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Light-induced oxidation of iron atoms in a photosensitive nitrile hydratase

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The photoactivation process of a photosensitive nitrile hydratase (NHase) from *Rhodococcus* sp. N-771 has been investigated by ^{57}Fe Mössbauer spectroscopy and magnetic susceptibility measurements in order to clarify the behavior of iron atoms in the enzyme. Mössbauer spectra of inactive NHase gave two symmetric-doublet components indicating the presence of two iron species, while that of the active NHase gave a single symmetric doublet indicating the presence of a single iron species. Magnetic susceptibility measurements of the inactive and active HNase both showed small effective magnetic moments. These results led us to conclude that one of the two iron atoms incorporated in the NHase is oxidized during photoactivation, namely from a low spin ferrous to a low spin ferric state. This is the first observation of an intramolecular photooxidation phenomena involving iron in a single protein molecule.

Nitrile hydratase; Mössbauer spectroscopy; Magnetic susceptibility; Photoactivation; Oxidation; Non-heme iron

1. INTRODUCTION

Nitrile hydratase (NHase, EC 4.2.1.84) is an enzyme which hydrates aliphatic nitrile compounds to the corresponding amides (Eq. 1). This enzyme from *Rhodococcus* sp. N-771 has a photosensitive property [1]. Its catalytic activity is enhanced by an exposure to near ultra-violet light, and this activation process (Eq. 2) is accompanied by a drastic change in absorption spectrum [2].

$$\text{R-CN} + \text{H}_2\text{O} \xrightarrow[\text{NHase}]{} \text{R-CONH}_2 \qquad \text{(Eq. 1)}$$

$$\text{Inactive NHase} \xrightarrow[h\nu]{} \text{Active NHase} \qquad \text{(Eq. 2)}$$

We presumed that this change in absorption is an indication of the two iron atoms incorporated per NHase molecule as non-heme irons [2], making some kind of electronic and/or structural change upon this photoactivation process. Thus we have undertaken the present investigation to clarify changes in the state of iron atoms in this enzyme.

Abbreviations: NHase, nitrile hydratase; SQUID, superconducting quantum interference device.

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Mössbauer spectroscopy is a technique which presents spectra reflecting the environment around a single atom (typically ^{57}Fe). Because of this, it offers unusually 'specific' information about the characteristics of iron atoms in biological macromolecules compared to, for example, absorption spectra which reflect properties of a complex or molecule as a whole. In this report, we will present the results of Mössbauer spectroscopy and magnetic susceptibility studies on both states of photosensitive NHase, and discuss the possible process that is occurring during photoactivation.

2. MATERIALS AND METHODS

2.1. *Preparation of ^{57}Fe-enriched nitrile hydratase for Mössbauer spectroscopy*

Rhodococcus sp. N-771 was precultivated twice prior to cultivation in order to decrease the ^{56}Fe content in the cells. Precultivation was performed at 30°C for 24 h in a 500 ml baffle flask containing 100 ml medium (pH 7.5) consisting of 10 g glucose, 5 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 5 g casamino acid, 0.002 g thiamine-HCl/l ultrapure water prepared with Milli-Q SP (MilliPore). All the chemicals used were of reagent grade. The precultivated seed culture was then inoculated into an 8-l mini jar fermenter containing 6 l of ^{57}Fe-enriched medium prepared as follows. $^{57}Fe_2O_3$ (enrichment 95.10%, Dupont de Nemours) was dissolved in a boiling HCl solution. Then an excess amount of $K_2C_2O_4$ was added to the solution to make ^{57}Fe complex with oxalate ions. 40 mg of this chelated ^{57}Fe and 3 g of antifoam (Pluronic L-61, Asahi Denka)/l was added to the growth medium mentioned above. Cultivation was carried out to an early stationary growth phase at 30°C with an aeration rate of 6 l/min and an agitation speed of 500 rpm. The cells obtained from 12 l medium (185 g wet cell

weight) were harvested and suspended in 900 ml of 20 mM phosphate buffer containing 10 mM butyric acid, pH 7.5. The cell suspension was incubated aerobically in the dark at 5°C for 24 h to inactivate the NHase in the cells. Then 1.8 g of lysozyme (grade I, Sigma) was added to the cell suspension and incubated at 25°C for about 12 hours to disrupt the cells. The cell suspension was centrifuged at 27,170 × g for 30 min. The supernatant was used as the crude cell-free extract. The crude cell-free extract was purified by ammonium sulfate fractionation (40–60% saturation), ion-exchange column chromatography (Whatman DE-52) and hydrophobic column chromatography (Toyopearl HW-65C) as described previously [2]. The NHase fractions were collected, dialyzed in 20 mM phosphate buffer and concentrated with an ultrafiltration module (Ultrafree PF, MilliPore). All purification procedures were carried out at 5°C in the dark. Active NHase was prepared by irradiating inactive NHase with a photoreflector lamp as previously described [1].

