JOURNAL OF PHYSICAL ORGANIC CHEMISTRY, VOL. 11, 512–518 (1998)

Review Commentary

Imperatives for enzymatic catalysis of isomerization of sugars and sugar phosphates

John P. Richard,* Juan Crugeiras and R. W. Nagorski

Department of Chemistry, University at Buffalo, SUNY, Buffalo, New York 14260–3000, USA

Received 15 September 1997; revised 16 January 1998; accepted 4 February 1998

ABSTRACT: The relative yields of the products of isomerization of D,L-glyceraldehyde in D_2O with intramolecular transfer of a hydride ([1-^{1}H]DHA) and with proton transfer ([1-^{2}H]DHA) were determined by high-resolution ^{1}H NMR analyses. A study of the catalysis of this isomerization by deuteroxide ion, buffer anions and Zn^{2+} established the following: (1) isomerization with proton and hydride transfer occurs at approximately equal rates in dilute solutions of sodium deuteroxide; (2) Brønsted bases catalyze isomerization with proton transfer in a bimolecular reaction; (3) Zn^{2+} catalyzes isomerization with hydride transfer in a bimolecular reaction; and (4) Zn^{2+} and acetate ion react in concert to catalyze isomerization with proton transfer in a termolecular reaction. These results show that the various pathways for isomerization with proton and hydride transfer proceed via transition states of similar energies, so that there is no strong imperative for enzymatic catalysis by any particular reaction mechanism. The relevance of these results to the mechanism of action of xylose isomerase is discussed briefly. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: sugar isomerization; sugar phosphate isomerization; enzymatic catalysis

INTRODUCTION

About 30 years ago, Jencks[1] compared biochemists who attempted to understand enzyme-catalyzed reactions through study of the mechanism of non-enzymatic reactions and their rate accelerations to 'the drunk on his hands and knees under the corner street light who, when approached by a citizen asking his intentions, replies that he is looking for his keys here, rather than in the poorly illuminated center of the block where they were lost, because the light is better at the corner.' This is a penetrating analogy, because studies of solution models for enzymatic reactions, which *are* intrinsically simpler than studies of enzymatic processes, cannot elucidate the events which occur at an enzyme active site. It is important, therefore, to emphasize what can be learned by 'looking where the light is better.' The determination of the mechanisms of the reactions of small molecule models for enzyme-catalyzed processes serves as a blueprint for the design and interpretation of the experiments to determine enzymatic reaction mechanisms. This is because there is a strong imperative for enzyme catalysts to follow the same mechanism as observed for the corresponding non-enzymatic reaction in water, and a close congruence between the mechanisms for solution and enzyme-catalyzed reactions. This is simply explained by a comparison of the enzymatic rate accelerations obtained from catalytic stabilization of the low-energy transition state for an observed solution reaction with that obtained from stabilization of a second higher energy transition state. Equal stabilization of these two transition states will always result in a larger catalytic rate acceleration for the favored reaction in water, because reduction of the barrier for the higher energy transition state cannot result in a rate acceleration until this barrier is first reduced below that for the favored transition state.

In this paper, we discuss the results of studies on the mechanism for non-enzymatic aldose–ketose isomerization in water which were conducted to resolve a possible exception to the imperative that enzyme-catalyzed aldose–ketose isomerization follows the same mechanism as observed for the reaction in solution. Instead, the results of this work provide a textbook illustration of the close parallels between the mechanisms for solution and enzyme-catalyzed reactions.

**Correspondence to:* J. P. Richard, Department of Chemistry, University at Buffalo, SUNY, Buffalo, New York 14260–3000, USA. E-mail: jrichard@acsu.buffalo.edu
Contract/grant sponsor: National Institutes of Health; *contract/grant number:* GM 47307; *contract/grant number:* GM 39754

