

# Notes

## NAD<sup>+</sup> Reduction by Ethanol Depending on Mitochondrial Surface

Jae Yang Lee, Sang Jik Lee,\* and Ji Young Song\*

*Department of Biochemistry, Yeungnam University, Gyongsan 712-749, Korea*

*Received May 25 2002*

**Key Words :** NADH, Ethanol, Mitochondria

One recognizes that all enzymatic reactions are reversible to some degree<sup>1</sup> and much of the respiratory chain is reversible.<sup>2</sup> Although the usual electron transport relying on the external electron-transport chain<sup>3</sup> of mitochondrial outer membrane gets the electrons out from exogenous NADH via NADH-cytochrome b<sub>5</sub> reductase<sup>4</sup> (abbr. to *fb<sub>5</sub>* below) for the final reduction of oxygen, one of the present reporters observed earlier that this NADH oxidation could be reversed by ferrocyanide, an artificial reductant, on the surface of a mitochondrion for NAD<sup>+</sup> reduction.<sup>5</sup> This observation strengthened the probability that the potential for the reduction exercised by a reductant was multiplied with the catalysis given by mitochondrial surface which is built with oxidoreductases. Some textbooks in organic chemistry describe the capacity of alcohols to reduce nicotinamide adenine dinucleotide.<sup>6</sup> The hydroxyl carbon of the reductant alcohol is considered to be functional for the reduction. The bovine-heart mitochondria, presenting an excellent oxidative catalysis for exogenous NADH,<sup>7</sup> were made to reduce NAD<sup>+</sup> for its reduced counterpart production with the support of ethanol in this study. Demonstrating such capacity given by ethanol for the mitochondrial surface would make us explain the energy production, a phenomenon caused by usual oral alcohol ingestion.

### Experimental Section

The catalyst mitochondria from bovine heart were separated making use of Ernster-Nordenbrand method<sup>8</sup> as follows: We got rid of fatty tissue etc. from the heart for the purpose of using cardiac muscle only. This muscle lump obtained was washed with cold 0.15 M KCl several times to be dipped in a modified Chappell-Perry medium of 0.05 M Tris-HCl buffer, pH 7.4 containing 0.1 M KCl and 0.005 M MgSO<sub>4</sub>. We prepared the modified medium by eliminating ATP and EDTA from true Chappell-Perry medium to except hydroxyl species which could participate in the present experiment as if it were ethanol. The muscle lump taken out of the modified medium was cut into pieces with a mincer. This minced muscle tissue was homogenized by a glass

homogenizer with a teflon pestle using an ice bath at 0 to 2 °C. An AC-driven stirrer homogenized the minced tissue with the speed of 2,000 rpm at this temperature. The homogenate formed was suspended into the modified medium for the suspension to be centrifuged 10 mins at 650 × g. The supernatant fraction was decanted into a new tube and recentrifuged as before. The resulting supernatant was centrifuged 10 mins at 14,000 × g for pelleting required mitochondrial fraction. To the mitochondrial fraction a small amount of the modified medium was added for another centrifugation at 14,000 × g for 10 mins. The cardiac muscle mitochondria repelleted were stored at -5 °C in a refrigerator. The biuret method introduced by Rendina<sup>9</sup> and Layne<sup>10</sup> was used for quantifying mitochondrial protein employing a calibration curve drawn with bovine serum albumin as a standard.

We read the NADH formations in terms of 340 nm-absorbency increases for 1 min. at 24 °C for the four nonmitochondrial systems containing 0.1 M Tris-HCl buffer (pH 7.4), NAD<sup>+</sup>, rotenone, and ethanol before reading those for the four mitochondrial systems composed of NAD<sup>+</sup>/rotenone/ethanol/identical buffer with the same manner as described below. Each of these eight systems just mentioned contained not only 20 μL of 40 mM NAD<sup>+</sup> but 10 μL of 0.5 mM rotenone identically. The nonmitochondrial systems from 1 to 4 contained 0 to 2.380 mM ethanol respectively and the mitochondrial systems from a to d also contained 0 to 2.380 mM ethanol respectively (Table 1). Mitochondrial b system, *e.g.*, contained 937.08 μL of 0.1 M Tris-HCl buffer, 20 μL of 40 mM NAD<sup>+</sup>, 10 μL of 0.5 mM rotenone, 2.92 μL of aqueous ethanol (displaying net ethanol concentration of 0.149 mM for b system shown in Table 1), and 10 μL of 58 mg/mL mitochondria. Thus, each of the mitochondrial and the nonmitochondrial reaction systems corresponded to 980 μL one. For each of the eight systems shown above, nonmitochondrial plus mitochondrial ones, five distinct redox-probable mixtures containing identical constituents were composed separately for the measurements of 340 nm absorbencies five times for each system. After laying aside one minute at 24 °C, the redox reactions destined to occur on the mitochondrial surface were terminated adding 1mM mersaly1,<sup>11,12</sup> a *fb<sub>5</sub>* inhibitor. These five determinations for the five mixtures of the identical constituents would bless us

