BIOORGANIC CHEMISTRY 16, 223–231 (1988)

Enzymatic *in Vitro* Reduction of Ketones

15. The Influence of Reaction Conditions on the Stereochemical Course of HLAD-Catalyzed Reductions: 3-Cyano-4,4-dimethylcyclohexanone as a Sensitive Probe

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Received July 24, 1987

The stereochemical course of the horse liver alcohol dehydrogenase (HLAD)-catalyzed reduction is studied on substituted cyclohexanones under varying reaction conditions. Temperature, pH, and ethanol concentration influence the stereochemical course of the reduction of some substrates. Moreover, the quantitative aspects of the stereochemistry of HLAD-catalyzed reductions seem to vary with the origin of the enzyme preparations. 3-Cyano-4,4-dimethylcyclohexanone is an appropriate and sensitive test substrate in the study of these influences.

INTRODUCTION

During our first HLAD[1]-catalyzed reductions of 3-cyano-4,4-dimethylcyclohexanone **4** (J. J. Willaert, results to be published) in an ethanol-coupled NAD(H) recycling system, we observed an important influence of pH on the *cis/trans* ratio of the alcohols. It is known for pig liver esterase (*1, 2*) that reaction conditions can influence the enantiomeric ratio of the reaction products.

These facts prompted us to investigate the influence of pH, temperature, and ethanol concentration on the stereochemical course of the reduction of **4** and some other well-documented substrates: cyclohexanone **1** (*3*), 3-methylcyclohexanone **2** (*4*), methyl 3-oxocyclohexanecarboxylate **3**[2b] (*5*), and 3-cyanocyclohexanone **5** (*6*).

[1] Abbreviations used: HLAD, horse liver alcohol dehydrogenase; Mops, 4-morpholinopropanesulfonic acid.

[2] For simplicity in this paper two deviations from the IUPAC nomenclature and the Cahn–Ingold–Prelog conventions are made: (a) For 3-cyano-4,4-dimethylcyclohexanone and -ol the chirality label (*R/S*) of carbon number 3 is reversed in respect to the Cahn–Ingold–Prelog rules in order to keep the same label assignment for all substrates under consideration. (b) Methyl 3-oxo- and 3-hydroxycyclohexanecarboxylate are renamed as 3-methoxycarbonylcyclohexanone and -ol to obtain the same ring numbering for all substrates and products, which means that the carbonyl or the alcohol function is given number 1 as locant.

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SETUP AND VALIDITY OF THE EXPERIMENTS

All HLAD-catalyzed reductions were performed with commercially available enzyme; this is *not* the purified EE-isoenzyme (*7*).

In order to maintain the highest consistency, we prepared for all experiments one single stock solution of HLAD in water, which was divided into standard aliquots of 0.50 ml. A nonbuffered solution allows us to use the enzyme stock in different buffers without influencing the pH of the reaction mixture.

Experimental errors were in general below 5% and can safely be regarded to be less than 10%. During 3 months of experimentation, and within a margin of 5%, the activity of the stock solution was found to be constant.

RESULTS AND DISCUSSION

In 1978 Van Osselaer *et al.* (*3*) described the kinetics of the HLAD-catalyzed reduction of ketones in an ethanol-coupled NADH recycling system. Under appropriate reaction conditions the initial reduction rate v_0 is given by

$$v_0 = \frac{\text{Et} \cdot k_{3B} \cdot [\text{ketone}]_0}{1 + [\text{EtOH}]_0/K_I} \qquad [1]$$

Et = Enzyme activity
k_{3B} = reaction rate constant of the catalytic reduction step
K_I = dissociation constant of the dead-end complex HLAD–NADH–EtOH ($K_I = 0.104$) (*3*).

For this publication we further define the normalized initial reduction rate v_0' which is independent of the ketone concentration as $v_0' = v_0/[\text{ketone}]_0$.

It is important to stress the difference between stereochemical data obtained from kinetic and from preparative scale experiments. In kinetic experiments, performed under initial rate conditions, the formation of reaction products is exclusively kinetically controlled by interactions of the substrates with the enzyme–coenzyme complex in the transition state. In preparative scale experiments, which proceed beyond the initial rate conditions, product isomer ratios can be partially controlled by the thermodynamic properties of substrates and products. Thus in this study on the influence of the reaction conditions on the enzymatic catalysis, all experiments are kept under strict kinetic reaction conditions.

