disulfonate complex was taken as 22 140 M^{-1} cm^{-1} (Blair & Diehl, 1961). Calibrations with standard $FeSO_4$ solutions confirmed this value. It was essential to carry out the assay at neutral pH, as the formation of the iron complex is not complete below pH 3 (Blair & Diehl, 1961).

Amino Acid Analyses. Ferredoxin samples (ca. 100 nmol in 0.4 mL) were passed through Sephadex G-25 columns (1.5 × 20 cm) equilibrated with NH_4HCO_3, 60 mM, pH 8.0, and their UV–visible spectra were recorded to determine the A_{390}/A_{280} ratios. The samples were subsequently split into fractions of ca. 20 nmol, dried in vacuo, hydrolyzed with HCl, 6 N, in sealed tubes (48 h at 110 °C), dried again, and kept in a desiccator. The amino acid analyses were carried out at the EMBL, Heidelberg Federal Republic of Germany, on a Durrum D500 analyzer. The amounts of protein were calculated from the analyses of alanine, valine, isoleucine, and glutamic acid by using the known amino acid composition (Rabinowitz, 1972).

Other Assays. Proteins were assayed with the Folin reagent (Lowry et al., 1951). Sulfide was determined as described by Chen & Mortenson (1977). For the determination of selenium, a known amount of $Na_2{}^{75}SeO_3$ was added to the Na_2SeO_3 solution used for the reconstitution of ferredoxin. ^{75}Se was measured on a Kontron MR480 γ counter. UV–visible spectra were recorded with a Cary 219 or with a Perkin-Elmer 557 spectrophotometer.

Results

Incorporation of Fe–Se Sites into Apoprotein. A standard procedure has been developed, which is similar to the one previously described (Meyer & Moulis, 1981). All operations were carried out under strictly anaerobic conditions. The apoprotein (1–2 mg/mL in 0.1 M Tris-HCl, pH 7.7) was preincubated for 30 min at room temperature with a 50-fold molar excess of dithiothreitol (DTT). Fe^{3+} (as an aqueous solution of $FeCl_3 \cdot 6H_2O$) was then injected, immediately followed by the addition of Se^{2-}, both in 32-fold molar excess over the apoprotein. Se^{2-} was prepared just before use by reducing SeO_3^{2-} with DTT: a 10-fold excess of DTT in aqueous solution (0.5 M) was added to solid Na_2SeO_3 (or to a solution when $Na_2{}^{75}SeO_3$ was used) and the mixture was allowed to react for 5 min. Upon addition of Se^{2-} to the apoprotein and Fe^{3+}, the reaction mixture turned dark brown, and a fine black precipitate appeared. After 30 min at room temperature, the turbid solution was loaded on a small DE-52 column (5 mL for 10–20 mg of protein) equilibrated with Tris-HCl, 0.02 M, pH 7.4, and NaCl, 0.1 M. The column was washed with 50–100 mL of the same buffer, which removed most of the free iron, selenide, and DTT. The black precipitate remained on top of the column and did not interfere with the subsequent elution of the ferredoxin, which was carried out with 0.4 M NaCl. The remaining free iron and selenide were then separated from the protein by gel filtration: the protein fraction from the DE-52 column (5–6 mL) was passed through a 2 × 50 cm column of Sephadex G-25 equilibrated with Tris-HCl, 50 mM, pH 8.0. The procedure for the reconstitution of [4Fe–4S] sites was identical, except for the replacement of Se^{2-} by S^{2-}, added as a solution of $Na_2S \cdot 9H_2O$, and for the use of apoprotein solutions buffered at pH 8.5 (Rabinowitz, 1972). The yields of the reconstitutions were calculated as follows: the amounts of iron or chalcogenide found in the reconstituted ferredoxins were divided by 8 and by the amount of apoferredoxin used as starting material. This is justified by the fact that the reconstitution procedure used here eliminates any iron or chalcogenide not belonging to the active sites (Meyer & Moulis, 1981; see also the extrusion experiments in Table II). The yields of the reconstitutions were in the 50–70% range, with no significant difference when Se^{2-} was used instead of S^{2-}.

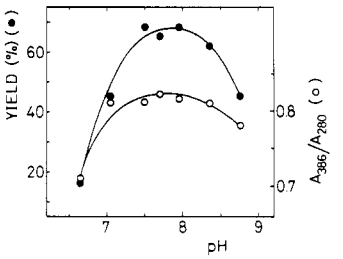

FIGURE 1: Effect of pH on the reconstitution of the 2[4Fe–4Se] ferredoxin. Aliquots of apoferredoxin (0.75 mg in 1.5 mL of 0.1 M Tris–0.1 M Mes) were incubated at the indicated pH values and reconstituted as described in the text with Fe^{3+} and Se^{2-} in 32-fold excess over the apoprotein. The ferredoxins were subsequently separated from the reaction mixtures by chromatography on 1-mL DE-52 columns (see the text), their UV–visible spectra were recorded, and they were assayed for iron and selenium. The yields (●) are the mole ratios of the 2[4Fe–4Se] ferredoxin obtained to the apoferredoxin used as the starting material. A_{386}/A_{280} ratios (○) were calculated from the UV–visible spectra.

Optimal Conditions for Preparation of Se-Substituted Ferredoxin. Urea as a chaotropic agent is generally used to facilitate the reconstitution of ferredoxins from the corresponding apoproteins (Rabinowitz, 1972). We have observed no favorable effect of urea either on the yields of the reconstitutions or on the A_{390}/A_{280} ratio of the reconstituted ferredoxins. Urea has subsequently been omitted from the reaction mixtures.

Sodium selenite, which was used as a source of selenium, had to be reduced to selenide in order to be reactive in the reconstitution experiments. β-Mercaptoethanol was found to be about 10 times less efficient than dithiothreitol as a reducing agent. Sodium borohydride reduces selenite, but in its presence the reconstitution yields were lower, probably due to side reactions of borohydride with the protein. We have tried to generate selenide from selenite in situ (Fee & Palmer, 1971), with apoferredoxin:DTT:Fe^{3+}:SeO_3^{2-} molar ratios of 1:250:24:24, but the reconstitution yields were 15–30% lower under these conditions than when selenite was reduced before being added to the reconstitution reaction mixture. The latter conditions have subsequently been used in the standard technique.

Elemental selenium (as a grey powder) can be reduced to selenide by borohydride (Klayman & Griffin, 1973), but the latter reductant is not convenient for protein reconstitution reactions (see above). As elemental selenium was not significantly reduced by DTT, we did not consider it further for the generation of selenide in ferredoxin reconstitution experiments.

