Vol. 103, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1981 Pages 667-673

REPLACEMENT OF SULFIDE BY SELENIDE IN THE [4Fe-4S] CLUSTERS OF THE FERREDOXIN FROM CLOSTRIDIUM PASTEURIANUM

Jacques MEYER and Jean-Marc MOULIS
Laboratoire de Biochimie (CNRS/ER 235 et INSERM U.191)
Département de Recherche Fondamentale, Centre d'Etudes Nucléaires,
85 X, 38041 Grenoble Cédex, France

Received September 13,1981

ABSTRACT: The sulfur atoms of the two [4Fe-4S] clusters present in the ferredoxin from C. pasteurianum have been replaced by selenium. The optical absorption spectrum of the Se-ferredoxin is slightly different from the spectrum of the native protein, but it displays the characteristic features of [4Fe-4X] (X = S, Se) clusters. The reduced Se-ferredoxin can reduce hydrogenase, and the oxidized Se-ferredoxin can be reduced by hydrogenase in the presence of molecular hydrogen. This is the first report of sulfide substitution by selenide in an iron-sulfur protein containing [4Fe-4S] active sites.

INTRODUCTION

Ferredoxins are small proteins (molecular weight 6,000 to 25,000) that serve as electron carriers in various systems. They contain iron and inorganic sulfur organized into [4Fe-4S] (1) or [2Fe-2S] (2) clusters. Recently, a [3Fe-3S] cluster has been evidenced in a ferredoxin from Azotobacter vinelandii (3). The Fe_2S_2 and Fe_4S_4 units have been extensively studied in proteins (1, 2) as well as in low molecular weight synthetic analogs (4). An interesting feature of these iron-sulfur cores is the possibility of substituting sulfur by selenium with retention of the overall structure and main physico-chemical properties. Indeed, $Fe_4Se_4(SR)_4^{2-}$ (5, 6) and $Fe_2Se_2(SR)_4^{2-}$ (7, 8) clusters have been synthesized chemically and characterized. In ferredoxins, however, only [2Fe-2Se] clusters have been assembled so far from the appropriate apoproteins, with iron and selenium reagents (9, 10, 11).

We here report the incorporation of two [4Fe-4Se] clusters into the ferredoxin from Clostridium pasteurianum (M.W. = 6,000), which contains two [4Fe-4S] clusters in its native state. It is thus established that the S/Se substitution is feasible in both the binuclear and tetranuclear sites of ferredoxins, as well as in the synthetic analogs.

MATERIALS AND METHODS

The growth of C. pasteurianum W5 (ATCC 6013) cells and the purification of ferredoxin were carried out as described by Rabinowitz (12). Hydrogenase was purified and assayed as described by Chen and Mortenson (13). Apoferredoxin

0006-291X/81/220667-07$01.00/0

was prepared by repeated (2 or 3 times) precipitations of the ferredoxin with 8% trichloracetic acid and redissolution in 0.5M Tris-Cl pH 8.5, followed by filtration on Sephadex G-25. Iron was assayed by using bathophenanthroline disulfonate (14). Sulfide was determined as described by Chen and Mortenson (15). For the determination of selenium, known amounts of $Na_2{}^{75}SeO_3$ (from the Radiochemical Center, Amersham, U.K) were added to the solutions of Na_2SeO_3 (99%, from Aldrich) used for the reconstitution of ferredoxin. ^{75}Se was measured on a Kontron MR480 gamma counter. UV-visible spectra were recorded with a Cary 219 spectrophotometer.