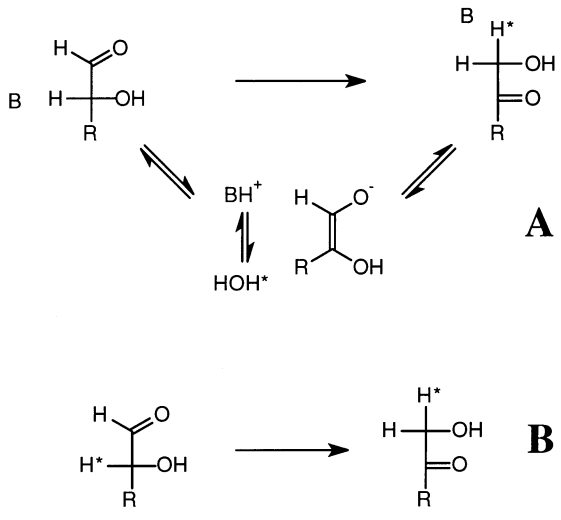
 CCC 0894–3230/98/080512–07 $17.50

ISOMERIZATION OF SUGARS AND SUGAR PHOSPHATES

Similar proton transfer mechanisms are observed for the enzyme-catalyzed aldose–ketose isomerization of sugar phosphates[2,3] and the non-enzymatic reactions in water [Scheme 1(A)].[4] This proton transfer reaction mechanism is favored by the relatively large acidities for the α-carbonyl hydrogens of aldehydes and ketones.[5]

By contrast, studies on the mechanism of action of xylose isomerase provide strong evidence that this enzymatic reaction proceeds by a different mechanism in which there is intramolecular transfer of a hydride ion from reactant to product [Scheme 1(B)].[6–13] A striking result was the observation that the transfer of tritium from solvent to the equilibrating reactant and products occurs only once every billion turnovers of xylose isomerase.[10] This virtual absence of mixing of solvent and reactant protons during turnover is required for a hydride transfer reaction mechanism. The possibility that isomerization occurs by transfer of a proton which is highly shielded by the protein catalyst from reaction with solvent has not been rigorously excluded; however, such shielding was shown to be unlikely because 'loosening' the protein structure by changing the reaction temperature or the pH or by the addition of guanidine hydrochloride did not affect the level of incorporation of tritium label into substrate and product.[10]

The observation that xylose isomerase and related sugar isomerases proceed by a hydride transfer mechanism would require either (1) that there is no *absolute* requirement that enzymatic reactions follow the same mechanism as for the uncatalyzed reaction in water or (2) that there are similar barriers for isomerization with proton transfer and hydride transfer in water, and no strong mechanistic advantage for enzymatic catalysis of aldose–ketose isomerization by either mechanism.



Scheme 1

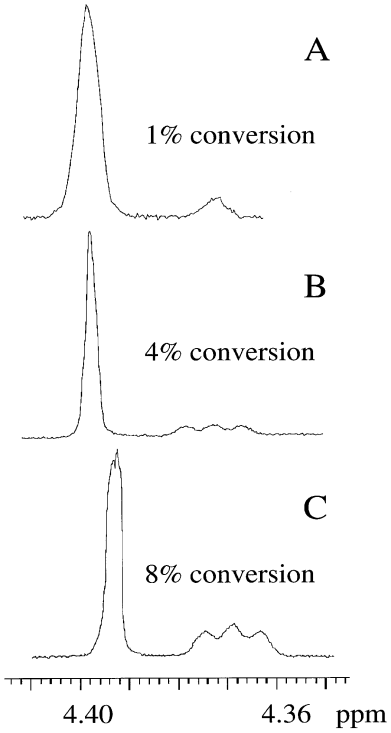
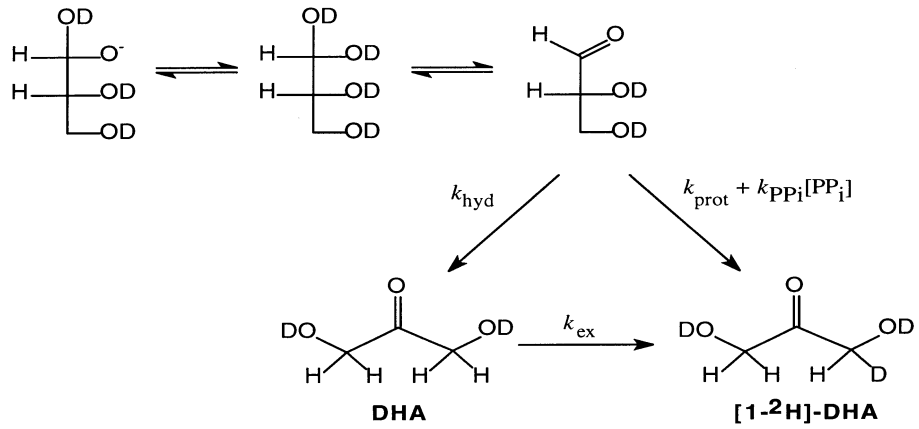