The reconstitution reaction did not depend on the redox state of the iron (ferrous or ferric) nor on the sequence of iron and selenide additions to the reaction medium.

The effect of pH on the Fe,Se reconstitution is shown in Figure 1. The best yields and highest A_{386}/A_{280} ratios were obtained between pH 7.5 and pH 8.2. At higher or lower pH values the yields decreased and the formation of heavier black precipitates was observed.

The effect of a varied excess of iron and selenium over apoprotein in the reconstitution reactions is shown in Figure 2. The molar ratio of iron to selenium was kept equal to 1.

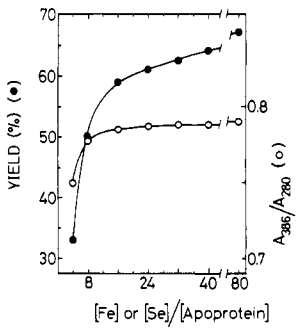

FIGURE 2: Reconstitution of 2[4Fe–4Se] ferredoxin with increasing amounts of iron and selenide. Aliquots of apoferredoxin (0.7 mg in 0.6 mL of Tris, 0.1 M, pH 7.4) were incubated and reconstituted as described in the text with the indicated amounts of Fe^{3+} and Se^{2-}. The reconstitution products were separated from the reaction mixtures by chromatography on 1-mL DE-52 columns, their UV–visible spectra were recorded, and they were assayed for iron and selenium. (●) Reconstitution yields; (○) A_{386}/A_{280} ratios.

The reconstitution yields were optimal from a 2-fold excess of iron and selenium (16 atoms of Fe or Se per mol of apoprotein) on up to much higher values (10-fold excess). With iron and sulfide, the reconstitutions work best with 2–4-fold excesses of metal and chalcogenide (Hong & Rabinowitz, 1970a), but in the presence of higher excesses the yield decreases significantly (our unpublished observations). The latter difference between sulfur and selenium is probably due to differences in the solubility of intermediary products of the reconstitution reactions.

After the reconstitution reaction, the DE-52 column removed most of the unreacted material. However, the filtration on Sephadex G-25 is a necessary step, as it increases significantly the A_{386}/A_{280} ratio of the reconstituted protein (from 0.77–0.80 to 0.81–0.83). Moreover, it removes small amounts of iron still bound to the protein: after the G-25 filtration, iron and selenium are always found in equal amounts in the reconstituted ferredoxin (Meyer & Moulis, 1981), whereas before that step iron is in excess (5–10%) over selenium.

It has previously been shown that the apoferredoxin can be separated from the 2[4Fe–4S] ferredoxin by chromatography on a DE-52 column with a linear gradient (0.15–0.5 M) of NaCl (Hong & Rabinowitz, 1970a). We have applied the latter procedure to the reconstituted 2[4Fe–4Se] ferredoxin, but the protein thus obtained did not show a higher A_{386}/A_{280} ratio than the protein obtained by our above described technique.

Characterization of Active Sites of Iron–Selenium Ferredoxin. The electronic absorption spectrum of the selenium substituted ferredoxin from *C. pasteurianum* has been shown elsewhere (Meyer & Moulis, 1981; see also Figure 7). It displays the two broad absorption bands at ca. 300 and 400 nm, which are a property common to the $[4Fe–4S]^{2+}$ cores of proteins (Sweeney & Rabinowitz, 1980) and of synthetic analogues in aqueous solutions (Hill et al., 1977).

The spectral features of the native and of the Se-substituted ferredoxins are displayed in Table I, together with those of water-soluble iron–sulfur and iron–selenium cubane clusters. The spectra of the ferredoxins are very similar to those of the corresponding analogues. Moreover, the substitution of sulfur by selenium results in band shifts that are the same in the ferredoxins and in the synthetic analogues. These data strongly suggest that the Se-substituted ferredoxin contains [4Fe–4Se] clusters and further evidence can be adduced by core extrusion of the active sites with benzenethiol (Que et al., 1975). The results of such experiments performed on the native and on the Se-substituted ferredoxins are displayed in Table II. The

Table I: Electronic Spectral Features of 2[4Fe–4S] and 2[4Fe–4Se] Ferredoxins and of Cubane Iron–Sulfur and Iron–Selenium Clusters in Aqueous Solutions[a]

	λ_{max} (nm)[b]	λ_{min} (nm)
2[4Fe–4S] Fd	300 (37 000), 388 (30 000)	354
2[4Fe–4Se] Fd	305 (42 500), 386 (32 000)	368
$[Fe_4S_4(SR)_4]^{2-}$	300 (21 600), 375 (16 600)	355
$[Fe_4Se_4(SR)_4]^{2-}$	306 (23 000), 373 (16 700)	366

[a] RSH = 3-mercaptopropionamide was synthesized as described by Miller et al. (1971). $(Et_4N)_2[Fe_4S_4(SR)_4]$ was synthesized as described by Christou & Garner (1979) for $(Me_4N)_2[Fe_4S_4(SR')_4]$, with R'SH = 2-mercaptoethanol. The same procedure was used for the synthesis of $(Et_4N)_2[Fe_4Se_4(SR)_4]$, using elemental selenium instead of elemental sulfur. The iron–sulfur and iron–selenium clusters were dissolved in oxygen-free aqueous solutions containing free thiol (40 mM, pH 8.9) in ca. 100-fold excess over the clusters. Under these conditions the spectra were stable for several hours at least. [b] The extinction coefficients (M^{-1} cm^{-1}) are in parentheses.

