Cofactor Recycling Mechanism in Asymmetric Biocatalytic Reduction of Carbonyl Compounds Mediated by Yeast: Which is the Efficient Electron Donor?

Ben-Li Zhang* and Sébastien Pionnier[a]

Abstract: In asymmetric reduction of carbonyl compounds mediated by microorganisms, the cofactors that transfer hydride should be regenerated by using a recycling system. In most cases, this recycling system consists of carbohydrate molecules, especially glucose or sucrose. Other molecules such as ethanol and acetate have been used as electron donors too. The reduction can even be conducted without added electron donors. To improve biocatalytic synthesis, it is important to understand the cofactor recycling mechanism. In this work, the hydride-transfer mechanism in cofactor regeneration, which takes place in bioreduction mediated by yeast, was studied by means of an isotope tracing technique. The results show that, when glucose was used, the NADH involved in the glycolysis was consumed directly in the formation of ethanol and was not used in the bioreduction. Hence, the regeneration of cofactors in the reduction is not coupled with glycolysis. Nevertheless, glucose is an efficient electron donor that transfers hydride through the hexose monophosphate (HMP) pathway in which the main hydrogen source is C-1 and C-3 hydrogen of glucose. Ethanol is not a good electron donor, since, when it was used, only a small quantity of hydrogen was transferred from this molecule, and the main hydrogen source was water. Therefore, the ethanol oxidation pathway may not be efficient. In the absence of added auxiliary substrates, the yeast cells may use electron donors stored in its cellules. However, in this case we observed that the main hydrogen source for cofactor recycling was water, while only very few hydrogen atoms were from unexchangeable sites. This is similar to the case in which ethanol is used, and is in contradiction with the HMP pathway if stored glucose was the electron donor. The question that remains to be investigated is "what is the efficient electron donor recycling mechanism in the yeast cellules?"

Keywords: cofactor regeneration · enzyme catalysis · hydrides · isotopic labeling · yeast

Introduction

The stereoselective reduction of carbonyl compounds mediated by yeast is an important method in organic synthesis.[1, 2, 3] Research in this field is very active. In organic chemistry, most studies were focussed on the search for new chiral molecule synthesis, or for the improvement of the reaction conditions in order to raise yield and optical purity of the products. In biochemistry, many enzyme–cofactor systems have been identified. In spite of this progress, the biochemical mechanism is not always well understood. One of the key problems in these mechanistic studies is cofactor recycling. In the biocatalytic reduction, the reductases in the microorganisms transfer a hydride to the carbonyl from the cofactors such as NADH (nicotinamide adenine dinucleotide) or NADPH (nicotinamide adenine dinucleotide phosphate), and transform the carbonyl compound into an alcohol. If the carbonyl compound is prochiral, the hydrogen atom of the hydride is bound to the carbonyl carbon in an asymmetric way, and a chiral alcohol can be formed. Since the cofactors in the microorganism exist only in catalytic quantities, after hydride transfer the cofactors should be regenerated. For the cofactor regeneration, auxiliary substrates or electron donors are needed. These substrates, together with certain enzymes, and the cofactors form a recycling system that ensures hydride transfer from the electron donors to the cofactors (Scheme 1). In most cases, the electron donors are carbohydrates, such as glucose or sucrose. Other molecules such as formate, ethanol, and so on, have also been used.[3] The reduction can also be carried out without the addition of auxiliary substrates.[4] How the cofactors are regenerated in the different cases is not very clear.

A 3-oxo ester, ethyl acetoacetate (EAA, ethyl 3-oxobutanoate), is one of the most studied carbonyl compounds in the

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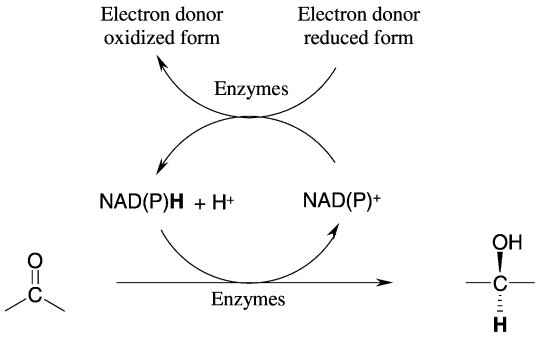

Scheme 1. Asymmetric reduction of carbonyl compounds and cofactor regeneration.

biotransformation mediated by bakers yeast, in which ethyl (*S*)-3-hydroxybutanoate (EHB), an intermediate in the synthesis of many important chiral molecules, can be obtained with high yield and high optical purity.[5, 6, 7, 8] In this work, we used the bioreduction of EAA as a model reaction, and studied the hydride transfer mechanism between the electron donors and the carbonyl compound in cofactor recycling by using hydrogen isotope tracing.

