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4 *Pro-R* Hydrogen of NADPH was Abstracted for Enzymatic Hydride Transfer by *N*-Ethylmaleimide Reductase of *Yarrowia lipolytica*

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MIZUGAKI, M., MIURA, K., YAMAMOTO, H., KAYABA-NAKAZAWA, M., EDO, K., TOMIOKA, Y. and HISHINUMA, T. *4 Pro-R Hydrogen of NADPH was Abstracted for Enzymatic Hydride Transfer by N-Ethylmaleimide Reductase of Yarrowia lipolytica.* Tohoku J. Exp. Med., 1997, **181** (4), 447-457 —— We studied the steric course of the reaction catalyzed by the *N*-ethylmaleimide (NEM) reductase of *Yarrowia* (*Candida*) *lipolytica* (*Y. lipolytica*), using 4*R*-[4-$^2H_1$]NADPH and 4*S*-[4-$^2H_1$]NADPH as cofactors and *N*-ethylcitraconimide as substrate. Active substrates and inhibitors of NEM reductase and its subcellular distribution were also investigated to clarify the biochemical properties of this enzyme. NEM reductase catalyzes the reduction of *N*-ethylmaleimide to *N*-ethylsuccinimide with NAD(P)H as the cofactor. Several maleimide and cyclopentenone derivatives tested were also active substrates for NEM reductase of *Y. lipolytica*. Some pyrazolone derivatives, particularly 1-phenyl-5-pyrazolone, were found to be effective inhibitors of NEM reductase. Subcellular localization of NEM reductase was carried out using protoplast formation and differential centrifugation. Ninety-eight percent of the NEM reductase activity was recovered in the cytosolic fraction, indicating that NEM reductase in *Y. lipolytica* was the cytosolic enzyme. We also determined the stereochemical specificity of the reduction of *N*-ethylcitraconimide by NEM reductase in *Y. lipolytica*, showing that 4 *Pro-R* hydrogen of NADPH was abstracted for enzymatic hydride transfer by NEM reductase, and two hydrogen atoms from NADPH and H_2O added to opposite faces of the double bond of *N*-ethylcitraconimide. —— *N*-ethylmaleimide metabolism; oxidoreductase antagonist and inhibitor; unsaturated fatty acids

The yeast *Yarrowia* (*Candida*) *lipolytica* (*Y. lipolytica*) is a typical organism that can utilize *n*-alkanes or long-chain fatty acids as the sole carbon source (Nyns et al. 1967). However, phylogenetic analysis using sequence data from cellular

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ribosomal RNAs or cloned 18S ribosomal genes indicated that the sequence of *Y. lipolytica* was quite distantly related to those of the other *Candida* species (Barns et al. 1991). Recently, several new and emerging yeasts have been isolated from blood cultures of the unusual yeast infection (Hazen 1995). At least one case of *Y. lipolytica* infection has also been reported (Walsh et al. 1989).

Maleimide derivatives, such as *N*-ethylmaleimide, have an active double bond and are known to be active alkylating agents for sulfhydryl, amino and imidazole groups (Gregory 1955; Morell et al. 1964; Leslie 1965; Shrapless and Flavin 1966). Thus, showdomycin, one of these derivatives of carbon-linked nucleoside antibiotics (Darnall et al. 1967) and first isolated from *Streptomyces showdoensis* by Nishimura et al. (1964), had effective antimitotic activity on bacterium cells (Titani and Tsuruta 1976).

Many species have various systems of unsaturated fatty acid degradation as part of their basal metabolism. In β-oxidation of unsaturated fatty acids, for example, there are differences between *Escherichia coli* (*E. coli*), *Y. lipolytica*, and rat. We previously suggested the existence of a NADPH-dependent *cis*-2-enoyl-CoA reductase pathway in *E. coli*, which is different from NADPH-dependent *trans*-2-enoyl-CoA reductase (Mizugaki et al. 1979).

We also noted *N*-ethylmaleimide reducing enzyme (NEM reductase) activity in the studies of *cis*-2-enoyl-CoA reductase on the unsaturated fatty acid metabolism (Mizugaki et al. 1979, 1981). NEM reductase of *Y. lipolytica* catalyzes the reduction of *N*-ethylmaleimide to *N*-ethylsuccinimide with NAD(P)H, has a molecular mass of 47,000 by SDS-PAGE and 43,000 by gel-filtration chromatography, and is active against five-membered ring compounds such as 4-cyclopenten-1, 3-dione (Mizugaki et al. 1989). However, it is not known whether NEM reductase acts on any endogenous substrate, and the physiological function of this enzyme is not clear. We speculated that NEM reductase has some functions for the metabolism related to unsaturated fatty acids because NEM reductase activity was detected with the supplementation of linoleic acid into culture medium for *E. coli* and *Y. lipolytica* (Mizugaki et al. 1981, 1989).

