Hydrogen–Deuterium Exchange of the C-2 Protons of Histidine and Histidine Peptides and Proteins

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Kinetic equations previously developed for the H–D exchange of the C-2 protons of an isolated imidazole group or for an imidazole group with an adjacent charged group of p*K* 6—12, have been analysed in detail. By measurement of the pseudo-first-order rate constant *k* for exchange in D_2O as a function of pD, it is possible to obtain the second-order rate constant k_2 and the p*K* of the histidine residue. A nearby charged group with a p*K* two or more units greater than that of the histidine residue, causes a double S shaped curve when *k* is plotted against pD, from which the p*K* of the nearby charged group may be determined. The size of the second step in the curve is related to the closeness of the charged group to the histidine residue. Kinetic equations have been developed for more complex cases involving (a) two adjacent imidazole rings and one adjacent charged group of p*K* 6—12 and (b) an imidazole group with two adjacent charged groups of p*K* 6—12. The equations involve numerous second-order rate constants and equilibrium constants which cannot be evaluated explicity using experimental data obtained for L-histidyl-L-histidine, L-histidyl-L-tyrosine, and L-histidyl-L-lysine. For histidine residues in proteins it is possible to determine their p*K* and k_2 values for H–D exchange. From the magnitude of these values one can infer the type of environment of the histidine residue. Thus the data for certain histidine residues in lysozyme, ribonuclease, myoglobin, and leghaemoglobin have been rationalised in terms of their known environments obtained from *X*-ray structural studies.

A DETAILED knowledge of the mechanism of kinetics of the hydrogen–deuterium (H–D) exchange and the closely related hydrogen–tritium (H–T) exchange of the C-2 protons of histidine and its compounds in peptides and proteins is important for studies that involve histidine residues in proteins. The studies on imidazole and substituted imidazoles [1-4] formed the basis for the development of a satisfactory mechanism [4] which has been subsequently applied in studies of proteins.

The exchange method has been used to determine p*K* values of histidine residues in peptides and in proteins [5-9] and of groups that are close to histidine residues and titrate in the alkaline pH range.[6,7] A simple example of the latter is L-histidine, which, when the first-order rate constant is plotted against pH, shows an S shaped curve centred around the p*K* of the imidazole ring and another S shaped curve centred around the p*K* of the α-amino-group.[7] The first-order rate constant for H–D exchange is determined by measuring the reduction in the area of the C-2 proton resonance by ^1H n.m.r. spectroscopy.[4,6,7,9,10] First-order rate constants for H–T exchange are determined by measurement of the amount of tritium label incorporated into the histidine residue.[5,8,10] The numerical value of the rate constants have been used to give information about (a) the likely proximity to the imidazole ring of charged, titratable groups [7] and (b) the accessibility of the histidine residue to the aqueous medium.[10-17]

Another very useful application of H–D exchange has been in the assignment of histidine H-2 resonances to specific histidine residues in proteins. Since the first application of this procedure to ribonuclease-A,[18] it has been used with various modifications on the same protein [9,11,12,19,20] because of doubt about the correct assignment. Numerous applications have also been made, with some modifications of procedures, to other protein systems.[13,15-17,21-23] It has proved to be useful as a means for distinguishing between resonances from histidine C-2 protons, which normally undergo H–D exchange, and histidine C-4 protons,[16,17] which are incapable of exchange except at temperatures >100 °C.[4]

Because of the considerable importance of the H–D exchange of C-2 protons of histidine residues in proteins, it is useful to consider in detail the kinetics of the exchange reaction in simple model compounds. A short account of some of this work has already been given.[7] It is the purpose of this paper to develop a systematic framework for the kinetic analysis of different types of histidine residues and to interpret results obtained from H–D exchange experiments.