Deuterium tracing is a very useful tool in the study of hydride transfer mechanisms mediated by cofactors.[9] Today with modern powerful analytic tools, the study can be performed in an easy and more efficient way. ^{2}H SNIF-NMR (site-specific natural isotope fractionation NMR) spectroscopy is a new isotope analysis tool.[10, 11] On the basis of this technique, we developed a method of quantitative isotope tracing close to natural abundance that has been successfully used in the study of natural isotope fractionation and deuterium transfer in biotransformations.[12, 13, 14, 15, 17] This method is described below.

At natural abundance, deuterium occurs in molecules of a compound essentially as monodeuterated isotopomers. The parameter measured by ^{2}H NMR is the specific isotopic ratio of site i of the sample molecule, $(D/H)_i$ in ppm [see Eq. (1)below][11]

$$(D/H)_i = N_{D_i}/(P_i N_H) \quad (1)$$

in which N_{D_i} is the number of site i deuterated isotopomers, P_i, is the stoichiometric hydrogen number at site i, and N_H is the number of unlabeled (totally protiated) molecules. $(D/H)_i$ at all sites of the sample compound can be determined when their ^{2}H NMR signals are sufficiently resolved.

In a biotransformation the site-specific isotopic ratios of a product, $(D/H)_i$, is a linear function of the site-specific isotopic ratios of the carbon-bound hydrogen positions of the substrate and of the medium [see Eq. (2)]:

$$(D/H)_i = a_{im}(D/H)_m + \sum_j a_{ij}(D/H)_j = \sum_k a_{ik}(D/H)_k \quad (2)$$

in which $(D/H)_m$ and $(D/H)_j$ are the isotopic ratios of the medium and the unexchangeable site, j, of the substrate, respectively. For most biotransformations, the medium is water. However, the added sugar contains a considerable quantity of hydroxyl hydrogen atoms that are in rapid exchange with the water of the fermentation medium. Consequently, if there is a large quantity of sugar, the $(D/H)_w$ value of the water medium is modified. When glucose was present, we used $(D/H)_m$ which is a weighted mean value of all hydroxyl molecules, calculated on the basis of the $(D/H)_{OH}$ of glucose and $(D/H)_w$, and the quantities of water and glucose in the medium.[15] When there was a small quantity of glucose, $(D/H)_w$ of water was used. The terms a_{im} and a_{ij} are isotope redistribution coefficients, m,j $\subset$ k. The coefficient a_{ik} characterizes the specific genealogies of the deuterium atoms and is related to the reaction mechanism. It also depends on the complex isotope effects during the biochemical reactions and yeast activity. The coefficient a_{ik} is determined by specifically and quantitatively labeling a position, j, of the substrate or the medium, and measuring the variation of $(D/H)_i$ values of the corresponding product. In the experiment with glucose, a commercial corn glucose was used as a reference. Its natural site-specific isotopic ratios were carefully determined by ^{2}H NMR spectroscopy.[15] The site-specific labeling of glucose was achieved by adding very small quantities of glucose deuterated at site j into the corn glucose. The addition of a site-j-deuterated glucose to the reference glucose only increased $(D/H)_j$ of this site, while the specific isotopic ratios of other sites remained unchanged. The reference glucose and all the labeled glucose molecules were fermented in a reference water, tap water, of natural deuterium abundance. The labeled water labeling molecules were prepared by adding small quantities of deuterated water into the reference water. In water experiments, only the reference glucose was used. Hence, by measuring the variation of $(D/H)_i$ of the product as a function of the $(D/H)_k$ of the substrate or medium, the value of a_{ik} (a_{im} or a_{ij}) can be evaluated from a linear equation [Eq. (3)]:

$$(D/H)_i = a_{ik}(D/H)_k + b \quad (3)$$

For the labeled site k, the slope of the equation is a_{ik} and the intercept is $\sum a_{ik'}(D/H)_{k'}$ ($k \neq k'$). For instance, in alcohol fermentation, ^{k}when k = m (water medium is labeled), a_{im} reflects the degree of deuterium transfer from the medium to site i of ethanol, whereas the intercept value in the equations corresponds only to the contribution of unexchangeable deuterium atoms of glucose at this site. The biotransformation of glucose to ethanol and glycerol have been studied in this way and important deuterium transfer information has been obtained.[13, 14, 15] Similarly, by comparing the site-specific hydrogen isotopic ratios of the auxiliary substrates with that of the α-carbon hydrogen atom from the hydroxy of the alcohol produced, the cofactor recycling mechanism can be studied in an easy way.

