Thermostable Enzymes in Organic Synthesis. 2.[1] Asymmetric Reduction of Ketones with Alcohol Dehydrogenase from *Thermoanaerobium brockii*

Ehud Keinan,[*†§] **Eva K. Hafeli,**[†] **Kamal K. Seth,**[†] **and Raphael Lamed**[*‡]

Contribution from the Department of Organic Chemistry, Weizmann Institute of Science, Rehovot, Israel, and the Center for Biotechnology, Tel Aviv University, Tel Aviv, Israel. Received October 9, 1984

Abstract: The asymmetric reduction of aliphatic acyclic ketones (C_4–C_{10} substrates) is efficiently achieved by using alcohol dehydrogenase from *Thermoanaerobium brockii* either as a homogeneous, heat-treated, cell-free extract or following immobilization on a solid support. Both methods are superior to the use of whole-cell fermentation. The experimental conditions for working with TBADH were studied and optimized in order to improve reaction rates and the optical purity of the product. An interesting substrate size-induced reversal of stereoselectivity was observed. The smaller substrates (methyl ethyl, methyl isopropyl, or methyl cyclopropyl ketones) are reduced to *R* alcohols, whereas the higher ketones form the *S* enantiomer.

The asymmetric reduction of small aliphatic ketones remains a major challenge in organic chemistry. The use of enzymatic systems[2] for this purpose, an obvious approach to the problem, has so far enjoyed only limited success. For example, reduction of aliphatic ketones with yeast alcohol dehydrogenase (YADH),[3] employing either whole cells or cell-free extracts, resulted in secondary alcohols with only moderate optical purity. The direct stereoselective reduction of acyclic ketones with horse liver alcohol dehydrogenase (HLADH)[4] is even less promising, as, except for aldehydes and primary alcohols, acyclic compounds are poor substrates of the enzyme. Furthermore, even the limited number of reduction reactions that can be achieved occur with low enantioselectivity.[2a] Jones,[4a,5] however, has elegantly circumvented this problem by designing heterocyclic substrates that are stereoselectively reduced by HLADH and subsequently transformed into the desired acyclic products. In addition, the practical use of these two commercially available enzymes, YADH and HLADH, is somewhat limited due to their temperature sensitivity as both are unstable above 30 °C. Also, they are quite sensitive to organic solvents and tend to lose their activity upon immobilization.[6] In contrast, enzymes from thermophilic bacteria have unique structural properties which engender higher thermal resistance, chemical stability, and rapid reaction rates. These enzymes are at present attracting much technological interest.[7]

Recently, we have isolated and characterized a novel alcohol dehydrogenase from *Thermoanaerobium brockii*,[8,9] which is a caldoactive (i.e., extremely thermophilic), saccharolytic, ethanologenic bacterium. This enzyme (TBADH) is remarkably stable at temperatures up to 85 °C and exhibits high tolerance toward organic solvents.[9] We have also found that reduction of ketones to alcohols can be catalyzed by TBADH, while its coenzyme (NADPH) is conveniently regenerated by the coupled-substrate approach,[10] in which 2-propanol is oxidized to acetone. 2-Propanol thus serves both as a reducing agent and a cosolvent (Scheme I).

In this paper, we set the stage for the asymmetric reduction of aliphatic acyclic ketones with the employment of TBADH either as a homogeneous, cell-free extract or by following immobilization on a solid support. Both methods are more general than reductions carried out with growing cells. The experimental conditions for working with TBADH have been studied and optimized in order to achieve higher rates of reaction and higher optical purity of product.

Results and Discussion

A. Reaction Conditions. Unlike the common catalysts used in organic chemistry, the activity of biocatalysts is extremely

Scheme I
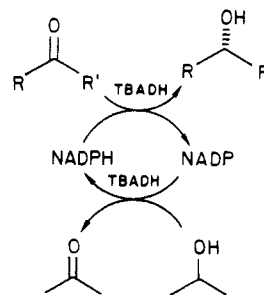
dependent on reaction conditions. The successful employment of enzymes, therefore, relies on a firm knowledge of that dependence, especially when working with newly discovered and not well-characterized enzymes from unusual sources, such as those of thermophilic bacteria. Therefore, before investigating the scope of the enantioselective reductions utilizing TBADH, it was essential to study the influence of reaction conditions on the enantiomeric purity of the product and on its rate of production. Surpisingly, such optimization studies are quite rare in the literature of enzymatic organic synthesis, although there is a growing awareness of the major advantages associated with optimization of enzymatic processes.[11]

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(7) Sonnleitner, B.; Fiechter, A. *Trends Biotechnol.* **1983**, *1*, 74.

(8) (a) Lamed, R.; Zeikus, J. G. *Biochem. J.* **1981**, *195*, 183. (b) Lamed, R.; Zeikus, J. G. *J. Bacteriol.* **1980**, *141*, 1251. (c) Lamed, R.; Zeikus, J. G. *Ibid.* **1980**, *144*, 569. (d) Ben-Bassat, A.; Lamed, R.; Zeikus, J. G. *Ibid.* **1981**, *146*, 192. (e) Zeikus, J. G.; Lamed, R. U.S. Patent 4 352 885, 1982.

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† Weizmann Institute of Science.
‡ Tel Aviv University.
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Table I. Dependence of the Reduction of 2-Pentanone on Reaction Time (or Conversion Rate)[a]

buffer	temp, °C	time, h	conversion	$[\alpha]_D$, deg
phosphate	25	3	24%	8.37
phosphate	25	8.5	47%	8.35
phosphate	25	24	83%	7.74
phosphate	25	47	92%	7.12
phosphate	25	144	quantitative	5.36
Tris–HCl	25	3	40%	8.47
Tris–HCl	25	5	48%	8.46
Tris–HCl	25	24	92%	7.59
Tris–HCl	25	47	92%	6.36
Tris–HCl	25	144	quantitative	3.48
glycine–NaOH	25	3	38%	8.60
glycine–NaOH	25	5	49%	8.59
glycine–NaOH	25	24	92%	7.68
glycine–NaOH	25	47	92%	6.55
glycine–NaOH	25	144	quantitative	3.44
glycine–NaOH[b]	37	4	41%	7.60
glycine–NaOH[b]	37	19	69%	6.65
glycine–NaOH[b]	37	43	69%	4.81
glycine–NaOH[b]	4	168	46%	9.18

[a] Serum bottles were loaded with 250 mL of a solution containing the following: 2-pentanone (2%, v/v), 2-propanol (20%, v/v), TBADH (1.0 unit/mL), NADP (0.05 mM), mercaptoethanol (3 mM), and a buffer (50 mM, pH 8). The buffers used were potassium phosphate, Tris–HCl, and glycine–NaOH. The bottles were stored at 25 °C, and the reduction was followed by GC. Samples (50 mL each) were taken at different times and worked up with a large excess of ammonium sulfate, followed by extraction with either hexane or CH_2Cl_2. The extract was dried over sodium sulfate or by a short silica gel column, followed by removal of solvent under reduced pressure. Pure samples of 2-pentanol were obtained by preparative GC (15% Carbowax on Chromosorb W), and their optical rotation was measured in $CHCl_3$. [b] 16% (v/v) 2-propanol, 8% pentanone.

