Effect of Copper(I) on Hydrogenase Activity from *Desulfovibrio vulgaris* (Miyazaki)

Toshiaki Kamachi and Ichiro Okura*

Department of Bioengineering, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226

(Received August 19, 1997)

Effect of copper ion on the activity of the hydrogenase from *Desulfovibrio vulgaris* (Miyazaki) was studied. When the hydrogenase was incubated with Cu(II) ion, the hydrogenase activity did not change. The hydrogenase activity was inhibited when the hydrogenase was incubated with copper ion in the presence of sodium ascorbate, suggesting the effect of Cu(I) ion. ESR studies indicate that the signal intensity attributed to [3Fe–4S] cluster decreased with decreasing the activity. Copper contents of hydrogenase increased, but the contents of iron and nickel did not change. Results obtained demonstrate that incorporated Cu(I) affects the hydrogenase a activity by the perturbation of [3Fe–4S] cluster.

The hydrogenase from *D. vulgaris* (Miyazaki) has been purified, and the catalytic and the molecular properties have been studied extensively.[1—5] In the presence of carbon monoxide, heavy metal ions or chelate compounds, hydrogenase activity is strongly inhibited.[6—8] Especially, copper ion and Hg ion are known to inhibit the hydrogenase strongly, but the inhibition mechanism is not clarified yet.

The effect of Hg(II) for *D. vulgaris* (Miyazaki) hydrogenase has been reported[9] and the mechanism of Fe extraction from iron sulfur cluster(s) by Hg(II) ion has been proposed. The effect of Cu(I) ion for *D. gigas* hydrogenase, which is the same family as the hydrogenase from *D. vulgaris* (Miyazaki) from the viewpoints of metal contents, subunit structure, and prosthetic groups, has been reported by Fernandez et al. They indicate that the treatment Cu(I) caused the decrease of Fe contents of hydrogenase and that the [4Fe–4S] clusters seemed to be mainly responsible for the loss of hydrogenase activity, but the precise inhibition mechanism remains unclear.[7]

In this study, we describe the effect of Cu(I) on the hydrogenase from *D. vulgaris* (Miyazaki) and discuss the inhibition mechanism.

Results and Discussion

The sample solution of the hydrogenase as isolated was incubated with 1.0 mM (1 M = 1 mol dm^{-3}) of Cu(II) under Ar at 30 °C, and the hydrogen evolution rate from reduced methyl viologen was measured. Effect of incubation time is shown in Fig. 1. Hydrogenase activity was not affected by Cu(II). When 5.0 mM of ascorbate was added to the above sample solution, as indicated by the arrow in the figure, the hydrogenase activity rapidly decreased. Almost all the hydrogenase activity was lost in 25 min after incubation with Cu(II) in the presence of ascorbate. The hydrogenase activity was not affected by ascorbate alone. The added ascorbate reduces Cu(II) to Cu(I). The inhibition of hydrogenase with

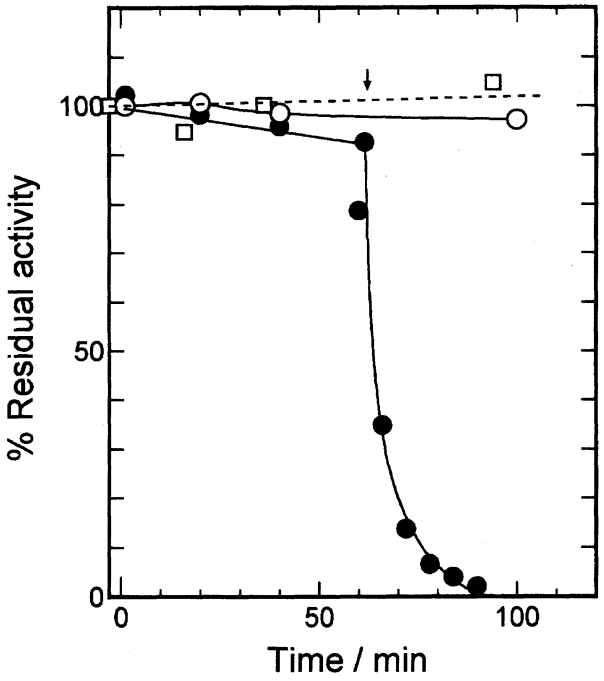

Fig. 1. Effect of Cu(II) on hydrogen evolution activity. Triton X-100 solubilized hydrogenase was incubated with 1.0 mM $CuCl_2$ (●) or without $CuCl_2$ (○) or sodium ascorbate alone (□) under Ar at 30 °C and after 60 min incubation, sodium ascorbate was added anaerobically with gas tight syringe to give final concentration of 5.0 mM.

copper ion in the presence of ascorbate was also observed under air, indicating that the inhibition was not caused by the hydroxyl radical (Haber–Weiss mechanism). The hydrogenase activity did not recover after removal of excess Cu(I) by gel filtration with Sephadex G-50. These results indicate that the hydrogenase from *D. vulgaris* (Miyazaki) was inhibited by Cu(I) irreversibly but not by Cu(II).