In the bioreduction of EAA, although many compounds are considered as electron donors, glucose, ethanol, and acetate are the most commonly used.[5] When glucose is used as an auxiliary substrate in yeast-mediated biotransformation, its main metabolic pathway is glycolysis and alcohol fermentation, in which the main products are ethanol (95–97%) and glycerol (3–5%). The hydride transfer and NADH recycling mechanism in the alcohol fermentation has been well studied.[13, 15, 17] The large quantity of NADH consumed in ethanol and glycerol formation is regenerated in glycolysis.

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How this metabolism couples with the reduction reaction should be understood. In this work, we studied the cofactor recycling mechanism in the bioreduction of ethyl acetoacetate with glucose as an electron donor and compared the results with those obtained in alcohol fermentation. The cases with ethanol and no electron donor were also studied.

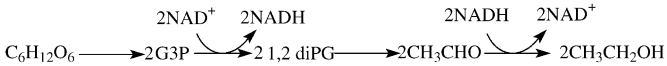
Results and discussion

In the first series of experiments, the reduction was carried out with corn glucose (reference) in waters of different isotopic compositions, then with glucose labeled with deuterium at position 1, 3, and 4 in tap water. The results are summarized in Table 1.

In our bioreduction procedure, we first used a dried commercial mixture of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (1:1) for wine production (lot 1). The use of this mixture is more efficient in sugar fermentation, since *S. cerevisiae* is very active at the early stages of fermentation, while *S. bayanus,* which is more alcohol-resistant, ensures the completion of fermentation. We have carried out tests in the bioreduction with pure *S. cerevisiae* and *S. bayanus* strains. In these cases, the conversion rates were somewhat lower than when the mixture was used. Later when the stock of lot 1 was exhausted, we prepared a 1:1 mixture of the two pure strains with purchased dried yeast (lot 2). The use of phosphate in the reduction was necessary, since, in its absence, the yield decreased slightly.

In a ^{2}H NMR spectrum of ethyl 3-hydroxybutanoate (EHB), the signal of the α-carbon H atom of hydroxyl overlaps that of the methylene of the ethyl. This rendered the direct analysis of EHB difficult. This was overcome by converting EHB to ethyl 3-acetoxybutanoate for which the NMR signals were well separated.

In alcohol fermentation (Scheme 2), a glucose molecule is transformed into two ethanol molecules after the reduction of acetaldehyde. The reduction step consumes two NADH molecules, which are regenerated in the step of oxidation of two glyceraldehyde-3-phosphates (G3P) to two 1,3-diphosphoglycerates (1,3 diPG).



Scheme 2. Ethanol formation in glucose fermentation with yeast.

The active hydrogen atom of NADH is abstracted from the carbonyl α-position of G3P. Most of the hydrogen atoms present during the G3P/DHAP interconversion, are those from water, and some of them are the remaining ones of C-4 of glucose due to stereospecificity of the reaction.[17, 18] In ethanol, the cofactor-transferred hydrogen atom is that at the pro-*R* position of the methylene group.[19, 20]

The ethanol molecules, produced simultaneously with the reduction, were also extracted and their site-specific isotope ratios were analyzed (Table 2). The results are in good agreement with those obtained in pure alcohol fermentation experiments.[13, 17] Once again, we observed the presence of deuterium from C-4 glucose and water at the methylene group pro-*R* position of ethanol. The relation between $(D/H)_{pro\text{-}RCH}$ and $(D/H)_m$ of the medium, and $(D/H)_4$ of glucose in alcohol

Table 1. Reduction of ethyl acetoacetate mediated by yeast with labeled water and glucose molecules.

Expt. no.	Yeasts Lot no.	Medium $(D/H)_m$/[ppm]	Labeled site j	Glucose $(D/H)_j$ of the reference[a][ppm]	$(D/H)_j$ of the labeled site[ppm]	Ethyl 3-hydroxybutanoate $(D/H)_{H-COH}$[ppm]
1	1	149.7	reference[a]			110.5(0.5)
2	1	236.9	reference[a]			125.3 (0.4)
3	1	327.6	reference[a]			140.6 (0.9)
4	1	149.7	1	173.8[b]	424.6	176.9 (1.2)
5	1	149.7	4	140.3[b]	454.6	113.4(1.4)
6	2	149.7	reference[a]			118.6(1.0)
7	2	149.7	1	173.8[c]	298.0	147.8(0.4)
8	2	149.7	3	146.4[c]	418.1	219.7(0.6)

[a] Commercial corn glucose as the reference.[16] [b] The $(D/H)_{H-COH}$ value of the corresponding ethyl 3-hydroxybutanoate was obtained in experiment 1. [c] The $(D/H_{H\text{-}COH}$ value of the corresponding ethyl 3-hydroxybutanoate was obtained in experiment 6.

Table 2. $(D/H)_i$ of ethanol molecules obtained in the bioreduction of ethyl acetoacetate by using glucose as electron donor with labeled water and glucose molecules.