2.2. *Mössbauer spectroscopy*

About 90 mg each of inactive and active NHase samples were placed in acrylic cells and subjected to Mössbauer measurement at room, liquid-nitrogen and liquid-helium temperatures by means of a conventional Mössbauer spectrometer (Ranger VT-700) using a ^{57}Co/Rh source. Mössbauer spectra of a metallic iron foil and sodium nitroprusside were used to calibrate the velocity scale, and the centroid of the former was taken as zero velocity. The spectra were analyzed by least-squares fitting using FACOM M380 and M780 computers, assuming Lorentzian line shapes.

2.3. *Magnetic susceptibility measurement*

The inactive and active NHase samples (native) containing 14.5 μg Fe each (equivalent to about 6 mg NHase) were prepared by the method previously described [2], and placed in pharmaceutical capsules. Magnetic susceptibility measurements were performed using SQUID (Hokusan model HSM-D) between 4.2K and 200K at $H = 730$ Gauss. Iron content in the NHase was determined quantitatively by inductively coupled radio frequency plasma spectrophotometer, Shimadzu ICPS-50.

3. RESULTS AND DISCUSSION

Mössbauer spectra of inactive NHase at 200K, 77K and 9K are shown in Fig. 1, and that of active NHase at 77K is shown in Fig. 2. Their Mössbauer parameters are shown in Table I. The isomer shift (centroid of the doublet) is related to the s-electron density at the iron nucleus which varies with the oxidation state and the bonding of the iron. The quadrupole splitting (the difference between the positions of two lines in a doublet) is influenced by the configuration of the electrons and ligands around the iron nucleus. Mössbauer spectrum for inactive NHase can be fitted with a superposition of two symmetric-doublet components. One of them (A) has a small isomer shift of 0.02 mm/s and a large quadrupole splitting of 1.51 mm/s. The other (B) has an isomer shift of 0.31 mm/s and a small quadrupole splitting of 0.36 mm/s. The spectrum for the active enzyme shows a single symmetric doublet (B) with an isomer shift of 0.33 mm/s and a small quadrupole splitting of 0.37 mm/s. These results clearly show that two iron species, A and B, exist in the inactive state of NHase, and after photoirradiation, species A is transformed to species B giving a single doublet of species B in the active NHase. The ratio of the areas of the two components A and B in the inactive enzyme spectra exhibits temperature dependency (Table I). This ratio is 1.16 when extrapolated to 0K. Discrepancy between the value and unity is ascribed to the following two facts: (1) a very small fraction of the molecules in the inactive NHase sample could have been in the active form, and (2) there can be a difference in recoil-free fractions of species A and B due to differences in the environment around each of the two iron atoms in the same NHase molecule. It is worthy of notice that an isomer shift of 0.02 mm/s (species A) and a quadrupole splitting of 0.36 mm/s (species B) are exceptionally low values compared with those of other non-heme irons hitherto observed in various proteins, according to our recent survey of Mössbauer parameters. This fact and the absorption