Figure 1. Representative partial 500 MHz ^{1}H NMR spectra (obtained at 25 °C in D_2O), in the region of the hydroxymethylene groups of dihydroxyacetone (DHA), of the reaction mixture from the isomerization of D,L-glyceraldehyde to give DHA in D_2O at 25 °C. (A) and (B) isomerization in 0.01 M KOD (I = 0.10, KCl); (C) isomerization in 150 mM potassium pyrophosphate buffer at pD 8.4. The singlets at *ca* 4.4 ppm are due to the two CH_2OD groups of unlabeled DHA and the singlet CH_2OD group of monodeuterated [1-^{2}H]DHA. The upfield-shifted singlet in the deuterium decoupled spectrum (A) and the triplets in the absence of decoupling (B and C) are due to the CHDOD group of monodeuteriated DHA. Reprinted with permission from *J. Am. Chem. Soc.* **118,** 7432–7433 (1996)

Intramolecular transfer of hydride ion from a metal alkoxide to a keto group is known to occur readily in organic solvents.[14a] However, previous experiments to determine whether isomerization with hydride transfer is a viable reaction mechanism in water have produced contradictory results. The small amount of intramolecular transfer of tritium observed during the *anaerobic* hydroxide ion-catalyzed epimerization of [2-^{3}H]-D-ribose to give arabinose may occur by consecutive isomerization reactions with hydride transfer.[14b] However, the interpretation of these data is clouded by uncertainties about the mechanism and possible contribution of a much faster aerobic pathway for intramolecular transfer of tritium.[14] No intramolecular transfer of tritium was detected during the isomerization of [2-^{3}H]-D-glucose to give D-fructose,[15] and there is evidence that the interconversion of D-glucose, D-mannose and D-fructose in alkaline D_2O proceeds through a common enolate reaction intermediate.[16] However, the former experiments lack the appropriate controls to determine

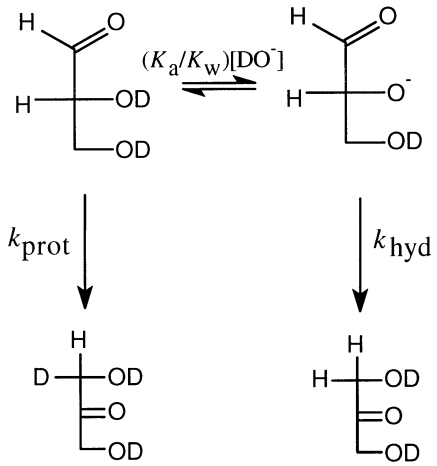


Scheme 2

whether the tritium-labeled product that might be formed by intramolecular hydride transfer is stable towards hydroxide ion-catalyzed transfer of tritium from product to solvent.

ISOMERIZATION OF D,L-GLYCERALDEHYDE (D,L-GA) TO DIHYDROXYACETONE (DHA)

^{1}H NMR is a convenient method to distinguish between the isomerization of D,L-GA in D_2O with intramolecular transfer of hydride to give unlabelled DHA from the isomerization with proton transfer to give [1-^{2}H]DHA (Scheme 1).[17] The use of ^{1}H NMR to determine the relative yields of [1-^{1}H]DHA (unlabeled) and [1-^{2}H]DHA from the isomerization of D,L-GA is illustrated in Fig. 1, which shows partial ^{1}H NMR spectra in the hydroxymethylene region of DHA after 1% [Fig. 1(A), deuterium decoupled spectrum] and 4% conversion [Fig. 1(B)] of D,L-GA in 0.01 M KOD in D_2O at 25 °C, and after 8% conversion of D,L-GA in 150 mM potassium pyrophosphate buffer in D_2O at pD 8.4 and 25 °C [Fig. 1(C)]. The singlet at 4.4 ppm in each spectrum is due to the two CH_2OD groups of unlabeled DHA and the single CH_2OD group of the monodeuterated [1-^{2}H]DHA (Scheme 2). The deuterium at the CHDOD group causes the signal for the remaining proton in this group to shift 0.024 ppm upfield from that for the CH_2OD groups.[18,19] This signal appears as either a singlet in a deuterium decoupled spectrum [Fig. 1(A)], or as a triplet in the absence of decoupling [Fig. 1(B) and 1(C)].