Expt. no.	Yeast Lot no.	Medium $(D/H)_m$ [ppm]	Labeled site j	Glucose $(D/H)_j$ of the reference[a]	$(D/H)_j$ of the labeled site[ppm]	Ethanol $(D/H)_{CH_3}$ [ppm]	$(D/H)_{CH_2}$ [ppm]	$(D/H)_{pro\text{-}RCH}$ [ppm]	$(D/H)_{pro\text{-}SCH}$ [ppm]
1	1	149.7	reference[a]			111.7(0.4)	127.5(0.4)	136.2(1.4)	118.8(1.2)
2	1	236.9	reference[a]			125.4(0.2)	199.7(0.3)		
3	1	327.6	reference[a]			140.6(0.3)	271.4(0.5)	283.8(1.4)	259.0(1.3)
4	2	149.7	1	173.8	297.8	128.3(0.4)	126.4(0.3)		
5	1	149.7	1	173.8[b]	427.0	144.6(0.3)	125.1(0.4)		
6	2	149.7	3	146.4	410.8	112.4(0.3)	127.6(0.3)		
7	1	149.7	4	140.3[b]	454.6	113.0(0.3)	137.3(0.3)	157.7(1.1)	116.8(0.8)

[a] Commercial corn glucose as the reference. [b] The $(D/H)_i$ values of the corresponding ethanol was obtained in experiment 1.

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fermentation during the reduction, can be characterized by the following linear equations [Eqs. (4) and (5)].

$$(D/H)_{pro\text{-}RCH} = 0.83(D/H)_m + 12 \quad (4)$$

$$(D/H)_{pro\text{-}RCH} = 0.07(D/H)_4 + 126.6 \quad (5)$$

Each of the two equations were established with two sets of data:[15] $(D/H)_m$ and $(D/H)_{pro\text{-}RCH}$ in experiments 1 and 3 for Equation (4), and $(D/H)_4$ and $(D/H)_{pro\text{-}RCH}$ in experiments 1 and 7 for Equation (5)(Table 2). These results are in good accord with those obtained in pure alcohol fermentation.[13, 17] Generally, the uncertainty of these type of equations is ± 0.02 for the slope (a_{ij}) and ± 5 for the intercept. Since the only source of the NADH-transferred hydrogen in glycolysis is the medium and the unexchangeable hydrogen of C-4 of glucose,[17] on the basis of Equations (4) and (5), the connection between $(D/H)_{pro\text{-}RCH}$, $(D/H)_m$, and $(D/H)_4$ of glucose can be established [Eq. (6)].

$$(D/H)_{pro\text{-}RCH} = 0.83(D/H)_m + 0.07(D/H)_4 \quad (6)$$

Equation (6) shows that the main source of pro-*R* deuterium is water, and that the contribution of the deuterium bound to C-4 of glucose is less significant. The NADH regenerated in this way is also used for the reduction of dihydroxyacetone phosphate (DHAP) into *sn*-3-glycerol phosphate in glycerol biosynthesis, since we observed the glucose C-4 deuterium was transferred to the C-2 site of glycerol to a similar degree.[15]

However, in ethyl acetoacetate (EAA) reduction, the relation between $(D/H)_{H\text{–}COH}$ and $(D/H)_m$ is given by Equation (7).

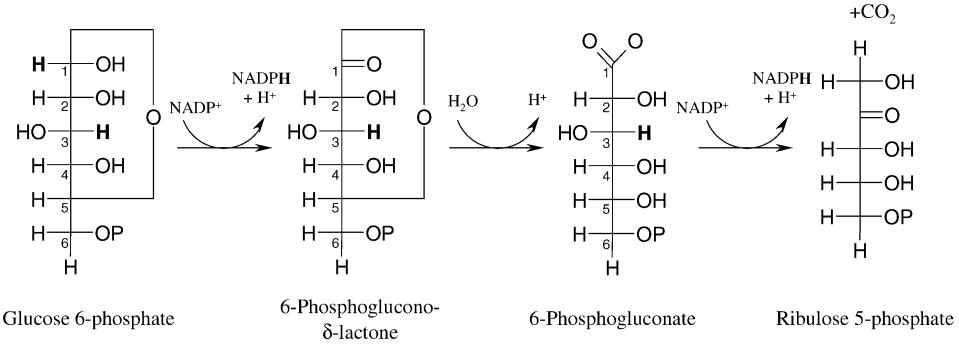
$$(D/H)_{H\text{–}COH} = 0.17(D/H)_m + 85.2 \quad (7)$$

This equation was established with three sets of data as shown in experiments 1–3 in Table 1. The small slope and big intercept values show that the deuterium introduced by cofactors comes mainly from glucose, and that water or the medium is not the main deuterium source. This isotopic difference should reflect the difference in mechanism. In addition, there was almost no H-4 of glucose on the α-position of the alcohol produced (Table 1). When $(D/H)_4$ increased from 140.3 to 454.6 ppm in experiment 5, $(D/H)_{H\text{–}COH}$ remained nearly the same (the difference between 110.5 and 113.3 ppm is very small and close to the precision limit). From the results, it can be concluded that the large quantity of NADH regenerated in glycolysis is not used by the reductases in EAA reduction. The two biotransformation pathways are not coupled through common cofactors.