In this study, to clarify the biochemical properties of NEM reductase, we studied the steric course of the reaction catalyzed by NEM reductase of *Y. lipolytica*, using 4*R*-[4-$^2H_1$]NADPH and 4*S*-[4-$^2H_1$]NADPH as cofactors and *N*-ethylcitraconimide and NEM as substrates. Active substrates and inhibitors of NEM reductase and its subcellular distribution were also investigated.

Materials and Methods

Yeast strain and culture

Yarrowia lipolytica strain IMR30002, provided by Dr. Kamio, Y., Faculty of Agriculture, Tohoku University, was used in this study. *Y. lipolytica* was precultured at 27°C with agitation at 150 reciprocations per min for 24 hr in medium A (0.5% yeast extract, 1% polypeptone and 2% dextrose) and then

cultured at 27°C for 18 hr in medium B (3% meat extract supplemented with 0.3% linoleic acid).

Chromatography

Cell-free lysates of *Y. lipolytica* were applied to a DEAE cellulose column equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA. A linear gradient of 0.05 to 0.3 M KCl was used to elute the bound protein, and NEM reductase activity of the fractions was assayed and pooled. The pooled fractions were concentrated, dialyzed and applied to Sephacryl S-200 in 50 mM sodium phosphate buffer (pH 7.2) and 0.5 M NaCl. The eluted fractions were assayed for NEM reductase and pooled. This enzyme solution was used in this study.

Chemicals and materials

N-Ethylmaleimide, *p*-quinone, menadione, L-ascorbic acid and uracil were purchased from Wako Pure Chemical Industries, Osaka. Maleimide derivatives (phenylmaleimide, butylmaleimide, cyclohexylmaleimide, carboxymethylmaleimide, *N*-ethylcitraconimide, *N*-phenylcitraconimide), unsaturated lactone, pyrimidone derivatives (such as 1, 3-diethylpyrimidine-2, 5-dione, and 1, 3, 4-triethylpyrimidine-2, 5-dione), pyridazinone derivatives (3, 6-pyridazinone, 4-ethylpyridazine-3, 6-dione) and pyrazolone derivatives (1, 2-dimethyl-5-pyrazolone, 1-phenyl-2-methyl-5-pyrazolone, 1-phenyl-2-ethyl-5-pyrazolone, 1-phenyl-5-pyrazolone, 1-phenyl-3-methyl-5-pyrazolone, 1-phenyl-2, 3-dimethyl-4-amino-5-pyrazolone, 1-phenyl-2, 3-dimethyl-4-dimethylamino-5-pyrazolone) were chemically synthesized (Mehta et al. 1960). G2201-C was a gift from Dr. Pole, Glaxo Co., Middlesex, UK. NADPH and NADH were purchased from Oriental Yeast, Tokyo. Showdomycin was obtained from Shionogi Pharmaceutical Co., Ltd., Osaka. Pentenomycin triacetate was obtained from Tanabe Pharmaceutical Co., Ltd., Osaka. Benzimidazolylphenylmaleimide (BIPM) and anilinonaphthylmaleimide (ANM) were gifts from Dr. Kanaoka, Y., Hokkaido University. All other reagents used were of analytical grade. Sephacryl S-200 was purchased from Pharmacia Fine Chemical, Uppsala, Sweden. DEAE-cellulose was purchased from Seikagaku Kogyou Ltd., Tokyo.

Preparation of deuterium-labeled NADPH

Two specimens of stereochemically deuterium-labeled NADPH were prepared according to the methods of Seyama et al. (1977) and Kawaguchi et al. (1980). Deuterium-labeled NADPHs, 4*R*-[4-$^2H_1$]NADPH and 4*S*-[4-$^2H_1$]NADPH, were made by reducing 4-deuterio-$NADP^+$ with glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase, respectively.

