Cu(II) Coordination Structure in NAD Glycohydrolase from Agkistrodon acutus Venom Studied by EPR Spectroscopy

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NAD glycohydrolase (NADase) from *Agkistrodon acutus* venom and several Cu(II) model complexes was studied by EPR spectroscopy. It was found that three nitrogen atoms may coordinate to Cu(II) ions and these nitrogen atoms might be from the imidazole groups of histidines in NADase molecule.

NAD glycohydrolase (NADase, EC 3.2.2.5) can catalyze the hydrolysis of the nicotinamide-ribose bond to form nicotinamide and adenosine diphosphoribose (ADP-Rib). NADase is present in various microorganisms,[1–4] animal tissues[5–8] and animal venom.[9–11] NADases are also found in association with the membranes of most cell types, including erythrocytes in mammals.[12–14]

Snake venom contains many kinds of proteins and enzymes. The presence of NADase in snake venom was first demonstrated by Tatsuki et al.[9] A NADase has been purified from *Agkistrodon acutus* venom, which was found to contain one Cu(II) ion per NADase molecule and the copper ion is essential for its activity.[11] In order to know the role of Cu(II) in the enzyme, it is necessary to further investigate the Cu(II) coordination structure in NADase molecule.

Electron paramagnetic resonance spectroscopy (EPR) is a very useful method and was often used to study the copper complexes.[15–17] In this letter, we report the coordination structure of Cu(II) ion in NADase molecule studied by EPR spectroscopy. It was found that at least three nitrogen atoms are coordinated to Cu(II) ion and the nitrogen atoms may be from the imidazole groups in histidines of NADase comparing to several Cu(II) model complexes.

NADase was purified to electrophoretic homogeneity according to the method of Huang.[11] X-band room-temperature and low-temperature EPR spectra were recorded with an ER-200D-SRC-10/12 EPR spectrometer. Microwave frequency was 9.01 GHz. The spectra was marked with 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) as an g-value standard, of which the g value is 2.0037 ± 0.0002. The frequency was monitored using a Microwave controller ER048H and the magnetic field was accurately determined by NMR Gaussmeter (BRUKER ER035M). The EPR spectroscopy of model complexes were recorded under modulation amplitude 3.2 Gauss and microwave power 20 mW at room temperature, and 6.3 G, 20 mW at 100 K, that of NADase under 10.0 G, 20 mW at room temperature, and 6.3 G, 20 mW at 100 K, respectively. The concentrations of model complexes were 4.8 mmol/L expect for $CuPyr_4$, 2.4 mmol/L, and NADase 1.0 mmol/L. All the samples were dissolved in a buffer solution containing 0.02 mol/L sodium acetate and 20%(v/v) dimethylformamide (DMF).

Figure 1 shows the EPR spectra of Cu(II) model complexes at room temperature (Figure 1A) and at low temperature (100 K, Figure 1B). The g and A values were calculated according to the g-value standard of DPPH (in Table 1).

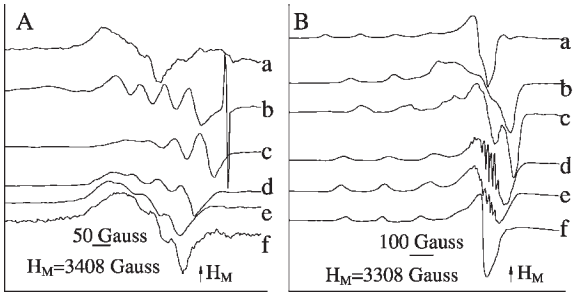

Figure 1. X-bands EPR spectra of Cu(II) complexes. A, room temperature. B, 100 K. a, $Cu(H_2O)_6^{2+}$; b, $Cu(NH_3)_6^{2+}$; c, CuHL; d, $CuImi_4$; e, $CuPyr_4$; f, CuEDTA. HL: 5,5,7,12,12,14-hexamethyl-1,4,8,11-tetraazacycloter-adecane-N′-acetic acid; Imi: imidazole; Pyr: pyridine; EDTA: ethylene diamine tetraacetic acid.

Table 1. Approximate EPR parameters of model complexes and NADase in solutions

Complex	g (298 K)	$g_\parallel$ (100 K)	$g_\perp$ (100 K)	g (100 K)	A^a (298 K)	$A^a_\parallel$ (100 K)	$A^a_\perp$ (100 K)
$Cu(H_2O)_6$	2.206	2.357	2.075	2.169	60.7	152	15.1
$Cu(NH_3)_6$	2.318	2.248	2.065	2.126	69.8	190	9.7
CuHL	2.119	2.225	2.056 2.019	2.100	74.2	185	18.8
$CuImi_4$	2.142	2.261	2.055	2.124	66.0	190	4.0
$CuPyr_4$	2.164	2.229	2.058	2.128	63.7	186	2.6
CuEDTA	2.157	2.290	2.067	2.141	65.4	160	18.1
NADase		2.231	2.0469 2.009	2.095		180	

[a]Unit: $\times 10^{-4}$ cm^{-1}.

