Natural stereospecific hydrogen isotope transfer in alcohol dehydrogenase-catalysed reduction

Ben-Li Zhang, Sébastien Pionnier

Abstract The enantiomeric purity of natural α-monodeuterated enantiomers, (*R*) and (*S*) ethanol-1-d_1, in the alcohol produced by sugar fermentation with yeast was studied by ^{2}H NMR using their esters derived from optical mandelic acid. The results of isotope tracing experiments show that the transfer pathways of the two enantiotopic hydrogens of the methylene group are different. It was observed that (*S*)-deuterium comes only from the medium water. The (*R*)-deuterium transfered by NADH in alcohol dehydrogenase reduction of the acetaldehyde is of complex origin. Some of them originates from carbon bound hydrogen of the sugar, especially from C(4) position of glucose and most of them comes from water. Only a small portion of the NADH deuterium is incorporated indirectly from water through enzyme catalysed exchange between the pro-*S* site of NADH and flavin. When a carbonyl compound (ethyl acetoacetate) was reduced under the same conditions during the alcoholic fermentation, among the NADH-transfered deuterium, only a small portion comes from water while most comes from the unexchangeable positions of the glucose.

Key words alcohol dehydrogenase • chiral isotopomers • deuterium • isotope tracing

B.-L. Zhang✉, S. Pionnier
Laboratoire d'Analyse Isotopique
et Electrochimique de Métabolismes,
CNRS UMR 6006, Université de Nantes,
2 rue de la Houssinière, 44322 Nantes, France,
Tel.: +33 2/ 51125714, Fax: +33 2/ 51125712,
e-mail: benli.zhang@chimbio.univ-nantes.fr

Received: 11 July 2001, Accepted: 30 November 2001

At natural abundance, the formation of *R*- and *S*-ethanol-1-d_1 in a sugar fermentation follows two different pathways respectively (Scheme 1). Due to the different hydrogen transfer mechanisms and isotope effects [1] related to the two pathways, the natural mixture of the two enantiomers in ethanol produced by fermentation is not racemic [3]. The enantiomeric excess (e.e.) can be determined by ^{2}H NMR when these enantiomers are converted to two diastereoisomers [ethanol-1-d_1 + (*S*)-mandelic acid → (*R*)- and (*S*)-ethyl-1-d_1 (*S*)-mandelate] [4]. We found that in ethanol obtained from corn glucose under natural conditions, e.e. = 8.3% for the *R*-enantiomer.

In order to examine the deuterium transfer pathway in a more quantitative way, further experiments were carried out. We have studied the variation of $(D/H)_{pro\text{-}RH}$ and $(D/H)_{pro\text{-}SH}$ as a function of $(D/H)_w$ of the medium water in the fermentation (Fig. 1). The unit of $(D/H)_i$ is ppm. We obtained

$$(D/H)_{pro\text{-}RH} = 0.779(D/H)_w + 18.5 \quad (1)$$

$$(D/H)_{pro\text{-}SH} = 0.775(D/H)_w - 4.0 \quad (2)$$

In these equations the slope reflects isotope effects and the intercept provides information on the site-specific deuterium transfer from the substrate to the product when $(D/H)_w = 0$ [7].

Glucose-4-d_1 was used in a labelling experiment (Table 1) and a correlation (Eq. (3)) can be established.

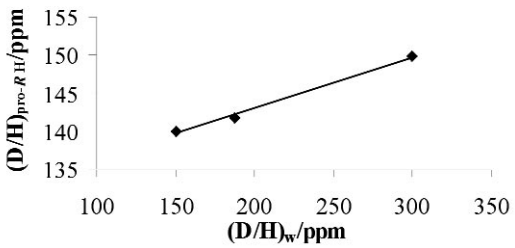


Fig. 1. $(D/H)_{pro\text{-}RH}$ and $(D/H)_{pro\text{-}SH}$ as a function of $(D/H)_{water}$.

(3) $$(D/H)_{pro\text{-}RH} = 0.09(D/H)_{G4} + 119$$

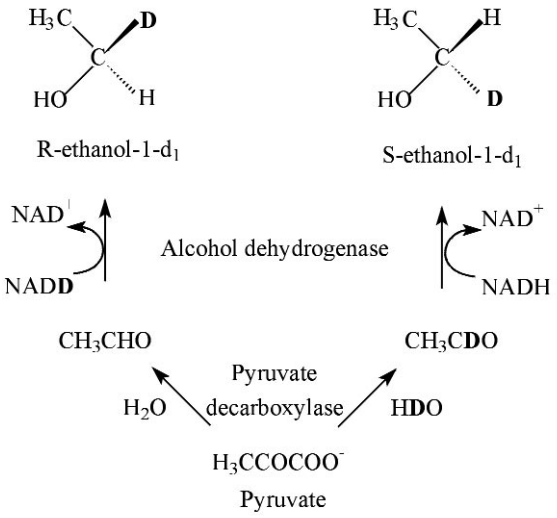
in which $(D/H)_{G4}$ is the specific isotopic ratio of hydrogen bound to C(4) of glucose.

