analytical chemistry

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"On-the-Fly" Kinetics of Enzymatic Racemization Using Deuterium NMR in DNA-Based Chiral Oriented Media

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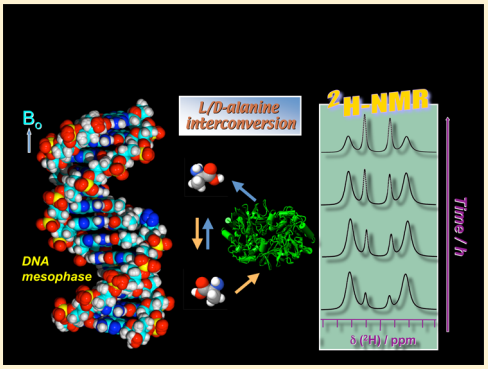
Supporting Information

ABSTRACT: We report the in situ and real-time monitoring of the interconversion of L- and D-alanine-d_3 by alanine racemase from *Bacillus stearothermophilus* directly observed by ^{2}H NMR spectroscopy in anisotropic phase. The enantiomers are distinguished by the difference of their ^{2}H quadrupolar splittings in a chiral liquid crystal containing short DNA fragments. The proof-of-principle, the reliability, and the robustness of this new method is demonstrated by the determination of the turnover rates of the enzyme using the Michaelis–Menten model.



Racemases recognize a chiral substrate such as L-alanine (L-Ala) and convert it into its enantiomer, i.e., D-alanine (D-Ala) and vice versa. Alanine racemase (AR) plays a vital role for certain bacteria, providing D-Ala for peptidoglycan cell-wall biosynthesis.[1] Elucidating the mechanism of enzymatic racemization is crucial for designing new inhibitors that may be useful as a novel class of antibiotics.[2,3] This requires techniques to distinguish L- and D-Ala enantiomers and follow their concentrations as a function of time, so that one can determine the kinetic parameters and study the effect of inhibitors. Although well-established methods such as circular dichroism (CD)[4,5] or UV spectroscopy[6], or chiral capillary electrophoresis[7] exist, we propose here a change of paradigm by using for the first time NMR spectroscopy as an analytical method.

NMR is routinely used to measure the time-course of chemical reactions, and enantiomers of amino-acids can be spectrally discriminated in DNA-based liquid crystals.[8,9] We show how one can simultaneously follow the time dependence of ^{2}H signals of L- and D-Ala-d_3 (i.e., alanine where the methyl group is selectively deuterated) in aqueous, chiral oriented solutions of DNA fragments in the presence of the AR. Since the signals in proton-decoupled deuterium spectra ^{2}H–{^{1}H} are proportional to the concentrations of the two enantiomers, one can determine the turnover numbers (k_{catL} and k_{catD}) of AR without requiring any chemical transformations of the substrate other than prior deuteration (Figure 1). Among the various known racemases, we

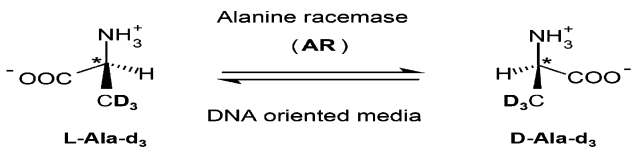


Figure 1. Racemization of Ala-d_3 by AR in DNA-based oriented media.

have focused attention on recombinant alanine racemase from *Bacillus stearothermophilus*, which can be easily expressed and purified and is stable over a wide range of temperatures up to 60 °C.[10,11]

In aqueous solutions, the challenge is to find a chiral environment that imposes a different average orientational order on two enantiomers, without perturbing the activity of the

Received: February 5, 2013
Accepted: April 2, 2013
Published: April 2, 2013

   dx.doi.org/10.1021/ac4004002 | *Anal. Chem.* 2013, 85, 4694–4697

enzyme and its ability to recognize its chiral substrates. Indeed, the latter may have different affinities for the orienting medium that could be in competition with their affinities for the enzyme.