RESULTS AND DISCUSSION

The following procedure was used for the incorporation of [4Fe-4Se] clusters into ferredoxin, strictly anaerobic conditions being maintained throughout. The apoprotein (0.5-1 mg/ml, in 0.1M Tris-Cl, pH 8.5) was preincubated 30 minutes at room temperature with a 50-fold molar excess of dithiothreitol (DTT). Fe^{3+} (as a solution of $FeCl_3.6H_2O$) 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 (10) : a ten-fold excess of DTT in aqueous solution was added to solid Na_2SeO_3 (or to a solution when ^{75}Se was used) under anaerobic conditions and the mixture was allowed to react for 5 minutes. Upon addition of Se^{2-}, the reaction mixture turned dark brown, and a fine black precipitate appeared. After one hour at room temperature, the solution was loaded on a small DE 52 column (5 ml for 10-20 mg of protein) equilibrated with Tris 0.02 M, pH 7.4 and NaCl 0.1M. 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 0.4M NaCl. The remaining free iron and selenide were then separated from the protein by filtration on Sephadex G-25. The procedure for the reconstitution of [4Fe-4S] clusters was identical, except for the replacement of Se^{2-} by S^{2-}, added as a solution of $Na_2S, 9H_2O$ (12). The yields of these reconstitutions varied between 55% and 75%.

The iron, sulfide, and selenide contents of the apoferredoxin and of the S^{2-} and Se^{2-} reconstituted ferredoxins are displayed on Table I. The Fe and S^{2-} contents of the apoferredoxin are not significantly different from zero, thus the [4Fe-4S] clusters have been quantitatively removed from the protein. The Fe and S^{2-} contents of the S^{2-} reconstituted protein are identical with those of the native ferredoxin, which indicates that the reconstitution procedure used here eliminates any iron or sulfide not belonging to the [4Fe-4S] clusters. The Se-ferredoxin contains equal amounts of iron and selenide, and practically no sulfide. 7 to 8 atoms of Fe and Se^{2-} per molecule of protein were found when the concentrations of iron and selenide were divided by the concentration of protein as determined by the assay of Lowry et al. (16). As the latter

Table I : Metal and chalcogenide contents, and optical absorption properties of the S- and Se-ferredoxins from C. pasteurianum

	Fe (atoms/molecule of protein)	S^{2-} (atoms/molecule of protein)	Se^{2-} (atoms/molecule of protein)	ε_M (λ_{max}) ($M^{-1} \times cm^{-1}$)	$A_{\lambda max} / A_{280}$
Apoferredoxin	0.3 ± 0.2 (4)[a]	0.04 ± 0.03 (9)	—	—	—
Fe, S reconstituted ferredoxin	8.3 ± 0.6 (6)	9.0 ± 1.1 (3)	—	30,000 ± 1,000[b] at 388 nm	0.82[c]
Fe, Se reconstituted ferredoxin	8.0 ± 0.3 (9)	0.03 ± 0.03 (8)	8.0 ± 0.3 (4)	32,000 ± 1,000[d] at 386 nm	0.80 - 0.82

a. In parentheses, number of determinations

b. This value is an average of those found in the litterature : 29,100 (ref. 13), 30,000 (ref. 21), 30,600 (ref. 12)

c. The corresponding ratio for the native protein was 0.83

d. Calculated for 8 Fe and 8 Se^{2-} per molecule of protein (see text).

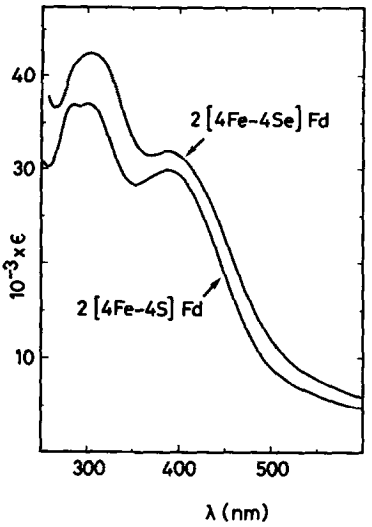

Figure 1 Optical absorption spectra of the ferredoxin from C. pasteurianum and of its selenium substituted analog. Both proteins are in their oxidized state, i.e. $2[4Fe\text{-}4X]^{2+}$ (X = S,Se). ε is the molar extinction coefficient in $M^{-1} \times cm^{-1}$. The wavelengths of the main features of the spectra and the extinction coefficients are to be found in Table I and in the text. Fd is an abbreviation for ferredoxin.

