HLADH-Catalyzed Reduction of Cyclohexanone with NADH Regeneration by Alcohols: Effects of Reaction Conditions

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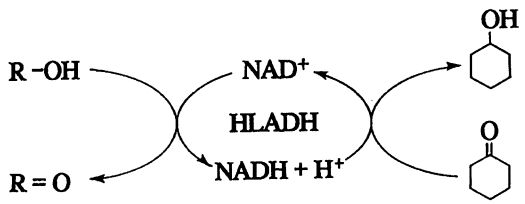
The effects of alcohol structure and reaction conditions on the horse liver alcohol dehydrogenase-catalyzed reduction of cyclohexanone were investigated with in situ regeneration of NADH by alcohols. The rate of coupled reduction of cyclohexanone was much more strongly affected by the structure of alcohols than the reduction rate of NAD measured in the absence of cyclohexanone. Diols such as 1,4-butanediol or 1,5-pentanediol were the reductants of choice in terms of reaction rate and the yield of cyclohexanol. The effects of pH and buffer concentration on coupled reduction of cyclohexanone were parallel to the effects on NAD reduction. However, the dependency of coupled reduction of cyclohexanone on reaction temperature reflected the temperature-dependency of cyclohexanone reduction by NADH in the absence of alcohols. These results were discussed in terms of the difference of sensitivity to the reaction conditions of each oxidoreduction and of coupled oxidoreductions.

Recently use of enzymes has become common for synthetic reactions which require high selectivity and specificity. In particular, alcohol dehydrogenases have been utilized for stereospecific conversions of aldehydes, ketones, and alcohols. Examples are the reduction of ketones or aldehydes by yeast or horse liver alcohol dehydrogenases.[1—4] In general, alcohol dehydrogenases require coenzymes such as NAD(P), and the regeneration of the coenzymes is essential for the practical applications of these enzymatic processes in large scale.

Relatively few reactions have been proposed for the in situ regeneration of NADH in the horse liver alcohol dehydrogenase (HLADH)-catalyzed synthetic reductions of carbonyl compounds to alcohols (Scheme 1).[5] Among these, coupled-substrate recycling of the coenzyme by a single enzyme HLADH seems to be most useful due to the simplicity of the reaction systems.[6—15] So far, ethanol has been used most extensively for NADH regeneration, but problems have often been encountered due to the inhibition or deactivation of the enzyme by ethanol or produced acetaldehyde.[6,16,17] The effects of the nature of alcohols and reaction conditions on the activity of HLADH have not been thoroughly investigated.

It may be reasonably assumed that each of the coupled reactions is differently affected by the reaction conditions and, therefore, has its own optimum conditions. For example, it was expected that, for the HLADH/NADH-catalyzed reduction of cyclohexanone by an alcohol, optimum pH would be different for the two component reactions, the reduction of cyclohexanone by NADH and the regeneration of NADH from NAD by the alcohol. The former consumes but the latter produces protons, and the pH-dependency of the coupled oxidoreductions may be governed by either of the component reactions.



Scheme 1.

The primary objective of this research is to elucidate the effects of the nature of alcohol and reaction conditions on the HLADH/NADH-catalyzed reduction of cyclohexanone by alcohols on the basis of the analysis of these effects on each of the component reactions. The results will be discussed in terms of rate-determining factors and the optimization of the reduction of carbonyl compounds by HLADH with in situ regeneration of NADH.

Experimental

Materials. Horse liver alcohol dehydrogenase (HLADH, EC 1.1.1.1,>98% protein, 1-2 units per mg protein) was purchased from Sigma. Chem. Co. β-Nicotinamide adenine dinucleotide (NAD) and β-nicotinamide adenine dinucleotide disodium salt (NADH) were the products of Kohjin Co., Ltd. Alcohols and cyclohexanone were obtained from Wako Pure Chem. Co. and dried on molecular sieves 3A.

Measurement of Reaction Rate. HLADH-catalyzed reductions of NAD by alcohols were done in 0.1 M Tris-HCl or phosphate buffer solutions in UV cells. The formation of NADH was followed by the measurement of the absorbance at 340 nm on a JASCO spectrophotometer Ubest-50. The temperature of the reaction solutions was controlled with a JASCO EHC-1 temperature controller equipped with a magnetic stirrer. HLADH-catalyzed reductions of cyclohexanone by NADH were done by a similar method. The reaction rate was determined by following the disappearance of NADH at 340 nm spectrophotometrically.