Dissolved in water or organic solvents, chiral polymers such as polypeptides, polysaccharides, or polynucleotides are known to form lyotropic chiral liquid crystals (CLCs) at concentrations that depend on their phase diagrams.[8,9,12−14] Among water-compatible aligning media, CLCs made of short-length double-stranded DNA helices (ca. 100−300 base pairs) can impose a different orientational order on enantiomers of various α-amino acids.[9] For selectively deuterated amino acids, one can observe distinct quadrupolar splittings for the two isomers in ^{2}H−{^{1}H} spectra.[9,15,16] Although nuclei such as ^{1}H or ^{13}C could also be observed in chiral oriented solvents, ^{2}H nuclei ($I = 1$) have three attractive features:[15,16] (i) their residual quadrupolar splittings $\Delta\nu_Q$ (specific for nuclei $I > 1/2$) are very sensitive to differences in orientational ordering; (ii) the analysis of ^{2}H−{^{1}H} 1D spectra (Supporting Information) is trivial for mono- or polydeuterated enantiomers since for each isotopically enriched site the two isomers lead to two distinct quadrupolar doublets centered on nearly the same chemical shift $\delta^{aniso}(^2H)$;[15] and (iii) since Overhauser effects are negligible, the integrals in ^{2}H quadrupolar doublets are directly proportional to the concentrations of the enantiomers, provided the recovery delays are long enough.[16] In the case of Ala-d_3, both enantiomers have the same T_1.[9] In this study, we have chosen Ala-d_3, but it would also be possible to use alanine deuterated on the stereogenic Cα carbon. Both molecules are commercially available and exhibit spectral enantiodiscrimination.[9] Notice however that, in the case of alanine deuterated at the Cα position (Ala-d_1), one would expect an isotope effect since deprotonation at the chiral center is known to be a rate-limiting step.[17,18] Kinetic isotope effects (KIEs) for the transformation of L- to D-alanine and vice versa were determined by circular dichroism. While the transformation starting from L-Ala-d_1 showed a KIE of 1.66, the reverse reaction exhibits a KIE of 1.57.[19] These isotope effects influence the turnover numbers and should be detectable with our new method. Details about ^{2}H−{^{1}H} NMR in chiral anisotropic phases can be found in Supporting Information

MATERIALS AND METHODS

DNA fibers and both L- and D-enantiomers of Ala-d_3 were purchased from Sigma Aldrich (India) and Eurisotop (France), respectively. The enzyme AR was produced and purified[20] with slight modifications (see Supporting Information). Sample No. 1 was prepared as follows: to 565 μL of DNA-based chiral phase, AR (89 nM) and 4 mg of Ala-d_3 powder (133 mM) were added. After addition of substrate or enzyme, 5−10 cycles of centrifugation at 500 rpm (corresponding to 0.5 g) during 5−10 s are necessary to obtain a homogeneous phase. The pH was found to be 7.5 after the third addition of Ala-d_3. All ^{2}H−{^{1}H} NMR spectra were recorded without sample spinning at 288 K on a 14 T (600 MHz for protons) Avance II NMR spectrometer equipped with a 5-mm ^{2}H cryoprobe (Bruker). However, since the substrate is isotopically enriched, a cryogenic probe is not essential. As no ^{2}H field-frequency lock was used, the magnetic field was shimmed by optimizing the FID. To ensure a quasi-continuous sampling of the enantiomeric concentrations until racemization is completed, 20 000 spectra, using 4 scans each and a recovery delay of 0.6 s between transients (total acquisition time of 2.7 s), were recorded in a total experimental time of 18 h. Protons were decoupled using Waltz-16 composite pulses. Neither filtering nor baseline correction were applied. The processed spectra were deconvoluted using Mathematica with a Pearson function showing an excellent agreement with the experimental data as shown in the Supporting Information. Sample No. 2 was prepared by adding 3.8 mg of L-Ala-d_3 to Sample No. 1. A further 4.1 mg of L-Ala-d_3 was added to prepare Sample No. 3.