When explaining these deuterium tracing results, it should be remembered that the degree of deuterium transfer should not necessarily be the same as that of protium, owing to kinetic and/or equilibrium isotope effects.[15] However, the behavior of deuterium during the biochemical reaction should reflect to a certain degree that of protium, even though it is not in a strictly quantitative way.

Moreover, the isotope effect may differ only in the step in which the $NAD(P)^+$ abstracts the hydrogen on different sites of the electron donors, and the effect is the same when NAD(P)H reduces the substrate.

Since the hydride comes mainly from the unexchangeable sites (except H-4) of glucose, what are these hydrogen atoms? NADPH is known as the coenzyme for most enzymes which can catalyze reduction.[5, 21] It has been proposed that the enzymatic reactions of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, in the hexose monophosphate (HMP) pathway (pentose phosphate pathway), is an efficient NADPH-regenerating mechanism[5](Scheme 3). In the conversion of glucose-6-phosphate into ribulose 5-phosphate, the H-1 and H-3 of the starting glucose were abstracted by $NADP^+$.



Scheme 3. Oxidative branch with NADPH regeneration of the HMP pathway.

When H-1- and H-3-labeled glucose were used in the reduction, an increase of $(D/H)_{H\text{–}COH}$ was observed; this is in contrast to alcohol fermentation in which no C-1 and C-3 deuterium can be found at the methylene pro-*R* site of ethanol. When yeasts of lot 1 were used, Equation (8) was

$$(D/H)_{H\text{–}COH} = 0.26(D/H)_1 + 64.9 \quad (8)$$

obtained, and when yeasts of lot 2 was used, we get Equations (9) and (10).

$$(D/H)_{H\text{–}COH} = 0.24(D/H)_1 + 77.7 \quad (9)$$

$$(D/H)_{H\text{–}COH} = 0.37(D/H)_3 + 62.6 \quad (10)$$

Each of the three equations was established with two sets of data: $(D/H)_j$ of the reference and the labeled glucose and $(D/H)_{H\text{–}COH}$ of their corresponding ethyl 3-hydroxybutanoate (EHB)(Table 1). If Equations (5)—(8) are combined, neglecting the small difference between the two lots of yeast, we get Equation (11):

$$(D/H)_{H\text{–}COH} = 0.17(D/H)m + 0.25\,(D/H)_1 + 0.37\,(D/H)_3 \quad (11)$$

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in which the coefficient for $(D/H)_1$ (0.25) is the mean value of Equations (8) and (9). From Equation (11), $(D/H)_m = 149.7$ ppm, $(D/H)_1 = 173.8$ ppm, and $(D/H)_3 = 146.4$ ppm for glucose,[16] (Table 1) the calculated value of $(D/H)_{H-COH}$ is 123 ppm, which is very close to the experimental value of 118.6 ppm. This shows that the hydrogen atom of the reduced carbon comes only from C-1 and C-3 of glucose and from the medium. The use of NADPH regenerated in further oxidation of the glucose in the HMP pathway was not observed. The presence of water in the site may be inevitable, since the NADPH-active hydrogen atoms can be in exchange in an indirect way with the water medium through flavine, even though the exchange degree is limited.[17, 19, 20b] These results confirm the HMP pathway in NADPH regeneration. However, it is surprising to observe that the contribution of D-3 is much higher than that of D-1. The cause of this difference is not clear. Isotope effect should be partially responsible, but may not be sufficient for a satisfactory explanation. Moreover, this observation seems in agreement with the results obtained by Seebach et al.,[22] that δ-gluconolactone was the most efficient electron donor among a set of reagents including fructose, lactate, glycerol, and others.

It can be concluded that when glucose was used as an electron donor, most of the glucose was transformed into ethanol, and only a small quantity of glucose took part in the HMP pathway and regenerated enough cofactors for the bioreduction.

Since ethanol was considered as an efficient electron donor,[5, 23] what is the role of ethanol? Ethanol was present even when glucose was used as an electron donor in the reduction. We performed experiments with ethanol as an auxiliary substrate. The quantity of added ethanol was 4 g per 5 g of EAA. Reduction was carried out with corn ethanol (reference) in water of different isotopic composition, and with ethanol labeled by deuterium at different sites. The isotope labeling experiment results are summarized in Table 3.