\*Present Address: Biotechnology Research Center, Pohang University of Science and Technology, Pohang 790-784, Korea

with just the same records if we were very lucky. The frequency for determining these NADH formations was in effect 40 times (5 times/system multiplied by 8 systems). This running was for the sake of statistical treatment (Table 1). The catalytic property of the mitochondrial surface oxidoreductases was tracked again by comparing the NADH formation for 0.59 mg/mL mitochondrial system (b system in Table 1) with that for the more diluted mitochondrial system (0.17 mg/mL mitochondria). This confirmational assay was also run 5 times separately not only for the concentrated mitochondrial system but also for the diluted one. We made any of these mitochondrial NAD<sup>+</sup> reduction systems contain at the beginning the components identically with those of mitochondrial(b) system listed in Table 1 except mitochondrial concentration.

### Results and Discussion

It is well admitted that ethanol yields energy when it is metabolized in the body.<sup>13</sup> The present experiment of ethanol oxidation depending on mitochondrial surface set the goal at explaining this energy production. Williams and Lansford<sup>14</sup> published an encyclopedia describing that once absorbed passively, alcohol is distributed throughout the body in proportion to the water content of the individual tissues. This description suggests what explains the inevitable contact between the diffusible alcohol molecules and the mitochondria within the cells all over the body when the liquor is drunk. It stands to reason that ethanol-mitochondrion contact once made could induce a chemical change which is imposed on ethanol molecule by any catalysis driven by mitochondrial surface. The NADH formation, *i.e.*, 340 nm-absorbency increase, rendered by the mitochondrial surface in the presence of ethanol does not involve the NADH dehydrogenase in mitochondrial inner membrane in the present study, because the activity of the dehydrogenase was always made to be blocked by rotenone.<sup>15</sup> When we measured the 340 nm-absorbency increases for 1 min. for the 5 mixtures per system of nonmitochondrial ethanolic NAD<sup>+</sup> systems, no supplement was observed in the absorbency increase in parallel with the increase in ethanol concentration (Table 1). Although ethanol concentration in nonmitochondrial ethanol-NAD<sup>+</sup> system increased from zero to 2.380 mM *via* 0.595 mM, the changes in 340 nm-absorbency increase were from 0.03620 to 0.03570 *via* 0.03544. There was virtually no change in absorbency increase. It may fairly be said that there was no potential within ethanol for NAD<sup>+</sup> reduction in the absence of

**Table 2.** Comparing NAD<sup>+</sup> Reductions by Ethanol Depending on Concentrated and Dilute Mitochondria

Mitochondrial systems (mg/mL)	NADH Productions $\pm$ Standard deviations
0.59	0.06416 $\pm$ 0.00193
0.17	0.04016 $\pm$ 0.00056

NADH production is in terms of 340 nm-absorbency increase. Refer to Table 1 and the text describing experimental methods for details.

mitochondrial catalysis after all. This finding was strengthened in respect that each of the 340 nm-absorbency increase values shown in Table 1 was the mean derived from five runnings using five separate mixtures. The base for strengthening this finding was also applicable to the mitochondrial measurements described below. The five determinations run for both mitochondrial and nonmitochondrial NADH-producing systems were also performed for examining the NADH production depending on mitochondrial concentration in ethanolic NAD<sup>+</sup> system (Table 2). In opposition to no mitochondrial intervention, the 340 nm-absorbency increases for mitochondrial ethanolic NAD<sup>+</sup> systems were subsequently measured. We witnessed the clear supplements in 340 nm-absorbency increases for mitochondrial NADH producing systems (Table 1). As the concentration of the ethanol brought into contact with mitochondria became larger from 0 to 2.380 mM, 340 nm-absorbency increase changed from 0.058 to 0.072 in this mitochondrial system of the ethanol oxidized. Ethanol did reduce an unknown oxidoreductase on mitochondrial surface for the production of reduced nicotinamide adenine dinucleotide.