EXPERIMENTAL

L-Histidine (Nutritional Biochemicals Corp.), L-histidyl-L-tyrosine, and L-histidyl-L-lysine (Fox Chemicals) and L-histidyl-L-histidine acetate (Cyclo) were checked for purity by ^1H n.m.r. spectroscopy. The compounds were lyophilised once from D_2O (>99.7%, Australian Atomic Energy Commission) to replace exchangeable protons with deuterons. The C-2 proton of L-histidine was deuteriated by reacting L-histidine in D_2O at pH 12 and 37 °C for seven days followed by lyophilisation and repetition of the treatment to ensure complete deuteriation.

Solution (2—5%) of L-histidine and histidine peptides in D_2O were prepared and the pH adjusted with NaOD or DCl (Stöhler) to the required value at the particular temperature using a small water jacketted vessel. The Beckman research pH meter was standardised using standard buffers at the temperature of the experiment.

Recorded values of pD are calculated by the equation pD = pH meter reading + 0.4 which applies in the range pD 2—12.[24,25] The solution was transferred to a tightly capped 5 mm n.m.r. tube and the ^1H n.m.r. spectrum determined either at 60 MHz with a Perkin-Elmer R-10 spectrometer or at 100 MHz using a JEOL MH-100 n.m.r. spectrometer. The solution was held at the desired temperature in a thermostat bath and the n.m.r. spectrum measured on successive days. The area (obtained by cutting out the peak and weighing it) and height of the

C-2 proton resonance decreased during the experiment and these values were normalised by comparison with the area and height of the C-4 resonance which did not undergo exchange.[6,7,9]

The height and area data was fitted by a least squares treatment to the straight line of best fit of the $\log_{10}$ (normalised height or area of resonance) plotted against time. The pseudo-first-order rate constant k was determined from the slope of the straight line. The rate constants obtained by the height and area methods agreed within 5% and the average error in the rate constants determined from the least squares treatment is 5%.

RESULTS AND DISCUSSION

(1) H–D *Exchange at an Isolated Imidazole Group.*— The mechanism for H–D exchange of the C-2 proton of an imidazole group is shown in Figure 1.[4] It involves

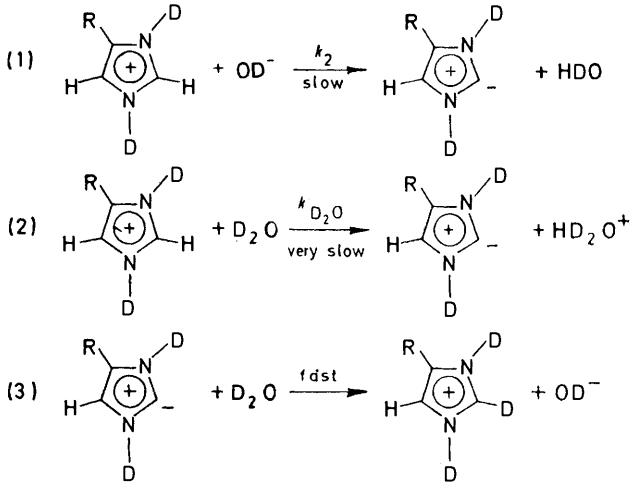

FIGURE 1 The ylide mechanism for H–D exchange at the C-2 position [4]

the attack of either OD^- or D_2O on the positively charged form of the ring to abstract a proton from the C-2 position and produce an ylide in a slow, rate-determining step. The ylide then reacts rapidly with D_2O with substitution of deuterium at the 2-position. The slow step appears to involve general base catalysis and this aspect is currently being studied. The kinetic equation developed for the simple case of an imidazole ring that is not interacting with any neighbouring group, *e.g.* a histidine residue interacting with solvent on the surface of a protein, is (1) where k is a pseudo-first-order rate constant that is determined experimentally for the H–D exchange by n.m.r. or for the H–T exchange by radiochemical techniques. In any particular exchange experiment k is a constant at constant pD, because of the constancy of $[OD^-]$ and $[His^+]$. $[His]_t$ and $[His^+]$ are the total concentration and the concentration of the positively charged form of the isolated histidine residue respectively; k_2 and k_{D_2O} are second-order rate constants, defined in Figure 1. Substitution of K, the apparent dissociation constant of the imidazole ring of the histidine in D_2O, and K_{D_2O}, the ionic product of D_2O and rearrangement gives equation (2).