Preliminary experiments[9] indicate that reduction rates of various aliphatic ketones and the optical purity of the alcohols produced are not equally sensitive to experimental conditions. The asymmetric reduction of 2-pentanone, for example, in contrast to that of higher ketones, has been found to be highly dependent on reaction conditions. We have, therefore, chosen this substrate to study the effects of various factors (reaction time, pH, temperature, concentration, ionic strength, etc.) on the enzymatic reduction. We assumed that findings of this study could be extrapolated to the reduction of other ketones. In order to check this assumption, most of the studies described below for 2-pentanone were repeated with 2-heptanone. Indeed, the general trends of dependence of optical yields and conversion rates on the reaction conditions were found to be similar for both substrates. However, the very narrow range of variations observed in optical yields of 2-heptanol (97%–99.5% ee) as well as the results concerning ketones higher than heptanone (vide infra, Table II) indicates that the reduction of higher ketones is more stereoselective with optical yields much less dependent on a given set of conditions.

Reaction Time. When the reaction was carried out for excessively long times, a decrease in optical yield was observed (Table I) due to an undesirable time-dependent racemization process. This may result from the reversible nature of the enzymatic reduction, the S-alcohol being oxidized more rapidly than the R enantiomer, leading to accumulation of the latter. It is expected, therefore, that optical yield will reciprocally correlate not only to reaction time, but, more generally, to the level of enzymatic activity. Indeed, the observed decrease in optical activity was faster in glycine–NaOH and Tris–HCl buffers than in phosphate buffer, use of phosphate buffer being associated with lower enzymatic activity (vide infra). Similarly, higher temperatures, which enhance reduction rate, also speeded up the decrease of optical purity.[12] Accordingly, increasing the ratio of substrate to 2-
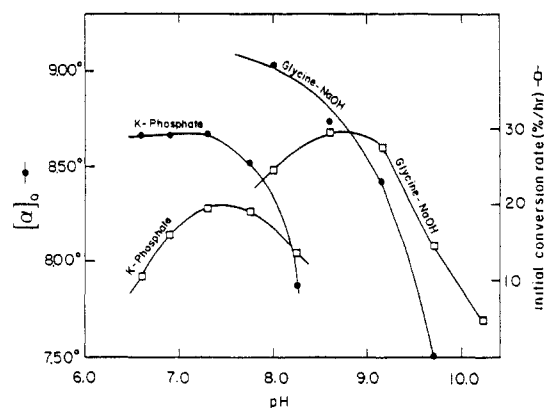

Figure 1. Reduction of 2-pentanone at various pH values. Ten serum bottles were loaded with 50 mL of aqueous solutions containing the following: 2-pentanone (2%, v/v), 2-propanol (20%, v/v), TBADH (0.8 unit/mL), NADP (0.05 mM), mercaptoethanol (3 mM), and either potassium phosphate or glycine–NaOH buffer (50 mM). The pH was measured by a pH meter (Radiometer Copenhagen PHM-62). The bottles were sealed under argon atmosphere and placed in a water bath at 36 °C. The reactions were followed by GC. All reactions were stopped at 50% conversion and worked up as described in Table I. (a) (□) Dependence of initial conversion rates on various pH values. (b) (●) The corresponding dependence of optical yield on pH.

propanol (last four entries of Table I) leads to an enhancement of the reverse reaction, resulting in pronounced racemization. It, therefore, seems practical to stop the reaction at less than 80% conversion, before equilibrium is reached. In our further studies, in order to minimize the time-dependent racemization processes, all reactions were interrupted at 50% conversion.

To determine whether optical purity of the product was a result of intrinsic TBADH enantioselectivity, rather than epimerization under the reversible conditions employed, the reaction was carried out in the absence of 2-propanol, in a coupled-enzyme cofactor-regenerating system. Thus, glucose-6-phosphate dehydrogenase was employed along with glucose 6-phosphate[13] for the reduction of 2-pentanone and 2-heptanone. The corresponding alcohols were obtained with optical yields essentially identical with those obtained with our coupled-substrate system at 50% conversion.

pH. Interestingly, the reaction is dependent not only on the pH but also upon the buffer used. The results given in Table I indicate that two out of the three buffer systems studied (glycine–NaOH and Tris–HCl) give similar results that differ from those of the potassium phosphate system. The difference is also apparent from the results given in Figure 1, which demonstrate the dependence of optical yield and reduction rate on pH with glycine–NaOH and potassium phosphate buffers. Table I and Figure 1 suggest that reduction would be best carried out at pH 7.5–8.0, where both glycine–NaOH and Tris–HCl buffers are superior to potassium phosphate buffer. The reason for lower enzymatic activity with the latter buffer is unknown. One may speculate that it arises from competitive binding of the phosphate anion to the enzyme cofactor-binding site.

Temperature. Figure 2 presents the dependence of optical yield on reaction temperature. Despite the inferior performance of phosphate buffer in comparison to other buffer systems, it was employed for these studies due to its superior pH stability over a wide range of temperatures (temperature coefficient = 0.03 pH units per 10 °C).

The effect of temperature on reduction rate and optical yield when using TBADH is of special interest, as the optimal temperature for growth of *T. brockii* is 65–70 °C. It is evident, however, from Figure 2 that the enzyme is active even at 7 °C.