The effect of Cu(I) on the hydrogenase was investigated

by ESR method to focus on the iron–sulfur center(s) and Ni center (Figs. 2 and 3). The intact hydrogenase shows a typical [3Fe–4S] ESR signal below 30 K as shown in Fig. 2a. By the treatment of hydrogenase with Cu(I) as mentioned in "experimental", the ESR signal generated from [3Fe–4S] cluster was eliminated and a new Cu(II) signal was generated as shown in Fig. 2b. At high temperature, the intact hydrogenase shows typical ESR spectra generated from Ni center as shown in Fig. 3a. The ESR signal corresponding to Ni center also changed by the Cu(I); as shown in Fig. 3b. These results demonstrate that the destruction of [3Fe–4S] cluster and/or Ni center occurs.

To reveal the inhibition mechanism by Cu(I), time dependence of ESR intensity corresponding to the [3Fe–4S] cluster was measured during incubation with Cu(I). The hydrogenase was incubated with 0.1 mM of Cu(II) in the presence of 5.0 mM of sodium ascorbate at 4 °C. And excess copper ions were also removed from the hydrogenase samples by using gel filtration. Results are shown in Fig. 4. The intensities of the doubly integrated ESR signal of the [3Fe–4S] cluster decreased time-dependently. And the hydrogenase activity decreased simultaneously. Without copper ions, the

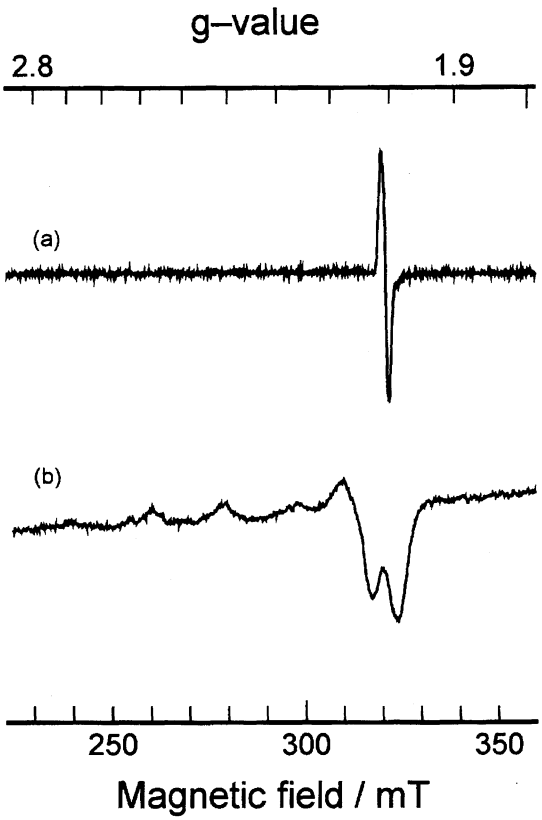

Fig. 2. Effect of Cu(I) on the ESR spectrum at low temperature. (a): Intact hydrogenase; (b): Cu(I) treated hydrogenase. ESR spectra were recorded (a) 8.9 K; (b) 9.0 K. Other experimental conditions; (a): microwave frequency: 9013.6 MHz, microwave power: 1 mW, modulation amplitude: 0.25 mT, gain: 500; (b): microwave frequency: 9050.2 MHz, microwave power: 1.00 mW, modulation amplitude: 0.2 mT, gain: 200.

