SELENIUM AS AN ACID LABILE SULFUR REPLACEMENT IN PUTIDAREDOXIN*

J.C.M. Tsibris, M.J. Namtvedt, and I.C. Gunsalus
Chemistry Department, University of Illinois, Urbana 61801

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Putidaredoxin is the iron-sulfide protein component of a system which hydroxylates methylene carbon five of (+)-camphor in Pseudomonas putida, strain CI (Cushman and Gunsalus, 1966). This enzyme contains in a molecular weight ca 12,000, two atoms each of acid-labile sulfur and iron and undergoes a one-electron oxidation-reduction with an E'_o -185 mVolts**. The sulfur and iron atoms can be removed from putidaredoxin by treatment with acid (TCA) in an argon atmosphere (Tsibris, et.al., 1968) or by treatment with mercurials (Malkin and Rabinowitz, 1966). As replacement elements, ^{32}S, ^{33}S, ^{56}Fe, and ^{57}Fe have been introduced, usually with a radioactive isotope, e.g., ^{35}S or ^{59}Fe.

We have now replaced the acid-labile sulfur with selenium of the natural isotopic abundance and with ^{80}Se labeled with the radioactive isotope ^{75}Se. The product is biologically active and possesses an altered iron-selenide active center as indicated by a shift in the visible spectrum to longer wavelength and by an electron spin resonance spectrum in the reduced state indicative of rhombohedral rather than axial symmetry as seen in the iron-sulfide enzyme. Changes observed in other physical properties will be reported subsequently.

METHODS

Putidaredoxin, specific activity 5, was prepared by a modification of the method of Cushman, Tsai, and Gunsalus (1967). The labile iron and sulfur

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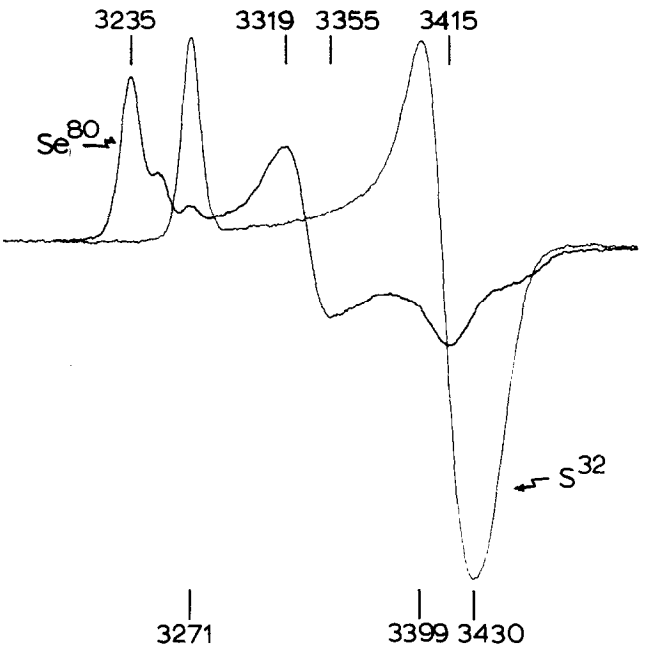
**Measurements of E'_o by Dr. G. S. Wilson, University of Arizona.

were removed after equilibration with an argon atmosphere at room temperature by dropwise addition of 100% TCA with constant agitation. Elemental selenium-80 (enrichment 96.87% obtained from Oak Ridge National Laboratory) was mixed with a small amount of elemental selenium-75 (1 mc/mg obtained from New England Nuclear Co.) and reduced to ammonium selenide by the procedure of Nitsche (1957). A 5 mg sample of the apo-putidaredoxin was treated in the argon atmosphere with a 10-fold molar excess of ammonium selenide and a 5-fold molar excess of ferrous iron in the presence of sulfide-free 10mM β-mercaptoethanol. The selenide putidaredoxin was recovered as described previously for the sulfide and iron reconstituted material. At this stage the iron and selenide contents were respectively 2.6 and 2.5 atoms per mole of enzyme (12,000 gm protein). After 5 hours dialysis versus 50 mM Tris buffer, pH 8.3, containing 10 mM β-mercaptoethanol, the iron and sulfide content was decreased to two atoms each per mole of enzyme, see table.