$$\text{rate} = k[His]_t = k_2[His^+][OD^-] + k_{D_2O}[His^+][D_2O] \quad (1)$$

$$k = (k_2K_{D_2O} + k_{D_2O}[D_2O][D^+])/(K + [D^+]) \quad (2)$$

(i) *Rate of* D_2O *mediated reaction* (2) (*Figure* 1). In order to allow separation of the two slow steps shown in Figure 1, we distinguish two extreme cases. At high pD, $[D^+] \ll K$, hence in the limit as $[D^+] \longrightarrow 0$, $k = k_2K_{D_2O}/K$. At low pD, $[D^+] \gg K$, and $k = k_{D_2O}[D_2O]$. The extent of the reaction (2) in Figure 1 can be determined from the first-order rate constant at low pD which should be independent of pD.

Experiments [6] carried out in D_2O with imidazole, L-histidine, *N*-acetyl-L-histidine, L-histidylglycine, L-alanyl-L-histidine, and β-alanyl-L-histidine at 37 °C and pD values of 1.2—5.2 showed a negligible amount of H–D exchange after 1.5—15 days. Indeed the maximum values of the rates of reaction at pD <5 and 37° were <2% of the values obtained at high pD and in the case of the 15 day deuteriation of L-histidine was <0.19% of the value at high pD (*i.e.* at pD 1.2, $k < 1.5 \times 10^{-8}$ s^{-1}).[6] In H–D exchange studies at temperatures <40 °C, it is therefore reasonable to neglect reaction (2) of Figure 1 and hence eliminate the second term on the right side of equation (2) which simplifies to (3).

$$k = k_2K_{D_2O}/(K + [D^+]) \quad (3)$$

The simplified equation is normally used,[5-9] but there is one case [10] in which the rate constants for H–T exchange are extremely small for two of the three histidine residues in bovine trypsin and one rate constant varies little over the whole range of pH values. The very slow rate of reaction is due to the inaccessibility of these histidine residues to solvent, an effect also observed with histidine 48 of ribonuclease-A.[9,12] Krieger *et al.*[10] used least squares to fit their experimental H–T exchange data to equation (2) from which they extracted values for K and the second-order rate constants k_2 and k_{D_2O}. However, our application of this treatment to the data of Matsuo *et al.*[5] has produced values of k_{D_2O} for *N*-acetyl-L-histidine and lysozyme, which are larger than the negligibly small values found experimentally at low pH by Matsuo *et al.*[5] and ourselves.

It is necessary to use the full treatment of equation (2) at temperatures in excess of 60 °C, because the rate constant for the deuteriation of imidazole at pD 0.3—2.9 is about one-tenth of that at high pD (see Table 1 of ref. 4). This indicates that reaction (2) of Figure 1, has a higher energy of activation than does reaction (1).

(ii) *Analysis of* OD^- *mediated reaction* (1) *of Figure* 1. At temperatures below 40°, equation (2) simplifies to (3) as described above and this gives rise to a sigmoidal curve as shown in Figure 2. The technique has been used to determine the pK of histidine residues in peptides and proteins.[5-9] As stated above at high pD, $K \gg [D^+]$ and equation (3) reduces to (4) where $k_{max.}$ is the constant value of k in the plateau region of the curve. The value of $k_{max.}$ is clearly proportional to k_2 and inversely proportional to K. It decreases ten-fold as the pK de-

creases from 7 to 6 and from 6 to 5 (see Figure 2). Thus very low values of k may result from values of pK $\leqslant 5$ and this may prevent the experimental determination of

$$k_{max.} = k_2 K_{D_2O}/K \qquad (4)$$

pK by this method, because of the problem of obtaining sufficiently accurate values of k. Furthermore, one must be careful not to assume that a low value of k is necessarily due to inaccessibility of a histidine residue in a protein, since it may simply be due to a low value of pK. Thus, the decreased rate constant for the exchange of histidine-107 as compared with histidine-68 in bovine α-lactalbumin may be explained in terms of a decrease in pK from 6.49 to 5.78, rather than as considered elsewhere [14] in terms of decreased accessibility of histidine

