^{13}C and ^{1}H NMR Line Broadening in Desferrioxamine B Spectra. Kinetics and Mechanism of Siderophore Chemistry

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Desferrioxamine B, H_3dfb, is a naturally occurring trishydroxamic acid used by numerous microorganisms as a strong and selective chelator for ferric ions.[1,2] In neutral aqueous media, H_3dfb is fully protonated (structure **I**). Deprotonation of its three hydroxamic acids leads to the formation of a hexadentate ligand that is capable to complete the coordination shell of iron(III). A complex between desferrioxamine B and ferric ion is a siderophore named ferrioxamine B.

Owing to desferrioxamine B's commercial availability,[3] the mechanism of hydrolysis of ferrioxamine B is probably the most extensively studied of all siderophores.[4−9] The mechanistic behavior forms a basis for understanding the biologically crucial unwrapping process in neutral or weak acid media, interchange processes,[10] and catalyzed hydrolyses.[11,12]

The hydrolysis of the tris(monohydroxamato)iron(III) complexes that were used as models for siderophores, proceeds through three distinct, proton-concentration dependent, kinetic steps.[13,14] Each step corresponds to the stepwise dissociation of one of the coordinated hydroxamate ligands and involves protonation of the hydroxamate N−O group. On the other hand, the results obtained in two independent laboratories agree that $Fe(Hdfb)^+$ undergoes full hydrolysis through at least four distinguishable kinetic steps.[5,8] The first, third, and fourth steps were found to be proton-concentration dependent, whereas the second step does not involve protonation of ferrioxamine B. It was suggested that dechelation of the second hydroxamato group in $Fe(Hdfb)^+$ proceeds in two steps.

Caudle and Crumbliss[14] have recently proposed that this discrepancy is caused by the slow rate of rotation around the middle hydroxamate C−N bond in partially unwrapped ferrioxamine B. As pointed out by the authors, the sizes of both substituents in the middle hydroxamato group of desferrioxamine B are maximized. Therefore, one might expect the rate of rotation around the middle C−N bond would be the slowest. The bulky substituents are also expected to stabilize the *E* conformation of the hydroxamato group in which two hydroxamate oxygens are not in the proper position for the chelation of iron(III) metal center. The possible slow rotation around the C−N bond was tested only on a model compound, *N*-methylacetohydroxamic acid (NMHA), by means of PMR.[14] The advantage of NMHA is that both the N and C substituents are also alkyl groups as in desferrioxamine B (alkyl groups increase the double bond character of the C−N bond[15,16] and therefore decrease the rate of rotation). The disadvantage of NMHA is, however, its small size which makes it inappropriate for comparison. Caudle and Crumbliss observed line broadening in ^1H spectra, and were able to indicate slow rotation around C−N bond in aqueous medium, whereas Bagno et al.[17] have observed only one set of signals in ^1H, ^{13}C, and ^{15}N spectra, indicating a fast exchange rate of *E* and *Z* forms. These two reports prompted us to present our attempt to address the question of the *E/Z* rotation by collecting direct evidence of the rotation around C−N bond in the natural siderophore H_4dfb^+ itself, using NMR line broadening experiments.

^{13}C and ^1H NMR spectra of H_4dfb^+, and their full assignment, were already reported.[18] Figure 1 shows that the ^1H spectrum of H_4dfb^+ changes considerably upon a temperature increase. An unequal pair of singlets at 2.12 and 2.10 ppm (the ratio of their integrals equals 4, respectively) assigned to the methyl-protons of the N-terminal hydroxamato group were significantly broadened at higher temperatures. They eventually coalesced into a single peak indicating a rapid *E/Z* interchange of the hydroxamato group. Figure 2 shows the spectra, the recorded and the calculated ones,[19] with corresponding rate constants that exhibited an excellent Eyring plot ($r = 0.998$). Due to its simplicity, we found temperature dependent ^{13}C spectra more suitable for studying the rotation around the remaining two C−N groups. Two main groups of carbons in desferrioxamine B are represented by the signals centered around 40 ppm (methylene carbons) and 170 ppm (carbonyl carbons) of the carbon spectrum. For the kinetic analysis carbonyl frequencies were chosen. The three peaks between 173.3 and 173.8 ppm in our spectra were already assigned to the hydroxamate carbonyls.[18] However, much weaker signals around 170 ppm were not discussed in the earlier study (Figure 8 in ref 18), but undoubtedly appear between 169.0 and 169.6 ppm in our spectra as well. Spectra of alkyl hydroxamic acids reveal that the carbonyl signal in *Z*-form is shifted about 5 ppm upfield compared to its *E* analogue.[20,21] Therefore, the weaker signals in desferrioxamine B spectra should correspond to carbonyls in *Z*-form. A higher intensity of the carbonyl peaks at lower fields suggests that *E* conformers of H_4dfb^+ prevails the equilibrium in aqueous medium. This finding is in accordance