(11) (a) Jones, J. B.; Takemura, T. *Can. J. Chem.* **1984**, *62*, 77. (b) Chen, C. S. Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294.
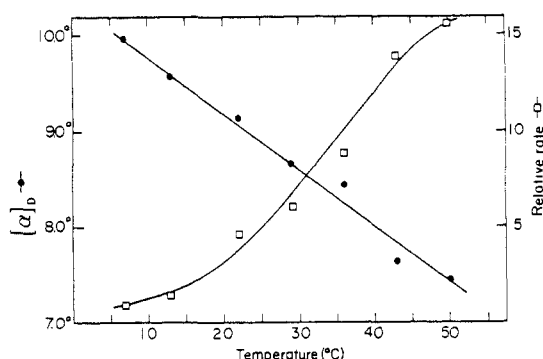
(12) At this point, we cannot exclude an alternative rationale for these phenomena which is based on the possibility of coexistence of several isozymes in the reaction mixture, having slightly different properties including different specificity as well as dissimilar stereoselectivity toward a given substrate.
(13) (a) Dutler, H.; Coon, M. J.; Kull, A.; Vogel, H.; Waldvogel, G.; Prelog, V. *Eur. J. Biochem.* **1971**, *22*, 203. (b) Wong, C. W.; Whitesides, G. M. *J. Am. Chem. Soc.* **1981**, *103*, 4890; **1982**, *104*, 3542.

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Figure 2. Reduction of 2-pentanone at different temperatures. Seven serum bottles were charged with 40 mL of aqueous solutions containing the following: 2-pentanone (2%, v/v), 2-propanol (20%, v/v), TBADH (0.8 unit/mL), NADP (0.05 mM), mercaptoethanol (3 mM), and a phosphate buffer (pH 7.4, 50 mM). The bottles were sealed under argon and placed in water baths at the following temperatures: 7, 13, 22, 29, 36, 43, and 50 °C. The reactions were followed by GC and interrupted at 50% conversion by addition of excess $(NH_4)_2SO_4$ and worked up as described in Table I. Optical rotations were measured in $CHCl_3$. (a) (●) Optical rotation. (b) (□) Relative rates of reduction (based on GC analysis, calculated from the time required to achieve 50% conversion).

In fact, when the reaction was carried out at lower tempertures, the optical purity of the product was enhanced substantially. Interestingly, the reciprocal relationship of optical purity to temperature seems to be linear within the range of temperatures studied. As expected, the rate of reduction decreases significantly with temperature but still allows for convenient work at temperatures between 10 and 50 °C.

Concentration. Two experiments were carried out in order to define optimal concentrations of the substrate and ancillary alcohol. In the first, the ratio of substrate to 2-propanol was varied, keeping the volume ratio of organic material to water constant. In the second, the substrate/2-propanol ratio was kept constant while the organic content was varied.

Assuming that racemizaton of the product alcohol occurs via the reverse reaction (vide supra), it would be expected that decreasing the ratio of substrate to 2-propanol would decrease racemization. Indeed, such an effect is apparent (see Experimental Section); however, it is rather small for pentanone, reflecting, perhaps, a higher affinity of the enzyme for 2-propanol than for 2-pentanol. The second experiment indicates that reductions can be carried out conveniently at organic contents as high as 30%, with only negligible loss of optical purity and a minor decrease in the reaction rate. Higher organic content may result in precipitation and/or destabilization of the enzyme.[8] It can be concluded that for practical synthesis, the optimal substrate/2-propanol ratio is about 1:10. Such a ratio allows both excellent conversion and high optical purity. However, for the sake of higher volumetric productivity, it is possible to increase this ratio up to 1:2 without significant loss of optical yield.

Salt Concentration. Increasing the ionic strength of the reaction medium by changing the concentration of potassium phosphate buffer somewhat decreased the optical yield. This effect was found to be rather negligible within a convenient buffer concentration range of 25–100 mM. However, at concentrations much higher than this practical range (e.g., 250 mM potassium phosphate), it was more pronounced. This may result from conformational changes in the enzyme caused by the substantial increase in ionic strength.

B. Immobilized Enzyme. Although the practical benefits of immobilizing enzymes are obvious[14] (e.g., their use in flow-system reactors), the generality and applicability of this approach depend largely on enzyme stability. For example, the most widely studied alcohol dehydrogenases, YADH and HLADH, have been used in their immobilized form only to a very limited extent,[6] due to

(14) (a) Klibanov, A. M. *Science (Washington, D.C.)* **1983**, *219*, 722. (b) Abbott, B. J. *Adv. Appl. Microbiol.* **1976**, *20*, 203. (c) Mosbach, K., *Methods Enzymol.* **1977**, *44*, 717–856.
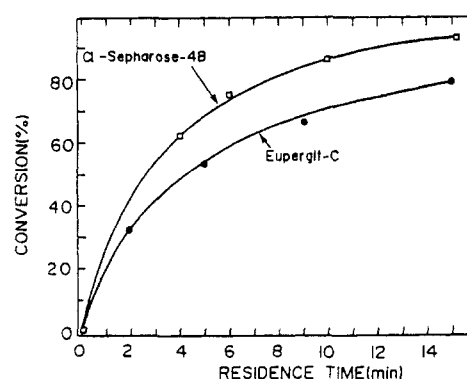

Figure 3. Effect of column residence–time on reduction of pentanone with immobilized TBADH. A solution containing 1% 2-pentanone, 10% 2-propanol, 0.025 mM NADP, 5 mM mercaptoethanol, and 50 mM Tris–HCl buffer (pH 8) was passed through two parallel columns containing either TBADH–Eupergit C or TBADH–CL–Sepharose–4B which were kept at 37 °C. The residence time was calculated from the flow rate which was controlled by a peristaltic pump. Conversion rates were determined by GC analysis.
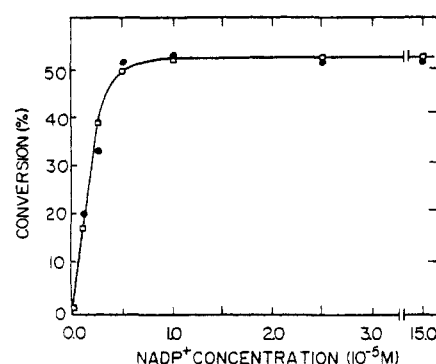

Figure 4. Effect of NADP concentration on pentanone reduction with immobilized TBADH. Experiments similar to those described in Figure 3 were performed with two columns of immobilized TBADH (Eupergit C, (●) and CL-Sepharose-4B, (□), except that the flow rate was kept constant and the concentration of NADP was changed.

problems of enzyme stability. In contrast, the outstanding heat and solvent stability of TBADH suggests that this enzyme may better withstand various immobilization procedures, and, more importantly, enzyme stability could well be retained, if not increased, by immobilization.

Indeed, most of the standard immobilization procedures tried have proved successful for TBADH. The most convenient methods were (a) coupling to cross-linked Agarose (CL-Sepharose-4B) preactivated by cyanogen bromide and (b) direct coupling to the epoxide-containing resin, Eupergit C. Both resulted in immobilized TBADH preparations which were successfully used in continuously operated reactors.