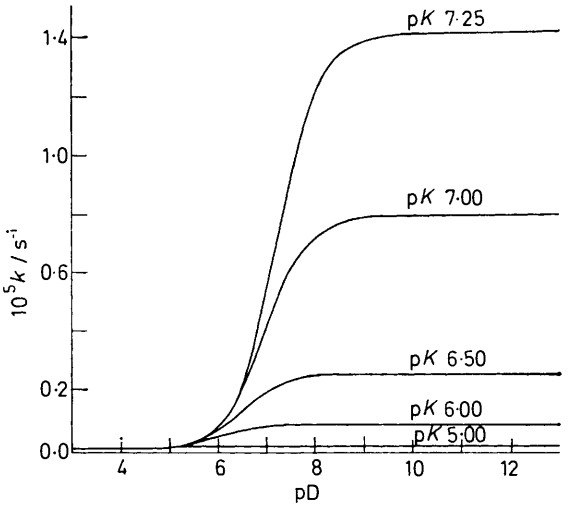

FIGURE 2 Computed curves of k *versus* pD using equation (3) to show the effect of variation of pK at a constant value of k_2 of 2.40 × 10^{-4} dm^3 mol^{-1} s^{-1}

107. On the other hand, histidine-107 and -32 of bovine α-lactalbumin both have similar values of k but the latter has the higher pK of 6.51, hence it may be somewhat inaccessible to D_2O.

At any value of pD the value of k is proportional to k_2, as shown by equation (3). The rate of attack of OD^- [see reaction (1) in Figure 1] should be increased by the presence of a nearby positively charged group and decreased by a nearby negatively charged group due to electrostatic effects. This is confirmed experimentally by the second-order rate constants for the deuteriation of histamine with the amino-group charged, for histamine with the amino-group uncharged and for L-histidine with the amino-group uncharged and the carboxy group charged, which are 400, 70, and 60 dm^3 mol^{-1} s^{-1}, respectively.[7] The value of k_2 will also be decreased by steric factors which cause inaccessibility of the imidazole ring to attack by OD^-. In proteins, inaccessibility is normally due to the presence of non-polar groups in the vicinity of the histidine side chain such that it occurs in a hydrophobic pocket.

These various environmental effects change both k_2 and pK and thereby k [by equation (3)] as summarised in Table 1. Thus, a non-polar environment decreases k_2 and may lead to a very slow rate of exchange.[10,16,17] The presence of a nearby positive change increases k_2 (see above) which tends to increase k, but also decreases pK by such a large amount, that the net effect is a decrease of k. For example, the maximum value of k ($k_{max.}$) for the H–D exchange of histidine (amino-group uncharged pK 7.6), is 8.0 × 10^{-6} s^{-1} whereas that of histidine (amino-group charged pK 6.6), is 3.4 × 10^{-6} s^{-1}.[7] On the other hand, a nearby negative charge decreases k_2 (see above) which thereby decreases k, but also slightly increases pK with a smaller increase of k; the net effect is a decrease of k. An example of this is obtained by comparing rate constants for imidazole and imidazolylacetic acid.[7]

The modifications of the environment of the histidine residues discussed in Table 1 all have the effect of decreasing k as compared with the unmodified case, imidazole for which $k_{max.}$ = 1.41 × 10^{-5} s^{-1} at 37 °C.[7] Imidazole would therefore appear to be a reasonable

TABLE 1

Effect of the environment of the histidine residue in a protein on its pK and rate of H–D exchange

	Environment of histidine residue [a]		
Effect of environment on	Non-polar environment causing inaccessibility	Positively charged group nearby	Negatively charged group nearby
pK	No change [b]	Decrease	Increase
k_2 dm^3 mol^{-1} s^{-1}	Decrease	Increase	Decrease
Net effect on k/s^{-1}	Decrease	Decrease	Decrease