Table II: Active Site Core Extrusions of Native and Se-Substituted Ferredoxins[a]

protein	protein concn (μM)[b]	[PhSH]/[Fe]	product $Fe_4X_4(SPh)_4^{2-}$ (X = S, Se) λ_{max} (nm)	μM[c]	$[Fe_4X_4(SPh)_4^{2-}]$/[protein]
2[4Fe–4S] Fd	9.6	1010	454	19.0	1.98
2[4Fe–4Se] Fd	18.3	532	466	37.7	2.06

[a] 0.1 mL of ferredoxin solution (in Tris-HCl, 0.05 M, pH 8.5) was injected into a 2-mm path length quartz cell fitted with a rubber septum and flushed with argon. 0.4 mL of degassed Me_2SO was then added and mixed with the aqueous solution of ferredoxin, followed by the indicated amount of benzenethiol. The UV-visible spectrum of the solution was recorded after stabilization of the absorbance at 460 nm (ca. 10 min after the injection of benzenethiol). [b] Calculated by taking $\epsilon_{390} = 30\,000$ M^{-1} cm^{-1} for the 2[4Fe–4S] Fd and $\epsilon_{386} = 32\,000$ M^{-1} cm^{-1} for the 2[4Fe–4Se] Fd (Meyer & Moulis, 1981). [c] Calculated with $\epsilon_{455} = 17\,200$ M^{-1} cm^{-1} for $[Fe_4S_4(SPh)_4]^{2-}$ (Gillum et al., 1977) and $\epsilon_{466} = 18\,100$ M^{-1} cm^{-1} for $[Fe_4Se_4(SPh)_4]^{2-}$ (Bobrik et al., 1978).

absorption maxima (454 and 466 nm for the native ferredoxin and the Se-substituted ferredoxin, respectively) as well as the shape of the spectra (not shown) of the extrusion products are identical with those of the synthetic $Fe_4X_4(SPh)_4^{2-}$ (X = S, Se) clusters in Me_2SO solutions (Bobrik et al., 1978). The concentrations of the extruded clusters were calculated by using the extinction coefficients given in the literature (Gillum et al., 1977; Bobrik et al., 1978) and were divided by the protein concentrations: in both cases two $[Fe_4X_4(SPh)_4]^{2-}$ clusters were obtained per protein molecule (Table II). So far our data show that the Se-substituted ferredoxin contains [4Fe–4Se] active sites, but they do not demonstrate that two such sites are present in each protein molecule: the stoichiometry obtained from the extrusion experiments is based on the assumption that the protein contains eight atoms of Fe and eight atoms of Se. If it contained only four atoms of each element, the extinction coefficient based on elemental analyses would be 16 000 at 386 nm, and the number of [4Fe–4Se] clusters obtained from the extrusion experiments would obviously be one per molecule of protein. However, we have several lines of evidence to show that the Se-substituted ferredoxin actually contains two [4Fe–4Se] clusters. First, the reconstitution yields based on active site recovery are similar for Fe,S and Fe,Se reconstitutions (average 60%, see above), and it has previously been shown (Hong & Rabinowitz, 1970a)

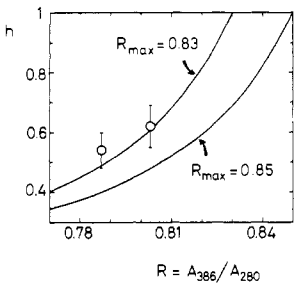

FIGURE 3: Calculated curves of h as a function of R. h is the mole fraction of 2[4Fe–4Se] holoferredoxin in a mixture of holo- and apoferredoxin. h is related to $R = A_{386}/A_{280}$ by eq 2 (see the text). R_{max} is the value of R for pure holoferredoxin. The R and h values of the experimental points (O) were calculated from spectrophotometric measurements and amino acid analyses (see the text).

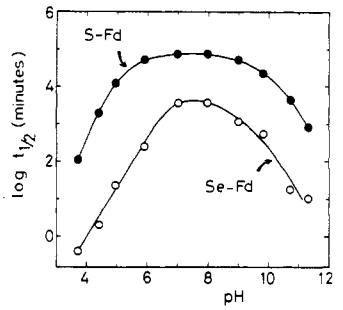

FIGURE 4: Stabilities of the native and Se-substituted ferredoxins as a function of pH. Ferredoxin solutions (50 μM) were incubated at 20° C under an atmosphere of argon at the indicated pH. The decrease of A_{390} was measured as a function of time in 2-mm path length cuvettes. Half-lives were determined from plots of log A_{390} as a function of time. The buffers used were a mixture of acetate, Mes, Mops, Tris, and borate, 0.1 M each, from pH 5 to pH 10, acetate, 0.5 M, below pH 5, and phosphate, 0.5 M, above pH 10. (●) Native ferredoxin; (O) Se-substituted ferredoxin.

that the Fe,S reconstitution is an all or none process, yielding only 2[4Fe–4S] ferredoxin. One may add that if only one [4Fe–4Se] cluster were present in the Se-substituted ferredoxin, the maximum theoretical yield of the reconstitution would only be 50% and probably only 30–40% in practice if one takes into account the two purification steps following the reconstitution reaction. Second, the Se-ferredoxin was assayed with the Folin reagent (Lowry et al., 1951). As this assay overestimates the ferredoxin concentration by a factor of 1.8 (Rabinowitz, 1972), the native ferredoxin was used as standard. For the best preparations of Se-ferredoxin (A_{386}/A_{280} = 0.82–0.83), seven to eight atoms of iron and selenium per protein molecule were found when the Fe and Se concentrations were divided by the protein concentration. Third, the amount of protein present in Se-ferredoxin preparations was measured by amino acid analyses. The samples had to be desalted on a G-25 column equilibrated with ammonium carbonate, which resulted in a significant loss of chromophore (decrease in the A_{386}/A_{280} ratio). However, the results could be extrapolated to samples of better quality in the following way: Taking R_{max} as the A_{386}/A_{280} ratio of a solution of 2[4Fe–4Se] ferredoxin containing no apoferredoxin, an extinction coefficient of 32 000 M^{-1} cm^{-1} at 386 nm for such a ferredoxin (Meyer & Moulis, 1981), and an extinction coefficient of 2000 M^{-1} cm^{-1} at 280 nm for the apoprotein (Bayer et al., 1969; our unpublished results), the $R = A_{386}/A_{280}$ ratio for any mixture of holoferredoxin (containing two [4Fe–4Se] cores) and apoferredoxin can be written

$$R = \frac{32000h}{2000a + 32000h/R_{max}} \quad (1)$$

where a and h are the mole fractions of apoferredoxin and holoferredoxin, respectively. Since $a + h = 1$, eq 1 can be written

$$h = \frac{1}{1 + 16(1/R - 1/R_{max})} \quad (2)$$

Thus, if R_{max} is known, the proportion of apoferredoxin contained in a given preparation can easily be calculated from the measured R ratio. Our best preparations of Se-substituted ferredoxin had R ratios of 0.83. If we take this as the R_{max} value, we obtain a theoretical $h = f(R)$ curve, which is shown in Figure 3 (upper curve). On the other hand, the experimental values obtained from the amino acid analyses and spectrophotometric measurements are $h = 0.54 \pm 0.06$ for $R = 0.787$ and $h = 0.62 \pm 0.07$ for $R = 0.803$. These points fall, within experimental error, on the curve corresponding to $R_{max} = 0.83$. We have also drawn the curve for $R_{max} = 0.85$, but it falls well below the experimental data. These calculations, together with the experimental data, show that a Se-substituted ferredoxin preparation with an A_{386}/A_{280} ratio of 0.82–0.83 contains less than 20% of apoprotein and that each protein molecule contains two [4Fe–4Se] clusters. The curves of Figure 3 deserve a more general comment, as they may be used for the native ferredoxin as well, with only minor changes: eq 2 becomes