Preparation of protoplasts and subcellular fractionation

Protoplasts from the cells of the exponential growth phase were prepared in 50 mM Tris-HCl buffer (pH 7.4) containing 0.8 M sorbitol and 0.1 M 2-mercaptoethanol with cell-wall-decomposing enzyme, Zymolyase 100,000 (125 μg/ml), by a similar method to Fukui et al. (1975). The following fractionation of particles was carried out at 0–4°C according to Fukui et al. (1975) and Mishina et al. (1978). Briefly, the protoplasts obtained from 4.0 g cells were suspended in 15 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.65 M sorbitol and 0.5 mM EDTA, then homogenized for 5 min under cooling in a teflon-glass homogenizer, and centrifuged for 10 min at 1,500 $\times$ g at 4°C. Pellets sedimented were pooled (debris), and the supernatant (supernatant 1) was centrifuged again at 25,000 $\times$ g for 60 min. The second supernatant (supernatant 2) was pooled, and pellets (heavy particulate fraction) were gently suspended in 50 mM Tris-HCl buffer (pH 7.4). The supernatant 2 was centrifuged again at 230,000 $\times$ g for 1 hr. The third supernatant (cytosol fraction) was pooled, and pellets (microsomal fraction) were gently suspended in 50 mM Tris-HCl buffer (pH 7.4).

Enzyme assay

NEM reductase activity was measured as previously described (Mizugaki et al. 1989). Briefly, the assay was performed in 50 mM potassium phosphate buffer (pH 7.0) containing 1 to 10 nmol of *N*-ethylmaleimide, 125 nmol of NADPH, and protein in a total volume of 0.8 ml. The reaction was initiated by the addition of the NEM solution, and the rates were measured by recording the oxidation of NADPH ($\varepsilon_{340} = 6.2\ mM^{-1} \cdot cm^{-1}$) with a spectrophotometer (type 557; Hitachi, Hitachi). *cis*-2-Enoyl-CoA reductase was determined as described before (Mizugaki et al. 1979). The coenzyme A derivatives were prepared according to the method of Weeks and Wakil (1968), and the concentration of acyl-CoA esters were determined according to the method of Ellman (1959). Each marker enzyme was measured according to the references; that is, catalase (Aebi 1984) and isocitrate lyase (Hirai et al. 1976) for peroxisomal marker, cytochrome c oxidase (Wharton and Tzagoloff 1967) for mitochondrial marker, NADPH-cytochorome c reductase (Kawamoto et al. 1977) for microsomal marker, and glucose-6-phosphate dehydrogenase (Hirai et al. 1976) for cytosolic marker.

Preparation and analysis by gas chromatography and gas chromatography/mass spectrometry

The enzyme reaction for NEM reductase was performed in 10 mM potassium phosphate buffer (pH 7.4) containing 2.2 μmoles of coenzyme (NADPH, 4*R*- or 4*S*-[4-$^2H_1$]NADPH) and 7 μmoles of substrate (NEM or *N*-ethylcitraconimide) in 1.5 ml of a total assay volume. After incubation for 1 hr at 37°C, each reaction mixture was extracted 3 times with 3 ml of ethyl acetate, then dried over anhy-

drous magnesium sulfate, and filtered. For gas chromatography, samples were applied to a glass column (2 m×3 mm) of OV-17, chromatographed at 120°C with nitrogen as the carrier gas at a flow rate of 60 ml/min, and then detected using the FID method. For gas chromatography/mass spectrometry, samples were applied to a DB-5 fused silica capillary column (15 m×0.25 mm i.d., 0.25-mm film thickness, J & W Scientific Co., Folsom, CA, USA) and then chromatographed at 100 or 70°C (16°C/min) at an electron accelerating voltage of 70eV, an ionizing current of 300 μA, and ion source temperature of 180°C or 150°C, respectively (DX303 GC-MS, Nippon Denshi Co., Tokyo).

Results and Discussion

Substrate specificity of NEM reductase

We previously reported that α, β-unsaturated five-membered ring compounds, such as NEM and 4-cyclopenten-1, 3-dione, act as good substrates. To further characterize the substrate specificity, we tested the maleimide derivatives and cyclopentenone derivatives. The substrate specificities of NEM reductase in *Y. lipolytica* is summarized in Table 1. α, β-unsaturated five-membered ring compounds, such as maleimide or cyclopentenone derivatives, were active substrates for NEM reductase of *Y. lipolytica*. It seemed that the α, β-unsaturated

Table 1. *Substrate specificities of N-ethylmaleimide reductase in Yarrowia lipolytica to N-substituted maleimide derivatives and cyclopentenone derivatives*