Immobilization on Eupergit C resulted in 15% residual activity, whereas coupling to CL-Sepharose-4B gave an improved figure of 30%. The dependence of conversion rates on residence time (Figure 3) seems to parallel the time dependence observed in the homogeneous system, indicating a similar behavior of the enzyme in both soluble and immobilized forms. As might be expected, based on the relative enzyme content of the two preparations, the TBADH-Sepharose is somewhat more active than the TBADH-Eupergit (Figure 3). However, for practical applications, we preferred the latter because of its ease of preparation and its superior mechanical properties, in particular high stability in both aqueous and organic solvents. Optimization of the coupling procedure is currently under way in our laboratories.

Paralleling the temperature dependence of pentanone reduction in homogeneous media (Figure 2), a nearly linear correlation between conversion rate and temperature, was also observed with the immobilized enzyme.

Studying the concentration effect of NADP (Figure 4) on conversion rates with the two above-mentioned immobilized

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Table II. Asymmetric Reduction of Aliphatic Ketones with TBADH[a]

entry	substrate	product	relative rate[b]	$[\alpha]_D$[c] deg	ee[d] (%)	abs config (ref)
1			12.0	−5.16	48 (12)	R (26)
2			3.0	−2.74	86 (61)	R (27)
3			0.8	−7.55	44 (13)	R (28)
4			3.3	+10.25	79[e] (6)	S (26b,c, 29)
5			1.0	+10.39	96 (85)	S (27a, 30)
6			0.3	+18.59	95	S (27a, 31)
7			0.1	+10.30	81[f]	2S,3R (18, 19)
8			0.9	+8.51	97	S (5, 30, 31)
9			0.9	+10.21	99	S (32)
10			0.2	+5.83	95	S (33)
11		no reaction				
12			0.6	+8.78	97	S (34)
13			0.3	+10.76	99	S (22, 23)
14			0.3	+7.96	98	S (35)
15		no reaction				
16			0.1	+6.08	99	S (36)
17		no reaction				
18		no reaction				
19			1.5[g]	+15.58	98	S (24)

[a] Serum bottles were loaded with 100-mL solutions containing the following: ketone substrate (2 mL), 2-propanol (20 mL), TBADH (2 units/mL), NADP (0.05 mM), mercaptoethanol (4 mM), and Tris–HCl buffer (pH 8, 50 mM). The mixtures were held at 37 °C, and the reactions were followed by GC. All reactions were interrupted at 50% conversion unless otherwise stated and worked up with ammonium sulfate, followed by extraction with either hexane or CH_2Cl_2. The resulting alcohols were further purified by preparative GC. For reductions carried out with either growing cells or immobilized enzyme, see Experimental Section. [b] Relative initial rates of reduction were determined by GC analysis, where the reduction rate of 2-hexanone was arbitrarily assigned unit value (the absolute value was 0.7 µmol/mL/min). [c] Optical rotations were measured in $CHCl_3$ in a 10-cm cell. [d] Enantiomeric excess was determined by HPLC as described in Figure 5. The values given in parentheses refer to reactions carried out with growing cells. [e] The reaction was carried out at 20 °C. [f] The reaction was interrupted at 25% conversion. [g] The reaction was carried out in a column of TBADH-Eupergit C operated at 37 °C. Relative rates of reduction were determined by comparing the column residence time required to achieve 50% conversion for different substrates.

preparations, we were delighted to find that only micromolar concentrations (less than 5×10^{-6} M) of this expensive coenzyme were required to maintain maximal conversion rates. This probably reflects the very high affinity of TBADH for its coenzyme.[8a] Accordingly, removal of NADP from the influent did not quench the reduction immediately but rather caused a very slow decay of enzymatic activity.

The stability of immobilized TBADH under operational conditions is indeed remarkable. Following 30 days of continuous operation at 37 °C, a TBADH-Eupergit C column used to reduce 2-pentanone suffered no apparent decrease in enzymatic activity. Moreover, changing the influent several times and varying the substrate undergoing reduction likewise had no effect on enzymatic activity.

C. Synthetic Applications. The establishment of a practical set of conditions for reducing 2-pentanone with TBADH in both homogeneous and heterogeneous systems enabled an exploration of the range of substrates reducible by this enzyme. A series of 19 aliphatic ketones was chosen as the first group to be studied. The results are summarized in Table II.

While the relative reaction rates of the various substrates were determined rather easily by GC analysis, optical purity of the product was more difficult to measure. Specific rotation determinations are of rather limited value, as their interpretation depends on the quality of earlier literature reports as well as on the chemical purity of the sample. Searching for a more reliable and independent method, we first tried an NMR approach assisted by chiral shift reagents. Proton NMR at 270 MHz employing a chiral europium shift reagent was found to be quite useful for determination of the optical purity of secondary alcohols with accuracies of up to 1%. Interestingly, the clearest enantiomeric splitting was observed for the signal corresponding to the ω-methyl group. For example, in the case of racemic 2-heptanol, the upfield triplet was split into well-separated triplets of equal intensity upon
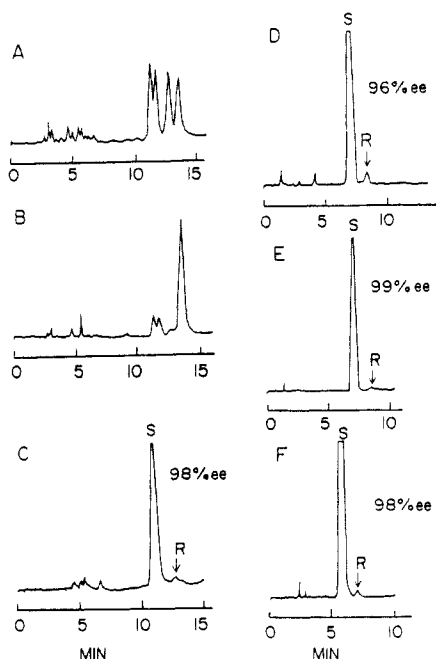
Figure 5. HPLC analyses of chiral secondary alcohols. The alcohols were mixed with equimolar quantities of (R)-$(-)$-1-(1-naphthyl)ethyl isocyanate[16] (Aldrich) in toluene and kept at 100 °C for 5 h and then analyzed by HPLC when using CH_2Cl_2 and a silica gel column, operated at a flow rate of 1 mL/min: (A) racemic 3-methylpentan-2-ol, (B) $(2S,3R)$-$(+)$-3-methylpentan-2-ol, (C) (S)-$(+)$-5-chloropentan-2-ol, (D) (S)-$(+)$-hexan-2-ol, (E) (S)-$(+)$-heptan-2-ol, (F) (S)-$(+)$-nonan-2-ol.
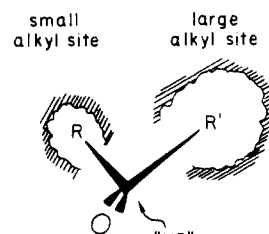
treatment with 25–35 mol % $Eu(tfc)_3$[15] in $CDCl_3$. Nevertheless, although useful in a limited number of instances, this approach was not found to be generally applicable to all substrates. In addition, the method is rather expensive and time-consuming, especially when required for routine analysis.