The Influence of pH and Nature of the Buffer

The substrates **1–5** were reduced in the pH range 6.0–10.0. Below pH 6.0 HLAD is slowly deactivated and above pH 10.0 it starts losing its zinc atoms (*8*). As can be seen from Table 1, the nature of the buffers used does not significantly influence the reaction rates.

The initial reduction rates v_0' for ketones **1–5** are summarized in Table 2. To

TABLE 1

Reduction of Cyclohexanone: Influence of the Buffer on the Reaction Rate v_0' ($10^{-4} \cdot s^{-1}$)

	pH		
Buffer	6	7	8
Phosphate	1.9	2.9	—
Mops	2.0	2.9	3.1
Tris/HCl	—	—	3.2

evaluate the pH dependence, a plot of $\ln(v_0')$ versus pH is given for each of the formed alcohols in Fig. 1. The profiles of the pH curves reveal three points:

1. The pH dependence of differently substituted cyclohexanones is different: e.g., cyclohexanone and (3*R*)-3-methoxycarbonylcyclohexanone.[2b]

2. The pH dependence of the reduction of enantiomeric ketones is different: e.g., (3*R*)- and (3*S*)-3-methylcyclohexanone.

3. The pH dependence differs for each diastereoisomeric alcohol derived from one single enantiomeric ketone: e.g., the alcohols obtained from (3*S*)-**4** and (3*S*)-**5**.

TABLE 2

Influence of pH on the Reduction Rate: Rates of Formation v_0' ($10^{-6} \cdot s^{-1}$)[a]

Substrate	Alcohol config.[b]	pH[c]						
		6	6.5	7	8	8.5	9	10
1	—	200	—	290	320	270	200	60
	1*S*,3*S*-*trans*	210	—	310	340	240	140	29
2	1*S*,3*R*-*cis*	1.7	—	2.4	3.4	3.5	3.2	2.3
	1*R*,3*R*-*trans*	0.055	—	0.098	0.15	0.14	0.12	0.073
3	1*S*,3*S*-*trans*[d]	240	240	150	130	120	82	32
4	1*S*,3*S*-*trans*[e]	14	—	22	28	26	24	17
	1*R*,3*S*-*cis*[e]	38	—	53	46	39	26	11
5	1*S*,3*S*-*trans*	65	—	160	200	200	140	48
	1*R*,3*S*-*cis*	34	—	47	42	30	16	5.7

[a] Enantiomerically pure ketone was used for (3*R*)-**2** only. In all other cases the ketone was racemic. The observed reaction rates of the racemic ketones were doubled to refer to pure (3*S*)-ketone.[b]

[b] The configurations are known from preceding preparative scale reductions (*4–6*; J. J. Willaert, results to be published).

[c] Buffers: 0.050 M Mops, pH 6.0 and 7.0; 0.050 M Tris/HCl, pH 8.0, 8.5, and 9.0; 0.050 M glycine/NaOH, pH 10.0.

[d] See footnote 2b in text.

[e] See footnote 2a in text.

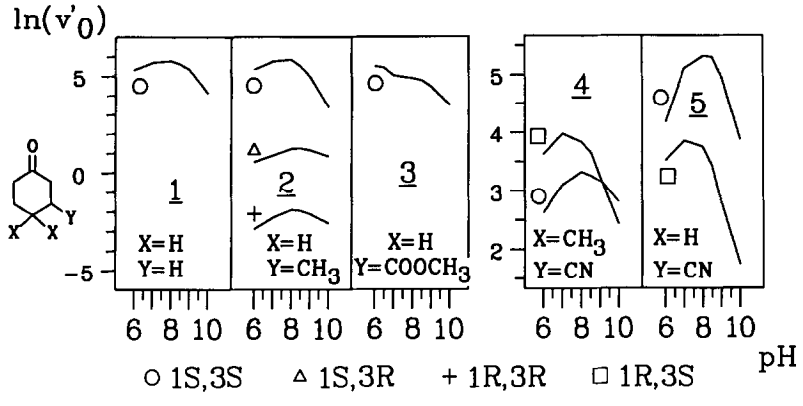

FIG. 1. Dependence on v_0' on pH.