The intercept of Equation (3) reflects deuterium labelling at the pro-*R* position when $(D/H)_{G4} = 0$. Qualitatively these results are in agreement with the glycolysis and fermentation mechanisms (Scheme 2) since we find only water deuterium in the *S*-enantiomer and deuterium of both water and C(4) site of glucose in the *R*-enantiomer.

There is an exchange between the ethanol *R*-deuterium and water due to an indirect exchange of the pro-*S* hydrogen of NADH with water via flavin catalysed by enzymes [2, 5]. The degree of the exchange was studied by incubation of an ethanol, of which the $(D/H)_{pro\text{-}RH}$ = 139 ppm in different waters in the presence of baker's yeast. At the end of the exchange, the ethanol in each water was extracted and its $(D/H)_{pro\text{-}RH}$ was determined (Fig. 2). The correlation between $(D/H)_{pro\text{-}RH}$ of ethanols and $(D/H)_w$ is described by Equation (4).

(4) $$(D/H)_{pro\text{-}RH} = 0.067(D/H)_w + 129.7$$

From the equation, the percentage of deuterium involved in the exchange can be evaluated as 7% =100×(139–129)/139. According to the glycolysis mechanism and the result of the exchange, there should be 53.5% of water deuterium and 46.5% of glucose C(4) deuterium in the *R*-enantiomer. The experimental data deviate considerably from the estimation



Scheme 1. Formation of the *R*- and *S*-ethanol-1-d_1 at natural abundance in glucose fermentation with *Saccharomyces cerevisiae*.

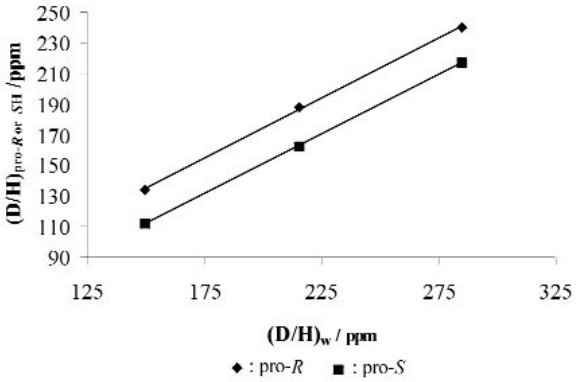
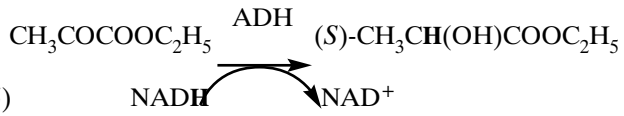


Fig. 2. Exchange between the pro-*R*H of ethanol methylene with water catalysed by alcohol dehydrogenase of baker's yeast. Influence of $(D/H)_w$ on $(D/H)_{pro\text{-}RH}$.

due to complex inter- and intra molecular D/H exchange and isotope fractionation.

A reduction of ethyl acetoacetate was carried out in the presence of the corn glucose and baker's yeast in different waters (Table 2 and Eq. (5)).



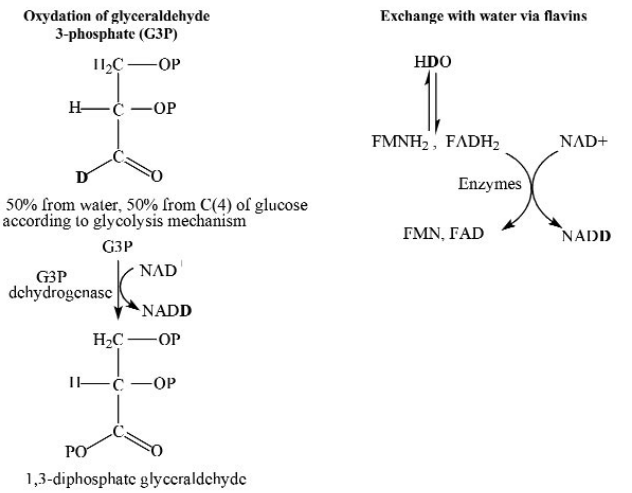
(5) $$(D/H)_H = 0.129(D/H)_w + 88.1$$

The intercept value of Equation (5) shows that a considerable quantity of the transfered deuterium comes from the substrate.