Scheme 3

The ratio of the yields of the products of isomerization with hydride and proton transfer, [DHA]/[[1-^{2}H]DHA], can be calculated from the ratio of the integrated areas of the peaks for the CH_2OD (A_{CH_2}) and CHDOD (A_{CHD}) groups according to the equation

$$[\mathrm{DHA}]/[[1-{}^2\mathrm{H}]\mathrm{DHA}] = (A_{CH_2} - 2A_{CHD})/(4A_{CHD}) = k_{hyd}/k_{prot} \qquad (1)$$

This ratio of product yields is equal to the rate constant ratio k_{hyd}/k_{prot}, where k_{hyd} and k_{prot} are the rate constants for isomerization with hydride and proton transfer, respectively [Eqn (1) and Scheme 2].

We analyzed the products of the isomerization of D,L-GA under a variety of reaction conditions. The following are the main conclusions of these product studies:

(1) The ratio of the integrated areas of the peaks for the CH_2OD and CHDOD groups of DHA in the spectrum in Fig. 1(C) is $A_{CH_2}/A_{CHD} = 1.9$. This result is consistent with the isomerization of D,L-GA to give a product which contains one CHDOD group and one CH_2OD group ([1-^{2}H]DHA), as is required for the exclusive isomerization of the substrate by a proton transfer mechanism catalyzed by pyrophosphate ion ($k_{PPi}[PP_i]$, Scheme 2).

(2) The values of $A_{CH_2}/A_{CHD} = 4.4$ and 3.8 determined from the spectra in Fig. 1(A) and (B), respectively, are smaller than $A_{CH_2}/A_{CHD} = 2$ that is observed when the isomerization of D,L-GA in D_2O occurs exclusively by a proton transfer mechanism. The excess hydrogen in product is due to a competing pathway for isomerization