Substrate	K_m (μM)	Vmax (nmol/min/mg protein)
N-Substituted maleimide derivatives		
N-Ethylmaleimide	2.2	179
Maleimide	8.7	216
N-Butylmaleimide	6.7	276
N-Phenylmaleimide	6.6	166
N-Cyclohexylmaleimide	23.0	52
N-Carboxymethylmaleimide	6.9	114
N-Benzimidazolylphenylmaleimide (BIPM)	2.0	57
N-Anilinonaphthylmaleimide (ANM)	11.0	25
N-Ethylcitraconimide	7.0	131
3-Methyl-*N*-phenylmaleimide	79.0	39
Cyclopentenone derivatives		
4-Cyclopenten-1, 3-dione	3.8	172
G2201-C	510	57
2-Cyclopentenone	140	30

carbonyl group was essential for NEM reductase as substrates. However, showdomycin [2-(β-D-ribofuranosyl)-maleimide] was not an active substrate for NEM reductase (data not shown). Steric hindrance may exist in the encounter between NEM reductase and showdomycin.

Inhibition of N-ethylmaleimide reductase by phenylpyrazolone derivatives

In *E. coli* K12, we reported that NEM reductase and *cis*-2-enoyl-CoA reductase were similar in terms of their molecular weight, the optical pH, the heat stability and the necessity of coenzyme (Mizugaki et al. 1979, 1981). Some pyrazolone derivatives, however, did not inhibit the activity of *cis*-2-enoyl-CoA reductase but did inhibit the NEM reductase (Mizugaki et al. 1981). Therefore, the sensitivities of NEM reductase to pyrazolone derivatives, which were chemical *N*-analogues of maleimide derivatives, were tested (Table 2). We first tested 1-phenyl-5-pyrazolone for inhibition of NEM reductase. At a concentration of 1 mM, activity of NEM reductase was decreased to 27% of the control. The activity was also decreased to 78% of the control activity at 10 μM and to 59% at 100 μM, suggesting that the pyrazolone derivatives were potent inhibitors of NEM reductase. Furthermore, 1-phenyl-5-pyrazolone was the most effective inhibitor of NEM reductase. The phenyl group at the position 1 of pyrazolone derivatives seemed to be important for the inhibition of NEM reductase. The inhibitory effect of 1-phenyl-3-methyl-5-pyrazolone was smaller than that of 1-phenyl-5-pyrazolone. Moreover, aminopyrine, which contains methyl-substituted group at the carbon-carbon double bond, did not inhibit NEM reductase, indicating that carbon-carbon double bond is critical for inhibition.

Subcellular fractionation of Y. lipolytica and determination of the subcellular localization of NEM reductase

The subcellular localization of NEM reductase in *Y. lipolytica* was examined using techniques of protoplast formation and differential centrifugation. We

TABLE 2. *The effects of the pyrazolone derivatives on the activities of the N-ethylmaleimide reductase in Yarrowia lipolytica*

Compound	% Inhibition		
	(M) 10^{-3}	10^{-4}	10^{-5}
1, 2-Dimethyl-5-pyrazolone	39	17	15
1-Phenyl-2-methyl-5-pyrazolone	25	10	N.E.
1-Phenyl-2-ethyl-5-pyrazolone	45	17	N.E.
1-Phenyl-5-pyrazolone	73	41	22
1-Phenyl-3-methyl-5-pyrazolone	5	N.E.	N.E.
1-Phenyl-2, 3-dimethyl-4-amino-5-pyrazolone	2	N.E.	N.E.

N.E., no effect.

fractionated the nuclei-free homogenate derived from 0.3% linoleic acid-grown cells to three fractions: heavy particulate (HP), microsomal (Ms) and cytosolic (S) fractions. As shown in Fig. 1, cytochrome c oxidase, known to be localized in mitochondria, was recovered in the HP fraction, while catalase, known to be localized in peroxisome, was detected in both the HP and the S fractions. This would be attributed, at least partly, to the degradation of particles during the preparation steps as mentioned previously (Tomioka et al. 1991). However, in yeast *Saccharomyces cerevisiae*, catalase T was localized in the cytosol, while the others (rat liver catalase, bovine liver catalase, human kidney catalase) were supposed to be localized in peroxisomes (Susani et al. 1976; Quan et al. 1986; Okada et al. 1987). These findings indicated that most of the peroxisomes and all of the mitochondria were recovered in the HP fraction. Glucose-6-phosphate dehydrogenase, known to be localized in the cytosol, was recovered mainly in the S fraction. However, NADPH-cytochrome c reductase, known to be localized in microsomes, was recovered in not only the Ms fraction but also in the HP and the S fractions. Ninety-eight percent of NEM reductase activity was recovered in the