Our approach comprises five steps: (i) preparation of a buffered (pH 6−7) CLC solution of short-length DNA that is transferred to an NMR tube (see experimental section and Supporting Information);[9] (ii) addition of a pure enantiomer (enantiomeric excess $ee = 1$) or of a scalemic mixture (i.e., a nonracemic mixture with $ee \neq 0$) of chiral substrates (here as a powder) to check the symmetry and linewidths of the ^{2}H doublets, the absence of isotropic signals, and (for scalemic mixtures) the spectral differentiation of the enantiomers that should have distinct quadrupolar splittings; (iii) addition of AR; (iv) monitoring of the racemic interconversion as a function of time; and (v) deconvolution of the partially overlapping ^{2}H doublets to determine the areas of the signals of the L- and D-isomers and hence their concentrations. From a practical point of view, the method appears to be very robust, since AR remains active in the CLC for at least 7 days at 288 K, and multiple additions of solid substrate do not perturb the properties of the CLC, since no isotropic signal appears.

This method relies on the preparation of homogeneous, uniformly aligned chiral media in order to record high-resolution ^{2}H−{^{1}H} spectra. Several cycles of mixing by centrifugation at moderate speed (0.5 g) of the NMR tube ensure the homogeneity of the CLC (elimination of isotropic domains and concentration gradients) and, more importantly, favor the homogeneous dispersion of the enzyme throughout the sample (see below). In ^{2}H−{^{1}H} spectra in short-length DNA-based CLCs, the range of quadrupolar splittings of deuterated solutes is typically between 0 and 1 kHz, and linewidths vary between 9 and 20 Hz.[9] To assign the ^{2}H doublets to the L- and D-isomers and to follow their interconversion as a function of time, we started with a scalemic mixture of Ala-d_3 ($ee \neq 0$), but the use of an enantiopure L- or D-substrate ($ee = 1$) is also possible if either enantiomer is not available (see Figure 1).

RESULTS AND DISCUSSION

In a first experiment (Exp. 1), we mixed 89 nM AR, 109 mM L-Ala-d_3, and 24 mM D-Ala-d_3 ($ee = 0.64$) before recording a series of NMR spectra at 288 K. The high substrate concentration (saturation of the enzyme) allows one to optimize the signal-to-noise of the ^{2}H−{^{1}H} spectra for a limited number of scans (here only 4 scans recorded at 14.1 T with a ^{2}H cryogenic probe), thus permitting one to monitor the time dependence of the concentrations in small time steps Δt.

Figure 2 shows the ^{2}H quadrupolar doublets of D- and L-Ala-d_3 and of HOD molecules at natural deuterium abundance (0.0156%) observed in a series of five representative ^{2}H−{^{1}H} spectra as a function of time after the introduction of the enzyme. The difference in linewidths of the outer doublet due to L-Ala-d_3 ($\Delta_{1/2} \approx 20$ Hz) and of the inner doublet of D-Ala-d_3 ($\Delta_{1/2} \approx 11$ Hz) results from ^{2}H−^{2}H dipolar couplings that are larger for L-Ala-d_3.[9] The ^{2}H doublet of HOD does not show any significant variation in either splitting or linewidth as a function of time, indicating that there is no degradation of the CLC during the overnight experiments. In particular, neither isotropic signals nor noticeable variations in order parameters were observed as a function of time.