Paramagnetic property of NADase in the solution form is shown at room temperature (Figure 2a) and hyperfine splitting signal was more distinct when the temperature decreased to 100 K (Figure 2b). The finer and more distinct splitting peaks were seen at low temperature than that at room temperature. At least seven turning points were obviously observed in low field region of $g_\perp$, and additional splitting were also found in the direction of $g_\parallel$. If the superhyperfine splitting was due to the nitrogen coordination

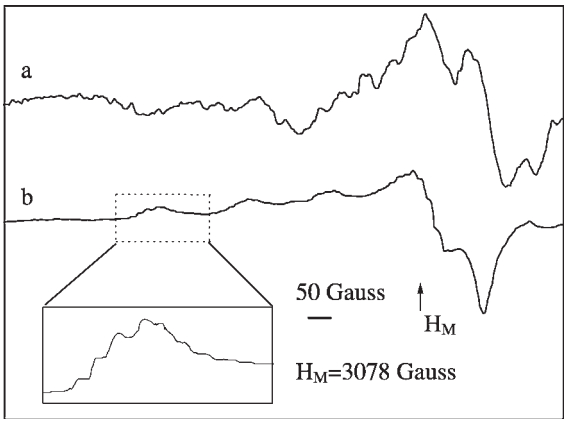

Figure 2. X-band of EPR spectra of NADase in solutions. (a) room temperature, (b) 100 K, Modulation amplitude (a) 10.0 G, (b) 6.3 G, both of the microwave power were 20 mW. The concentration of NADase was 1.0 mmol/L in the buffer solution containing 0.02 mol/L sodium acetate and 20%(v/v) DMF. Inset shows the expanded spectra of dotted frame in line b.

with Cu(II), it could be inferred that at least three atoms are coordinated to Cu(II) ion in the equatorial plane because I of ^{14}N atom is 1 and the number of hyperfine splitting peaks was more than 7 according to $(2nI + 1)$ rule.

The width between hyperfine splitting peaks in the low field region of $g_\perp$, viz. hyperfine splitting constant of N atom, $A_{N(\perp)} = 1.4$ mT, $A_{N(\|)} = 2.0$ mT, in the direction of $g_\|$, which was due to hyperfine splitting of N atoms coordinating to Cu(II) in the equatorial plane. In addition, EPR spectrum of NADase showed more anisotropic, that is, $g_x \neq g_y$, which suggested that coordination structure around Cu(II) ion in the enzyme molecule deviate from the square plane and no D_{4h} axial symmetry exist. Type I (pseudo-tetrahedral or blue) and type II (nearly square planar) of copper ions in copper proteins were classified according to the values of hyperfine constant of copper ions.[18,19] The copper ion belongs to type II when A value is more than 140×10^{-4} cm^{-1}, and type I when less than 140×10^{-4} cm^{-1}. $A_{Cu(II)} = 180 \times 10^{-4}$ cm^{-1} (in Table 1), indicated that the copper in NADase belongs to type *II*. According to the $g_\|$-$A_\|$ diagram, the EPR parameters of NADase also suggested that the copper site in NADase is a type II copper site with the coordination structure of CuN4 or CuN2O2.[20]

Comparing the EPR parameter of NADase with that of small molecular coordination complexes of Cu(II) in Table 1 and others,[21,22] it was found that the EPR parameters of NADase were much closer to that of CuHL and $CuIm_4$ and $Cu(P2A\text{-}R)_2$ (R = Norb, $g_\| = 2.206$, $g_\perp = 2.060$, $g = 2.109$, $A_\| = 168 \times 10^4$ cm^{-1}; R = Dma, $g_\| = 2.186$, $g_\perp = 2.053$, $g = 2.097$, $A_\| = 188 \times 10^4$ cm^{-1})[22] than the others, which implied that it is N atoms in imidazoles of histidines on the side chains coordinate to Cu(II) in NADase molecule. Coordination structure of Cu(II) ion in NADase may be also similar to that of CuHL, which is the mixture coordination of N and O atoms. Anisotropism shown in EPR spectrum of NADase was in accordance with that of CuHL, which implied that the coordination structure of Cu(II) ion in NADase molecule be much similar to that of CuHL.

In summary, the EPR spectra on the NADase at room temperature and low temperature have been used to study the coordination structure of Cu(II) in the NADase enzyme. It was found that three N atoms may be bound to Cu(II) ion and the structure is the distorted square plane. From the comparison with several Cu(II) model complexes, it could be inferred that the nitrogen atoms coordinated to Cu(II) may be from the imidazole groups of histidines in NADase molecule and the ligands coordinated to Cu(II) perhaps contain O atoms.

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