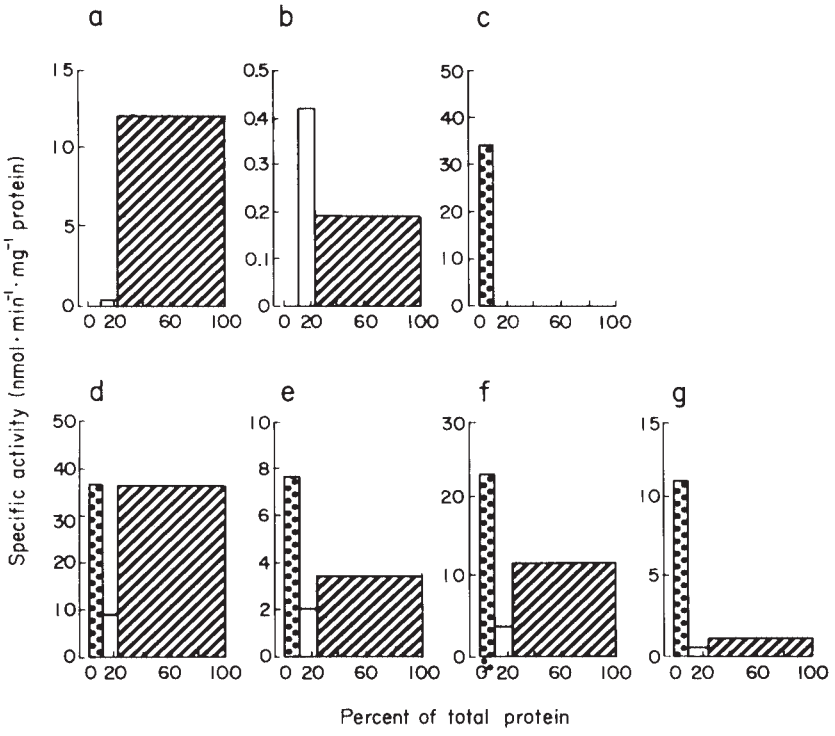

Fig. 1. Fractionation by differential centrifugation of the post nuclear fraction from *Yarrowia lipolytica*.
The activities of *N*-ethylmaleimide reducing enzyme (a), *cis*-2-enoyl-CoA reductase (b), cytochrome c oxidase (c), glucose-6-phosphate dehydrogenase (d), NADPH-cytochrome c reductase (e), catalase (f), and isocitrate lyase (g) were measured. Fractions are represented by their relative protein contents. ▨ soluble fraction; □ microsomal fraction; ⊡ heavy particulate fraction.

S fraction, and about 22% and 77% of the NADPH-*cis*-2-enoyl-CoA reductase activity were recovered in the Ms and S fraction, respectively. This suggested that NEM reductase in *Y. lipolytica* was the cytosolic enzyme and NADPH-*cis*-2-enoyl-CoA reductase was the microsomal enzyme.

The stereospecificity of the reduction of NEM by NEM reductase

NEM reductase from *Y. lipolytica* requires the presence of NADPH or NADH as a coenzyme in reducing NEM (Mizugaki et al. 1989). To determine the stereospecificity of this reduction, NADPH or H_2O labeled with deuterium as described by Seyama et al. (1977) were used instead of NADPH or H_2O in the standard incubation mixture, and the reduced products were analyzed by gas chromatography/mass spectrometry (GC/MS) monitoring the specific molecular ions. The m/z 127 and 128 fragments were the molecular ions of *N*-ethylsuccinimide and one deuterium-substituted *N*-ethylsuccinimide, respectively. (a) The reaction was performed in 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NADPH and 1 mmol *N*-ethylmaleimide, and m/z 127 was detected. (b) In 4*R*-[4-$^2H_1$]NADPH instead of NADPH, m/z 128 was detected. (c) In 4*S*-[4-$^2H_1$]NADPH, m/z 127 was detected. (d) In ^2H_2O instead of H_2O, m/z 128 was detected. When G2201-C, an antibiotic produced by *Streptomyces cattleya*, was used as the substrate for the experiment of (b) and (d), the m/z 243 fragment shifted to m/z 244 (Table 3); that is, hydride from the 4 *pro-R* position of NADPH and proton from H_2O were used for the reduction of NEM. The effect of deuterium substitution of NADPH on the rate of the reduction of NEM or G2201-C by NEM reductase were also investigated. The initial reaction rates of 4*R*-[4-$^2H_1$]NADPH/H_2O, 4*S*-[4-$^2H_1$]NADPH/H_2O, and NADPH/^2H_2O were compared spectrophotometrically with a non-deuterated mixture (NADPH/H_2O) (Table 3). The calculated relative initial reaction rates suggested that there were measurable isotopic effect, especially the rate decrease to 28% when 4*R*-[4-$^2H_1$] NADPH was added as a cofactor.