It seemed to us that a chromatographic approach based on detection of easily formed diastereomers could be more practical. Indeed, employment of a chiral isocyanate to form a diastereomeric mixture of carbamates according to Pirkle's procedure[16] followed by HPLC on silica gel proved to be a simple and a general method for determining optical purity. The chromatograms in Figure 5 represent typical traces from which optical purities were easily calculated.
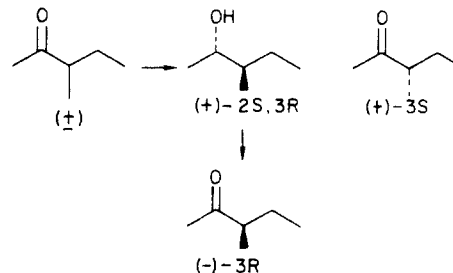
The potential of using TBADH in organic synthesis is clearly illustrated by its generality and high enantioselectivity of reduction of acyclic ketones (see Table II). A most striking feature is the substrate-size-induced reversal of stereoselectivity. When both alkyl groups attached to the carbonyl are small, e.g., in methyl ethyl, methyl isopropyl, or methyl cyclopropyl ketones (entries 1–3), the enzyme directs hydride attack at the *si* face of the carbonyl, leading to preferential formation of alcohols with an *R* configuration. However, with larger-size ketones, there is a clear reversal in stereoselectivity. Reduction of 2-pentanone (entry 4) involves hydride attack at the *re* face of the carbonyl to give an *S*-alcohol, whose enantiomeric excess is highly dependent on the reaction conditions. The *S* selectivity increases significantly and becomes absolute for higher ketones, which are reduced with excellent optical yield.

Similar inversions of enzyme stereospecificity within a structurally related series of substrates have previously been observed.[17] Such behavior may raise interesting speculations concerning the

(15) Tris(3-(trifluoromethylhydroxymethylene)-*d*-camphorato)europium-(III) (Aldrich, No. 17649-4).
(16) Pirkle, W. H.; Hoekstra, M. S. *J. Org. Chem.* **1974**, *39*, 3904.
(17) (a) Zhou, B.; Gopalan, S.; VanMiddlesworth, F.; Shieh, W. R.; Sih, C. J. *J. Am. Chem. Soc.* **1983**, *105*, 5925. (b) Sabbioni, G.; Shea, M. L.; Jones, J. B. *J. Chem. Soc., Chem. Commun.* **1984**, 236. (c) Schwartz, H. M.; Wu, W. S.; Marr, P. W.; Jones, J. B. *J. Am. Chem. Soc.* **1978**, *100*, 5199. (d) Boter, H. L.; Ooms, A. *J. Biochem. Pharmacol.* **1967**, *16*, 1563. (e) frater, G. *Helv. Chim. Acta* **1979**, *62*, 2825, 2829.

Scheme II

Scheme III

structure of the active site in the enzyme.[12] For example, the above-described observations may be explained by the following hypothetical structure of the TBADH substrate-binding site (Scheme II). The carbonyl function should occupy a defined position in the protein in close proximity to the nicotinamide moiety of the coenzyme, making possible efficient hydride transfer. Concomitantly, the two alkyl groups on the carbonyl occupy two hydrophobic sites which differ from one another in volume and also in their affinity toward the alkyl groups, the smaller site having the greater affinity. The *R* selectivity shown in entries 1–3 suggests that the "small alkyl site" can accommodate alkyl groups as small as methyl, ethyl, isopropyl, and cyclopropyl but not *n*-propyl and more bulky substituents. Selectivity will therefore be inverted in the case of 2-pentanone in which the *n*-propyl moiety is forced into the "large alkyl site" and the methyl occupies the "small alkyl site".

This hypothesis is further supported by comparing the three isomers of *n*-heptanone (entries 9–11). 2-Heptanone or 3-heptanone contain alkyl groups (methyl or ethyl, respectively) which are sufficiently small to fit into the "small alkyl site". Both are found to be good substrates of TBADH. 4-Heptanone, however, has two *n*-propyl groups which are too large to fit in the "small alkyl site". Reduction of 4-heptanone at 37 °C was, as would be predicted, slower than the reduction of 3-heptanone (5%) and even slower than that of 2-heptanone (1%). The same trend is even more pronounced when a similar comparison is made between the two isomers of *n*-nonanone (entries 14 and 15). 2-Nonanone having methyl and heptyl groups is reduced by TBADH at a reasonable rate, whereas 5-nonanone with two *n*-butyl groups was not reduced even under forcing conditions of 2 weeks at 37 °C in the presence of high enzyme concentrations.

On the basis of their relative rates of reduction, the substrates studied may be roughly divided into three categories. The first group comprises small substrates (C_4–C_5 ketones, entries 1–3) that are rapidly reduced with variable optical yields, depending on reaction conditions. The second group comprises C_6–C_9 ketones that are reduced at a moderate rate (1.0–0.3 on the relative scale). The third group represents the upper limit of the substrate range, which includes C_{10}–C_{11} ketones. The latter's small reduction rates may reflect either poor recognition by the enzyme and/or low solubility of these compounds in the reaction medium. When the relative rates given in Table II are calculated, the fact that reductions of the higher ketones were carried out in suspension rather than in homogeneous solution is not taken into account.