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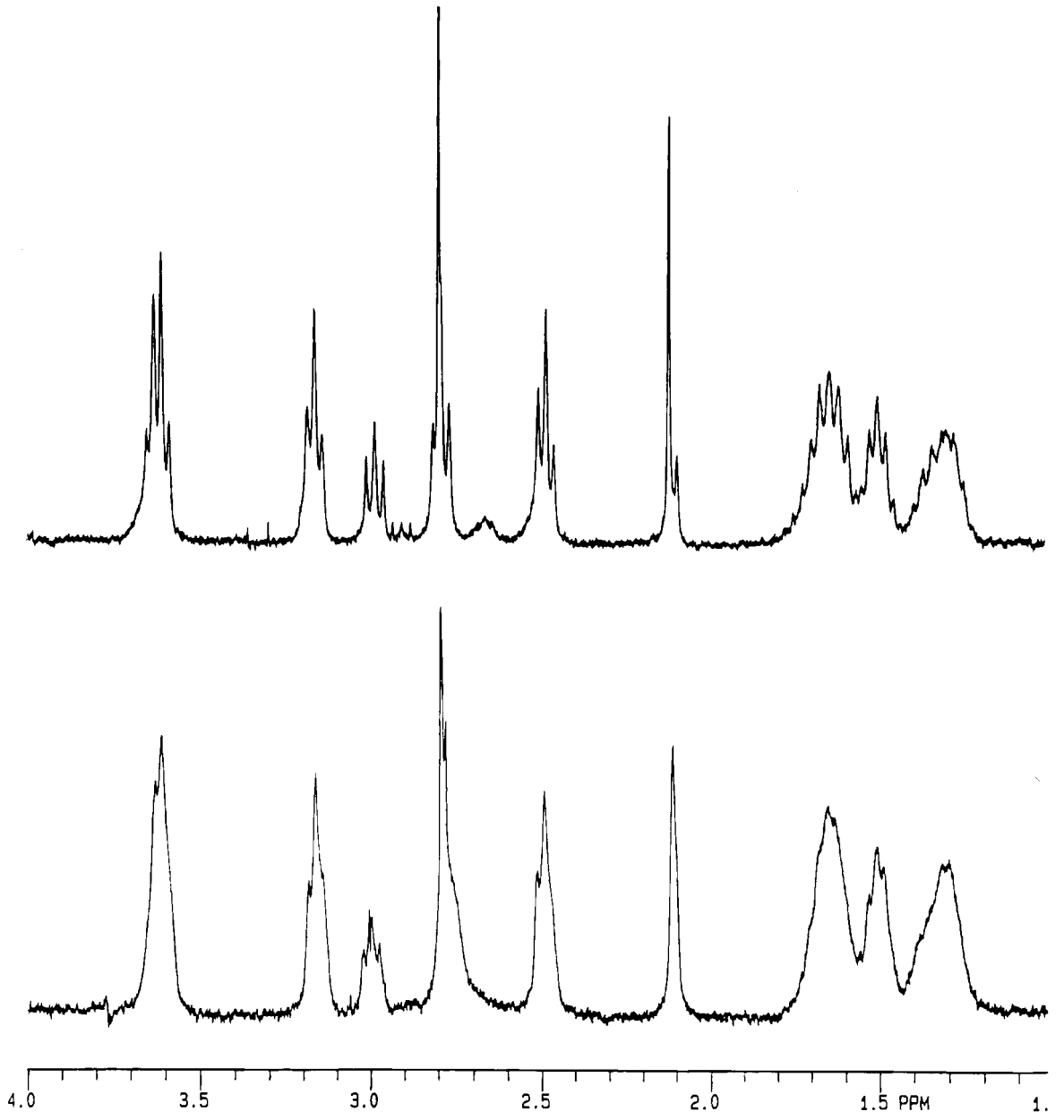
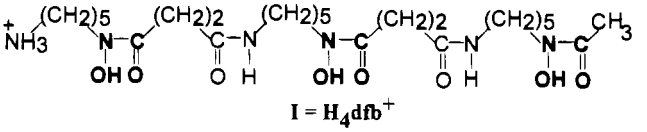