HLADH/NADH-catalyzed reductions of cyclohexanone with NADH regeneration (coupled reductions of cyclohexanone) by alcohols were performed in 0.1 M Tris-HCl

or phosphate buffer solutions in glass vials with constant magnetic stirring. The reaction rate was determined by following the formation of cyclohexanol on a Hitachi gas-liquid chromatograph with a column packed with diethylene glycol succinate on Uniport B. Total reaction volume was 10 ml, and reaction temperature was 30 °C unless otherwise stated. The reaction rate was expressed as the amount (μmol) of the product or reacted substrate per minute and per milligram of HLADH.

Results and Discussion

Effects of Alcohol Structure. It is known that the HLADH/NADH-catalyzed conversion of a carbonyl compound to the corresponding alcohol is the coupling of two reactions, the reduction of carbonyl compound by NADH and the regeneration of NADH from NAD by an alcohol (Scheme 1). Both reactions are catalyzed by the single enzyme HLADH, and this provides synthetic chemists with a facile method for preparing a variety of chiral alcohols from carbonyl compounds.

It was assumed that the overall rate of the reduction of a carbonyl compound is determined by either one of the coupled reactions; the slower reaction would be rate determining for the overall reaction. Therefore, we considered that the study on the reaction conditions for the two independent reactions would give information on the rate determining factors for the optimization of the overall reaction.

Initially, we investigated the effects of ethanol content on NAD reduction. It was found that, as shown in Fig. 1, the reaction rate decreased sharply with an increase in ethanol content. This may be the consequence of substrate inhibition or denaturation of HLADH by

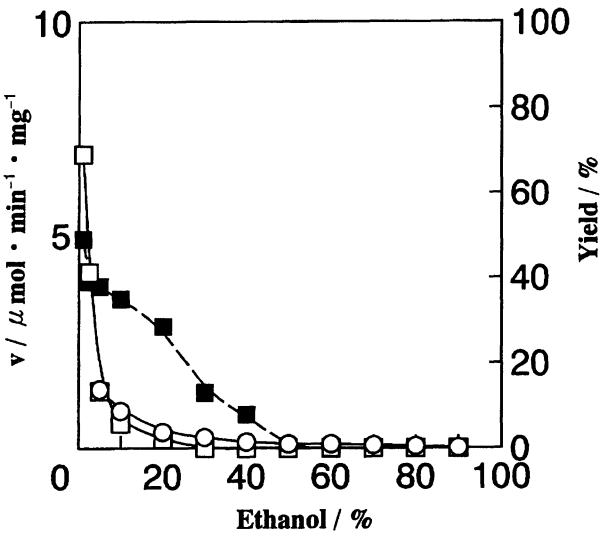


Fig. 1. NAD reduction and coupled cyclohexanone reduction by ethanol. HLADH 0.1 mg, NAD 0.25 mM (1 M=1 mol dm^{-3}), cyclohexanone 50 mM, Tris-HCl buffer (0.1 M, pH 9.0), 30 °C. ○: NAD reduction rate; □: cyclohexanone reduction rate; ■: cyclohexanol yield (48 h).

ethanol. As expected from this result, coupled reduction of cyclohexanone by ethanol was also severely retarded with an increase in ethanol content (Fig. 1). Therefore, 100 mM (about 0.58% by volume) of ethanol was used throughout this work.

Table 1 summarizes the results of the reduction of NAD and coupled reduction of cyclohexanone by several alcohols and diols. The reduction rate of cyclohexanone by NADH was measured separately and determined to be 12 μmol min^{-1} mg^{-1} under the same reaction conditions. It can be seen that the rate of NAD reduction was much less dependent on the structure of the alcohols than the rate of coupled reduction of cyclohexanone. The ratio of the rate of coupled cyclohexanone reduction to that of NAD reduction (v'/v in Table 1) was also a function of alcohol structure. It seems that the effect of alcohol structure is amplified in the coupled reduction of cyclohexanone; there is a tendency that the larger the reduction rate of NAD, the larger the rate ratio of the two reactions.

It is noteworthy that, contrary to the expectation that the coupled reaction rate should be limited by the slower step of the two component reactions, the coupled reduction of cyclohexanone was faster than NAD reduction for all the alcohols except for 2-propanol and ethylene glycol.