There are several special cases in Table II which deserve further discussion. 3-Methylpentan-2-one (entry 7) is the only chiral substrate studied, which in theory may yield, after reduction, four diastereomers. In fact, nonenzymatic reduction of this substrate

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with NaBH₄ followed by treatment with Pirkle's isocyanate[16] yield four compounds in equal quantities, appearing as two pairs of peaks on the HPLC chromatogram (Figure 5A). The reduction with TBADH was found to be not only stereoselective, with respect to the newly formed carbinol center, but also stereospecific, with respect to the existing asymmetric center. One product out of the four was formed preferentially (Figure 5B). The absolute configuration of this alcohol was found to be 2S,3R (Scheme III) based on the following: The reduction of 3-methylpentan-2-one was interrupted at 25% conversion, and both alcohol product and unreacted ketone were separated by preparative GC. The recovered ketone was enriched with the 3S-(+) enantiomer,[18] suggesting that the 3R-(−) ketone is preferentially reduced by the enzyme. Indeed, chromic acid oxidation of the product, 3-methylpentan-2-ol, back to the corresponding ketone yielded the 3R-(−) enantiomer. The relative configuration of the two asymmetric centers in the alcohols was determined by ¹³C NMR spectra. The major isomer (87%) was found to have a threo configuration, whereas the minor product (13%) had a spectrum identical with that reported for the erythro isomer.[19] On the basis of both HPLC and ¹³C NMR data, the four diastereomers of 3-methylpentan-2-ol—2S, 3S; 2R, 3R; 2R, 3S, and 2S, 3R,—were produced in a ratio of 9:9:2:80, respectively. This implies that the differentiation ability of TBADH with respect to the reduction of (R)- and (S)-3-methylpentan-2-one is 89:11, a fact that may be used for kinetic resolution of such racemic ketones.[20] Remarkably, the enantioface differentiation ability,[20] favoring hydride attack at the re face of the carbonyl, is quite similar in both of these enantiomeric ketones.

Interestingly, reduction of α,β-unsaturated ketones is not catalyzed by TBADH, as no reaction was observed with 5-methylhex-3-en-2-one (entry 18). Moreover, this substrate, as well as other conjugated enones tested, was found to irreversibly inhibit enzymatic activity. This observation suggests that α,β-unsaturated ketones react irreversibly with a certain nucleophilic residue in the active site of the enzyme, a fact which can be utilized for mapping the active site of TBADH.[21]

In contrast, the presence of a nonconjugated olefin in the substrate molecule had no inhibitory effect. This is illustrated by the reduction of 6-methylhept-5-en-2-one (entry 13) to the essentially optically pure (S)-(+)-sulcatol, the aggregation pheromone produced by males of *Gnathotrichus sulcatus*.[22] The direct reduction with TBADH represents, by far, the shortest synthesis of this natural product.[23]

Obviously, the employment of an immobilized enzyme, which may be packed in columns for convenient flow-system operation, is much more advantageous for large-scale syntheses than the use of the same enzyme in a batchwise solution operation. In general, the superiority of either system depends largely on the optical purity of the products obtained. Fortunately, in the case of TBADH, both the homogeneous and heterogeneous systems gave very similar results from that standpoint, as alcohols were obtained with reproducible optical purity, independent of the system used. The immobilized enzyme approach turned out to be of crucial importance in the special cases of unstable substrates or products such as chloro ketones and chloro alcohols,[24] which have limited lifetimes in a homogeneous reaction medium. This stability problem, found for both 5-chloropentan-2-one and its product, 5-chloropentan-2-ol (entry 19), was successfully overcome by carrying out the reduction on a column of immobilized TBADH.[24]

Reduction with Growing Cells. Reduction of several aliphatic ketones occurred also in cultures of *T. brockii*, with the same general order of substrate reactivity and same enantioselectivity in both the whole-cell and cell-free systems, all suggesting that the same enzyme operates in both. However, optical yields in the fermentation process were found to be disappointingly lower than those of the cell-free reactions, especially with the group of small substrates (C₄–C₅ ketones), the reductions of which are more susceptible to reaction conditions (entries 1–5, Table II). This poor stereoselectivity may reflect the presence of several alcohol dehydrogenases in the bacterium.[8] Additionally, the relatively high temperature necessary for bacterial growth may also account for the poor optical yield (vide supra). As expected, the reduction of higher ketones should be less sensitive to the method used and therefore they may be reduced with high enantioselectivity in both systems. Indeed, this was found to be the case for 2-hexanone. Unfortunately, ketones higher than hexanone are not accepted by the growing bacterium. Moreover, they seem to act as growth inhibitors of *T. brockii*, and therefore, they cannot be practically reduced via a fermentation process. Although bacterial fermentation is an attractive technological approach for its operational simplicity, it is apparently not always the method of choice for enantioselective reductions.[25]

Experimental Section

General Methods. Infrared spectra were recorded on a Perkin-Elmer 467 grating spectrometer and are given in units of inverse centimeters. Proton NMR spectra were measured in deuteriated chloroform on a Varian FT-80A or Bruker WH-270 NMR spectrometers. ¹³C NMR spectra were measured on a Bruker WH-270 NMR spectrometer. All chemical shifts are reported in δ units downfield from Me₄Si. Optical rotations were measured by a Perkin-Elmer 141 polarimeter, using a 1-dm (1 mL) cell. Mass spectra were recorded on a Varian Mat 731 spectrometer or on a Finnigan 4500 GC-MS. Measurements of pH were carried out on a Radiometer-Copenhagen PHM-62. Thin-layer chromatography (TLC) was performed on aluminum sheets precoated with silica gel (Merck, Kieselgel 60, F-254, art 5549). Column chromatographic separations were performed on silica gel (Merck, Kieselgel 60, 230–400 mesh, Art. 9385) under a pressure of 1 atm (flash chromatography). Preparative TLC was performed on glass plates precoated with silica gel (Merck, Kieselgel 60 F-254 Art 5717). Distillations of products were performed in a Kugelrohr apparatus; the temperatures given are pot temperatures. GLC analyses were performed on a Spectra-Physics SP-7100 (FI detector) equipped with a 0.125 in. × 6 ft column packed with 10% Carbowax 20 on Chromosorb w. Preparative GLC separations

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Table III

total vol, mL	concn of 2-pentanone, mL	2-propanol/ pentanone (v/v)	time to achieve 30% conversion, min	$[\alpha]_D$, deg
10	1 (10%)	2%	95	8.42
25	1 (4%)	5%	60	8.59
50	1 (2%)	10%	60	8.96
125	1 (0.8%)	25%	100	8.99
250	1 (0.4%)	50%	150	9.01
500	1 (0.2%)	100%	335	9.01

were performed on a Varian Aerograph 90P (TC detector) equipped with a $^1/_2$ in. × 12 ft column packed with 10% Carbowax 20 on Chromosorb w.

Preparation of Crude TBADH Solution. Dry acetone powder of *Thermoanaerobium brockii*[8,9] (ATCC 33075, DSM 1457) (10 g) was mixed with 150 mL of aqueous phosphate buffer solution (pH 7.0–7.5, 0.5 M) and 50 μL of mercaptoethanol. The mixture was stirred at room temperature for 1 h and then heated to 80 °C for 3 min. The resulting milky suspension was cooled to room temperature and centrifuged at 15 000 rpm for 15 min. The clear yellow supernatant was collected and used without further purification. The concentration of alcohol dehydrogenase in this solution (40 units/mL) was determined by following the increase in absorbance at 340 nm, using 2-propanol as a substrate (1%, v/v) at 40 °C in Tris–HCl buffer (pH 8) containing 0.5 mM NADP. One unit is defined as the amount of enzyme required to convert 1 μmol of substrate per min.