Figure 1. ^{1}H NMR spectra at 300 MHz, of 5 mM desferrioxamine B in D_2O at 23 (upper) and 53 °C.



I = H_4dfb^+

with Brown et al.'s[20] results showing that in methanol (the single protic solvent used in their study) *E* form is also more stable. It should be noted that the ^{13}C NMR spectra of desferrioxamine B also confirm that the *E* form is more stable than the *Z* form (all the carbonyl signals are observed above 173 ppm, which was found to be characteristic for *E* form in all hydroxamic acids studied).

Line broadening was observed for all ^{13}C NMR carbonyl signals, as is presented in Figure 3. The observed line broadening of the highest field carbonyl peak (173.3 ppm) and its counterpart (169.0 ppm), corresponding to the C-terminal hydroxamate group, gives essentially the same *Z/E* exchange rate constant as obtained in study of ^{1}H spectra. Since at the higher temperature the signals at 173.6 and 173.8 ppm strongly overlap (as well as their counterparts at 169.57 and 169.64 ppm), they had to be fitted simultaneously. Despite the fact that line shape of the stronger signal is generally less sensitive to the exchange rate than the line shape of its weak-signal counterpart, fitting was applied to the line-broadening of 173.3, 173.6 and 173.8-ppm signals.

Calculated rate constants related to the *E/Z* isomerization of the N-terminal, middle, and C-terminal hydroxamato groups of H_4dfb^+ at 25 °C in aqueous medium are 9 s^{-1}, 15 s^{-1} and 12 s^{-1} (^{1}H and ^{13}C average value), respectively. The last value is fairly consistent with the izomerization rate constant obtained with NMHA.[14] The similarity of these values suggests that the rates of rotation around C–N bonds are almost independent of the hydroxamate position in H_4dfb^+ molecule. Plausible explanations for this are the following: (i) A rotational barrier of the isomerization is primarily determined by the partial double bond character of the hydroxamate C–N bond, which is mainly affected by the electronic properties of C- and N-substitu-