TABLE 3. *Relative initial reaction of NADPH consumption with NADPH/H_2O, 4R-[4-$^2H_1$]NADPH/H_2O, 4S-[4-$^2H_1$]NADPH/H_2O and NADPH/^2H_2O and fragment ions derived from N-ethylsuccinimide (m/z 127) and deuterium-added N-ethylsuccinimide (m/z 128) or derived from hydrated G2201-C (m/z 243) and deuterium-added hydrated G2201-C (m/z 244)*

Reaction cofactor and solvent	NEM		G2201-C	
	m/z	Catalytic rate (%)	m/z	Catalytic rate (%)
NADPH/H_2O	127	100	243	100
4*R*-[4-$^2H_1$]NADPH/H_2O	128	28	244	26
4*S*-[4-$^2H_1$]NADPH/H_2O	127	44	243	68
NADPH/^2H_2O	128	64	244	45

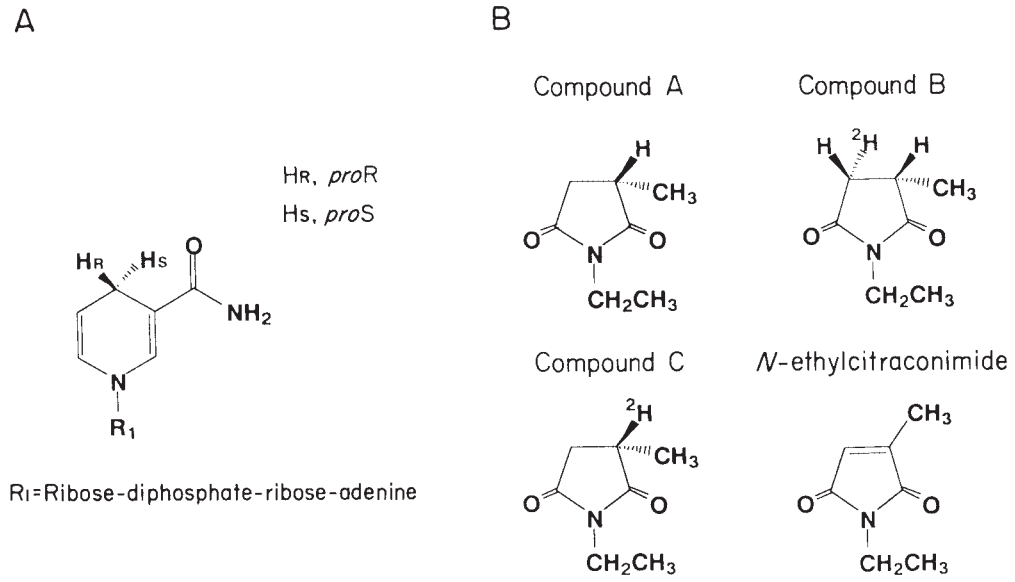

Fig. 2. (A) Common structure of NADPH. Designations HR and Hs refer to *pro*-R and *pro*-S, respectively. (B) The chemical structures of *N*-ethylcitraconimide and its derivatives, compound A, compound B and compound C.

