BIOORGANIC CHEMISTRY 8, 1–8 (1979)

Mechanism of Action of Chalcone Isomerase

MICHAEL J. BOLAND AND EDMON WONG

Department of Scientific and Industrial Research, Palmerston North, New Zealand

Received October 3, 1977

The pH profile of the rate of isomerization of 4,2′,4′-trihydroxychalcone catalyzed by chalcone isomerase shows dependence on the basic form of a group with a pK of 7.25. The same pH dependence is seen for the reverse reaction. Enzyme activity is lost in the presence of diethylpyrocarbonate at pH 6.0. In the presence of 20% formamide in imidazole buffers, the pK for the forward reaction is modified by a second pK of 7.1. This behavior represents a perturbed pK of a neutral acid group and is attributable to the 2′ hydroxyl of the chalcone substrate. These results suggest a mechanism of enzyme action involving nucleophilic addition of an imidazole group in the active site to the double bond followed by nucleophilic attack by the 2′ phenolate group, resulting in ring closure with inversion of configuration at C-2.

INTRODUCTION

The enzyme chalcone isomerase (EC 5.5.1.6) has been purified from soya bean, and its steady-state kinetic behavior has been studied (*1*). The catalytic mechanism has not been established, although the stereochemistry of the enzyme-catalyzed isomerization of isoliquiritigenin [4,2′,4′-trihydroxychalcone, (I)] has been determined and a possible mechanism has been suggested (*2*).

Useful information about catalytic mechanism of enzymes can often be obtained from the pH dependence of the maximum velocity, V_{max}. The pH profile of V_{max} for the enzyme-catalyzed isomerization of isoliquiritigenin shows a sharp decrease at low pH, of the shape expected for dependence on the basic form of an ionizing group with p$K \simeq$ 6.9 in phosphate buffer (*1*). We have suggested (*1*) that the group concerned might be an imidazole side chain of a histidine residue, and some independent evidence from inactivation studies with iodoacetamide (*1*) indicates that a histidine residue is essential for the reaction. Alternatively, the observed pH dependence could be due to ionization of a phenolic hydroxyl group in the substrate (which is a moderately acidic phenol), particularly the 2′ hydroxyl group, which is involved in the isomerization reaction.

It is possible to distinguish between ionizations of the neutral acid type ($AH \rightarrow A^- + H^+$), such as that of a phenolic group, and those of cation acid type ($BH^+ \rightarrow B + H^+$), as exhibited by an imidazole group, by the effect on pK of added organic solvents (*3*). The dependence of reaction rate of ionizing groups of the enzyme, but without the effect of the ionizing 2′ phenolic hydroxyl group in the chalcone substrate, can be determined by investigation of the pH dependence of the back reaction, i.e., the enzyme-catalyzed conversion of liquiritigenin (II) to isoliquiritigenin (I). A pH profile showing a pK around 7 for this reaction will implicate a catalytic imidazole group of the enzyme,

0045-2068/79/0081-1-08$02.00/0

and the direction of the pH dependence will indicate whether this group is acting as an acid–base catalyst or as a nucleophile.

EXPERIMENTAL

Materials. Chalcone isomerase was prepared from soya bean as previously described (*1*). Formamide was "Unilab" grade (Ajax Chemicals Ltd.), and diethylpyrocarbonate was obtained from Sigma Chemical Co. All other reagents were "Analar" or equivalent grade.

Methods. Imidazole–HCl buffers (0.1 *M*) were made up in the range pH 6.0–8.5. For the forward reaction, mixtures contained 0.075 *M* buffer, 30 μM isoliquiritigenin added in 10μl of 2-ethoxyethanol, and 10 μl of enzyme solution, or 50 μl of enzyme solution and 200 μl of formamide, in a total volume of 1 ml. The wavelength of maximum absorbance was determined before the addition of enzyme (which was added last), and the rate of disappearance of absorbance at this wavelength was recorded. Over the pH range studied the wavelength of maximum absorbance was found to change, but the initial absorbance at λ_{max} remained constant within experimental error ($\pm$5%).

The pH dependence of the wavelength of maximum absorbance above pH 8 was determined using solutions of isoliquiritigenin in 10 m*M* Tris–HCl buffer : 1% 2-ethoxyethanol, and λ_{max} at pH 5.2 was obtained from a solution of isoliquiritigenin in water : 1% 2-ethoxyethanol.