assay overestimates ferredoxin concentrations by a factor of 1.8 (12), native ferredoxin was used as a standard for the determination of Se-ferredoxin concentrations. These assays, and other data shown below, demonstrate that the Fe-Se reconstituted ferredoxin contains two [4Fe-4Se] clusters. A molar extinction coefficient of 32,000 $M^{-1} \times cm^{-1}$ at 386 nm was calculated, based on 8 Fe and 8 Se^{2-} per molecule.

The UV-visible absorption spectra of the native and selenium-substituted ferredoxins are shown in Figure 1. The two spectra are very similar, both having the two broad absorption bands at ca. 400 and 300 nm, characteristic of [4Fe-4S] clusters in proteins (1). Nevertheless, a few small but significant differences are observed : First, the low energy band of the Se-ferredoxin is blue-shifted by 2 nm compared to the native ferredoxin (from 388 to 386 nm). This is somewhat in disagreement with the observations made with synthetic Fe_4S_4 cores, where the substitution of S by Se causes a red shift of 8 nm (6) to 13 nm (5). Even larger red shifts have been reported in selenium substituted [2Fe-2S] ferredoxins (10, 17). There is one example, however, of an iron-sulfide-thiolate complex where the replacement of sulfide by selenide results in a blue shift of 10 to 15 nm (18). Second, the high energy band in the Se-ferredoxin

is red shifted (from 302 to 305 nm), which results in a significant red shift of the well between the two peaks : 368 nm for the Se-ferredoxin, versus 354 nm for the native protein. Third, the Se-ferredoxin has a higher molar extinction coefficient (ε_M) than the native protein at ca. 390 nm (32,000 versus 30,000 $M^{-1} \times cm^{-1}$) and at ca. 300 nm (42,500 versus 37,000 $M^{-1} \times cm^{-1}$). Of the two studies on synthetic analogs, one reports a 3% increase (6), the other a 8% decrease (5) of ε_M (at ca. 470 nm) when sulfide is replaced by selenide.

The biochemical properties of the Se-substituted ferredoxin from C. pasteurianum will be detailed elsewhere. Preliminary experiments have shown that the dithionite-reduced Se-ferredoxin is nearly as efficient an electron donor to the hydrogenase from C. pasteurianum as the native ferredoxin : the maximum velocity of H_2 evolution is decreased by less than 30% when the former is used instead of the latter. In addition, like the native ferredoxin, the Se-ferredoxin is reduced by hydrogenase in H_2-saturated solutions. These results are in good agreement with previous observations that the biological function of [2Fe-2S] ferredoxins is only slightly impaired by the Se/S substitution (9, 10, 17). Furthermore, in synthetic Fe_4X_4 (X = S, Se) cores, the redox potential of the $Fe_4X_4^{2+}/Fe_4X_4^{+}$ couple, which is operating in the ferredoxin from C. pasteurianum, changes by less than 30 mV upon replacement of S by Se (6).

We have observed that when reconstitution reactions are carried out with mixtures of S^{2-} and Se^{2-}, both chalcogenides are incorporated into the apoprotein. Recent chemical studies (8) suggest that various hybrid clusters of the series $Fe_4S_{4-n}Se_n$ (n = 0, 1, 2, 3, 4) may be present in such reconstituted ferredoxins. It should also be mentionned that a mixed species of the type Fe_2SSe has previously been obtained when adrenodoxin was reconstituted with Se^{2-} and S^{2-} (11, 17).