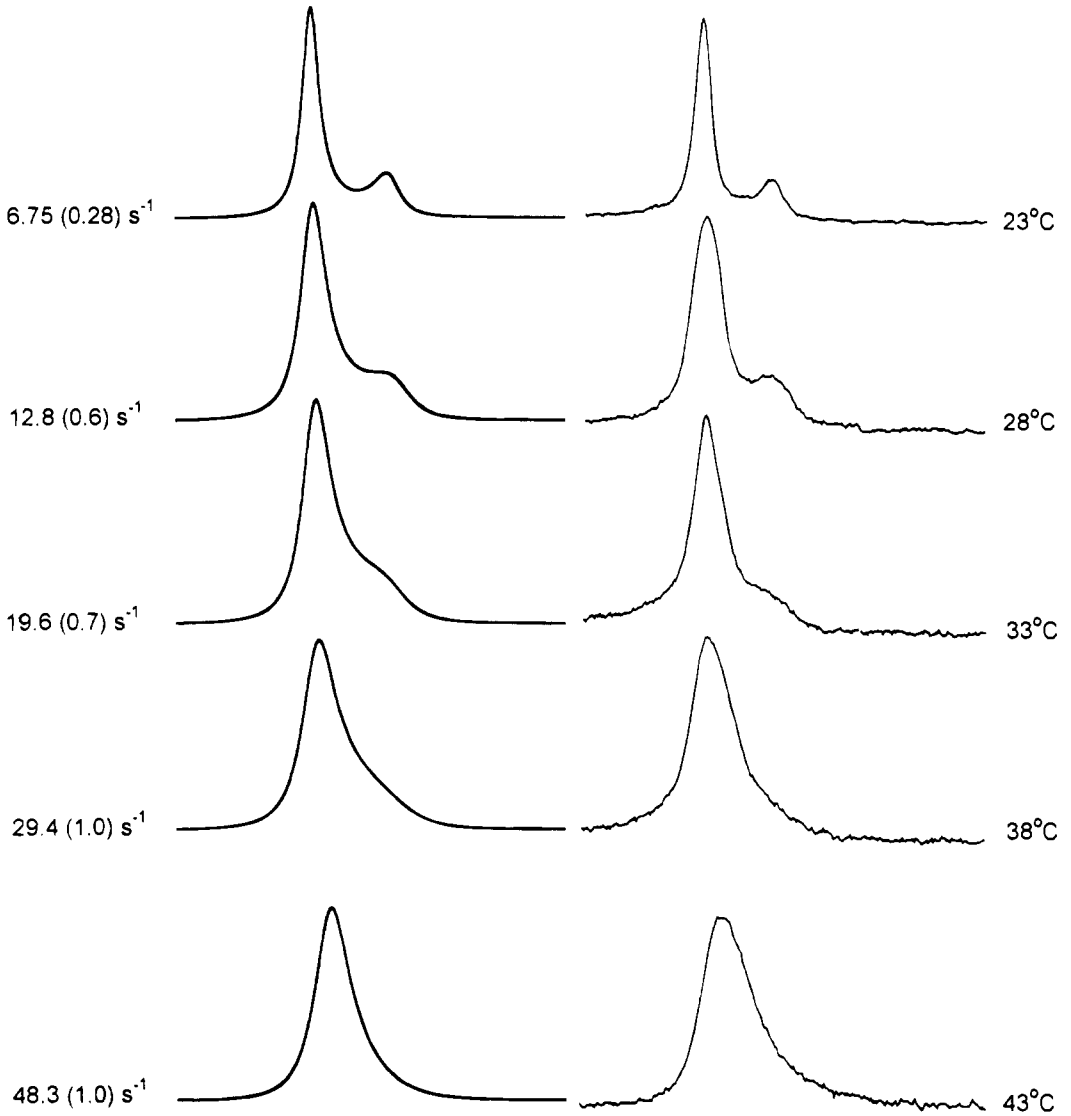
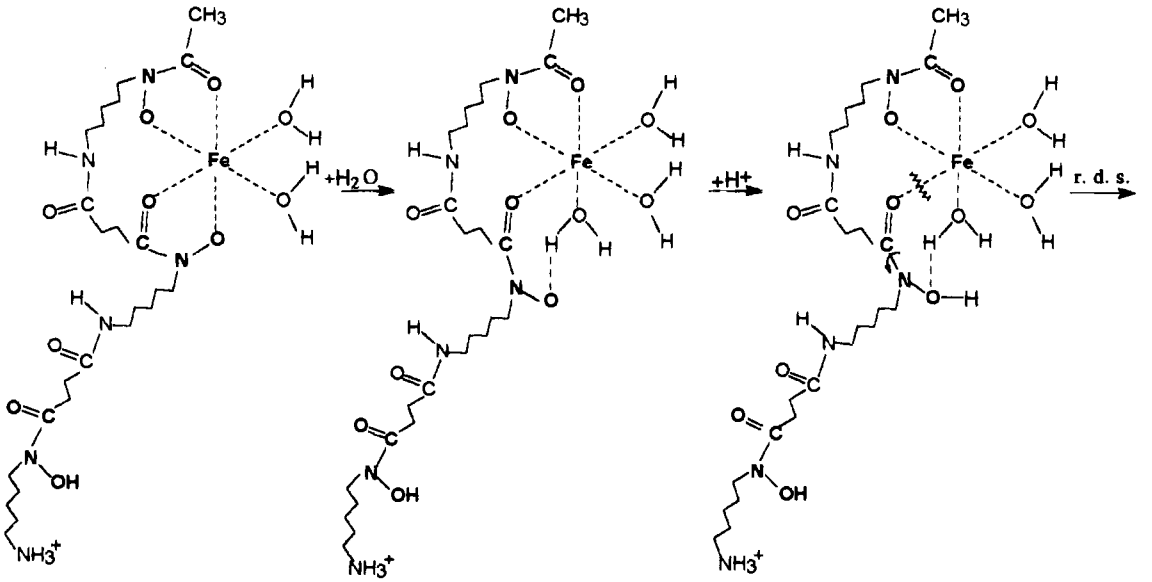