The Influence of the Ethanol Concentration

Ethanol is the coupled substrate recycling NAD^+ to NADH. Under our experimental conditions, the recycling is much faster than the reduction and has no direct influence on the reduction rate.

Nevertheless several indirect effects are observed. First, ethanol forms a dead-end complex HLAD–NADH–ethanol (*3*). From Eq. [1] it is clear that the reduction rate decreases when the ethanol concentration is raised. Further, in preparative scale experiments, where higher ethanol concentrations are required to maximize product yield, ethanol is a substantial part of the medium and can exert a solvent effect on the enzyme. In this indirect way an influence of ethanol on the rate and eventually the stereochemical course of the reduction reaction can be expected.

Therefore the formation rates of (1*S*,3*S*)- and (1*R*,3*S*)-3-cyano-4,4-dimethylcyclohexanol[2a] were measured for ethanol concentrations ranging from 0.050 up to 5.0 M, the latter containing about 30% (v/v) of ethanol.

A plot of the inverse initial reduction rate versus the ethanol concentration shows a nearly linear relationship for concentrations up to 2.0 M (Fig. 2). This linear relationship can be ascribed to the formation of the dead-end complex HLAD–NADH–EtOH. Above 2.0 M the reaction rate drops rapidly below its theoretically predicted value.[3]

The *cis*/*trans* ratio varies from 1.4 at 0.10 M ethanol to 0.6 at 5.0 M ethanol. Thus (1*S*,3*S*)-3-cyano-4,4-dimethylcyclohexanol[2a] changes from a minor compound at lower ethanol concentrations to a major compound at higher concentrations.

[3] The decrease of enzyme activity due to ethanol concentrations higher than 2.0 M is not due to irreversible enzyme denaturation since the reduction rate is constant during the experiment (5.0 M ethanol: 3 days).

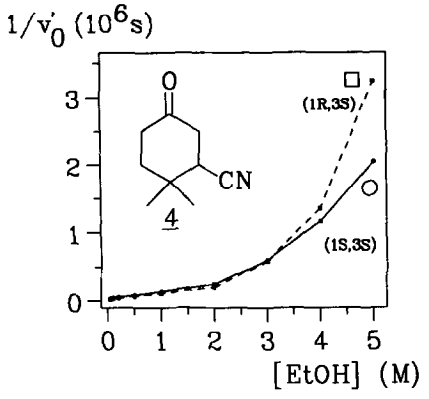

FIG. 2. Dependence of v_0' on ethanol concentration.

More general, our results suggest that the addition of organic solvents not only can change the rate of HLAD-catalyzed reactions (*9*, *10*) but also can influence their stereochemical course.

The Influence of Temperature on the Stereochemistry of HLAD Reductions

We checked the influence of temperature on the reduction rates of 3-cyano-4,4-dimethylcyclohexanone. Figure 3 shows the relation $\ln(v_0')$ versus $1/T$ measured at pH 8.5. The (1*S*,3*S*)/(1*R*,3*S*)[2a] ratio of formation varied from 0.5 at 5°C to 0.9 at 45°C.

Inspection of the activation parameters provided by T. Van Osselaer (*4*, *11*) shows that also for his substrates, the alcohol isomer composition varies with reaction temperature.

Stereochemical Differences Due to the Use of Different Enzyme Batches

When comparing the data of Table 2 with earlier results from our laboratory some remarkable discrepancies appear. The ratio of the initial formation rates for

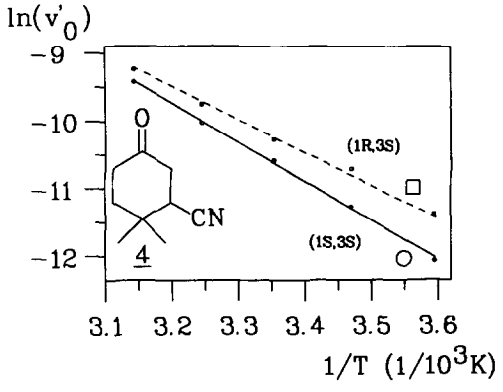

FIG. 3. Dependence of v_0' on temperature.