$$h = \frac{1}{1 + 15(1/R - 1/R_{max})} \quad (3)$$

(taking ϵ_M = 30 000 M^{-1} cm^{-1} at 388 nm for the native ferredoxin). It is worth noticing that the $h = f(R)$ curves are very steep at the higher values of R; thus small decreases of R correspond to large decreases of h. For example, with R_{max} = 0.83, a solution of native or Se-substituted ferredoxin having an A_{390}/A_{280} ratio of 0.80 will contain ca. 40% of apoprotein (Figure 3). It therefore appears that care should be taken when using the A_{390}/A_{280} ratio as a criterion of purity or of active site integrity.

Stability of Active Sites of Se-Substituted Ferredoxin. The pH dependence of the stability of the [4Fe–4S] and [4Fe–4Se] active sites is shown in Figure 4. The kinetics of the decrease in absorbance at 390 nm were measured at 20 °C in the absence of oxygen and were used to calculate the half-lives of the iron–chalcogenide clusters. As previously observed (Maskiewicz & Bruice, 1977), the native ferredoxin is stable over a wide range of pH values, with a half-life of 1300 h at pH 7.0. The Se-substituted ferredoxin is less stable than the native ferredoxin (its half-life is 60 h at pH 7.0) and more so at extreme pH values: the half-lives of the two ferredoxins differ by a factor of 20 at neutral pH and by a factor of more than 200 below pH 6 or above pH 9 (Figure 4).

The inactivation of the native and of the Se-ferredoxin by air has been quantitated at pH 8 and at 20 °C by two methods: First, by measuring the decrease in absorbance at 390 nm we found half-lives of 160 and 9.5 h for the native and for the Se-substituted ferredoxin, respectively. Second, by measuring the decreases in ferredoxin activity in the hydrogenase-catalyzed hydrogen evolution, we have found half-lives of 200 and 4.5 h, respectively. Thus, if the ferredoxins are kept in the presence of air instead of oxygen-free atmospheres, their half-lives are reduced 6–8-fold for the native ferredoxin and 6–20-fold for the Se-substituted ferredoxin.

The effect of temperature was not investigated systematically. However, we found both ferredoxins to be more stable at 0–4 °C than at room temperature, as previously observed

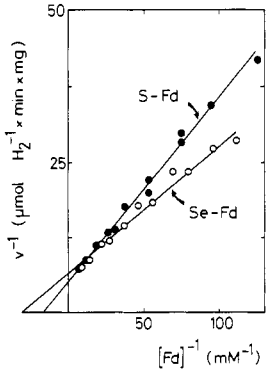

FIGURE 5: Double-reciprocal plots of hydrogenase activity vs. ferredoxin concentration. Hydrogenase activity was measured as described under Materials and Methods. Each vial contained 4.7 μg of hydrogenase. The pH was 8.0. The straight lines were obtained from the data points by least-squares analysis. (●) Native ferredoxin; (○) Se-substituted ferredoxin.

Table III: Kinetic Constants of 2[4Fe-4S] and 2[4Fe-4Se] Ferredoxins as Electron Donors to *C. pasteurianum* Hydrogenase[a]

	2[4Fe-4S] Fd		2[4Fe-4Se] Fd	
pH	V_{max}[b]	K_m[c]	V_{max}[b]	K_m[c]
8.5	120	62	104	43
8.0	202	62	162	35
7.4	210	26	160	18
7.0	186	15	196	10

[a] Experimental conditions were as described under Materials and Methods and in the legend of Figure 5. The V_{max} and K_m values were determined by least-squares analysis of at least six data points at each pH value. [b] In μmol of H_2 min^{-1} (mg of H_2ase)$^{-1}$. [c] In μM.

for other ferredoxins (Lode et al., 1976a; Maskiewicz & Bruice, 1977).

Kinetic Properties of the Se-Substituted Ferredoxin as an Electron Donor to Hydrogenase. The major physiological role of ferredoxin in *C. pasteurianum* is the transfer of electrons from the phosphoroclastic enzyme system to hydrogenase and nitrogenase. In vitro, the enzymatic activity of ferredoxin is most conveniently assayed by measuring its ability to transfer electrons from dithionite to hydrogenase (Adams et al., 1981). We have used the latter reaction to compare the biological activities of the native and the Se-substituted ferredoxin. Hydrogenase activities as a function of ferredoxin concentrations are shown in Figure 5 as double-reciprocal plots. For both ferredoxins, straight lines are obtained, which intersect at a point corresponding to a concentration of 70 μM. The K_m for the native ferredoxin is 62 μM, in good agreement with the previously published value of 51 μM (Chen & Mortenson, 1974). The K_m and V_{max} values obtained at four different pH values are given in Table III. In most cases (except at the lowest pH value), the maximal velocity in the presence of native ferredoxin is about 20% higher than in the presence of Se-substituted ferredoxin. On the other hand, the K_m for the Se-ferredoxin is 30–50% lower than for the native ferredoxin. For both ferredoxins the maximal velocities tend to increase and the Michaelis constants decrease when the pH is shifted toward more acidic values (Table III). As the native and Se-substituted ferredoxins have similar properties as electron donors to hydrogenase, we have also observed that they are both reduced by hydrogenase in the presence of molecular hydrogen. The latter reaction has been used below to measure their redox potentials.

Redox Properties of the Se-Substituted Ferredoxin. Reductive (with dithionite) and oxidative (with thionine) titration

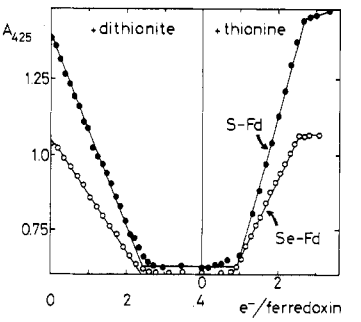

FIGURE 6: Reductive and oxidative titrations of the native and the Se-substituted ferredoxins. The titrations were carried out as described under Materials and Methods. Proteins, dithionite, and thionine were in Tris-HCl, 0.05 M, pH 8.0. The initial concentrations of the proteins were 270 μM for the native ferredoxin (●) and 175 μM for the Se-substituted ferredoxin (○). Dithionite, 0.9 mM, was used for the reductive titrations (left part of the figure) and thionine, 0.67 mM, for the oxidative titrations (right part of the figure). The absorbance at 425 nm was corrected for dilution and for excess thionine present at the end of the reoxidation (see Materials and Methods).