The pH dependence of the reverse reaction was investigated using 0.1 *M* imidazole buffers and 0.08 *M* imidazole buffers containing 20% formamide, with reaction mixtures containing 0.5 m*M* (–)-liquiritigenin (50 μl of 10 m*M* solution in 2-ethoxyethanol) and 40 μl of enzyme solution in a total volume of 1 ml. Appearance of chalcone was observed at the wavelength of maximum absorption for that pH. These experiments were carried out at 25°C.

The effect of diethylpyrocarbonate was shown by diluting 100 μl of enzyme with 0.2 *M* phosphate buffer (1 : 1), pH 6.0, and adding 5 μl of diethylpyrocarbonate which had been diluted with water to the required concentration immediately prior to addition. The lowest concentration of diethylpyrocarbonate was nominally 0.25 m*M* in the enzyme solution. The enzyme was assayed at pH 7.5 as described above and before and 60 sec after addition of diethylpyrocarbonate.

Determination of pK values. The pH dependence of the maximum velocity, *V*, of an enzyme-catalyzed reaction dependent on the basic form of an ionizing group, is given by (*4*):

$$\log V = \log V_0 - \log(1 + [H^+]/K_h),$$

where V_0 is the maximum velocity when the ionizing group is fully in the basic form, and K_h is the dissociation constant for the group concerned, i.e. $pK = -\log K_h$.

The shape of this function was calculated and plotted for a value $V_0 = 1$ from $pK - 2$ to $pK + 2$. The curve was fitted to experimental points by superimposition to give a best fit. Determinations of p*K* using this method have an estimated accuracy of $\pm$0.1.

RESULTS

The pH profile of V_{max} for the chalcone isomerase-catalyzed isomerization of isoliquiritigenin is shown in Fig. 1. The reaction in aqueous imidazole buffer shows dependence on the basic form of a group with pK 7.25. The pK observed here is higher than that (6.9) for the same reaction in phosphate buffer (*1*). The reaction in 20% formamide shows a dependence on pH much sharper than is expected for a single ionizing group (*4*). If a second ionizing group is involved, the effects will be

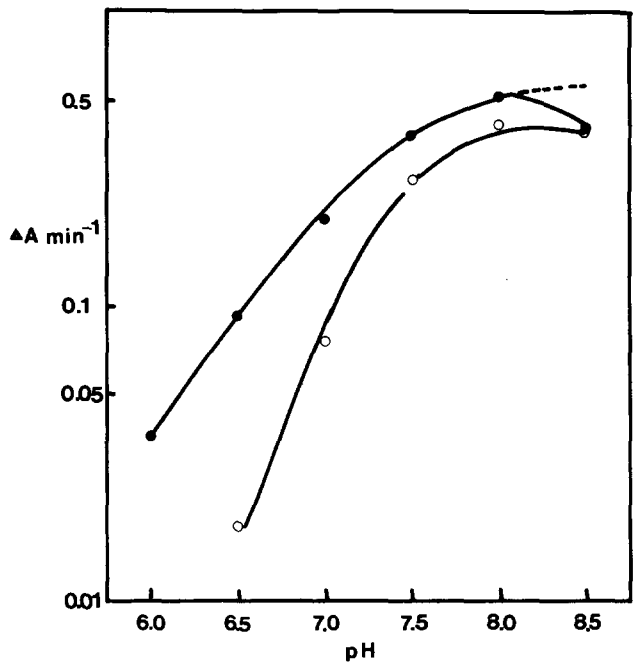


FIG. 1. Log-scale pH dependence of initial rate of reaction of chalcone isomerase with isoliquiritigenin in 0.075 M imidazole–HCl buffer, 10 μl of enzyme solution (●), and in the same buffer containing 20% formamide, 50 μl of enzyme solution (○), 25°C. Initial substrate concentration was 30 μM (7 × K_m in aqueous buffer). Since reaction rates in formamide buffers were as linear as those obtained in aqueous buffers, it can be assumed that K_m values were safely exceeded. The line drawn for the pH dependence of the reaction in aqueous buffer fits the equation: $\log V = \log 0.6 - \log(1 + [H^+]/\{5.6 \times 10^{-8} M\})$, up to pH 8.0. The dashed line is an extrapolation to pH 8.5 of this line. The observed pH profile above 8.0 shows the influence of another ionizing group. The equation for the fitted line up to pH 8.0 gives a pK of 7.25.

multiplicative; since only one ionization is seen in the absence of formamide, the superimposed effect of the second group can be separated by plotting the pH dependence of $V_{formamide}/V_{aqueous}$. This interpretation is shown in Fig. 2 and fits a calculated line for a group with pK 7.1. The pH profile of the reaction of chalcone isomerase with liquiritigenin is shown in Fig. 3. This representation shows dependence on the basic form of a group with pK 7.4 in both aqueous and formamide-containing imidazole buffers.