Reduction of 2-Pentanone at Various Ionic Strengths. Four serum bottles were loaded with 50 mL of solution containing the following: 2-pentanone (1 mL), 2-propanol (10 mL), TBADH (0.80 unit/mL), NADP (0.1 mM), mercaptoethanol (3 mM), and potassium phosphate buffer at the following concentrations: (1) 25 mM, (2) 50 mM, (3) 100 mM, and (4) 250 mM. The bottles were placed in a water bath at 36 °C. The reduction was followed by GC and interrupted at 50% conversion with a subsequent workup as described in Table I. The following optical rotations were recorded for 2-pentanol obtained from these four experiments, respectively: (1) 8.75°, (2) 8.64°, (3) 8.60°, and (4) 7.75°.

Reduction of 2-Pentanone at Various Substrate Concentrations. Six serum bottles were charged with 10–500 mL of aqueous solutions containing 2-pentanone (1 mL), TBADH (40 units), NADP (0.01 mmol), 2-propanol (20%, v/v), mercaptoethanol (3 mM), and potassium phosphate buffer (pH 7.4, 25 mM). The bottles were placed in a water bath at 36 °C. The reduction was followed by GC and interrupted at 30% conversion. The reaction mixtures were treated as described in Table I. Optical rotations were taken in CHCl₃. The results obtained are given in Table III.

Reduction of 2-Pentanone in Media of Varying Organic Content. Four serum bottles were loaded with the following: 2-pentanone (1 mL), 2-propanol (10 mL), and TBADH (40 units). The mixtures were diluted to total volumes of 32, 50, 100, and 200 mL with an aqueous solution containing phosphate buffer (pH 7.4, 50 mM), NADP (0.05 mM), and mercaptoethanol (3 mM). The bottles were sealed under argon and placed in a water bath at 36 °C. The reductions were monitored by GC and interrupted at 50% conversion. The mixtures were treated as described in Table I. The relative rates of reduction (initial rates) were determined by GC, and optical rotation of the product was measured in CHCl₃. The following were observed (organic content, initial rate (mmol/h), $[\alpha]_D$): 5%, 3.3, 8.26°; 10%, 2.7, 8.25°; 20%, 2.3, 8.36°; 30%, 1.9, 8.23°.

Preparation of Immobilized TBADH. A. Preparation of TBADH—Eupergit C. Eupergit C (Rohm-Pharma) (3.3 g) was mixed with crude TBADH solution (15 mL) under N₂ atmosphere. The mixture was left to stand at room temperature for 1 day and was then loaded into a glass column and washed with aqueous NaCl (1 M), followed by aqueous Tris–HCl buffer (pH 7.5, 50 mM) containing 10 mM mercaptoethanol. This column was then ready for use and exhibited residual synthetic activity of 15%. The columns could be stored for several months when kept wet and closed at 4 °C. Catalytic activity was determined from the residence time required to achieve 50% conversion of pentanone to pentanol in a continuous flow system operated at 36 °C using a solution containing the substrate, NADP, mercaptoethanol, and 2-propanol and a buffer concentration similar to that used in a batch reaction with the soluble enzyme.

B. Preparation of TBADH-CL-Sepharose-4B. CL-Sepharose-4B gel (Pharmacia) (10 mL) was suspended in aqueous K₂CO₃ (1 M, 20 mL) and cooled to 4 °C. A solution of cyanogen bromide (1 g) in acetonitrile (1 mL) was added, and the mixture was stirred for 1.5 min, followed by washing with cold water. A crude solution of TBADH (10 mL) was

added, and the mixture was shaken at 4 °C for 16 h. It was then loaded into a glass column and washed with 1 M NaCl solution and then with Tris–HCl buffer (50 mM, pH 7.5) containing 10 mM mercaptoethanol. Recovered synthetic activity was 30%.

Operational Stability of Immobilized TBADH. A. The two columns containing the above-described immobilized TBADH were continuously operated at 37 °C with pentanone as the substrate. Some loss of activity (less than 10%) was observed in both columns during the first day of operation. However, no further change in activity could be detected within the next 30 days of continuous operation.

B. A column containing TBADH-Eupergit C was operated continuously at 37 °C for 2 weeks with 2-pentanone (1%) as a substrate and 10% 2-propanol. The flow rate was 0.75 mL/min, which yielded a constant conversion rate of 50% over that period of time. The reaction mixture was then replaced by a new mixture containing a different substrate (2-heptanone) and a different concentration of 2-propanol (20% instead of 10%). After being operated at 37 °C for 1 week at a flow rate of 0.25 mL/min (which gave 50% conversion), the original solution was reintroduced. The column was operated at a flow rate of 0.75 mL/min, giving a constant level of 50% conversion.

Effect of Temperature on Pentanone Reduction with Immobilized TBADH. A reaction mixture identical with that described in Figure 3 was passed through a TBADH-Eupergit C doubled-walled column at constant flow rate. The column temperature was controlled by circulating water. Conversion rates were determined by GC. The following conversions were observed: 25 °C, 44%; 37 °C, 54%; 42 °C, 60%; 53 °, 70%.

General Procedure for Asymmetric Reductions of Ketones Using Immobilized TBADH. A wet powder of TBADH immobilized on Eupergit C (10 mL) was packed in a double-walled glass column that was kept at 37 °C by circulating water. The reaction mixture (0.2–5 L of aqueous solution containing 1% substrate, 10% 2-propanol, 50 mM Tris–HCl buffer (pH 8), 0.01 mM NADP, and 3 mM mercaptoethanol) was passed through the column by using a peristaltic pump at a flow rate (usually between 0.2 and 1.0 mL/min) adjusted to achieve 50% conversion of ketone to alcohol (as indicated by GC analysis). In cases where sensitive substrates were to be reduced (e.g., chloropentanone, Table II, entry 19), the whole system was placed in a cold room to assure that all components except the column itself were at 4 °C. The mixture was collected and worked up as described in Table I. In cases of substrates having only limited solubility, the concentration of 2-propanol was raised to 20–30% with no apparent decrease in enzymatic activity.