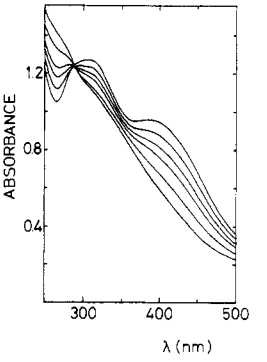

FIGURE 7: Stepwise reduction of the 2[4Fe-4Se] ferredoxin with dithionite. The oxidized 2[4Fe-4Se] ferredoxin (52 nmol in 0.4 mL of Tris-HCl, 0.05 M, pH 8) was injected into a 2-mm path length quartz cell flushed with argon and fitted with a rubber septum. The spectrum was recorded (upper trace), and the ferredoxin was titrated with dithionite, 10 mM, in 0.05 M Tris-HCl, pH 8. The number of electron equivalents added per mole of ferredoxin was 0, 0.38, 0.74, 1.03, 1.56, and 2.0, respectively, for the spectra taken in the order of decreasing A_{425}. The last spectrum (lowest trace) was not modified above 380 nm when more dithionite was added.

curves of the native and the Se-substituted ferredoxins are shown in Figure 6. The number of electrons transferred per molecule of protein is the same for both ferredoxins: 2.3 during the reductive step and 1.75 during the oxidative step. Values of 1.9–2.3 have previously been obtained from reductive and oxidative titrations of the native ferredoxin from *C. pasteurianum* (Sobel & Lovenberg, 1966; Mayhew et al., 1969). The involvement of less electrons for the oxidation than for the reduction may be due to a systematic error or to a partial loss of the active site. The latter explanation is less likely, as one would then expect to have a larger difference for the Se-ferredoxin, which is much less stable than the native ferredoxin (see above). In addition, reduction of the Se-ferredoxin with dithionite seems to take place without denaturation of the active sites: the spectra in Figure 7 clearly show an isosbestic point at 288 nm, which is an indication that the reduction is proceeding cleanly, with no irreversible damage to the chromophore. We cannot, however, rule out the possibility of a partial inactivation of the ferredoxins during the reoxidation by thionine: This would at least in part account for the lower number of electrons measured in the oxidative titration. Nevertheless, the number of electrons involved in the reductive and oxidative titrations (Figure 6) differ from two by no more than 15%, and one is thus justified to conclude that the Se-

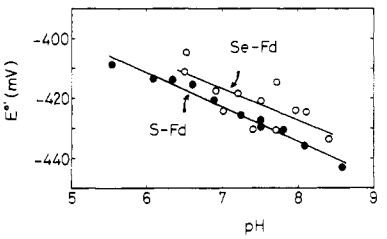

FIGURE 8: pH dependence of the apparent redox potentials of the native and the Se-substituted ferredoxins. The measurements were carried out as described under Materials and Methods, at 25 °C, in 0.025 M Mes–0.025 M Tris buffer. The straight lines are least-squares fits of the experimental data for the 2[4Fe–4S] (●) and the 2[4Fe–4Se] (○) ferredoxins.

ferredoxin, like the native ferredoxin, is a two-electron carrier. The redox titrations (Figure 7) have also been used to calculate the absorbance ratios of the fully reduced to the fully oxidized ferredoxins at 425 nm. These ratios did not depend on the procedure used for the reduction (small successive additions of dithionite or larger amounts added in one time as a solid or as a concentrated solution). We found $(A_{red}/A_{ox})_{425}$ values of 0.445 for the native ferredoxin and 0.562 for the Se-substituted ferredoxin. Values of 0.454, 0.45, and 0.435 have previously been reported for the native ferredoxin (Eisenstein & Wang, 1969; Stombaugh et al., 1976; Lode et al., 1976b).

The reduced to oxidized absorbance ratios were used to determine the redox potentials of the ferredoxins after equilibration with the H^+/H_2 couple via hydrogenase (see Materials and Methods). The redox potentials of both ferredoxins have been calculated from the Nernst equation (Stombaugh et al., 1976) and plotted as a function of pH (Figure 8). The experimental points corresponding to the Se-ferredoxin are much more scattered than those corresponding to the native ferredoxin. We have no straightforward explanation for this discrepancy, as both series of measurements were carried out under identical conditions. For the Se-ferredoxin, $E^{0'}$ measurements could not safely be carried out below pH 6.5, due to the instability of the protein at acidic pH (Figure 4). Straight lines were derived from the experimental data by least-squares analysis (Figure 8), and the following redox potentials were obtained, at pH 7.0: −423 ± 1.5 mV for the native ferredoxin and −417 ± 4.5 mV for the Se-ferredoxin. The variations of $E^{0'}$ with pH are −11.5 mV/pH unit for the native ferredoxin and −10.5 mV/pH unit for the Se-substituted ferredoxin.

Taking into account the fact that the partial pressure of hydrogen was kept constant in our experiments, the Nernst equation for the H^+/H_2 and ferredoxin (reduced/oxidized) couples (Stombaugh et al., 1976) can be written as

$$\log ([Fd_{red}]/[Fd_{ox}]) = n(a + 1)pH + K$$

where K is a constant and a is the slope of the $E^{0'} = f(pH)$ curve, in volts per unit. Since $a \simeq 0.01$ (see above), it may be neglected and the plot of $\log ([Fd_{red}]/[Fd_{ox}])$ as a function of pH (Figure 9) is a straight line with a slope equal to n. The n values have been determined from Figure 9 and are equal to 0.85 for both ferredoxins. Thus the n value in the Nernst equation must be taken as equal to 1, which means that in both ferredoxins the electrons are accepted or donated one at a time and independently.