The pH dependence of wavelength of maximum absorption by isoliquiritigenin is shown in Fig. 4 for aqueous imidazole buffers and also for 20% formamide imidazole buffers. The effect of adding formamide is to increase the apparent pK values of the hydroxyl groups as predicted. The amplitude of the shift of individual pK values is difficult to estimate, but a shift of at least 0.3 is indicated below pH 7.5.

At pH 8.0 addition of formamide to the reaction mixture causes a decrease in rate of reaction proportional to the amount of formamide added, at least up to 20% formamide. This behavior is shown in Fig. 5.

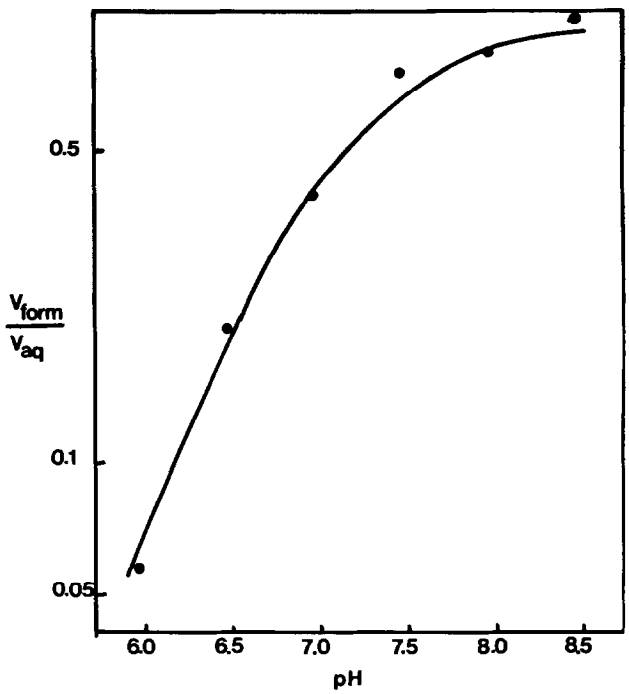


FIG. 2. Log-scale pH dependence of (reaction rate in 20% formamide/reaction rate in aqueous solution) for reaction of chalcone isomerase with isoliquiritigenin, 0.075 M imidazole buffers, 25°C. The line drawn is described by the equation: $\log(V_{\text{formamide}}/V_{\text{aqueous}}) = \log 0.92 - \log(1 + [H^+]/\{8 \times 10^{-8}\ M\})$, corresponding to a p$K$ of 7.1.

Diethylpyrocarbonate at a nominal concentration of 0.25 mM in the enzyme solution caused loss of measurable enzyme activity (>99% inactivation).

DISCUSSION

The reaction of chalcone isomerase shows dependence on an imidazole side chain of the enzyme, and the 2′ hydroxyl group of the chalcone substrate is probably required in the anionic form for the reaction to proceed.

In the imidazole buffer used, cation acid groups should show no perturbation of pK in the presence of formamide, but pK values of neutral acids should be increased (*3*). From the data in Figs. 1 and 2 it can be seen that the pH dependence of reaction in formamide buffer shows the effect of the ionizing group pK 7.25 (seen in aqueous buffer) modified by the effect of another group, the apparent pK of which must be below 6.5 in aqueous buffer but is perturbed to 7.1 in 20% formamide. Such a group must be of the neutral acid type. This effect is unlikely to be associated with a catalytic group on the enzyme since the only groups of this type in enzyme molecules are carboxyl groups of glutamic