TABLE 3

Competition Experiments for Enzyme Batches

	Enzyme batch		
	1	2	3
v_0' CHL ($10^{-4} \cdot s^{-1}$)	2.5	2.5	2.7
v_0'(1*S*,3*S*)-3-Me-CHL ($10^{-4} \cdot s^{-1}$)	2.2	2.2	2.2
v_0'(1*S*,3*S*)-3-Me-CHL/v_0'CHL	0.89	0.88	0.82

Note. CHL, cyclohexanol.

(1*S*,3*S*)-3-methylcyclohexanol versus cyclohexanol is now found to be 0.9, in contrast to 0.5 published in 1980 (*4*). The ratio for (1*S*,3*S*)- versus (1*R*,3*S*)-3-cyanocyclohexanol is now found to be 6.6, in contrast to 2.5 reported before (*6*). The ratio for (1*S*,3*S*)-3-methoxycarbonylcyclohexanol[2b]/cyclohexanol was reported to be 0.3 (*5*) and now 0.4 is found.

Since these differences can hardly be explained by statistical errors, we wondered whether enzyme composition could be responsible for these discrepancies. Analogous results were reported for pig liver esterase (*1*). So we decided to compare the stereochemical behavior of three different HLAD batches. Two tests were applied on each of them.

First a competition experiment was set up between equimolar amounts of cyclohexanone and 3-methylcyclohexanone. In the same reaction vessel both ketones are reduced simultaneously. The ratio of reduction rates for both ketones is determined for each enzyme stock (see Table 3). The reaction rate ratios are the same within error margins for all three stocks. This certainly does not prove that all enzyme batches behave identically.

In a second experiment 3-cyano-4,4-dimethylcyclohexanone was reduced and the ratio of v_0' for the formation of the (1*S*,3*S*)[2a] and (1*R*,3*S*)[2a] alcohols was determined. Table 4 gives the results. In contrast with the former experiment, a

TABLE 4

Reduction of 3-Cyano-4,4-dimethylcyclohexanone: Stereochemical Differences between Enzyme Batches

	Enzyme batch		
	1	2	3
v_0'(1*S*,3*S*)DMCHL[a] ($10^{-6} \cdot s^{-1}$)	25	48	34
v_0'(1*R*,3*S*)DMCHL[a] ($10^{-6} \cdot s^{-1}$)	33	49	40
v_0'(1*S*,3*S*)DMCHL[a]/v_0'(1*R*,3*S*)DMCHL[a]	0.76	0.98	0.86
v_0'(1*S*,3*S*)DMCHL[a]/v_0'CHL	0.099	0.19	0.13

Note. DMCHL, 3-Cyano-4,4-dimethylcyclohexanone; CHL, cyclohexanol.

[a] See footnote 2a in text.

significant difference can be observed for each of the enzyme stocks. First, (1*S*,3*S*)/(1*R*,3*S*)[2a] alcohol ratios vary from 0.76 for enzyme stock 1 to 1.0 for enzyme stock 2. Moreover, the reduction rate ratios for (3*S*)-3-cyano-4,4-dimethylcyclohexanone[2a] versus cyclohexanone differ as much as a factor of 2 between enzyme batches 1 and 2. This clearly illustrates the fact that substrate preferences can differ when different enzyme stocks are used.

CONCLUSIONS

From our results it is quite clear that "the" stereochemical course of HLAD-catalyzed reactions does not exist since it can be changed by varying reaction circumstances. 3-Cyano-4,4-dimethylcyclohexanone has been shown to be a very sensitive tool to detect the influence of the reaction medium on HLAD catalysis.

This finding has two important consequences. Whenever HLAD is used to prepare a chiral product, and the stereoselectivity of the reaction is not extreme, enantiomeric purities of the products can eventually be optimized by changing the reaction conditions. Further it means that all existing HLAD reaction models (*12–15*) are valid only for the reaction conditions used in the reactions from which the models are built up.