It has been proposed that the oxidation of acetaldehyde to acetic acid, catalyzed by NADP-dependent acetaldehyde dehydrogenase, may be a cofactor recycling pathway [Eq. (12)].

$$CH_3CHO + NADP^+ \rightarrow \text{acetic acid} + NADPH \quad (12)$$

According to the mechanism, when ethanol is used as an electron donor, it is oxidized in a set of reactions: ethanol → acetaldehyde → acetic acid → CO_2, and at the same time NAD(P)H is regenerated through hydride transfer from the carbon-bound hydrogen sites of methylene and methyl groups.[5, 23] This mechanism implies that acetate can be considered as an electron donor too.

When ethanol partially labeled at the methyl and pro-*R* methylene sites was added as an electron donor, no increase of $(D/H)_{H-COH}$ was observed. Even when methyl bideuterated ethanol was used, this value remained unchanged (experiments 4–7, Table 3). Only when all the three unexchangeable sites (methyl, pro-*R* and pro *R*-methylene) of ethanol were labeled at a high degree, a small increase of $(D/H)_{H-COH}$ was observed (experiments 1 and 2, Table 3). If the ethanol oxidation mechanism was efficient, $(D/H)_{H-COH}$ would be sensitive to ethanol labeling. The results show this is not the case. When ethanol was added in differently labeled waters, we obtained the following equation [Eq. (13)].

$$(D/H)_{H-COH} = 0.35(D/H)_w + 20.6 \quad (13)$$

This equation was established with two sets of data: $(D/H)_w$ and the corresponding $(D/H)_{H-COH}$ in experiments 3 and 4 (Table 3). The relatively big slope value and the small intercept value indicate that the contribution from unexchangeable hydrogen from electron donors is limited in contrast to adding glucose. In this case the main hydrogen source was water.

It has been shown that the bioreduction can be carried out without an electron donor in the presence of a large amount of yeast.[4, 24] In this work we realized the reduction with a small amount of yeast. The conversion rate depends on the quantity of yeast. For 5 g of EAA in 300 mL of solution, the conversion rates corresponding to 5, 10, and 16 g of dried yeast (lot 1) are 70, 78, and 97%, respectively, according to GC analysis. As a standard condition in this work we used 16 g of dried yeast for 5 g of EAA in 300 mL of water. When no auxiliary substrate was added, we performed reductions in a set of water with a different isotopic composition.(Table 4) On the basis of the $(D/H)_w$ and the corresponding $(D/H)_{H-COH}$ values for yeast of lot 1 in Table 4, we obtained Equation (14).

$$(D/H)_{H-COH} = 0.45(D/H)_w + 7.5 \quad (14)$$

The big slope value and the very small intercept value show that only very few unexchangeable hydrogen atoms were transferred, and the cofactor-transferred hydrogen is mainly that of water. The active hydrogen of NADH or NADPH is not exchangeable in the medium, and the indirect exchange

Table 3. $(D/H)_{H-COH}$ of ethyl 3-hydroxbutanoate obtained in the bioreduction with ethanol as electron donor.

Expt. no.	Yeast Lot no.	Water $(D/H)_w$/[ppm]	Ethanol $(D/H)_{CH_3}$[ppm]	Ethanol $(D/H)_{CH_2}$[ppm]	Ethanol $(D/H)_{Pro\text{-}RCH}$[ppm]	Ethanol $(D/H)_{Pro\text{-}SCH}$[ppm]	Ethyl 3- hydroxybutanoate $(D/H)_{H-COH}$[ppm]
1[a]	1	149.7	111.1(03)	123.2(0.3)	134.2(1.5)	112.2(1.2)	88.8(1.4)
2	1	149.7	196.3(0.4)	499.0(1.3)	505.7(2.5)	492.3(2.5)	103.3(0.4)
3[a]	2	149.7	111.1(03)	123.2(0.3)	134.2(1.5)	112.2(1.2)	72.5(1.5)
4[a]	2	248.7	111.1(03)	123.2(0.3)	134.2(1.5)	112.2(1.2)	106.8
5	2	149.7	153.7(0.2)	123.1(0.5)			68.2(1.3)
6	2	149.7	111.8(0.3)	138.8(0.4)	162.6(0.6)	115.0(0.4)	69.9(0.4)
7	2	149.7	166.6(0.2)[b]	123.6(0.3)			69.0(0.3)

[a] With the reference ethanol of natural abundance. [b] With ethanol (2.8 g) added, containing methyl bideuterated isotopomers.

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Table 4. $(D/H)_{H-COH}$ of the ethyl 3-hydroxbutanoate obtained by bioreduction in water of different isotopic compositions when no auxiliary substrate was added.