Active-Site Reconstitutions with Mixtures of Sulfide and Selenide. We have reconstituted the active sites of the ferredoxin from *C. pasteurianum* in the presence of various mixtures of sulfide and selenide and subsequently measured the amounts of each chalcogenide present in the [4Fe–4X] (X = S, Se) clusters. The data displayed in Figure 10 show that

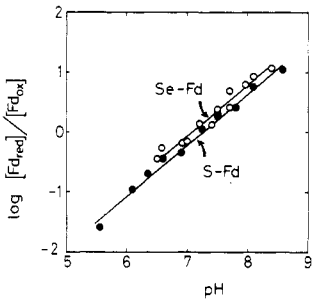

FIGURE 9: Determination of the value of n in the Nernst equation for the native and the Se-substituted ferredoxins. The experimental data are those of Figure 8. Straight lines were obtained by least-squares analysis. The slopes (n values) are 0.85 for both the native (●) and the Se-substituted (○) ferredoxins.

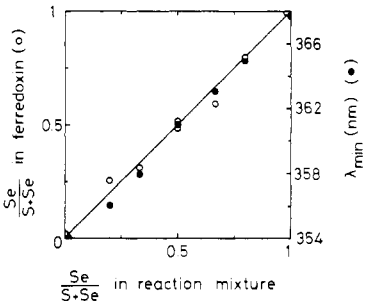

FIGURE 10: Reconstitutions with mixtures of sulfide and selenide. The reconstitutions were carried out with iron and chalcogenide (sulfide plus selenide) in 32-fold excess over the apoprotein (0.8 mg in 1.25 mL of Tris-HCl, 0.1 M, pH 8.5). The reconstituted proteins were separated from the reaction mixtures on 1-mL DE-52 columns (see Results, first section), their UV–visible absorption spectra were recorded, and they were analyzed for iron, sulfide, and selenide. The mole fractions of selenide in the reconstituted ferredoxins (○) and the absorption minima of the latter (●) are plotted as functions of the mole fraction of selenide present in the reaction mixture.

the relative amounts of sulfide and selenide found in the reconstituted proteins are about equal to those present in the reconstitution mixture. In other words, the two elements are equally reactive toward reconstitution of the active site. The results of mixed reconstitutions, which are most accurately analyzed by assaying sulfide and selenide in the reconstituted proteins, can also be evaluated spectrophotometrically: we have previously observed (Meyer & Moulis, 1981) that when sulfide is replaced by selenide, the well between the two peaks of the UV–visible spectrum is shifted from 354 to 368 nm. The latter bathochromic shift is shown in Figure 10 to be proportional to the relative amount of selenide present in the reconstituted ferredoxin.

Exchange of Active-Site Chalcogenide with Free Chalcogenide. The exchanges of active site sulfide with free selenide or vice versa are another way of preparing hybrid ferredoxins. It has previously been shown that iron–sulfur or iron–selenium core atoms can be exchanged in ferredoxins [iron and sulfur exchanges (Hong & Rabinowitz, 1970b)] and in synthetic clusters [sulfur and selenium exchanges (Reynolds & Holm, 1981)]. We have measured the exchange of sulfide from the native ferredoxin with free selenide and the exchange of selenide from the Se-substituted ferredoxin with free sulfide (Table IV). Both exchanges are stimulated in the presence of urea, as previously observed for iron and sulfur exchanges (Hong & Rabinowitz, 1970b). It is worth noting that the exchange of active site selenide with free sulfide is faster than the exchange of active site sulfide with free selenide. The latter observation is in apparent contradiction with the former observation (Figure 9) that selenide and sulfide are equally re-

Table IV: Exchange of Active Site Sulfide or Selenide with Free Selenide or Sulfide, Respectively, in Native and Se-Substituted Ferredoxins[a]

	sulfide replacement by selenide (%) in the 2[4Fe–4S] ferredoxin[b]	selenide replacement by sulfide (%) in the 2[4Fe–4Se] ferredoxin[c]
no urea	0	22
8 M urea	28	87

[a] Ferredoxins were incubated 1 h at 25 °C in the presence of DTT, Fe^{3+}, and chalcogenide and then separated from the reaction mixtures on a 1-mL DE-52 column. Their UV-visible absorption spectra were recorded and their contents in iron, sulfide, and selenium were analyzed. [b] The native ferredoxin (57 nmol in 1.5 mL of Tris-HCl, 0.1 M, pH 8.0) was incubated in the presence of a 50-fold molar excess of DTT and an 80-fold molar excess of Se^{2-} and Fe^{3+}. [c] The Se-substituted ferredoxin (46 nmol in 1.1 mL of Tris-HCl, 0.1 M, pH 8.0) was incubated in the presence of an 80-fold molar excess of DTT, S^{2-}, and Fe^{3+}.

active in ferredoxin active site reconstitutions (see Discussion).

Discussion

The preparation and characterization of the Se-substituted ferredoxin from *C. pasteurianum* (Meyer & Moulis, 1981; this report) is an extension to [4Fe–4S] protein active sites of the S/Se substitutions previously carried out in Fe_4S_4 (Bobrik et al., 1978; Christou et al., 1978) and Fe_2S_2 (Reynolds & Holm, 1980) synthetic cores and in [2Fe–2S] protein active sites (Tsibris et al., 1968; Fee & Palmer, 1971; Mukai et al., 1973).

The method used here for the incorporation of inorganic selenium into the ferredoxin from *C. pasteurianum* was basically similar to the techniques used by others for the reconstitution of iron–sulfur (Malkin & Rabinowitz, 1966; Rabinowitz, 1972) or iron-selenium clusters (Tsibris et al., 1968; Fee & Palmer, 1971; Mukai et al., 1973) in various ferredoxins. We shall only point out a few parameters that must be carefully controlled in order to achieve optimal reconstitutions of [4Fe–4Se] clusters. First, strictly anaerobic conditions must be maintained throughout the reconstitution procedure, as selenide and the Se-substituted ferredoxin are more oxygen sensitive than the corresponding sulfur compounds. Second, the pH should be kept in the range 7.5–8.0 (Figure 1), i.e., about 1 pH unit more acidic than for iron–sulfur reconstitutions (Rabinowitz, 1972). A similar shift of the optimum pH had previously been observed in a comparative study of iron–thiol–sulfide and iron–thiol–selenide complexes in aqueous solution (Sugiura et al., 1975). Third, selenide is conveniently generated in solution by reducing selenite with excess DTT (Fee & Palmer, 1971). We have obtained higher reconstitution yields when selenite was reduced prior to the reconstitution reaction, rather than in the presence of the apoprotein (Fee & Palmer, 1971).