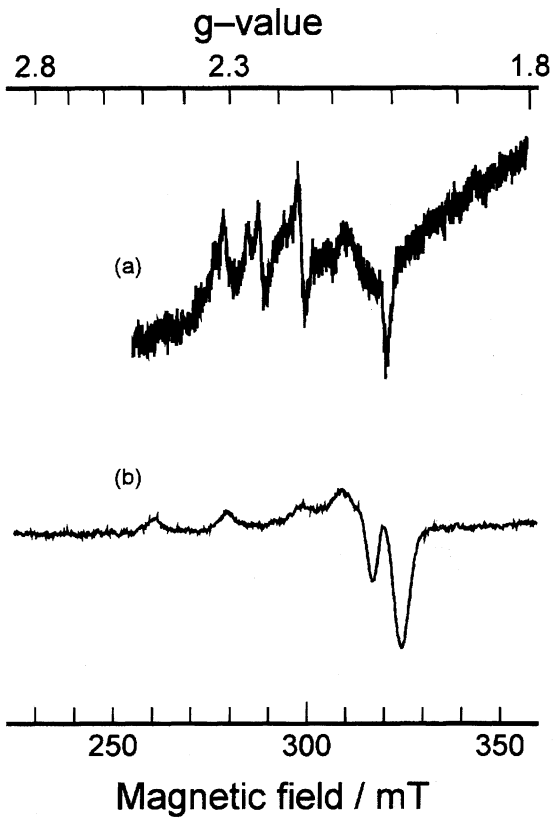

Fig. 3. Effect of Cu(I) on the ESR spectrum at high temperature. (a): Intact hydrogenase; (b): Cu(I) treated hydrogenase. ESR spectra were recorded at (a) 113.5 K; (b) 118.6 K. Other experimental conditions; (a): microwave frequency: 9029.9 MHz, microwave power: 10 mW, modulation amplitude: 1 mT, gain: 2000; (b): microwave frequency: 9004.6 MHz, microwave power: 1 mW, modulation amplitude: 0.25 mT, gain: 200.

ESR signal intensity of [3Fe–4S] cluster did not change during treatment of sodium ascorbate. These results indicates the inhibition of the hydrogenase with Cu(I) was caused by the destruction of [3Fe–4S] cluster. These data indicate that either the iron atom(s) in the cluster and/or the nickel atoms may be displaced by copper ions, or Cu(I) may strongly interact with the above center(s).

The metal contents of the hydrogenase was evaluated by Inductively Coupled Plasma (ICP) emission spectrometry. Figure 5 shows the time dependence of metal contents and hydrogenase activity during incubation with 0.1 mM of $CuCl_2$ and 5.0 mM of ascorbate at 4 °C under Ar. One molecule of the native hydrogenase contains about 13 Fe ions and one Ni ion. The Cu contents of the hydrogenase increased simultaneously with the loss of hydrogenase activity during incubation with copper ion and ascorbate. But the Fe contents and Ni contents were consistent during copper ion treatments. These results indicate that the copper ions did not replace the iron and nickel from the hydrogenase. Our previous study[9] reveals that Hg ions inhibit hydrogenase by extraction and exchange Fe with Hg.

Since the copper ion is known to be chelated by amide

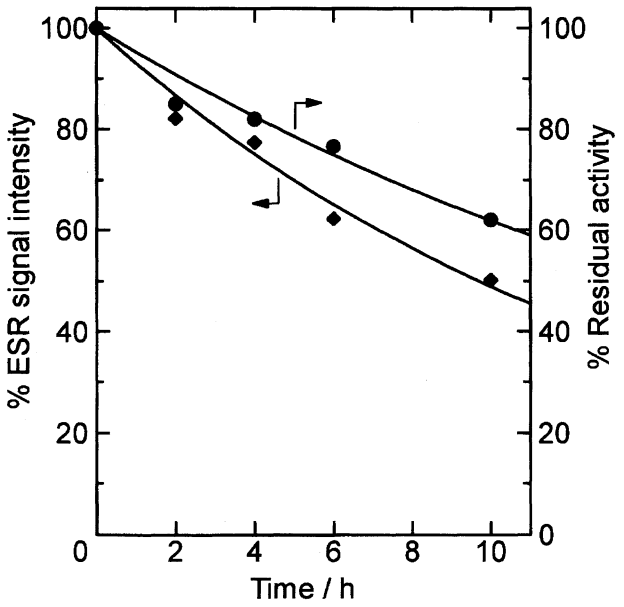

Fig. 4. Time dependence of [3Fe–4S] cluster ESR signal intensity (◆) and hydrogenase activity (●) during incubation with 0.1 mM $CuCl_2$ and 5.0 mM sodium ascorbate at 4 °C under Ar.