Yeasts		Lot 1		Lot 2
$(D/H)_w$[ppm]	149.1	248.7	338.8	149.1
$(D/H)_{H-COH}$[ppm]	79.2(1.2)	126.0(1.1)	170.1(0.6)	70.2(0.4)

through flavin is limited.[17] There are glucose and other carbohydrate molecules stored in the yeast cells.[25] It was proposed that these carbohydrate molecules may be used as electron donors in the absence of added electron donors.[5] In this case, if the electron donors were carbohydrate molecules, such as glucose, stored in the yeast cells, the deuterium would come mainly from unexchangeable sites of the carbohydrate molecules, as shown in experiments with labeled glucose molecules. However, the experimental results contradict the HMP pathway. The results are close to those obtained when ethanol was used, and may imply that the cofactor recycling mechanisms in the two cases are very similar although the intercept of Equation (13) is slightly higher than that of Equation (14), showing that only a few unexchangeable hydrogen atoms of ethanol were transferred. This may indicate that when ethanol was added, the main electron donor was those stored in the yeast cells, like when no electron donor was added, therefore ethanol is not an efficient electron donor. In fact, when ethanol was used, the yield was lower than when glucose was used and nothing added (see Experimental Section).

In all experiments, $(D/H)_{H-COH}$ was always lower than $(D/H)_w$; this implies a global normal isotope effect during the transfer of water hydrogen. Similar isotope effects have been observed in ethanol and glycerol formation in fermentation mediated by yeast.[13, 15, 17] With the available data, it is difficult to evaluate the isotope effect related to complex reactions, since the effect may occur during either the reduction of $NADP^+$ or the indirect exchange with water.[17b]

The two lots of yeasts used in this work maybe produced in different conditions, especially with carbon sources of different origin in the culture medium, and they may be somewhat different in activity. The difference in carbon source may influence the isotopic composition of the carbohydrate molecules stored in the yeast cells. The slight difference in yeast activity may modify the isotope effects on deuterium transfer to a certain degree.[26] When the two lots of yeasts were used under the same condition, for example, with ethanol and no electron donor, in tap water the $(D/H)_{H-COH}$ value was bigger for lot 1 than lot 2. However, when glucose was used, the value of lot 2 was bigger than lot 1. This influence can also be shown in Equations (7) and (8) for which the slopes are very close, but the intercepts are different for lot 1 and lot 2. The influence of the yeast strain on the isotopic data of ethanol is negligible. This is confirmed by the agreement of the results here, and in previous work.[17]

How the cell-stored carbohydrate molecules take part in the cofactor recycling is an interesting question. In the bioreduction performed with no electron donor, we observed a mass loss of the reaction mixture due to the formation of CO_2, and detected the formation of some ethanol. This shows that there is a metabolism process that we call "self fermentation" of sugars stored in the yeast cells. The mass loss was 2.2 g for 16 g of dried yeasts. If the metabolic process was pure alcohol fermentation, the CO_2 would correspond to a consumption of about 4.5 g of glucose. However, the quantity of ethanol was lower than the theoretical value of 2.5 g. The same mass loss was also observed in the case of adding ethanol (2.2 g), and even in a blank experiment in which only the same quantity of yeast was added to a solution of phosphate ($1.7\,gL^{-1}$) without EAA and electron donor (2.34 g). Therefore, the reduction hardly modified the quantity of CO_2 produced. According to these observations, there seems to be no relationship between this metabolism and the reduction.

Why the main hydrogen source was water when ethanol or nothing was used remains an open question. As discussed above, when the hydride is transferred by NADH, regenerated in glycolysis, the transferred hydrogen comes mainly from water due to a complex exchange process. However, the reductase molecules do not use NADH as a cofactor, as shown above. Furthermore, the redistribution coefficient (slope) of Equation (4) is much higher than that of Equation (13). This also proves that NADH regenerated in glycolysis of cellule-stored sugar should not be the cofactor. With the available data, it is difficult to precisely identify the mechanism of cofactor recycling in the yeast cellule in which no electron donors were added; further research is necessary.

Conclusion

We studied the mechanism of cofactor regeneration in asymmetric reduction mediated by yeast, by using quantitative isotope tracing close to natural abundance. Ethanol is not an efficient electron donor. The role of glucose and its hydride transfer pathway in the cofactor recycling were confirmed. Herein, we only focused on the hydride transfer mechanism, the stereochemical aspect was not examined. Since part of the important results obtained in the study remained unexplainable, new perspectives of the research are opened up.