A characteristic effect of the replacement of sulfur by selenium is the appearance of bathochromic shifts of 5–40 nm in the electronic spectra of iron–sulfur clusters (Bobrik et al., 1978; Christou et al., 1978; Fee & Palmer, 1971; Tang et al., 1973; Mukai et al., 1974). No such clear-cut effect is observed in the ferredoxin from *C. pasteurianum*: the high-energy absorption band undergoes a bathochromic shift (from 300 to 305 nm) whereas the low-energy absorption band shows a hypsochromic shift (from 388 to 386 nm) when sulfide is replaced by selenide (Table I; Meyer & Moulis, 1981). Similar shifts are observed with synthetic Fe_4S_4 and Fe_4Se_4 clusters in aqueous solutions (Table I). The latter result suggests that the unusual effects of the S/Se substitution on the electronic spectrum of *C. pasteurianum* ferredoxin are due to intrinsic properties of Fe_4X_4 (X = S, Se) clusters in aqueous solution rather than to an effect of the polypeptide chain on the [4Fe–4X] (X = S, Se) active sites. The substitution of sulfur by selenium has also been reported to have hyperchromic effects (Bobrik et al., 1978). We have observed such effects in the ferredoxin from *C. pasteurianum* as well as in water-soluble synthetic analogues (Table I).

The features of the UV–visible absorption spectrum of the Se-substituted ferredoxin from *C. pasteurianum* witness the presence of [4Fe–4Se] clusters but do not definitely rule out the presence of other structures, for example, products of oxidative damage of the active sites (Thomson et al., 1981). We have therefore carried out core extrusions of the active sites with benzenethiol, which yielded quantitatively $[Fe_4Se_4(SPh)_4]^{2-}$ clusters (Table II), thus establishing that iron and selenium are assembled in [4Fe–4Se] clusters in the Se-substituted ferredoxin.

Another important issue, concerning both the biological and the physicochemical properties of the Se-substituted ferredoxin, is the number of [4Fe–4Se] clusters present in each protein molecule. A number of measurements, including reconstitution yields, elemental analysis of iron and selenium, spectrophotometry, protein assay, and amino acid analysis, have established that Se-substituted ferredoxin preparations having A_{386}/A_{280} ratios of 0.82–0.83 contain two [4Fe–4Se] clusters per molecule of protein.

Selenium-substituted [2Fe–2S] proteins have previously been reported to be more sensitive to irreversible oxidation and to pH variations than their native homologues (Orme-Johnson et al., 1968; Fee et al., 1971). However, no quantitative evaluations of the selenium-induced lability have been brought forth. We have here compared the stabilities of the 2[4Fe–4S] and 2[4Fe–4Se] ferredoxins over a wide range of pH values under anaerobic conditions (Figure 4) and under air at neutral pH. Under the mildest conditions (neutral pH, anaerobic conditions), the Se-substituted ferredoxin has a 20 times shorter half-life than the native ferredoxin. At alkaline or acidic pH, the difference increases up to 500-fold. The lower stability of the Se-substituted ferredoxin may be explained as follows: First, studies on synthetic analogues have shown that Fe_4Se_4 clusters are intrinsically less stable than Fe_4S_4 clusters (Bobrik et al., 1978). Second, due to its larger size (Bobrik et al., 1978), the [4Fe–4Se] cluster is probably less efficiently accommodated and protected by the polypeptide chain. The relative contributions of these two factors to the lability of the Se-ferredoxin could perhaps be estimated by comparing the rates of solvolysis (Maskiewicz & Bruice, 1977) of water-soluble $[Fe_4X_4(SR)_4]^{2-}$ (X = S, Se) synthetic analogues with those of the native and Se-substituted ferredoxins.

The replacement of sulfide by selenide in several [2Fe–2S] ferredoxins has been reported to decrease the enzymatic activity of these proteins by less than 20–30% (Tsibris et al., 1968; Orme-Johnson et al., 1968; Fee & Palmer, 1971). In agreement with these reports, we have found that the native and Se-substituted ferredoxins from *C. pasteurianum* have very similar properties when used as electron donors to *C. pasteurianum* hydrogenase (Figure 5; Table III). The kinetics, measured at several pH values, indicate, however, that the two ferredoxins are slightly but significantly different, the native ferredoxin having the higher maximum velocities and the Se-substituted ferredoxin having the lower K_m values (Table III). We cannot provide any straightforward explanation for these differences. On the other hand, some properties are common to both ferredoxins: the maximum velocities tend

to increase at lower pH values, which represents a general property of the ferredoxin–hydrogenase H_2-evolving system (Adams et al., 1981), and the K_m values decrease considerably, about 4-fold, when the pH is decreased from 8.5 to 7.0. In connection with the similar enzymatic activities of the native and Se-substituted ferredoxins, it should be mentioned that $[Fe_4X_4(SCH_2CH_2OH)_4]^{2-}$ (X = S, Se) synthetic clusters have been shown to transfer electrons from dithionite to *C. pasteurianum* hydrogenase (Adams et al., 1980): The sulfur and selenium clusters display similar kinetics at concentrations lower than 10^{-4} M, but the sulfur cluster is a more efficient electron donor at higher concentrations.