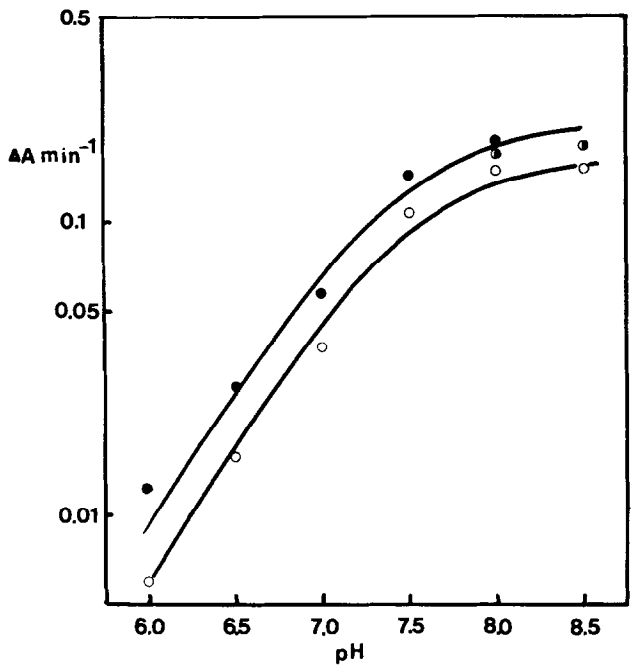


FIG. 3. Log-scale pH dependence of initial rate of reaction of chalcone isomerase with liquiritigenin in 0.1 M imidazole (●), Tris (◑), and 0.08 M imidazole–20% formamide buffers (○), 25°C. Rates in buffers not containing formamide have been multiplied by 0.2 for convenience in presentation. Both lines fit the equation: $\log V = \log V_0 - \log(1 + [H^+]/\{4 \times 10^{-8}\ M\})$, with V_0 values of 0.21 A min^{-1} for aqueous buffers and 0.18 A min^{-1} for formamide buffers. This corresponds to a pK of 7.4.

and aspartic acid residues, which typically have pK values of 3.0–4.5, and the phenolic hydroxyl group of tyrosine, which has a pK of about 10. A large perturbation would be required to arrive at the observed pK value of 7.1. On the other hand the 2′ hydroxyl group of the chalcone substrate can well account for this effect. Ionization of this group at pH greater than about 6.5 is apparently necessary for reaction, and such an ionization is suggested by the data presented in Fig. 4.

Because the pK at 7.25 is not shifted upward in the presence of formamide, it must be due to a group of the cation acid type. This behavior is consistent with the expected involvement of an imidazole group at the active site, functioning in its unprotonated

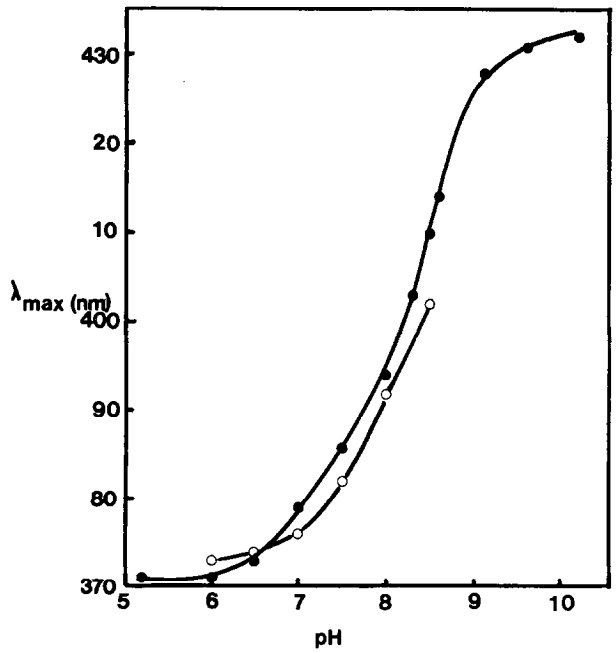


FIG. 4. The pH dependence of the wavelength of maximum absorbance of isoliquiritigenin (4,2′,4′-trihydroxychalcone) in 0.075 *M* imidazole–HCl buffer, 10 m*M* Tris–HCl buffer, or water (●), and in 0.075 *M* imidazole–HCl buffer containing 20% (v/v) formamide (○).

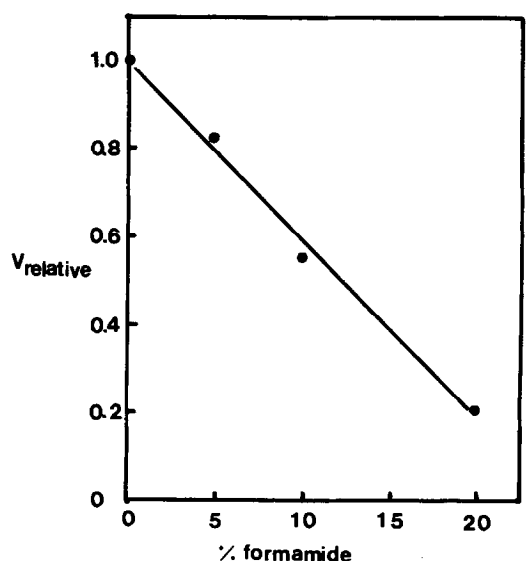


FIG. 5. Effect of increasing formamide concentration on initial rate of reaction of chalcone isomerase with isoliquiritigenin, pH 8.0, 0.075 *M* imidazole–HCl buffer at 25°C.