Changes of the stereochemical course induced by pH, temperature, and ethanol concentration can be explained by changes of enzyme conformation. Indeed all factors controlling the protonation equilibria of acidic or basic groups of amino acids can change the polarity (*16*) and geometry of the active sites of the enzyme.

The variability of the stereochemistry of HLAD-catalyzed reactions for different enzyme preparations is probably due to varying enzyme compositions of the enzyme batches. It is a well-documented fact that naturally occurring HLAD consists of up to 12 isoenzymes (*7, 17*). It is also known that different isoenzymes have different substrate specificities (*18*). Until now no experimental data describe the stereochemical course of reactions catalyzed by each of the pure isoenzymes. Whether all these isoenzymes are actually participating when HLAD reductions of substituted cyclohexanones are performed remains an open question, but from our experiments we conclude that at least two HLAD variants must be active in the reduction of cyclohexanones. Moreover, these two HLAD variants must show a different behavior toward the reduction of a ketone. In this way the changing HLAD composition may account for differences between enzyme batches and also for the contradictory results obtained by different authors. Further, it should be noted that performing reductions with HLAD isoenzyme mixtures also may, at least partially, account for the different pH profiles of different substrates.

EXPERIMENTAL

Enzyme and coenzyme. All the HLAD used in our experiments was obtained from Sigma in portions of 200 units crystallized and lyophilized enzyme (A 6128).

The lot numbers were stock 1, 66F-8120; stock 2, 96F-8220; stock 3, unknown. Enzyme stock 1 was used in all experiments on the influence of reaction conditions. NAD^+ was also purchased from Sigma (N-7004).

Substrates. Cyclohexanone: Aldrich C10,218-0 (redistilled); racemic 3-methylcyclohexanone: Aldrich M3,860-5 (used as such); (R)-(+)-3-methylcyclohexanone: Aldrich M3,858-3 (used as such); 3-cyanocyclohexanone: synthesized as described earlier (*6*); 3-methoxycarbonylcyclohexanone:[2b] synthesized as described earler (*5*); and 3-cyano-4,4-dimethylcyclohexanone: prepared in a manner analogous to that of 3-cyanocyclohexanone.

Preparation of the enzyme stock. One hundred units of HLAD was dissolved in 50 ml of distilled water. The solution was stirred gently for about 20 min to ascertain homogenization. Then this stock was divided into portions of 0.50 ml in small reaction tubes. These standard aliquots were stored under liquid nitrogen.

Control of enzyme activity. At different time intervals during the series of experiments 1 unit of HLAD was used to perform a kinetic experiment under standard conditions, i.e., the reduction at 25°C of 0.010 M cyclohexanone in 0.050 M Tris/HCl buffer, pH 8.5, using 0.50 M ethanol (*3*). Samples of the reaction were taken every 3 min.

Kinetic experiments. In a typical experiment the initial rate was determined as follows: In a reaction tube were added successively 100 μmol ketone, 8.50 ml of the appropriate buffer, 1.00 ml of a 1.00 M solution of ethanol in the same buffer, and 1.3 mg of NAD^+. Then the reaction mixture was allowed to reach 25°C in a thermostatted water bath. The reaction was started by adding one standard aliquot of the enzyme stock. The ethanol concentration was 0.10 M in all experiments, except the experiments on the influence of the ethanol concentration.

At regular time intervals, a 1.0-ml sample of the reaction mixture was taken and immediately added to a sample tube containing 50 μl of perchloric acid. This sample was saturated with 0.7 g of ammonium sulfate and extracted with 2 ml of ether or CS_2. Nine samples were taken in each experiment.

Analysis and calculations. All samples were analyzed on a Carbowax 20M column (3% on Chromsorb GHP 100/200 mesh, 3 m, 2.5 mm i.d.) in a Varian 3700 gas chromatograph controlled by a Varian CDS 402 computing integrator. All samples were injected three times.

An unweighted linear regression was performed on the results. For each experiment the linearity was checked. In those cases where curvature was observed, the measurements beyond initial reaction conditions were rejected or the experiment was repeated over a shorter time interval.

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