In agreement with their very similar biological activities, the native and the Se-substituted ferredoxins display nearly identical redox properties. Reductive and oxidative titrations have shown that both proteins are two-electron carriers (Figure 6), which is a well-established property of clostridial ferredoxins (Sobel & Lovenberg, 1966; Mayhew et al., 1969; Stombaugh et al., 1976). We have measured the redox potentials of the ferredoxins by equilibrating them with the H^+/H_2 couple via hydrogenase (Tagawa & Arnon, 1968). The latter reaction is clean, reversible, and unaffected by ill-defined or irreversible reactions with artificial mediators and electrodes (Stombaugh et al., 1976; Eddowes & Hill, 1981). The redox potential of the Se-substituted ferredoxin is 6 mV more positive than that of the native ferredoxin (Figure 8). Somewhat larger positive shifts have been observed upon replacement of sulfide by selenide in synthetic clusters: 30 mV (Bobrik et al., 1978) and 10–60 mV (Reynolds & Holm, 1980) for the $[Fe_4X_4(SR)_4]^{2-/3-}$ and $[Fe_2X_2(SR)_4]^{2-/3-}$ (X = S, Se) couples, respectively. In contrast, the Se/S substitution in [2Fe–2S] proteins results in shifts of variable sign, which may at least in part result from the use of different techniques: +38 mV for parsley ferredoxin (Fee et al., 1971), −10 mV for putidaredoxin (Wilson et al., 1973), and −14 mV for adrenodoxin (Mukai et al., 1974). The redox potentials of the native and the Se-substituted ferredoxins from *C. pasteurianum* both decrease by 11 mV when the pH increases by 1 unit (Figure 8). Values of −11 mV/pH unit (Stombaugh et al., 1976) and −16 mV/pH unit (Lode et al., 1976b) have previously been reported for the native ferredoxin from *C. pasteurianum*. The small pH dependence of the redox potentials shows that no free proton is directly involved in the redox reactions of either the native or the Se-substituted ferredoxin. The value of n in the Nernst equation is equal to 1 for both ferredoxins (Figure 9), in agreement with most previous reports (Lode et al., 1976b; Stombaugh et al., 1976). Thus, in both ferredoxins, the electrons are accepted or donated one at a time and independently. The redox potential we have measured for the native *C. pasteurianum* ferredoxin (−423 mV at pH 7.0, Figure 8) is somewhat lower than the values reported in the literature at the same pH: −406 mV (Sobel & Lovenberg, 1966), −405 mV (Lode et al., 1976b), and −403 mV (Stombaugh et al., 1976). Part (5–10 mV) of this discrepancy can be accounted for by our use of more diluted buffer solutions (0.05 M) than those used by other authors (0.1 M), as the redox potentials decrease with decreasing ionic strength (Lode et al., 1976b; Sweeney & Magliozzo, 1980). The use of ferredoxin preparations containing various amounts of inactivated material, which is known to increase the measured redox potential (Stombaugh et al., 1976), may bring additional contributions to the aforementioned differences: we used ferredoxin preparations with an A_{390}/A_{280} ratio of 0.83, whereas the corresponding values reported in the literature were 0.80–0.81 (Stombaugh et al., 1976; Lode et al., 1976b). As shown in Figure 3, such differences in the A_{380}/A_{280} ratios, though small, reflect large differences in the relative proportions of holoferredoxin and apoferredoxin present in the preparations.

The many similar properties of the Se-substituted and the native ferredoxin are due to the closely related chemistries of sulfur and selenium and suggest that both chalcogenides may be incorporated simultaneously in [4Fe–4X] (X = S, Se) clusters of *C. pasteurianum* ferredoxin. Indeed, hybrid iron–sulfur–selenium clusters have previously been evidenced in a [2Fe–2S] protein by EPR spectroscopy (Mukai et al., 1973, 1974) and in Fe_2X_2 and Fe_4X_4 (X = S, Se) synthetic cores by ^1H NMR (Reynolds & Holm, 1981). We have carried out active site reconstitutions with mixtures of sulfide and selenide and found that the two chalcogenides are equally reactive in the incorporation of [4Fe–4X] (X = S, Se) clusters into *C. pasteurianum* ferredoxin (Figure 10). In contrast, similar experiments with adrenodoxin seemed to indicate that selenide is incorporated more easily than sulfide into [2Fe–2X] (X = S, Se) active sites (Mukai et al., 1974). When *C. pasteurianum* ferredoxin is reconstituted with mixtures of sulfide and selenide, it most likely contains hybrid $[Fe_4–S_{4-n}–Se_n]$ (n = 1, 2, 3) clusters similar to those evidenced in solutions of synthetic analogues (Reynolds & Holm, 1981). The latter hypothesis has been confirmed by resonance Raman spectroscopy: A hybrid ferredoxin containing 50% S and 50% Se displays vibronic features that are different from those of the native and the Se-substituted ferredoxins and also different from those of an equimolar mixture of the two latter proteins [collaboration with M. Lutz (unpublished experiments)].

Ferredoxins containing hybrid iron–sulfur–selenium clusters can also be prepared by exchanging active site chalcogenide atoms (Table IV). It had previously been shown that active site sulfide from *C. pasteurianum* ferredoxin is exchangeable with free sulfide at alkaline pH and in the presence of urea (Hong & Rabinowitz, 1970b). Furthermore, sulfide and selenide atoms may be exchanged between $[Fe_4X_4(SR)_4]^{2-}$ (X = S, Se) synthetic clusters in acetonitrile solution (Reynolds & Holm, 1981). We have extended these studies by demonstrating exchanges of active site sulfide or selenide with free selenide or sulfide, respectively, in the ferredoxin from *C. pasteurianum*. The replacement of cluster selenide by free sulfide appears to be easier than the replacement of cluster sulfide by free selenide (Table III). As the two chalcogenides are equally reactive for the reconstitution of active sites in the apoprotein (Figure 10), the results shown in Table IV would perhaps be best explained by a greater exposure of the [4Fe–4Se] clusters to the solvent (see above).

The present report establishes the general feasibility of the S/Se substitution in iron–sulfur clusters of small ferredoxins. The replacement of sulfide by selenide in well-characterized proteins may contribute to the understanding of why Se has been selected rather than S at the active sites of a few enzymes (Stadtman, 1980). It should be noticed, however, that the presence of inorganic selenium in a native enzyme has been reported only once (Andreesen & Ljungdahl, 1973), whereas in all other cases selenium is present as selenocysteine (Stadtman, 1980). An interesting extension of the present work would be the replacement of sulfur by selenium in larger and more complicated iron–sulfur proteins and the subsequent determination of the effect of selenium on more sophisticated enzymatic activities, e.g., hydrogenase or nitrogenase, than electron transfer. Such experiments, however, will require the elaboration of gentler S/Se exchange techniques than those described here and previously. Selenium-substituted iron–sulfur proteins have also proved to be useful for the elucidation

of the physicochemical properties of iron–sulfur active sites. Indeed, several physical techniques have been fruitfully applied to [2Fe–2Se] ferredoxins (Bertrand & Gayda, 1980, and references cited therein), and their use is now being extended to a [4Fe–4Se] ferredoxin. However, although the Se-substituted ferredoxins are very similar to the corresponding native proteins, the replacement of S by Se is obviously more than an isotopic substitution: selenium has a larger covalent radius than sulfur (+15%) and is less electronegative and its atomic weight is more than twice that of sulfur. The latter differences are reflected more or less intensely in the various properties of the iron–sulfur and iron–selenium clusters, thus establishing selenium as a valuable intrinsic probe of iron–sulfur clusters.

Acknowledgments

We thank Dr. A. C. Dianoux and J. J. Scheffler for the amino acid analyses, Dr. P. M. Vignais and Dr. J. C. Willison for critically reading the manuscript, and J. J. Bournet for typing the manuscript.

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