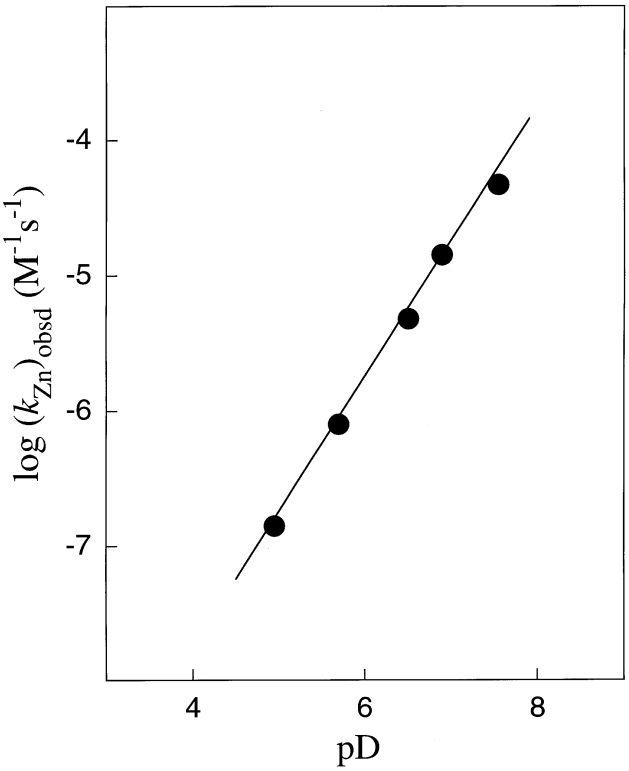


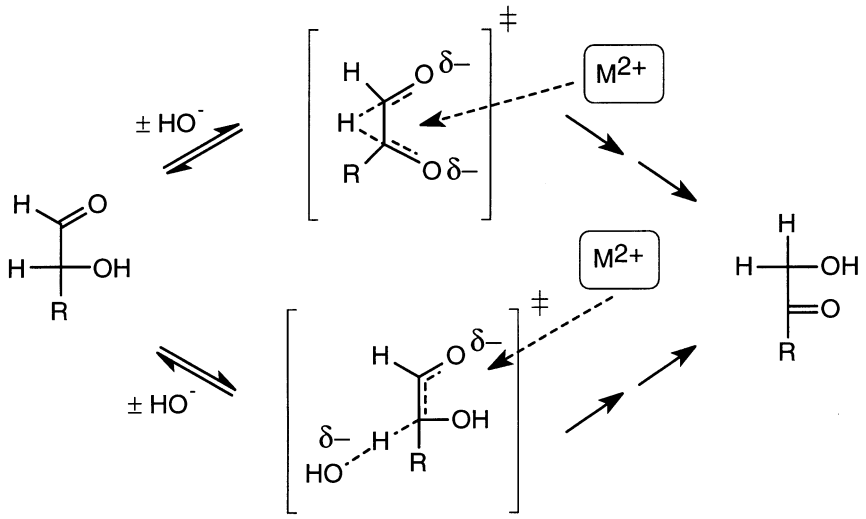
Figure 2. The logarithmic dependence of $(k_{Zn})_{obsd}$, the observed second-order rate constant for the Zn^{2+}-catalyzed isomerization of D,L-GA to give dihydroxyacetone in D_2O at 25 °C, on the pD of the reaction mixture

with transfer of hydride. These values of A_{CH_2}/A_{CHD} can be substituted into Eqn (1) to give [DHA]/[[1-^{2}H]DHA] = 0.60 and 0.45 for the relative yields of the products of isomerization with hydride and proton transfer, respectively, from the reactions in Fig. 1(A) and (B).

(3) There are decreases in A_{CH_2}/A_{CHD} at increasing times for the isomerization of D,L-GA in the presence of 0.01 M KOD. This decrease is due to the base-catalyzed exchange of deuterium from solvent into DHA and [1-^{2}H]DHA (k_{ex}, Scheme 2). The ratio of the yields of the initial products of isomerization with hydride and proton transfer, [DHA]/[[1-^{2}H]DHA] = k_{hyd}/k_{prot} = 0.63 was determined by extrapolation of the observed product yields to zero reaction time.

(4) The ratio of product yields [DHA]/[[1-^{2}H]DHA] = 0.63 remains constant as the concentration of deuteroxide ion is increased from 0.01 to 0.10 M. This observation that k_{hyd}/k_{prot} = 0.63 for isomerization of D,L-GA is independent of [DO^-] requires that the observed first-order rate constants k_{hyd} and k_{prot} show the same dependence on [DO^-]. Therefore, the increase in k_{prot} from the DO^--catalyzed deprotonation of D,L-GA must be exactly balanced by an increase in k_{hyd} from catalysis by DO^- of the isomerization with hydride transfer. The mechanism for catalysis of the isomerization with hydride transfer probably involves preequilibrium deprotonation of D,L-GA at O-2 to form the oxyanion which is much more reactive than the neutral substrate towards intramolecular transfer of hydride (Scheme 3).

Our experimental results provide a solid chemical precedent for aldose–ketose isomerization with intramolecular transfer of a hydride, so that there is no obvious advantage for aldose–ketose isomerization by either a proton or a hydride transfer reaction mechanism. We conclude that the evolution of enzymes which catalyze aldose–ketose isomerization by these two different reaction mechanisms is probably a consequence of this lack of a strong imperative for catalysis by either mechanism.



Scheme 4

METAL ION CATALYSIS OF ALDOSE–KETOSE ISOMERIZATION

The coulombic interaction between a metal ion and a developing negative charge will stabilize the transition states for both isomerization with hydride transfer and base-catalyzed isomerization with proton transfer. Stronger metal ion catalysis is predicted for hydride compared with proton transfer, because there is a full negative charge to interact with a metal cation at the transition state for the former reaction, but only a partial negative charge at the transition state for proton transfer (Scheme 4).

A metal ion cofactor is required by all enzymes known to catalyze the isomerization of sugars with hydride transfer[20] and, in the case of xylose isomerase, two enzyme-bound metal ions have been shown participate in catalysis.[11] By contrast, metal ion analyses of six enzymes which catalyze aldose–ketose isomerization by a proton transfer mechanism show that only mannose isomerase is a metalloenzyme.[20,21] These observations are consistent with the notion that there is a greater advantage in using a metal ion in catalysis of isomerization with hydride transfer, where the metal ion interacts with a full negative charge at the reaction transition state, than for catalysis of isomerization with proton transfer.