dx.doi.org/10.1021/ac4004002 | Anal. Chem. 2013, 85, 4694−4697

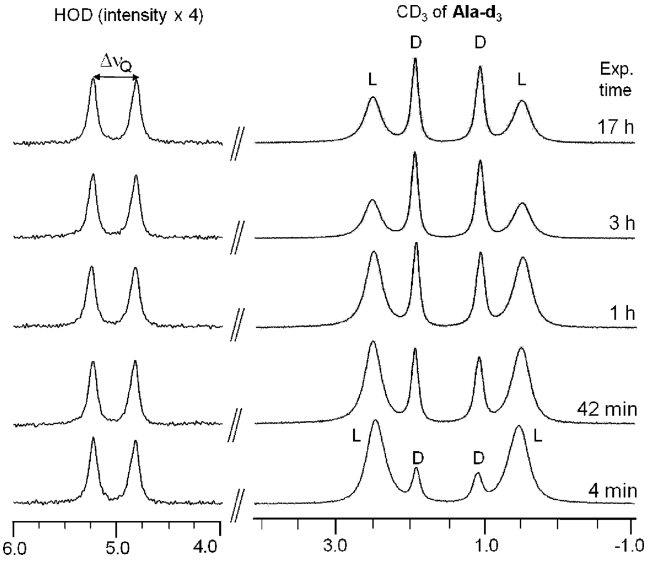


Figure 2. Examples of $^2H-\{^1H\}$ spectra plotted on the same scale showing HOD resonances (left) and signals of L- and D-Ala-d_3 (right) at different intervals after the introduction of the enzyme. The small differences in intensities and lineshapes between the high- and low-field components of each doublet are due to imperfect homogeneity of the CLC.

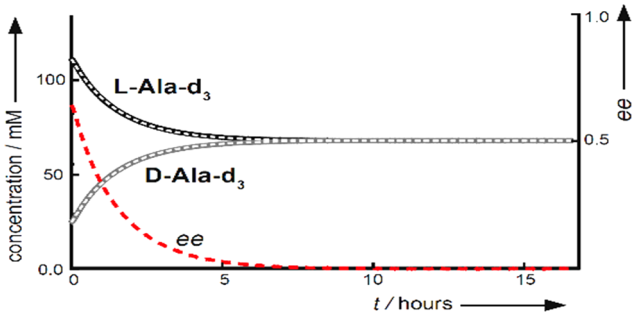


Figure 3. Enantiomeric excess (*ee*, red dashed line) and experimental (black and gray continuous lines) and fitted (white dashed lines) concentrations (in mM) of L- and D-Ala-d_3 in Sample No. 1 as a function of time (in hours) after the introduction of the enzyme. Note the excellent agreement between the fitted and experimental curves.

The time-dependent concentrations (see Figure 3) allowed us to evaluate the turnover numbers of the enzyme (k_{catL} and k_{catD}) for a reversible Michaelis–Menten model described by the coupled differential eqs 1 to 2. We used the Michaelis constants obtained by circular dichroism experiments elsewhere (K_M = 2.1 mM and K'_M = 1.6 mM at pH = 6.9).[18] The resulting values of k_{catL} and k_{catD} and the initial concentrations L_0 and D_0 for the different experiments are reported in Table 1. Racemization is complete after 7 h when the enantiomeric excess drops to zero. Note that the turnover numbers k_{catL} and k_{catD} are not identical because of different Michaelis constants.

$$\frac{d[L-Ala-d_3]}{dt} = -\frac{k_{catL}\cdot[AR]\cdot[L-Ala-d_3]/K_M}{1 + [L-Ala-d_3]/K_M + [D-Ala-d_3]/K'_M} + \frac{k_{catD}\cdot[AR]\cdot[D-Ala-d_3]/K'_M}{1 + [L-Ala-d_3]/K_M + [D-Ala-d_3]/K'_M} \quad (1)$$

Table 1. Kinetic Parameters Determined by Fitting[a]

exp. nb.	Ala-d_3 (mg)	[AR] (mM)	L_0 (mM)	D_0 (mM)	k_{catL} (s^{-1})	k_{catD} (s^{-1})
1	6.9	8.9×10^{-5}	109	24	166	126
2	10.7	8.9×10^{-5}	129	76	390	302
3	14.8	8.9×10^{-5}	174	111	376	286