The mechanism of HLADH/NADH-catalyzed reduction of carbonyl compounds has been proposed, which includes the binding of substrates and NAD(H) to HLADH at different binding sites.[18] It is known that the dissociation of HLADH–NADH complex is slow compared to the reduction of bound NAD by an alcohol,[6,8,16] and this may be responsible for the relatively small dependency of NAD reduction rate on the structure of alcohols. The apparent acceleration of NAD reduction in coupled reduction of cyclohexanone may be ascribed to the fact that the dissociation of HLADH–NADH is not required for recycling of the coenzyme during the reaction. For the reduction of cyclohexanone by bound NADH, the slow dissociation of HLADH–NADH complex is advantageous, and this would accelerate the overall cyclohexanone reduction. In this case, the effects of alcohol structure on coupled reduction rate would be amplified.

It is noteworthy that, as seen in Table 1, 1,4-butanediol and 1,5-pentanediol were effective reductants for the reduction of cyclohexanone. Ethanol and short chain diols were not satisfactory for the preparative purposes because of the low yields of cyclohexanol, probably due to the inhibition or deactivation of HLADH. The reason for the high efficiency of 1,4-butanediol and 1,5-pentanediol is not clear, but there is a possibility that produced ω-hydroxyalkanals form cyclic hemiacetals by intramolecular addition reactions. This would eliminate the aldehyde groups which would impair the catalytic function of HLADH.

Figure 2 shows the time course of coupled reduc-

Table 1. Effects of Alcohol Structure on the Rate of NAD Reduction and Cyclohexanone Reduction with NADH Regeneration[a)]

Alcohol	Reduction rate			Cyclohexanol
	NAD (v)	Cyclohexanone (v')	v'/v	%[b)]
Ethanol	1.5	13	8.4	44
2-Propanol	1.1	0	—	58
2-Butanol	0.78	2.1	2.7	58
3-Pentanol	1.2	5.0	4.1	86
Cyclopentanol	0.53	1.2	2.2	65
Ethylene glycol	0.98	0.05	0.05	26
1,3-Propanediol	1.5	5.5	3.6	42
1,4-Butanediol	2.3	14	6.3	88
1,5-Pentanediol	2.5	16	6.3	87

a) v or v'=μmol min^{-1} mg^{-1}, HLADH 0.1 mg, NAD 0.25 mM, alcohol 100 mM, cyclohexanone 50 mM, Tris-HCl buffer (0.1 M, pH 9.0), 30 °C. b) HLADH 0.5 mg, NAD 1.25 mM, alcohol 200 mM, cyclohexanone 100 mM, Tris-HCl buffer (0.1 M, pH 9.0), 30 °C, 24 h.

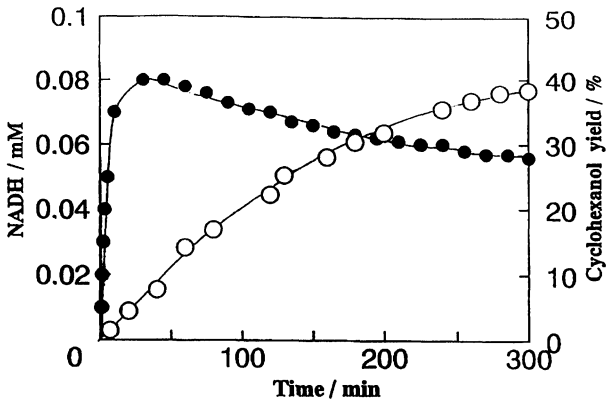


Fig. 2. Time course of coupled reduction of cyclohexanone. HLADH 0.1 mg, NAD 0.25 mM, ethanol 100 mM, cyclohexanone 50 mM, Tris-HCl buffer (0.1 M, pH 9.0), 30 °C. ○: cyclohexanol yield; ●: NADH concentration.