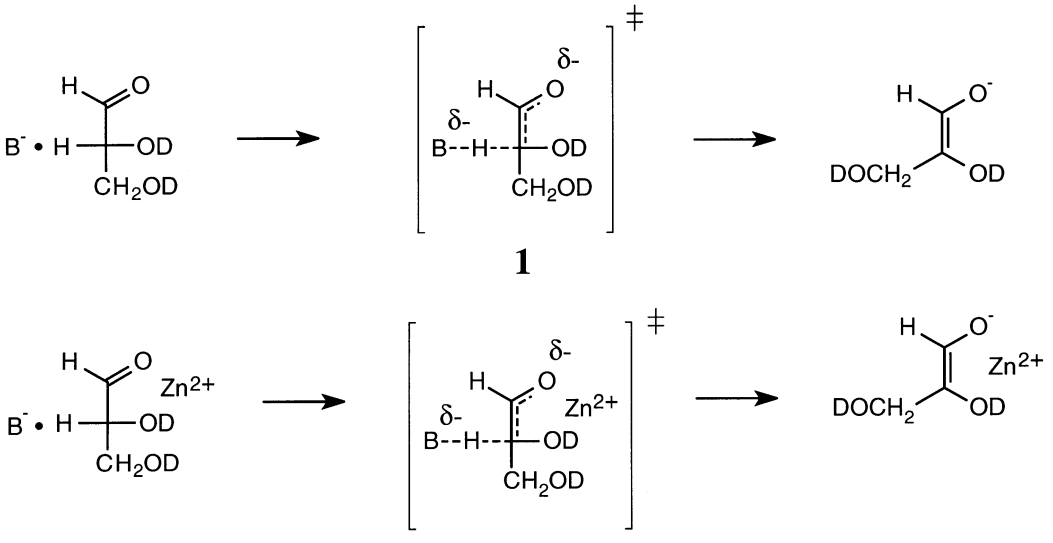
It has been shown that Zn^{2+} is a very effective catalyst of the isomerization of mannose-6-phosphate to give fructose-6-phosphate in water;[22] however, it was not determined whether this isomerization occurs by a hydride or proton transfer mechanism. We examined the effect of Zn^{2+} on the observed first-order rate constants for the isomerization of D,L-GA in D_2O, on the yields of the products of isomerization with hydride transfer (DHA, Scheme 2) and with proton transfer ([1-^{2}H]DHA) and on the rate of deprotonation of acetone. The following are the main conclusions from these studies:

(1) Only a singlet due to unlabeled DHA was detected by ^{1}H NMR analysis of the products of isomerization of D,L-GA in D_2O at pD = 5.7 (36 mM acetic acid buffer) and 25 °C containing 90 mM Zn^{2+}. Therefore, isomerization with intramolecular transfer of hydride is the dominant pathway under these reaction conditions.

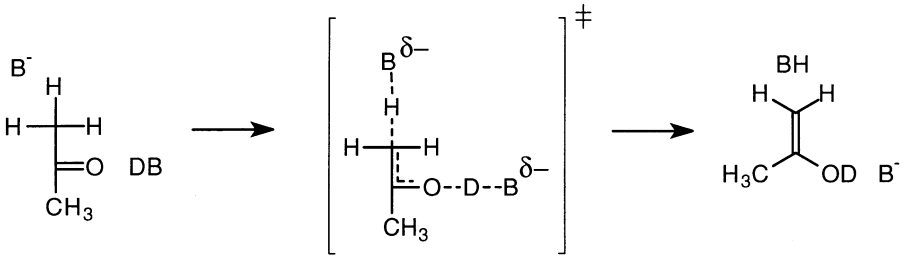
(2) Increasing the concentration of Zn^{2+} for the reaction of D,L-GA in D_2O at pD = 5.7, but at a higher concentration of acetate buffer (0.3 M), results in an increase in the velocity of formation of both unlabeled DHA from isomerization with hydride transfer and [1-^{2}H]DHA from isomerization with proton transfer. There are two pathways for isomerization of D,L-GA with proton transfer: (i) a bimolecular reaction of acetate ion, which corresponds to direct deprotonation of substrate by this Brønsted base (transition state **1,** Scheme 5), and (ii) a termolecular reaction which corresponds to Zn^{2+}-assisted deprotonation of D,L-GA by acetate ion (transition state **2,** Scheme 5).