Experimental Section

Materials: The corn glucose (reference) was obtained from Prolabo. The (1-$^2H_1$)glucose, (2-$^2H_1$)glucose, and (6,6-$^2H_2$) glucose were purchased from Aldrich. The (3-$^2H_1$)glucose and (4-$^2H_1$) glucose were purchased from Omicron. The isotopic purity of these isotopically substituted glucose molecules were at least 97% determined by NMR spectroscopy. D_2O (99.95%) was purchased from Eurisotop. The ethyl acetoacetate was a Sigma–Aldrich product.

The dried yeasts *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, and their mixture (50/50), used in wine production, were purchased from Val-Oeno. The number of living cells was $2.4 \times 10^{10}\,g^{-1}$, number of wild cells: none, bacteria number: $3.9 \times 10^5\,g^{-1}$. Two lots of yeast were used. Lot 1 was the commercial mixture of *S. cerevisiae* and *S. bayanus*. Lot 2 was a 50/50 mixture of the two pure strains purchased from the same supplier mixed by us.

Ethyl acetoacetate reduction: [27] The reduction medium was composed of yeast ($54\,gL^{-1}$) and Na_2HPO_4 ($1.7\,gL^{-1}$) dissolved in water (300 mL). Glucose ($270\,gL^{-1}$) or ethanol ($17\,gL^{-1}$) was added to the medium for bioreduction for cofactor regeneration. Glucose samples, slightly enriched

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at specific positions, were prepared by adding small quantities of the specific deuterium-substituted glucose (10–25 mg) to the corn glucose (80 g) dissolved in tap water (300 mL). The variation of the $(D/H)_j$ of glucose was evaluated on a mass basis of the added enriched glucose obtained by weighing with a correction of its purity. The water medium of different (D/H) values was prepared by adding D_2O to tap water. The $(D/H)_w$ of both tap water and the labeled waters were determined by isotopic ratio mass spectrometry (IRMS).[9] The labeled ethanol molecules were obtained in alcohol fermentation reactions with different labeled glucose molecules or slightly deuterated water. Their site-specific isotopic ratios $(D/H)_i$ were measured precisely. The bioreduction reactions were performed either in tap water or in water with different isotopic composition under anaerobic condition. The incubation temperature was $30 \pm 1\,^{\circ}C$ for seven days. At the end of the biotransformation, the medium was centrifuged to remove the biomass. When glucose was used in the reductions, the ethanol produced was extracted by fractional distillation on a spinning-band column. The residue was saturated with NaCl and extracted five times with diethyl ether. Impure 3-hydroxybutanoate was obtained after distillation of ether. The impure product was further purified by vacuum distillation. GC analysis of the reaction mixture at the end of the reaction showed that there was no unreacted ethyl acetoacetate when glucose was added (yield: 79–83%), whereas we found 10% and 3–4% unreacted reactant when ethanol was added (yield: ~70%), and with no electron donor (yield: 78–80%).

Acetylation of 3-hydroxybutanoate: Ester 3-hydroxybutanoate (3–5 g) was acetylated with two equivalents of acetic anhydride dissolved in pyridine (4 mL). The mixture was stirred for 2 days at room temperature. The excess acetic anhydride and pyridine were removed by co-evaporation with toluene. Pure ethyl 3-acetoxybutanoate was obtained after vacuum distillation. The acetylation reaction was quantitative.

Synthesis of ethyl mandelate: *S*-(+)-mandelic acid was used. The synthesis of ethyl mandelate was performed according to ref. [17].

^{2}H NMR measurements: The deuterium NMR spectra were recorded at 61.4 MHz under broad-band proton decoupling by using a Bruker DPX 400 spectrometer equipped with a ^{19}F lock device. Other conditions were: frequency window 1200 Hz, memory size 16 K, scan number 500 for ethanol and 14000 for ethyl 3-acetoxybutanoate, and an exponential multiplication corresponding to a line broadening of 0.5 Hz for ethanol, ethyl mandelate, and ethyl 3-acetoxybutanoate. Three spectra were recorded for each sample, and an average $(D/H)_i$ value was calculated from these three measurements.

The $(D/H)_i$ values were determined by using an external reference TMU (tetramethylurea), of which the isotopic ratio $(D/H)_R$ was precisely calibrated by IRMS. $(D/H)_i$ was calculated from the following equation [Eq. (15)].

$$(D/H)_i = (D/H)_R P_R m_R M_S S_i / (P_i f m_S M_R S_R) \quad (15)$$

In which P_i and P_R are the stoichiometric numbers of hydrogen atoms in site i and in the reference. M_S, m_S and M_R, m_R are the molecular weight and mass of the sample and the reference, respectively, f is the purity of the sample in mole fraction, and S_i and S_R correspond to the surface area of the signals of the site i monodeuterated molecule, and the reference in the ^{2}H NMR spectrum, respectively. The quantitative evaluation of the surface areas was performed by using a curve-fitting program (Interlis from Eurofins, Nantes, France).[28]

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