The enzymatic activity was measured by the fixed time (product formation) method of Cushman, et.al. (1967), and by the kinetic DPNH-oxidation method of Katagiri, et.al., (1968).

RESULTS AND DISCUSSION

The properties of putidaredoxin in the native form and after replacement of sulfide by selenide-^{80}Se are as shown in the table. The removal of the two sulfides contained per mole of native enzyme on formation of the apo-protein is greater than 95%, as indicated by decrease in sulfide-^{35}S, the absence of absorption bands at 415 and 455 mμ, and the lack of their restoration by Fe^{++} in the presence of β-mercaptoethanol without added sulfide. Upon displacement of selenide-80 + 75 from the reconstituted sample with ^{32}S, less than 4% of the ^{75}Se counts remain and the UV spectrum is restored to that of the native enzyme. The iron and selenide contents of the reconstructed enzyme shown in the table are comparable to the values obtained with ^{32}S and ^{33}S replacement (Tsibris, et.al., 1968). In the selenide sample, the principal visible absorption maxima of the oxidized enzyme are shifted about 20 mμ to longer wavelength--the 325 maximum is shifted only about 5 mμ. The biological activity is comparable to the sulfide enzyme in both the product formation assay and the more recent substrate-dependent DPNH oxidation of Katagiri (1968). At higher concentration, the selenide putida-



EPR Spectra of Reduced Putidaredoxin.

Native form, curve ^{32}S; selenide exchanged ^{80}Se.
The two curves represent equal numbers of spins.

Conditions of EPR spectroscopy: microwave power, 27 mWatt; frequency 9,223 MHz modulation amplitude 3 Gauss; scanning rate, 100 Gauss per min; time constant 1 sec; temperature, 83°K.

Ordinate is an arbitrary measure of the first derivative of the EPR absorption curve. Field markers (abscissa) are in Gauss; upper set refers to the major excursion of the ^{80}Se-putidaredoxin curve and the lower set to the native (^{32}S) putidaredoxin.

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redoxin is inhibitory in the DPNH oxidation assay.

The electron spin resonance spectrum*** of the selenide putidaredoxin is shown superimposed on an earlier spectrum of sulfide-^{32}S enzyme, see figure. The substitution of 2 atoms of ^{80}Se (spin 0 isotope) into the native protein induces a dramatic change in the EPR spectrum of the reduced species. The active center of the native material, $g = 2.01$ and $g = 1.93$, is distorted in the Se-enzyme to a rhombohedral complex with $g_z = 2.04$, $g_y = 1.98$,

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***Measurements by Drs Beinert and Orme-Johnson, Institute for Enzyme Research, University of Wisconsin.

and $g_x = 1.93$. This suggests that the selenium atoms are not positioned in the paramagnetic complex in exactly the same way as the sulfur atoms which they replace and that asymmetric distortions of the complex accompany the substitution. The minor bands near the low and high field ends of the selenium enzyme spectrum are as yet unexplained. They may result in part from residual ^{32}S in the preparation. Nevertheless, this profound effect on the EPR spectrum underlines strongly what was found earlier by Tsibris, et.al., (1968), by substitution of ^{32}S with the spin = 3/2 isotope ^{33}S, namely, that the electron in the g = 1.94 interacts with the nuclei of acid-labile sulfur atoms which therefore participate as primary lagands in this paramagnetic complex. It should prove interesting to compare ^{77}Se (spin = 1/2) with the ^{80}Se-putidaredoxin spectra in an effort to establish how many Se (and by analogy S) atoms participate in the g = 1.94 center, as has been done with iron (Tsibris, et.al., 1968).

Selenide-80 Putidaredoxin: Properties and Activity

Measured	Enzyme	
	Native	^{80}Se-replaced
	atoms/mole enzyme	
Acid labile Fe[1]	2.17	1.85
" " chalcogenide	1.85[2]	1.85[3]
Activity	units/mg protein	
Product formation[4]	5	3.1
Coupled DPNH oxidation[5]	4	5-7
Absorption spectra	mμ E_{mM}	mμ E_{mM}
	455 9.6	477 8.6
	415 10.	433 8.9
	325 15.	330 16.
	280 22.5	

1. Fischer, Price, 1964. 2. Fogo, Popowsky, 1949. 3. Radiochemical ^{75}Se.
4. Cushman, 1966. 5. Katagiri, nmoles DPNH oxidized, $min^{-1}ml^{-1}$.

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