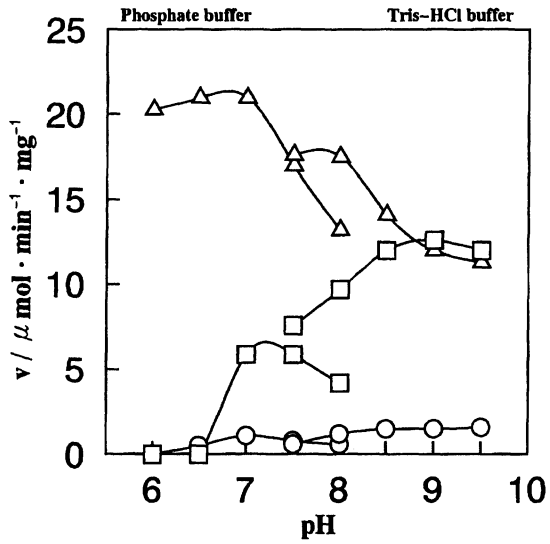


Fig. 3. Effects of pH on NAD reduction, NADH oxidation, and coupled cyclohexanone reduction. HLADH 0.1 mg, NAD or NADH 0.25 mM, ethanol 100 mM, cyclohexanone 50 mM, phosphate buffer (0.1 M, pH 6.0—8.0), Tris-HCl buffer (0.1 M, pH 7.5—9.5), 30 °C. ○: NAD reduction; △: NADH oxidation; □: cyclohexanone reduction.

tion of cyclohexanone by ethanol. It can be seen that at the initial stage of the reaction, the concentration of NADH increased rapidly and then decreased gradually. The initial rate of NADH formation was 0.4 μmol min^{-1} mg^{-1}, which was about 1/4 of the reaction rate measured in the absence of cyclohexanone. The concentration of NADH after the initial burst of the reaction was 0.05—0.08 mM, which was 1/5—1/3 of the initial concentration of NAD. These results suggest that the dissociation of HLADH–NADH is not very slow, but is comparable to oxidoreduction of cyclohexanone and ethanol. This would rationalize the study on the rate-determining factors for coupled cyclohexanone reduction by probing each of the component reactions independently.

Effects of Reaction Conditions. The effects of pH on the three reactions are illustrated in Fig. 3. There are some inconsistencies in pH dependency of reaction rate in Tris-HCl and phosphate buffer solutions. However, there is a tendency that, with an increase in pH, the rate of NAD reduction by ethanol increased but the rate of cyclohexanone reduction by NADH decreased. The result is understandable because the former reaction forms and the latter reaction consumes protons. Similar to NAD reduction, the rate of coupled reduction of cyclohexanone by ethanol increased with an increase in pH. This suggests that the rate of coupled reduction of cyclohexanone is mainly governed by NAD reduction rate rather than by the rate of cyclohexanone reduction by NADH. When 3-pentanol was used as a reductant, the situation was very similar to the case of ethanol, as shown in Fig. 4. In both cases, at pH above 9, the rate of coupled reduction of cyclohexanone became comparable to the rate

of cyclohexanone reduction by NADH, probably due to the acceleration of NAD reduction by alcohols.

As shown in Fig. 5, the concentration of Tris-HCl buffer solution also affected the rate of oxidoreductions. Similar to the effects of pH, the rate of coupled reduction of cyclohexanone by ethanol increased with buffer concentration in parallel with the increase of NAD reduction rate by ethanol.

The above results suggest that the rate of coupled reduction of cyclohexanone by alcohols is primarily gov-

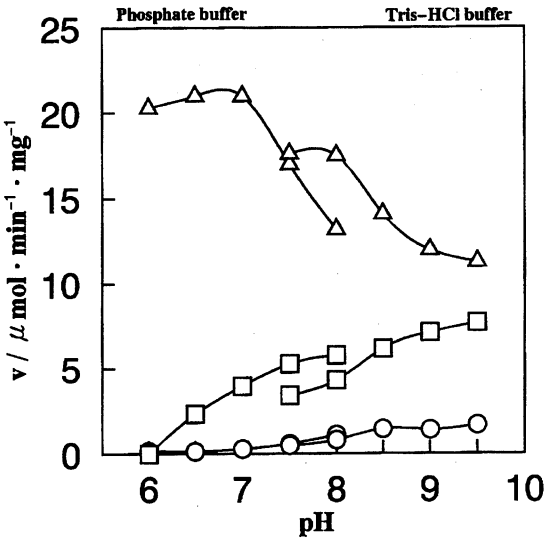


Fig. 4. Effects of pH on NAD reduction, NADH oxidation, and coupled cyclohexanone reduction. Conditions and symbols are the same as in Fig. 3 except that 100 mM of 3-pentanol was used as a reductant.