Figure 2. Computed and observed ^{1}H-DNMR spectra of 5 mM desferrioxamine B in D_2O with corresponding rate constants.

Scheme 1. Dechelation of the Middle Hydroxamato Group of Ferrioxamine B



ents.[16,20,21] In all three hydroxamate groups of H_4dfb^+ the substituents are alkyl groups which are expected to have nearly equal electron donating ability. (ii) Brown and co-workers[20] have pointed out that steric interaction between the OH group and the N-substituent has a dominant impact on the *Z/E* ratio, whereas interaction between the N- and C-substituents is less important. If the kinetics and the equilibrium of *E/Z* isomerization are similarly affected by N-substituents, similar rates

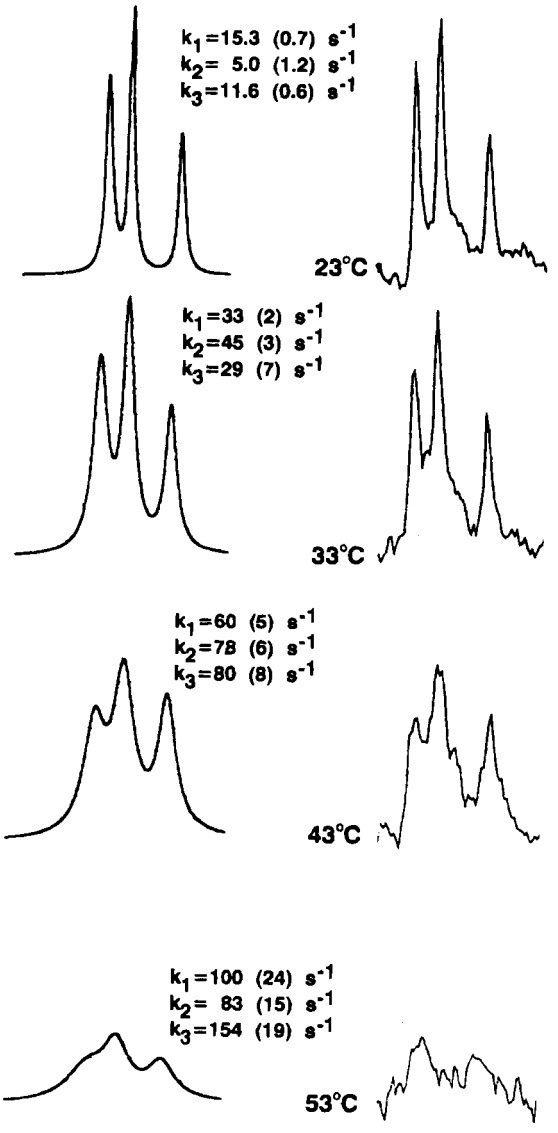


Figure 3. Computed and observed ^{13}C-DNMR spectra (at 75 MHz) of 0.4 M desferrioxamine B in water. The calculated rate constants, k_1, k_2 and k_3, corespond to the $E \leftrightarrow Z$ rotation of the C-terminal (173.3 ppm), the middle (173.6 ppm) and the N-terminal (173.8 ppm) hydroxamates, respectively.

are expected for all three hydroxamate groups for, neglecting the differences beyond the fifth methylene group, all three hydroxamates of H_4dfb^+ have essentially the same N-substituent which may be viewed as a pentyl radical. (iii) Different sizes of the substituents, and their distinct bulkiness, have no significant effect on the isomerization rate because the rotation around single bonds in the rest of H_4dfb^+ is fast, making the molecule flexible enough to avoid the effect of chain rigidity on the rotation of C−N bonds in hydroxamate groups.