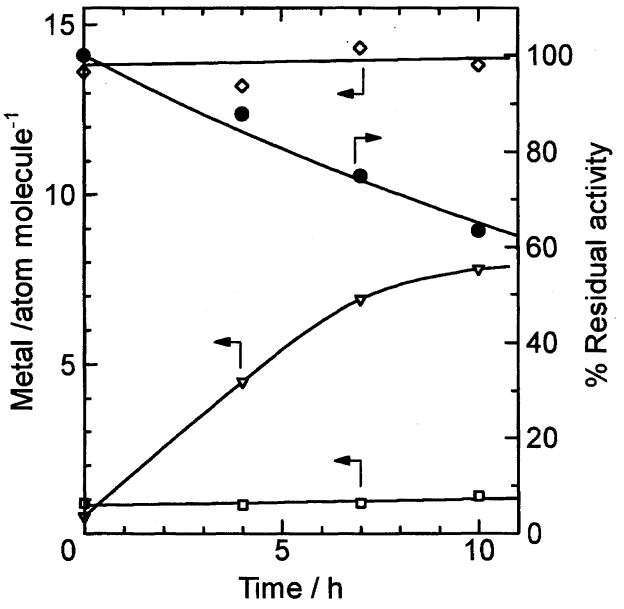

Fig. 5. Time dependence of metal contents and hydrogenase activity during incubation with 0.1 mM $CuCl_2$ and 5.0 mM sodium ascorbate at 4 °C under Ar. ●: Residual hydrogenase activity, ◇: Fe, □: Ni, ▽: Cu.

nitrogen, imidazole nitrogen in histidine or cysteine sulfur, it is probable that these amino acid residues in the hydrogenase chelate to copper ions and inhibit the activity. As soft acids prefer to coordinate to soft bases, Cu(I) may coordinate to a soft base such as cysteine residue. Our previous study revealed that Hg(II), a soft acid, inhibited hydrogenase. On the other hand, hard acids such as Fe(III), Ni(II), and Cu(II) did not affect hydrogenase activity. The soft acid metal ions seem to attack the cysteine residues to inhibit the hydrogenase activity. As the inhibition of *D. gigas* hydrogenase by Cu(I) is caused by the extraction of Fe ion by Cu(I), the inhibition mechanism may be different.

Experimental

Desulfovibrio vulgaris (Miyazaki) was grown anaerobically at 30 °C according to the literature,[4] and stored at −80 °C before use.

The hydrogenase was solubilized from membrane fraction using nonionic detergent Triton X-100 and was purified to homogeneity as reported previously.[10] The purified hydrogenase showed single band on native-PAGE.

The hydrogenase activity was measured by the hydrogen evolution rate in the presence of dithionite-reduced methylviologen in a 5.0 ml test tube sealed with Septa at 30 °C. The reaction mixture (4.0 ml) contains 50 mM of Tris-HCl buffer (pH 7.4), 3 mM of methylviologen and 20 mM of dithionite.

Protein concentration was determined by modified Lowry protein assay[11] with bovine serum albumin as a standard.

The hydrogenase was incubated with 20 mM of $CuCl_2$ in the presence of 20 mM ascorbate at 4 °C under Ar atmosphere. The copper ion-treated hydrogenase was applied to Sephadex G-25 gel chromatography to remove excess copper ion and ascorbate in order to conduct ESR measurements. ESR spectra were recorded with JEOL FE3XG spectrophotometer equipped with a variable temperature cryostat (Air Products Heli-tran LTR-3-110).

ICP spectra were recorded with Seiko SPS1500VR ICP emission spectrophotometer.

This work was partially supported by a Grant-in Aid for Scientific Research on Priority Area "Photoreaction Dynamics" No. 08231226 from the Ministry of Education, Science, Sports and Culture.

References

1) Y. Higuchi, N. Yasuoka, M. Kakudo, Y. Katsube, T. Yagi, and H. Inokuchi, *J. Biol. Chem.*, **262**, 2823 (1987).

2) H. Deckers, F. R. Wilson, and G. Voordouw, *J. Gen. Microbiol.*, **136**, 2021 (1990).

3) M. Asso, B. Guigliarelli, T. Yagi, and P. Bertrand, *Biochim. Biophys. Acta*, **1122**, 50 (1992).

4) T. Yagi, K. Kimura, H. Daidoji, F. Sakai, S. Tamura, and N. Inokuchi, *J. Biochem.*, **79**, 661 (1976).

5) C. E. Hatchikian, M. Bruschi, and J. LeGall, *Biochem. Biophys. Res. Commum.*, **82**, 451 (1978).

6) W. Van der Zwaan, S. P. J. Albracht, R. D. Fontijn, and W. B. M. Roelfs, *Biochim. Biophys. Acta*, **872**, 208 (1986).

7) V. M. Fernandez, M. L. Rua, P. Reyes, R. Cammack, and C. Hatchikian, *Eur. J. Biochem.*, **185**, 449 (1989).

8) T. F. Fagan and G. Mayhew, *Biochem. J.*, **293**, 237 (1993).

9) T. Kamachi, S. Uno, T. Hiraishi, and I. Okura, *J. Mol. Catal.*, **96**, 329 (1995).

10) T. Kamachi, S. Uno, T. Hiraishi, and I. Okura, *J. Mol. Catal.*, **95**, 93 (1995).

11) A. Bensadoun and D. Weinstein, *Anal. Biochem.*, **70**, 241 (1976).