Reduction of Pentanone and Heptanone with TBADH Using Glucose-6-phosphate Dehydrogenase for Coenzyme Regeneration. Two serum bottles were loaded with 200-mL solutions containing the following: glucose-6-phosphate dehydrogenase (Sigma, G-8878, 2 mg), TBADH (1 unit/mL), glucose 6-phosphate (0.1 M), MgCl₂·6H₂O (0.03 M), Tris–HCl buffer (pH 8, 0.2 M), NADP (0.0001 M), and 2 mL of either 2-pentanone or 2-heptanone. Each mixture was held at 20 °C for 24 h and then extracted with ether, and the alcohol product was purified by preparative GC. The observed optical purities of (+)-2-pentanol and (+)-2-heptanol (as determined by HPLC with Pirkle's isocyanate) were 80% and 99% ee, respectively.

Reduction of Aliphatic Ketones by Growing *T. brockii* Cells. *T. brockii* was grown in TYEG medium[8] (100 mL) supplemented with the ketone substrate (500 mg) at 60 °C for 24 h in 200-mL serum bottles. The mixture was extracted and worked up as described in Table I. Chemical yields were determined by GC. The optical rotation and enantiomeric excess of the resulting alcohols were determined as described above.

Structure Determination of 3-methylpentan-2-ol. Reduction of racemic 3-methylpentan-2-one was carried out as described in Table II, and the reaction was interrupted after reaching 25% conversion. Separation by preparative GC yielded (+)-3-methylpentan-2-ol, $[\alpha]_D$ +10.30° (chloroform) and recovered (+)-3-methylpentan-2-one, $[\alpha]_D$ +2.50° (chloroform).

(+)-3-Methylpentan-2-ol (180 mg) was mixed with pyridinium dichromate (1.5 equiv) and pyridine (20 mg) in 2 mL of CH₂Cl₂. The mixture was stirred at room temperature for 3 h, filtered through silica gel with CH₂Cl₂, and then purified by preparative GC to give (−)-3-methylpentan-2-one, $[\alpha]_D$ −7.18°, as a mixture of threo and erythro isomers in a ratio of 87:13 (by integration of the ¹³C NMR spectrum). Optical rotations of both (S)-(+)- and (R)-(−)-3-methylpentanone are lower than expected,[18] possibly due to their partial racemization which could occur upon purification: ¹³C NMR (threo isomer) (measured in CS₂, numbers in parentheses are the literature values[19]) δ 70.31 (70.36), 41.72 (41.84), 25.60 (25.55), 20.29 (20.22), 14.00 (14.02), 12.12 (12.02); ¹³C NMR (erythro isomer) δ 70.68 (70.68), 41.72 (41.84), 25.60 (25.61), 19.32 (19.24), 14.19 (14.12), 11.96 (11.86).

Reduction of (+)-3-methylpentan-2-one with NaBH₄ followed by HPLC analysis similar to that shown in Figure 5A and 5B allowed the

following assignment of the carbamates of the four diastereomers of 3-methylpentan-2-ol (in order of increasing retention time): 2*S*, 3*S*; 2*R*, 3*R*; 2*R*, 3*S*, and 2*S*, 3*R*.

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Direct Quantitative Analysis of Enzyme-Catalyzed Reactions by Two-Dimensional Nuclear Magnetic Resonance Spectroscopy: Adenylate Kinase and Phosphoglyceromutase

George L. Mendz,* Gae Robinson, and Philip W. Kuchel

Contribution from the Department of Biochemistry, University of Sydney, NSW 2006 Australia. Received May 2, 1985

Abstract: The back-transformation method for the analysis of two-dimensional nuclear magnetic resonance cross-relaxation and exchange data has been employed to obtain the rate constants of the phosphoglyceromutase and adenylate kinase catalyzed reactions in vitro. The results are in excellent agreement with those obtained by other methods. It is shown that a single two-dimensional experiment for each catalyzed reaction is sufficient to calculate the rate constants. The study of the adenylate kinase reaction demonstrates the potential of the method for direct analysis of higher order reactions without having to simplify them in terms of pseudo-first-order steps.

I. Introduction

Complete characterization of metabolic pathways requires the determination of the rate constants of enzyme-catalyzed reactions. Nuclear magnetic resonance (NMR) spectroscopy is a particularly important method for the study of steady-state enzyme kinetics because of its ability to follow noninvasively in vivo intracellular processes and because of its potential to monitor individual metabolites. One-dimensional (1D) and two-dimensional (2D) Fourier transform NMR techniques have been applied to the measurements of the rates of reactions; saturation and inversion transfer of magnetization are the most widely used 1D methods. They are employed to measure "intermediate-to-slow" rate constants by following the direct transfer of magnetization between exchanging chemical species. ^{31}P NMR has been used to study the adenylate kinase[1,2] and phosphoglyceromutase-catalyzed[3] reactions involving high-energy phosphate esters. However, the application of these 1D methods presents difficulties; the most important one is the limit in the selectivity that can be achieved by irradiating a set of resonances in a crowded or complex spectrum.

Jeener et al.[4] designed a 2D technique, the exchange experiment, to measure chemical exchange and cross-relaxation rates. Ferretti and co-workers have demonstrated that it can be applied to the study of in vitro[5,6] and in vivo[1] enzyme-catalyzed reactions. A fundamental advantage of this technique is that under suitable conditions species which have undergone chemical or magnetic exchange are connected by off-diagonal cross-peaks on a two-dimensional spectral map whose diagonal represents the one-dimensional spectrum.

Thus, in principle, the limitation imposed by crowded spectral regions on selective irradiation can be overcome and all exchange pathways are observed simultaneously in a single experiment. However, the lack of a suitable method of analysis of the exchange experiment has limited the obtaining of quantitative information to very simple cases[4,8] and has made necessary a large number of experiments.

Kumar et al.[9] examining the variation of NOESY (Nuclear Overhauser enhancement spectroscopy) cross-peaks as a function of mixing time demonstrated a qualitative correlation between the time rate of increase of cross-peak intensity and the distance between atoms found from X-ray data. Bodenhausen and Ernst[10,11] devised a new type of exchange experiment, accordion spectroscopy, capable of measuring cross-relaxation rates. In *J*-coupled systems this technique requires the elimination of zero-quantum effects by means other than the standard random variation of the mixing time, using, for instance, a field-gradient pulse.

Rate constants for first-order reactions can be obtained by carrying out a number of experiments at different mixing times and fitting the relevant mathematical expression to the cross-peak volumes vs. mixing time data. The parameters thus fitted are employed to calculate the rate constants from the analytically solved rate equations. This method was used for the yeast phosphoglucose isomerase reaction with excellent results,[5] but *it required 12 experiments*. The analyses of more complex reactions

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