To clarify the stereochemistry of NEM reductase, we used *N*-ethylcitraconimide (NEC, Vmax = 131 nmol/min/mg and Km = 7.0 mM), which has a methyl group at 3-position of NEM, as the substrate (Fig. 2). The resulting product was *N*-ethyl-3-methylsuccinimide from ^{1}H-NMR analysis. Each reaction mixture was extracted with ethyl acetate, purified on thin layer chromatography and analyzed by ^{1}H-NMR (Fig. 2). Compound A (EI-MS m/z; 127[M^+]; ^{1}H-NMR (300 MHz, C^2HCl_3) δ: 1.17 (3H, *t*, J = 7.0 Hz), 1.34 (3H, *d*, J = 7.0 Hz), 2.29 (1H, *dd*, J = 17.0 and 4.0 Hz), 2.83 (1H, *m*), 2.90 (1H, *dd*, J = 17.0 and 4.0 Hz), 3.55 (2H, *q*, J = 7.0 Hz)) was detected from the experiment of (a) and (c). Compound B (EI-MS m/z, 128[M^+]; ^{1}H-NMR (300 MHz, C^2HCl_3) δ: 1.17 (3H, *t*, J = 7.0 Hz), 1.34 (3H, *d*, J = 7.0 Hz), 2.83 (1H, *dq*, J = 9.0 and 7.0 Hz), 2.90 (1H, *dt*, J = 8.7 and 2.2 Hz), 3.55 (2H, *q*, J = 7.0 Hz)) was detected from the experiment of (b). Compound C (EI-MS m/z, 128[M^+]; ^{1}H-NMR (300 MHz, C^2HCl_3) δ: 1.17 (3H, *t*, J = 7.0 Hz), 1.34 (3H, *s*), 2.29 (1H, *d*, J = 18.0 Hz), 2.90 (1H, *d*, J = 18.0 Hz), 3.55 (2H, *q*, J = 7.0 Hz)) was detected from the experiment of (d). These results indicate that hydrogen from NADPH added to the C-4 position of *N*-ethylcitraconimide and hydrogen from H_2O added to the C-3 position of *N*-ethylcitraconimide, and the two hydrogen atoms from NADPH and H_2O added to opposite faces of the double bond of *N*-ethylcitraconimide.

References

1) Aebi, H. (1984) Catalase in vivo. In: *Methods in Enzymol. Oxygen Radicals in Biological Systems*, edited by L. Packer, Academic Press, New York, **105**, 121–126.
2) Barns, S.M., Lane, D.J., Sogin, M.L., Bibeau, C. & Weisburg, W.G. (1991) Evolution-

ary relationships among pathogenic *Candida* species and relatives. *J. Bacteriol.*, **173**, 2250-2255.
3) Darnall, K.R., Townsend, L.B. & Robins, R.K. (1967) The structure of showdomycin, a novel carbon-linked nucleoside antibiotic related to uridine. *Proc. Natl. Acad. Sci. USA*, **57**, 548-553.
4) Ellman, G.L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82**, 70-77.
5) Fukui, S., Kawamoto, S., Yasuhara, S., Tanaka, A., Osumi, M. & Imaizumi, F. (1975) Microbody of methanol-grown yeasts: Localization of catalase and flavin-dependent alcohol oxidase in the isolated microbody. *Eur. J. Biochem.*, **59**, 564-566.
6) Gregory, J.D. (1955) The stability of *N*-ethylmaleimide and its reaction with sulfhydryl groups. *J. Am. Chem. Soc.*, **77**, 3922-3923.
7) Hazen, K.C. (1995) New and emerging yeast pathogens. *Clin. Microbiol. Rev.*, **8**, 462-478.
8) Hirai, H., Shiotani, T., Tanaka, A. & Fukui, S. (1976) Intracellular localization of several enzymes in *Candida tropicalis* grown on different carbon sources. *Agr. Biol. Chem.*, **40**, 1979-1985.
9) Kawaguchi, A., Tsubotani, S., Seyama, Y., Osumi, T., Hashimoto, T., Kikuchi, T., Ando, M. & Okuda, S. (1980) Stereochemistry of dehydrogenation catalyzed by acyl-CoA oxidase. *J. Biochem.* (*Tokyo*), **88**, 1481-1486.
10) Kawamoto, S., Tanaka, A., Yamamura, M., Teraishi, Y. & Fukui, S. (1977) Microbody of n-alkane-growth yeast. Enzyme localization in the isolated microbody. *Arch. Microbiol.*, **112**, 1-8.
11) Leslie, J. (1965) Spectral shifts in the reaction of *N*-ethylmaleimide with protein. *Anal. Biochem.*, **10**, 162-167.
12) Mehta, N.B., Phillips, A.P., Fu Lui, F. & Brooks, R.E. (1960) Maleamic and citraconamic acids, methyl esters, and imides. *J. Org. Chem.*, **25**, 1012-1015.
13) Mishina, M., Kamiryo, T., Tashiro, S., Hagihara, T., Tanaka, A., Fukui, S., Osumi, M. & Numa, S. (1978) Subcellular localization of two long-chain acyl-coenzyme-A synthetases in *Candida lipolytica*. *Eur. J. Biochem.*, **89**, 321-328.
14) Mizugaki, M., Unuma, T. & Yamanaka, H. (1979) Studies on the metabolism of unsaturated fatty acids. II. Separation and general properties of reduced nicotinamide adenine dinucleotide phosphate dependent *cis*-2-enoyl-coenzyme A reductase from *Escherichia coli* K-12. *Chem. Pharm. Bull.*, **27**, 2334-2337.
15) Mizugaki, M., Unuma, T., Shiraishi, T., Nishimaki, T. & Yamanaka, H. (1981) Studies on the metabolism of unsaturated fatty acids. IV. *N*-ethylmaleimide reducing activity in *Escherichia coli* K-12. *Chem. Pharm. Bull.*, **29**, 570-573.
16) Mizugaki, M., Nakazawa, M., Yamamoto, H. & Yamanaka, H. (1989) Purification and characterization of *N*-ethylmaleimide reducing enzyme from *Candida lipolytica*. *J. Biochem.* (*Tokyo*), **105**, 782-784.
17) Morell, S.A., Ayers, V.E., Greenwalt, T.J. & Hoffman, P. (1964) Thiols of the erythrocyte. I. Reaction of *N*-ethylmaleimide with intact erythrocytes. *J. Biol. Chem.*, **239**, 2696-2705.
18) Nishimura, H., Mayama, M., Komatsu, Y., Kato, H., Shimaoka, N. & Tanaka, Y. (1964) Showdomycin, a new antibiotic from a *Streptomyces sp.* *J. Antibiotics* (*Tokyo*), **17**, 148-155.
19) Nyns, E.J., Auquiere, J.P., Chiang, N. & Wiaux, A.L. (1967) Comparative growth of *Candida lipolytica* on glucose and *n*-hexadecane. *Nature*, **215**, 177-178.
20) Okada, H., Ueda, M., Sugaya, T., Atomi, H., Mozaffar, S., Hishida, T., Teranishi, Y., Okazaki, K., Takechi, T., Kamiryo, T. & Tanaka, A. (1987) Catalase gene of the yeast *Candida tropicalis*: Sequence analysis and comparison with peroxisomal and cytosolic catalases from other sources. *Eur. J. Biochem.*, **170**, 105-110.
21) Quan, F., Korneluk, R.G., Tropak, M.B. & Gravel, R.A. (1986) Isolation and characterization of the human catalase gene. *Nucleic Acids Res.*, **14**, 5321-5335.