(3) The results of a study of the catalysis of deprotonation of acetone in D_2O at 25 °C show that the third-order rate constant for the Zn^{2+}-assisted deprotonation of acetone by acetate ion (5.6×10^{-7} l^2 mol^{-2} s^{-1}) is not much larger than that (2.1×10^{-7} l^2 mol^{-2} s^{-1}) for the reaction where electrophilic assistance is instead provided by acetic acid (transition state **3,** Scheme 6).[23]

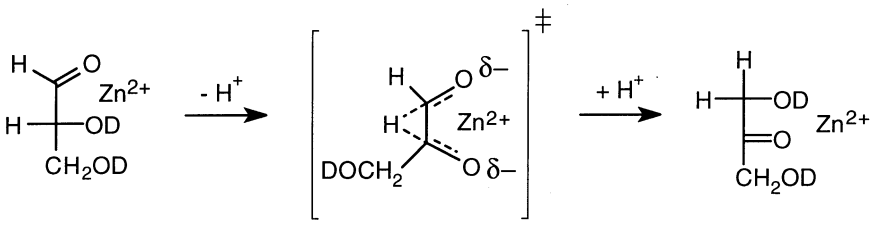
(4) The observed second-order rate constants for the reaction catalyzed by Zn^{2+}, $(k_{Zn})_{obsd}$, increase with increasing concentration of deuteroxide ion (Fig. 2).



Scheme 5



3



4

Scheme 6

This shows that the transition state (**4,** Scheme 6) for isomerization with intramolecular transfer of hydride ion contains one less proton than the neutral reactant D,L-GA.

(5) A value of $k_T = 1100\ l^2\ mol^{-2}\ s^{-1}$ was determined for the third-order rate constant for the Zn^{2+}-assisted deuteroxide ion-catalyzed isomerization of D,L-GA with hydride transfer in D_2O at 25 °C. This third-order rate constant may be used to calculate observed second-order rate constants for the Zn^{2+}-catalyzed isomerization at any concentration of DO^-.

It is significant that Zn^{2+} is only marginally more effective than acetic acid at providing stabilization of the transition state for deprotonation of acetone because, if there is no large advantage to electrophilic catalysis of deprotonation of α-carbonyl compounds by metal ions over Brønsted acids, then there can be no great advantage to the the evolution of metalloenzymes to catalyze these proton transfer reactions. This provides a simple rationalization for why, in general, there is no metal ion requirement for the enzyme-catalyzed deprotonation of -carbonyl compounds.

A rate acceleration of *ca* 10^7-fold for the xylose phosphate isomerase-catalyzed isomerization of xylose at pD = 8.0 is obtained from a comparison of the observed second-order rate constants for the Zn^{2+}-assisted isomerization of D,L-GA at pD 8.0, $(k_{Zn})_{obsd} = (1100\ l^2\ mol^{-2}\ s^{-1})(10^{-6}\ mol\ l^{-1}) = 1.1 \times 10^{-3}\ l\ mol^{-1}\ s^{-1}$, and $k_{cat}/K_m = 10^4\ l\ mol^{-1}\ s^{-1}$ for the metalloenzyme-catalyzed reaction.[20] Similar comparisons at pD < 8.0 and at pD > 8.0 would have resulted in larger and smaller calculated enzymatic rate accelerations, respectively, since $(k_{Zn})_{obsd}$ for the solution reaction is first order in the concentration of deuteroxide ion. This modest rate acceleration is probably the result of a combination of factors which include (a) direct stabilization of the Michaelis complex by interactions between the enzyme and bound substrate and (b) the creation of an open, or weakened, coordination site at the metal ion upon its formation of a binary complex with the protein catalyst, which is then occupied by a hydroxyl group of the bound sugar substrate. This would then allow for the full expression of this metal–hydroxyl interaction at the transition state for enzyme-catalyzed isomerization, whereas for the solution reaction, the stabilizing interactions between the metal ion and the substrate are offset by the energetic price 'paid' to free a coordination site at the metal ion.

In fact, xylose isomerase uses two metal ions in the catalysis of aldose–ketose isomerization.[11] The recruitment of a pair of metal ions to catalyze isomerization with hydride transfer suggests that the overriding requirement for catalysis of this reaction is stabilization of an enzyme-bound α-carbonyl hydroxyl oxyanion. This idea is supported by the results of an elegant analysis of the x-ray crystal structures of Mg^{2+}-activated xylose isomerase and complexes of this enzyme with the substrate glucose,[11] the substrate analog 3-*O*-methyl-D-glucose[11] and the tight-binding inhibitor D-threonohydramic acid.[12] What is not clear is whether generation of the enzyme-bound oxyanion alone is sufficient to account for the catalytic rate acceleration of xylose isomerase, or whether the enzyme also acts to accelerate the intramolecular transfer of hydride at this bound oxyanion.