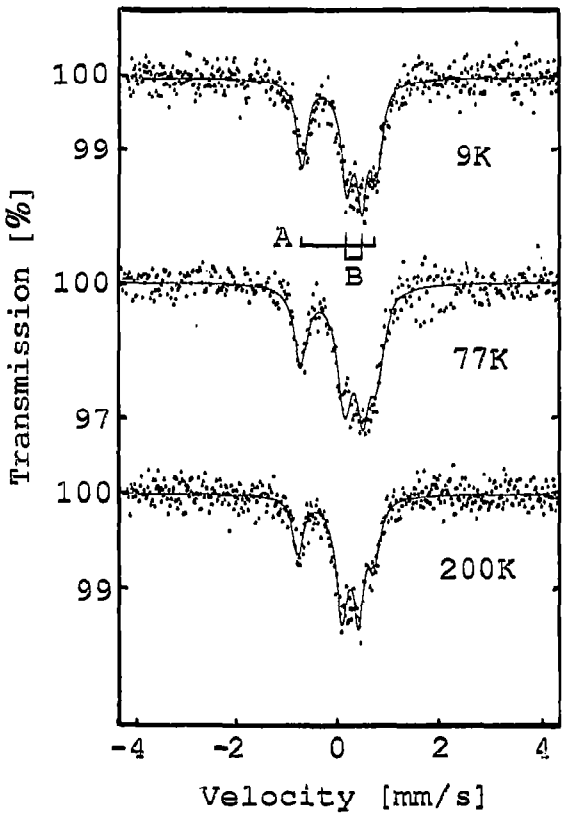

Fig. 1. Mössbauer spectra of inactive NHase at 9K, 77K and 200K.

Table I

Mössbauer parameters of NHase

	Inactive NHase						Active NHase	
Temperature (K)	9		77		200		77	
Species	A	B	A	B	A	B	A	B
Isomer shift (mm/s)	0.02	0.31	0.02	0.31	−0.02	0.25	–	0.33
Quadrupole splitting (mm/s)	1.49	0.31	1.51	0.36	1.50	0.33	–	0.37
Ratio of peak area (B/A)	1.20		1.45		1.96		–	

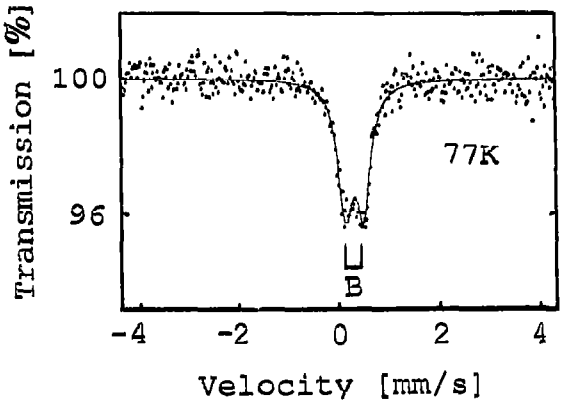

Fig. 2. Mössbauer spectrum of active NHase at 77K.

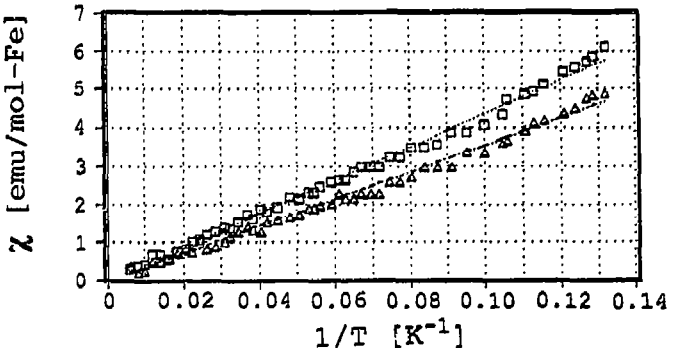

Fig. 3. $1/T$–χ plot of inactive (△) and active (□) NHases using SQUID. Assumed background values for buffer are subtracted.

spectra of this enzyme [2] manifest a possibility of the iron atoms in this enzyme being integrated in an unreported structure.

From Mössbauer parameters, species A can be assigned to either low spin ferrous (Fe^{2+}) or low spin ferric (Fe^{3+}). Species B can be assigned to either low spin or high spin Fe^{3+}, or low spin Fe^{2+} [3]. However, Mössbauer parameters observed here are not sufficient to determine the valencies of the species A and B unambiguously. Thus, in order to determine to which iron states these species can be assigned unambiguously, a magnetic susceptibility study has been performed. Fig. 3 shows the temperature dependence of magnetic susceptibility of the inactive and active NHases. Considering the diamagnetism of the protein being temperature-independent and its paramagnetism being proportional to the inverse of absolute temperature (Curie's law), the following correlation can be obtained:

$$\chi_{total} = \chi_{para} + \chi_{dia} = \frac{C}{T} + \chi_{dia} \qquad \text{(Eq. 3)}$$

where: χ_{total}= total magnetic susceptibility (of the enzyme sample), χ_{para}= paramagnetic susceptibility component, χ_{dia} = diamagnetic susceptibility component, C = Curie's constant and T = absolute temperature in Kelvin.