Other tracing experiments using glucoses deuterated at C(1), C(2), C(3), and C(6,6') in the fermentation showed that no deuterium was transferred from these sites to the pro-*R*H position of ethanol. The isotopic connection between glucose, water and the methylene of fermentation ethanol can be established:

(6) $$(D/H)_{pro\text{-}RH} = 0.78\ (D/H)_w + 0.09(D/H)_{G4}$$

(7) $$(D/H)_{pro\text{-}SH} = 0.78\ (D/H)_w$$



Scheme 2. Origin of **D** (deuterium) in NAD**D** at natural abundance.

Table 1. Results of isotope tracing with glucose-4-d_1.

$(D/H)_i$ /ppm	Glucose	Ethanol		
	$(D/H)_{G4}$	$(D/H)_{CH_3}$	$(D/H)_{pro\text{-}RH}$	$(D/H)_{pro\text{-}SH}$
Corn glucose (reference)	140.3	111.1 (0.3)	134.2 (1.5)	112.2 (1.2)
Corn glucose + glucose-4-d_1(G4)	317.2	118.8 (0.3)	162.6 (0.6)	115.0 (0.4)

Table 2. Reduction of ethyl acetoacetate catalysed by the baker's yeast in different waters.

$(D/H)_w$ /ppm	150.2	237.4	238.1
$(D/H)_H$ /ppm	107.7 (0.5)	118.5 (0.4)	130.7 (0.8)

Conclusion

Since the slopes of Eqs. (1) and (2) are both <1 and the pro-*R* hydrogen comes partially from water, it can be concluded that the global isotope effect which is composed of complex equilibrium and kinetic effects during the transfer of hydrogen from water to the two enantiomers is normal and is larger for the *S*-enantiomer formation. The α-hydrogen of acetadehyde forming during pyruvate decarboxylation comes only from the medium water. The deuterium transferred by NADH in alcohol dehydrogenase reduction of the acetaldehyde is of complex origin, mainly during its regeneration. Some of them (10%, calculated based on homogeneous distribution of deuterium in the starting water and substrate) are derived from carbon bound hydrogen of the sugar, especially from C(4) position of glucose and most of them (90%) come from some intermediates, of which the hydrogen was introduced from water during their formation, especially the α-hydrogen of glyceraldehyde 3-phosphate. Only a small portion (7%) of the methylene deuterium is in exchange with water due to an indirect exchange of the pro-*S* hydrogen of NADH with water via flavin catalysed by enzymes. Similar deuterium afilliation was observed in the reduction of DHAP to glycerol-3-phosphate during the glycerol formation [6]. However, when a carbonyl compound (ethyl acetoacetate) was reduced under the same conditions during the alcoholic fermentation, surprisingly, only a small portion (20%) of deuterium of water was transferred by NADH while most deuterium (80%) transferred by this co-factor are derived from the unexchangeable positions of the glucose.

Acknowledgments We thank Prof. M. L. Martin for helpful suggestions.

References

1. Cook PF, Blanchard JS, Cleland WW (1980) Primary and secondary deuterium isotope effects on equilibrium constants for enzyme-catalyzed reactions. Biochem 19:4853–4858
2. Günther H, Alizade MA, Kellner M, Biller F, Simon H (1973) Preparation of (1*R*)- and (1*S*)-1-^{2}H labeled alcohols by exchange reactions catalysed by yeast or a couple enzyme system. Z Naturforsch 28c:241–246
3. Martin ML, Zhang BL, Martin GJ (1983) Natural chirality of methylene sites applied to the recognition of origin and to the study of biochemical mechanisms. FEBS Letters 158:131–133
4. Rabiller C, Mesbahi M, Martin ML (1990) ^{2}H NMR resolution of the methylenic isotopomers of ethanol applied to the study of stereospecific enzyme-catalysed exchange. Chirality 2:85–89
5. Simon H, Kellner M, Günther H (1968) Synthesis, on a preparative scale, of ethanol stereospecifically labeled on hydrogen. Angew Chem Internat Edit 7;11:892
6. Zhang BL, Buddrus S, Martin ML (2000) Site-specific hydrogen isotope fractionation in the biosynthesis of glycerol. Bioorg Chem 28:1–15
7. Zhang BL, Yunianta Martin ML (1995) Site-specific isotope fractionation in the characterization of biochemical mechanisms. J Biol Chem 270:16023–16029