Everyone recognizes that a drunken fellow breathe hard for the sake of more oxygen consumption.<sup>16</sup> This oxygen inhaling might be explained with the more NADH production by ethanol and subsequent electron transport from the nucleotide up to corresponding oxygen quantity. Everyone also recognizes that this ethanol inducing reduction of oxygen is inevitably coupled with ATP synthesis. The difference in NADH formation occurred between concentrated and diluted mitochondrial systems of NAD<sup>+</sup> plus ethanol is tabulated at Table 2. We could confirm that NAD<sup>+</sup> reduction by more concentrated mitochondrial system produced more NADH depending on ethanol from Table 2. Confirming the catalytic property of mitochondrial surface for ethanol oxidation was further emphasized by this observation. NADH production was accelerated by mitochondrial contact with ethanol as we were already aware. Ethanol was in favor of energy production in this respect, but it was felt anxious that the over-

**Table 1.** The Effect of Ethanol on NAD<sup>+</sup> Reduction in Terms of 340-Absorbency Increase in the Presence or Absence of Mitochondria

Notations for the 8 systems	Without mitochondria				With mitochondria			
	1	2	3	4	a	b	c	d
Ethanol levels (mM)	0	0.149	0.595	2.380	0	0.149	0.595	2.380
Absorbency increases	0.03620	0.03638	0.03544	0.03570	0.05802	0.06416	0.070940	0.071920
$\pm$ Standard deviations	$\pm$ 0.000829	$\pm$ 0.001033	$\pm$ 0.001137	$\pm$ 0.000792	$\pm$ 0.003994	$\pm$ 0.00193	$\pm$ 0.003815	$\pm$ 0.001134

Refer to the text describing experimental methods for the notations and the others.

production of NADH by ethanol could give rise to the disruption of a homeostasis.<sup>17</sup>

**Acknowledgement.** We thank Miss Chun, Hyun-Ju, Department of Biochemistry in Yeungnam University, for her labor concerning the present experiment.

### References

1. Palmer, T. In *Understanding Enzymes*; Ellis Horwood Ltd.: 1995; p 6.
  2. Nicholls, D. G. In *Bioenergetics*; Academic Press: London, New York, 1982; p 99.
  3. Mokhova, E. N.; Skulachev, V. P.; Zhigacheva, I. V. *Biochimica et Biophysica Acta* **1977**, *501*, 423.
  4. Mokhova, E. N.; Skulachev, V. P.; Zhigacheva, I. V. *Biochimica et Biophysica Acta* **1977**, *501*, 416.
  5. Yu, M. H.; Lee, S. J. *Bull. Korean Chem. Soc.* **2001**, *22*(1), 13.
  6. Solomons, T. W. G. In *Fundamentals of Organic Chemistry*; John Wiley/Sons: New York, 1982; p 385.
  7. Lee, S. J.; Lee, J. Y. *Korean Biochem. J.* **1992**, *25*, 719.
  8. Emster, L.; Nordenbrand, K. *Methods in Enzymology* **1967**, *10*, 86.
  9. Rendina, G. In *Experimental Methods in Modern Biochemistry*; W. B. Saunders Co.: Philadelphia, 1971; pp 75-77.
  10. Layne, E. *Methods in Enzymology* **1957**, *3*, 450.
  11. Skulachev, V. P. In *Membrane Bioenergetics*; Springer-Verlag: Berlin, Heidelberg, 1988; p 284.
  12. Mokhova, E. N.; Skulachev, V. P.; Zhigacheva, I. V. *Biochimica et Biophysica Acta* **1977**, *501*, 421.
  13. Wilson, E. D.; Fisher, K. II.; Fuqua, M. E. In *Principles of Nutrition*; John Wiley and Sons Inc.: New York, 1975; p 45.
  14. Williams, R. J.; Lansford, E. M. In *Encyclopedia of Biochemistry*; Robert E. Krieger Pub. Co.: Huntington, New York, 1981; p 24.
  15. Stryer, L. In *Biochemistry*; W. H. Freeman and Co.: New York, 1995; p 544.
  16. Lieber, C. S. *Nutr. Rev.* **1988**, *46*(7), 241.
  17. Lieber, C. S. *Am. J. Clin. Nutr.* **1993**, *58*, 430.
-