CONCLUSIONS

Our experimental results show that the various pathways for isomerization with proton and hydride transfer proceed via transition states of similar energies, so that there is no strong imperative for enzymatic catalysis by any particular reaction mechanism. We conclude that the diversity in reaction mechanisms observed for enzyme-catalyzed aldose–ketose isomerization and for abstraction of α-carbonyl hydrogens may occur simply because there is no strong natural selection for enzymes which follow any one of these reaction mechanisms.

Acknowledgment

This work was supported by grants GM 47307 and GM 39754 from the National Institutes of Health.

REFERENCES

1. W. P. Jencks. *Catalysis in Chemistry and Enzymology*, p. 5. Dover, New York (1967)
2. S. V. Rieder and I. A. Rose. *J. Biol. Chem.* **234,** 1007–1010 (1959).
3. J. K. Knowles and W. J. Albery. *Acc. Chem. Res.* **10,** 105–111 (1977).
4. J. P. Richard. *J. Am. Chem. Soc.* **106,** 4926–4936 (1984).
5. J. R. Keeffe and A. J. Kresge. in *The Chemistry of Enols*, edited by Z. Rappoport, pp. 399–480. Wiley, Chichester (1990).
6. C. A. Collyer and D. M. Blow. *Proc. Natl. Acad. Sci., USA* **87,** 1362–1366 (1990).
7. C. A. Collyer, K. Henrick and D. M. Blow. *J. Mol. Biol.* **212,** 211–235 (1990).
8. C. A. Collyer, J. D. Goldberg, H. Viehmann, D. M. Blow, N. G. Ramsden, G. W. J. Fleet, F. J. Montgomery and P. Grice. *Biochemistry* **31,** 12211–12218 (1992).
9. G. K. Farber, G. A. Petsko and D. Ringe. *Protein Eng.* **1,** 469–466 (1987).
10. K. N. Allen, A. Lavie, G. Farber, A. Glasfeld, G. A. Petsko and D. Ringe. *Biochemistry* **33,** 1481–1487 (1994).
11. K. N. Allen, A. Lavie, A. Glasfeld, T. N. Tanada, D. P. Gerrity, S. C. Carlson, G. K. Farber, G. A. Petsko and D. Ringe. *Biochemistry* **33,** 1488–1494 (1994).
12. K. N. Allen, A. Lavie, G. Petsko and D. Ringe. *Biochemistry* **34,** 3742–3749 (1995).
13. A. Lavie, K. N. Allen, G. A. Petsko and D. Ringe. *Biochemistry* **33,** 5469–5480 (1994).
14. (a) C. I. F. Watt. *Adv. Phys. Org. Chem.* **24,** 57–112 (1988); (b) W. B. Gleason and R. Barker. *Can. J. Chem.* **49,** 1433–1440 (1971).
15. H. S. Isbell. *Adv. Chem. Ser.* **117,** 70–87 (1973).
16. C. Kooyman, K. Vellenga and H. G. de Wilt. *Carbohydr. Res.* **54,** 33–44 (1977).
17. R. W. Nagorski and J. P. Richard. *J. Am. Chem. Soc.* **118,** 7432–7433 (1996).
18. T. L. Amyes and J. P. Richard. *J. Am. Chem. Soc.* **114,** 10297–10302 (1992).
19. T. L. Amyes and J. P. Richard. *J. Am. Chem. Soc.* **118,** 3129–3141 (1996).
20. G. K. Farber, A. Glasfeld, G. Tiraby, D. Ringe and G. A. Petsko. *Biochemistry* **28,** 7289–7297 (1989).
21. R. W. Gracy and R. E. Noltmann. *J. Biol. Chem.* **243,** 4109–4116 (1968).
22. R. W. Gracy and R. E. Noltmann. *J. Biol. Chem.* **243,** 5410–5419 (1968).
23. A. F. Hegarty and W. P. Jencks. *J. Am. Chem. Soc.* **97,** 7188–7189 (1975).