22) Seyama, Y., Kasama, T., Yamakawa, T., Kawaguchi, A. & Okuda, S. (1977) Stereochemical studies of hydrogen incorporation from nucleotides with fatty acid synthetase from *Brevibacterium ammoniagenes*. *J. Biochem.* (*Tokyo*), **81**, 1167–1173.
23) Sharpless, N.E. & Flavin, M. (1966) The reactions of amines and amino acids with maleimides. Structure of the reaction products deduced from infrared and nuclear magnetic resonance spectroscopy. *Biochemistry*, **5**, 2963–2971.
24) Susani, M., Zimniak, P., Fessl, F. & Ruis, H. (1976) Localization of catalase A in vacuoles of Saccharomyces cerevisiae: Evidence for the vacuolar of isolated "yeast peroxisomes". *Hoppe-Syler's Z. Physiol. Chem.*, **357**, 961–970.
25) Titani, Y. & Tsuruta, Y. (1974) Some chemical and biological characteristics of showdomycin. *J. Antibiot.*(*Tokyo*), **27**, 956–962.
26) Tomioka, Y., Aihara, K., Hirose, A., Hishinuma, T. & Mizugaki, M. (1991) Detection of heat-stable Δ^3, Δ^2-enoyl-CoA isomerase in rat liver mitochondria and peroxisomes by immunochemical study using specific antibody. *J. Biochem.* (*Tokyo*), **109**, 394–398.
27) Walsh, T.J., Salkin, I.F., Dixon, D.M. & Hurd N.J. (1989) Clinical, microbiological, and experimental animal studies of *Candida lipolytica*. *J. Clin. Microbiol.*, **27**, 927–931.
28) Weeks, G. & Wakil, S.J. (1968) Studies on the metabolism of fatty acid synthesis. 18. Preparation and general properties of the enoyl acyl carrier protein reductases from *Escherichia coli*. *J. Biol. Chem.*, **243**, 1180–1189.
29) Wharton, D.C. & Tzagoloff, A. (1967) Cytochrome c oxidase from beef heart mitochondria. In: *Methods in Enzymol. Oxidation and Phosphorylation*, edited by R.W. Estabrook & M.E. Pullman, Academic Press, New York, **10**, 245–250.