For practical purposes, it has to be emphasized that the Se-ferredoxin is much less stable than the native ferredoxin. Indeed, in air-saturated dilute solutions (10-100µM) the former is inactivated about 10 times faster than the latter. Even under argon, at 0°C or at room temperature, the Se-ferredoxin loses its active sites over a period of 20-30 hours, whereas the native protein is stable for many days. [2Fe-2Se] ferredoxins have also been reported to be less stable than the corresponding native proteins (10). The lability of the Se-ferredoxin may be due either to the greater sensitivity of Se^{2-} (versus S^{2-}) towards oxidation, or to the larger size of the [4Fe-4Se] cluster (6), which would thus be less efficiently accomodated and protected by the protein than the native [4Fe-4S] cluster.

The replacement of sulfur by selenium at specific sites in proteins is of great interest for comparative purposes, as such investigations may contribute

to the understanding of why some enzymic processes do not distinguish selenium from sulfur while others show a specific requirement for one or the other of these elements (19). In addition, selenium is of advantage for some magnetic resonance studies, since ^{77}Se has a nuclear spin of 1/2, which causes a simpler pattern of hyperfine splitting than ^{33}S (spin = 3/2), for example (20).

ACKNOWLEDGEMENTS

This research was supported in part by grants from the Centre National de la Recherche Scientifique (PIRDES) and the EEC Solar Energy Research and Development Program.

REFERENCES

1. Sweeney, W.V. and Rabinowitz, J.C. (1980) Ann. Rev. Biochem. 49 139-161.
2. Fukuyama, K., Hase, T., Matsumoto, S., Tsukihara, T., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K. and Matsubara, H. (1980) Nature 286 522-524.
3. Ghosh, D., Furey, W., Jr., O'Donnell, S., and Stout, C.D. (1981) J. Biol. Chem. 256 4185-4192.
4. Holm, R.H. and Ibers, J.A. (1977) In : Iron-Sulfur proteins (Lovenberg, W., ed.) Vol III pp.205-281 Academic Press, New-York
5. Christou, G., Ridge, B. and Rydon, H.N. (1978) J. Chem. Soc. Dalton 1423-1425.
6. Bobrik, M.A., Laskowski, E.J., Johnson, R.W., Gillum, W.O., Berg, J.M., Hodgson, K.O. and Holm, R.H. (1978) Inorg. Chem. 17 1402-1410.
7. Reynolds, J.G. and Holm, R.H. (1980) Inorg. Chem. 19 3257-3260.
8. Reynolds, J.G. and Holm, R.H. (1981) Inorg. Chem. 20 1873-1878.
9. Tsibris, J.C.M., Namtvedt, M.J. and Gunsalus, I.C. (1968) Biochem. Biophys. Res. Comm. 30 323-327.
10. Fee, J.A. and Palmer, G. (1971) Biochim. Biophys. Acta 245 175-195.
11. Mukai, K., Huang, J.J. and Kimura, T. (1973) Biochim. Biophys. Res. Comm. 50 105-110.
12. Rabinowitz, J.C. (1972) In : Methods in Enzymology (San Pietro, A., ed.) Vol.24B, pp.431-446, Academic Press, New-York.
13. Chen, J.S. and Mortenson, L.E. (1974) Biochim. Biophys. Acta 371 283-298.
14. Blair, D. and Diehl, H. (1961) Talanta 7 163-174.
15. Chen, J.S. and Mortenson, L.E. (1977) Anal. Biochem. 79 157-165.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193 265-275.

17. Mukai, K., Huang, J.J. and Kimura, T. (1974) Biochim. Biophys. Acta 336 427-436.

18. Sugiura, Y., Ishizu, K., Kimura, T. and Tanaka, H. (1975). Bioinorg. Chem. 4 291-302.

19. Stadtman, T.C. (1980) Ann. Rev. Biochem. 49 93-110.

20. Orme-Johnson, W.H., Hansen, R.E., Beinert, H., Tsibris, J.C.M., Bartholomaus, R.C. and Gunsalus, I.C. (1968) Proc. Natl. Acad. Sci. USA 60 368-372.

21. Sobel, B.E. and Lovenberg, W. (1966) Biochemistry 5 6-13.