The above mentioned second (proton independent) hydrolysis step of ferrioxamine B was found to proceed by the rate constant 9.9 s^{-1} [8] or 14.1 s^{-1}.[5] Considering the different techniques used in these two studies, these values are consistent with our value of the rate constant of *Z/E* isomerization for the middle hydroxamate group (correction by the percentage of *E* form gives value of $k_{Z\rightarrow E} = 12\ s^{-1}$). The simplest mechanism that accounts for this fact is presented in Scheme 1.

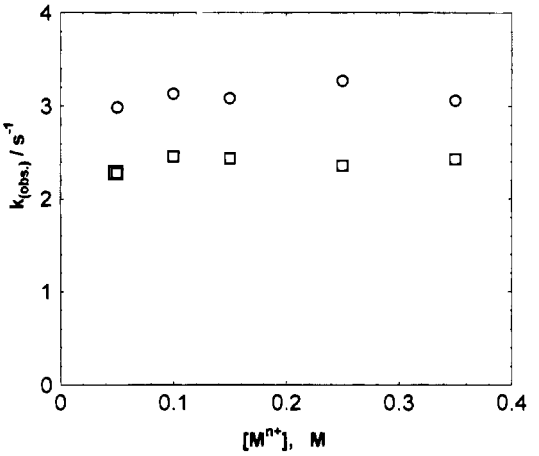


Figure 4. Plots of k_{obs} *vs* $[M^{n+}]$ for reactions of H_4dfb^+, at 25 °C: (□) $[M^{n+}] = [La^{3+}] \times 50$, $[H_4dfb^+] = 0.1$ mM, $I = 0.1$ M ($NaClO_4$), pH 7.55 (Hepes buffer); (○) $[M^{n+}] = [Cu^{2+}]$, $[H_4dfb^+] = 5$ mM, $I = 2$ M ($NaClO_4$), pH 4.35 (acetate buffer).

Crumbliss and co-workers proposed that the hydrolysis of iron(III)−hydroxamate complexes is initiated by a ring opening at protonated oxime oxygen, followed by the rate determining cleavage of the iron−carbonyl oxygen bond. Unless the mechanism is associative ($\Delta V^{\neq}$ of the hydrolysis of bis(acetohydroxamato)bis(aqua)iron(III) indicates an I_d mechanism),[22] the water molecule cannot enter the inner-coordination shell of iron before the dechelated carbonyl moiety is removed away. An efficient way to expel the carbonyl moiety from the coordination shell of iron is the rotation of the C−N bond that corresponds to the *Z/E* isomerization of free H_4dfb^+. Since the hydrogen bonding does not impose a severe barrier to the rotation of NH_4^+ ion in water,[23] we expect that the isomerization in the activated complex shall not be hindered by hydrogen bonding.

Our results indicate that ca. 80% of C-terminal hydroxamate is in the *E* form, which is an improper form for the bidentate binding of metal ions. Therefore, in a high molar excess of metal ions over H_4dfb^+ one may expect that the complex formation rate will be limited by the rate of isomerization (the latter can be estimated as 20% of the observed *E/Z* rate constant, *i.e.* 2.4 s^{-1}). Although experiments were carried out with Fe^{3+}, VO^{2+},[24] Al^{3+}, and Ga^{3+},[25] these metals were never in the excess over H_4dfb^+, in order to prevent their polymerization. Nevertheless, using La^{3+} and Cu^{2+}, we were able to design an experiment that supports the above mentioned consideration. Preliminary stopped-flow results reveal that the formation of La^{3+} and Cu^{2+} complexes with acetohydroxamic acid, AHA, is completed in less than 2 ms. One may expect that, as with Fe^{3+} and Al^{3+} ions, the complexation with H_4dfb^+ proceeds comparably fast as with AHA. Figure 4 shows that leveling of k_{obs} occurred at 2.5 s^{-1} for La^{3+} and 3.0 s^{-1} for Cu^{2+}. These values agree fairly well with the rate constant of $E \rightarrow Z$ isomerization of the C-terminal hydroxamato group, confirming that the rate of $E \rightarrow Z$ isomerization is the rate determining step in complex formation kinetics.

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