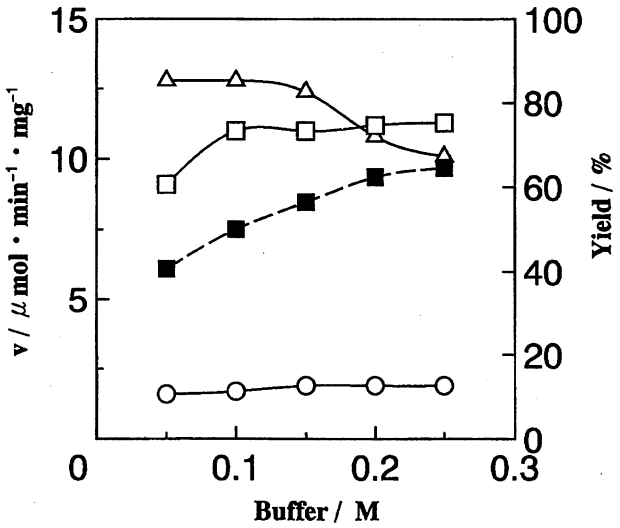


Fig. 5. Effects of buffer concentration on NAD reduction, NADH oxidation, and coupled cyclohexanone reduction. HLADH 0.1 mg, NAD or NADH 0.25 mM, ethanol 100 mM, cyclohexanone 50 mM, Tris-HCl buffer (pH 9.0), 30 °C. ○: NAD reduction rate; △: NADH oxidation rate; □ cyclohexanone reduction rate; ■: cyclohexanol yield (48 h).

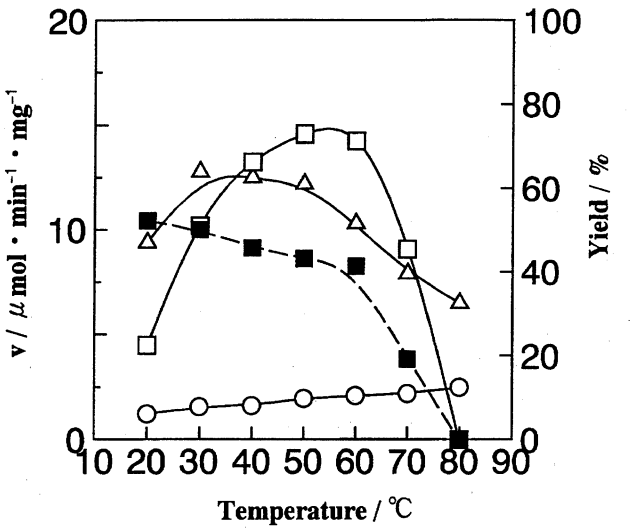


Fig. 6. Effects of reaction temperature. HLADH 0.1 mg, NAD or NADH 0.25 mM, ethanol 100 mM, cyclohexanone 50 mM, Tris-HCl buffer (0.1 M, pH 9.0). ○: NAD reduction rate; △: NADH oxidation rate; □: cyclohexanone reduction rate; ■: cyclohexanol yield (48 h).

erned by the rate of NAD reduction. However, the result of the temperature dependency was quite different. As shown in Fig. 6, NAD reduction was accelerated by an increase of the reaction temperature up to 80 °C, but the reduction of cyclohexanone by NADH was retarded above 40 °C. The rate of coupled reduction of cyclohexanone seemed to follow the latter reaction profile, but was more severely retarded at higher temperatures. A more comprehensive study is required for further refinement of the coenzyme recycling systems, but these findings enabled some rational selections of the reaction conditions for the efficient reduction of cyclohexanone by HLADH/NADH. The use of low concentrations of alcohols or diols, high pH, high buffer concentrations, and ambient temperatures are advantageous.

In conclusion, the rate of HLADH-catalyzed reduction of cyclohexanone with in situ regeneration of NADH by alcohols is strongly affected by the structure of alcohols. 1,4-Butanediol and 1,5-pentanediol were the best among the alcohols or diols used in this study. The results may be associated with slow dissociation of HLADH–NADH complex as revealed by the analysis of the effects of these alcohols on NAD reduction. Analysis of the effects of reaction conditions on each of the coupled reactions is also useful for optimization of coupled reduction of cyclohexanol. These findings may be extraporated to the reduction of other carbonyl compounds by HLADH/NADH.

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