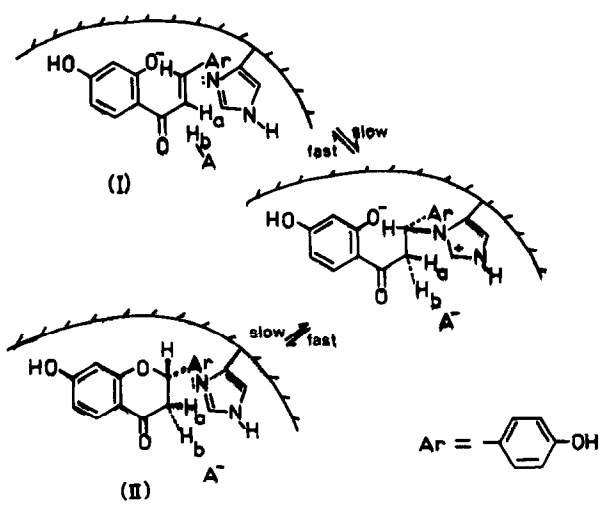
form in the rate-limiting step. The total loss of activity caused by diethylpyrocarbonate provides further evidence for an essential histidine residue (*5*).

Alternative explanations of the effect of formamide on the pH dependence of the reaction, such as change in rate-limiting step or alteration of the conformation of the enzyme, are considered unlikely, because they would be expected to show a break point in the straight line of Fig. 5. The linear decrease in reaction rate with increasing formamide concentration probably reflects changes in the solvation of reaction intermediates (*3*).

In studies on the enzyme-catalyzed isomerization of liquiritigenin (back reaction), the flavanone concentration was more than 25 × K_m at pH 7.6 (*1*). The pH-dependent rate decrease must therefore be due to an effect on V_{max}. It is probable that the ionizing group responsible is the same imidazole group seen in the forward reaction involved in the rate-limiting step for the reverse reaction. In the presence of formamide, however, the pH dependence is not modified in the same way as that of the forward reaction (Fig. 3; cf. Fig. 1). It is therefore evident that the group responsible for the additional pH dependence in the forward reaction (pK 7.1) either does not take part in the back reaction or takes part in a non-rate-limiting step. This would be true of the 2′ hydroxyl of isoliquiritigenin, as this group is not present as such in the flavanone and hence could not be limiting the rate of reaction.

The role of the imidazole group cannot be that of an acid–base catalyst, as this would cause opposite pH dependences of the forward and reverse reactions. This group must therefore act as a nucleophile, which would require the presence of the unprotonated form for both forward and reverse reactions.

The stereochemical course of the enzyme-catalyzed isomerization of isoliquiritigenin has previously been studied by Hahlbrock *et al.* (*2*). The absolute configuration of the (−)-flavanone product formed is shown in (II), representing a net *cis* addition to the double bond. In the light of the additional findings in the present work, we propose a mechanism of chalcone isomerase action which is consistent with this stereochemical data.



In the scheme proposed the role of the imidazole is that of a nucleophile, initiating conjugated addition and displacement in the forward and backward reactions, respectively. The requirement for an ionized 2′ hydroxyl group is apparent. An acidic group, A-H_b, is also involved. The nature of this group is unknown; it may be an acidic side chain of the enzyme or simply a water molecule.

The essential feature of this scheme is that isomerization is the result of enzyme-mediated *trans* addition to a double bond, followed by internal nucleophilic displacement by the *ortho* phenolate ion with resultant cyclization and inversion of configuration at C-2.

REFERENCES

1. M. J. BOLAND AND E. WONG, *Eur. J. Biochem.* **50**, 383–9 (1975).
2. K. HAHLBROCK, H. ZILG, AND H. GRISEBACH, *Eur. J. Biochem.* **15**, 13–18 (1970).
3. D. FINDLAY, A. P. MATHIAS, AND B. RABIN, *Biochem. J.* **85**, 139–44 (1962).
4. W. W. CLELAND, "The Enzymes" (P. D. Boyer, Ed.), 3rd ed., Vol. 2, pp. 52–54. Academic Press, New York, 1970.
5. A. MUHLRAD, G. HEGYI, AND M. HORANYI, *Biochim. Biophys. Acta* **181**, 184 (1969).