[a]Errors in the amounts of Ala-d_3 are estimated to be 0.10, 0.14, and 1.7 mg for Samples Nos. 1, 2, and 3, respectively, and the error in the concentration [AR] is $1.0.10^{-5}$ mM (11%). Since there is almost no dispersion with respect to the fitted curves, the errors in k_{cat} are dominated by the errors in [AR].

$$\frac{d[D-Ala-d_3]}{dt} = +\frac{k_{catL}\cdot[AR]\cdot[L-Ala-d_3]/K_M}{1 + [L-Ala-d_3]/K_M + [D-Ala-d_3]/K'_M} - \frac{k_{catD}\cdot[AR]\cdot[D-Ala-d_3]/K'_M}{1 + [L-Ala-d_3]/K_M + [D-Ala-d_3]/K'_M} \quad (2)$$

While in Sample No. 1, L-Ala-d_3 was dissolved before adding the enzyme, further amounts of substrate were added for Samples Nos. 2 and 3 without adding any AR (final pH = 7.5). The k_{cat} values determined for Sample No. 1 are lower by ca. 50% than those of Samples Nos. 2 and 3. This discrepancy suggests that in Sample No. 1 the results are affected by the imperfect dispersion of AR throughout the liquid crystal, thus highlighting the importance of achieving good homogeneity.

In contrast, the values of k_{catL} (390 and 376 s^{-1}) and of k_{catD} (302 and 286 s^{-1}) for Sample Nos. 2 and 3 lie between those at pH 6.9 (k_{catL} = 210 s^{-1} and k_{catD} = 180 s^{-1}) and pH 8.9 (k_{catL} = 1300 s^{-1} and k_{catD} = 1000 s^{-1}) determined by circular dichroism.[18] These results demonstrate the reliability of our approach for evaluating kinetic parameters. The use of different batches of DNA-based liquid crystals may affect the resolution of the spectra, but if the homogeneity of the sample is good, the same catalytic constants should be obtained.

CONCLUSIONS

In this preliminary work, we have shown that the racemization of selectively deuterated L-Ala-d_3 by alanine racemase (AR) from *Bacillus stearothermophilus* can be monitored "on-the-fly" by exploiting $^2H-\{^1H\}$ NMR spectra in chiral liquid-crystalline media containing short DNA fragments. The resulting rate constants k_{catL} and k_{catD} are similar to those observed by circular dichroism obtained under slightly different conditions. This illustrates the reliability and robustness of NMR in anisotropic phase for monitoring chiral enzymatic transformations.

This approach opens new prospects for investigating enzymatic reactions of chiral substrates in aqueous media: (i) applications to various kinds of enzymes transforming chiral substrates other than amino acids; (ii) study of other water-based CLCs in order to widen the range of applications to enzymes that are not compatible with DNA-based CLCs; (iii) extension of the method using substrates with deuterium in natural abundance.[15,16,21]

The method should provide new mechanistic insight and a better understanding of enzymatic reactions, in particular for AR. To date, no inhibitors have been found with high specificity and low toxicity that would be suitable for clinical use. Far from being a mere complement to existing methods, the new approach can help to elucidate enzymatic mechanisms through its ability to

dx.doi.org/10.1021/ac4004002 | Anal. Chem. 2013, 85, 4694–4697

discriminate enantiomeric products of enzymes in the presence of inhibitors. Hence, it could become a new standard for enzyme reactivity studies.

■ ASSOCIATED CONTENT

Supporting Information

Theoretical aspects, experimental details, and supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

P.L., L.D., P.P., and G.B. thank the CNRS for continued financial support. M.C.-H. thanks the ANR (project FastSpinProts ANR-09-BLAN-0111-01) for financial support and the Protein and antibody platform of the Curie institute (Dr. A. El Marjou) for protein purification.

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