Taking the range of 165–7.5K in the calculation of slope (because of the good linearity in this range), C was determined using the least-squares method (Fig. 3). This gave values 0.365 for C of the inactive NHase and 0.437 for C of the active NHase (based on the molar iron content of the samples). From this, effective number of Bohr magnetons can be calculated using the following relation.

$$n^2 = \frac{3\,k\,C}{N\mu_B^2} \qquad \text{(Eq. 4)}$$

where: n = effective Bohr magneton number, k = Boltzmann constant, N = Avogadro number and μ_B = Bohr magneton.

We have taken n^2 as a criteria for valency and spin state determination. The above calculations after sample volume correction gave the n^2 values of 5.68 per molecule for the inactive NHase and 7.00 per molecule for the active NHase. The spin-only values of iron in various states are shown in Table II, together with empirical values. As already mentioned, species B can be assigned to low-spin Fe^{2+}, low-spin Fe^{2+} or high-spin Fe^{3+} which have the spin-only n^2 values of 0, 3 or 35, respectively (Table II). For the active NHase, there is only a single iron species B involved, and its n^2 value of one iron atom (since there are two iron atoms per NHase molecule [2]) is 3.50. Thus we can safely assign species B to be low spin Fe^{3+}, and conclude that the iron atoms exist as a low spin ferric pair in the active NHase. As for species A, Mössbauer parameters tell us that it can be assigned to either low-spin Fe^{2+} or low-spin Fe^{3+}. The n^2 value of inactive NHase molecule is 5.68, and since the Mössbauer parameters of species B is almost identical in the inactive and active NHases, the n^2 value of species B in the inactive enzyme should also be 3.50. The n^2 value of species A will then be 5.68 −3.50 = 2.18. This value is too small to assign species A to be low-spin

Table II

n^2 values of iron in various states

	State	n^2
Spin-only [4]	LS Fe^{2+}	0
	LS Fe^{3+}	3
	HS Fe^{2+}	24
	HS Fe^{3+}	35
(Heme iron)		
Hemoglobin [4]	LS Fe^{3+}	4.93
	HS Fe^{3+}	34.60
(Non-heme iron)		
Ferredoxin (oxidized) [5]	2(LS Fe^{3+})*	0.99
Ferredoxin (reduced) [5]	LS Fe^{2+}, LS Fe^{3+}	3.86
Soy bean lipoxygenase [6]	HS Fe^{2+}	26.01
Rubredoxin [7]	HS Fe^{3+}	34.22

LS, low spin; HS, high spin; numbers in brackets indicate reference numbers; *antiferromagnetic coupling.

Table III
The behavior of iron atoms in the photosensitive NHase

	Inactive NHase				Active NHase			
Species	A	B	C	Total	B	B	C	Total
Iron state	(Fe^{2+})	(Fe^{3+})			(Fe^{3+})	(Fe^{3+})		
n^2	0	3.50	2.18	5.68	3.50	3.50	0	7.00

Fe^{3+} (Table II). Thus it is more reasonable to assign species A to be low-spin Fe^{2+} ($n^2 = 0$), and consider an existence of some other component (let us name this species C) which exhibits paramagnetic susceptibility with an n^2 value of 2.18. The behavior of iron atoms in the photoactivation process is summarized in Table III. As is evident from Table III, one of the two iron atoms in the NHase is photooxidized from Fe^{2+} to Fe^{3+}, and the electron is apparently transferred to species C, an electron acceptor, which cancels out its magnetic susceptibility. Also, this phenomenon is observed independent of the presence of oxygen (data not shown) indicating that oxygen is not an electron acceptor in this system. This interpretation is reasonable because the ESR spectrum of the active NHase from *Brevibacterium* sp., which is believed to be the same enzyme as that from *Rhodococcus* sp. [8], only shows characteristic low-spin Fe^{3+} spectrum [9]. An electron transfer similar to this type is well observed in protein complexes such as photosynthetic reaction center [10], but this type of intramolecular photooxidation involving iron in a single protein molecule is, to our knowledge, the first to be reported.

Studies are in progress to determine the structure of the reaction/photosensitive center(s) of the NHase by X-ray crystallography [11] which is excepted to give a better insight into the behavior of iron center in this enzyme.

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