[a] Hydrogen bonding the histidine residue as in histidine-48 of ribonuclease [26] causes little change in pK (pK' 6.0) [8] but a great reduction in k[9] due to inaccessibility. The hydrogen bonding of the charge relay system of the serine proteinases causes further complex effects.[10,15] [b] To a first approximation the non-polar environment does not alter the pK of the histidine ring.[27]

model for an unmodified histidine residue in a protein and gives a much greater rate of exchange than does *N*-acetyl-L-histidine methylamide (k = 3.3 × 10^{-6} s^{-1} at 35 °C and pD 9.5) which was used as a model by Takesada *et al.*[14]

By measurement of pK and k as a function of pD for the H–D exchange of the C-2 proton of a histidine residue, k_2 can be calculated using equations (3) and/or (4). By use of Table 1, it is possible to distinguish between the three types of environment listed in Table 1, because each produces a different effect on pK and k_2. Using the data of Matsuo *et al.*[5] on lysozyme it is clear that pK is low (5.2), $k_{max.}$ = 5.5 × 10^{-6} s^{-1} is less than that for imidazole and this gives rise to a high value for k_2. Reference to Table 1, shows that this behaviour is consistent with the presence nearby of a positively charged group. This is indeed the case since the NH(2) of arginine-14 is less than 0.4 nm away from the C-2 atom [CE(1)] of histidine-15.[28] The same general result is obtained with histidines-12 and -119 of ribonuclease-A which both exhibit low values of pK,[29] low values of $k_{max.}$[9] and high values of k_2 calculated by equation (4).

This is in agreement with the crystallographic studies [26,30] which show that both these histidine residues in the active site are in the vicinity of lysines-41, -7, and -66. The presence of aspartic acid-121 *ca.* 0.5 nm from histidine-119 would cause an increase of its pK above that of histidine-12 (as experimentally observed using the new assignment [12,19,20]) and may be the cause of its larger value of $k_{max.}$.[9] The observation of the very slow rate of exchange for the C-2 proton of the distal histidine residue in haem proteins (carbon monoxide, Fe^{II}, sperm whale myoglobin, and leghaemoglobin) is due to its low pK [16,17] and its inaccessibility to solvent, due to the non-polar environment of the haem pocket in myoglobin [31] and leghaemoglobin.[32]

(2) H–D *Exchange at an Imidazole Group Adjacent to One Charged Group with* pK 6—12.—In this case the kinetics are complicated because of the different rate constants for reaction with the two reactive forms which may be designated $N^+D_3Im^+D(His^{2+})$ and $ND_2Im^+D(His^+)$.* At temperatures $\leqslant$40 °C the rate of exchange with D_2O can be neglected as compared with OD^- (see Figure 1) and the rate of reaction is simply the sum of reaction of OD^- with the two reactive forms. Thus equation (5) obtains where k_2 [defined previously,

$$\text{rate} = k[\text{His}]_t = [O\text{D}^-](k_1[\text{His}^{2+}] + k_2[\text{His}^+]) \quad (5)$$

see equation (1)] and k_1 are the second-order rate constants for reactions of OD^- and His^+ and His^{2+}, respectively. Acid dissociation constants are defined by the equations $N^+D_3Im^+ \rightleftharpoons N^+D_3Im + D^+(K_1)$, $ND_2Im^+D \rightleftharpoons ND_2Im + D^+(K_2)$, $N^+D_3Im \rightleftharpoons ND_2Im + D^+(K_3)$. Substitution for $[His^{2+}]$ and $[His^+]$ in equation (5) in terms of K_1, K_2, K_3, and K_{D_2O} gives equation (6).[7]

$$k = \frac{k_1K_{D_2O}}{K_1 + [D^+] + \frac{K_1K_3}{[D^+]} + \frac{K_1K_3}{K_2}} + \frac{k_2K_{D_2O}}{K_2 + [D^+] + \frac{K_2[D^+]}{K_3} + \frac{K_2[D^+]^2}{K_1K_3}} \quad (6)$$

Previously,[7] experimental values of k were determined at various values of pD, and by substitution of values of K_1, K_2, K_3, and K_{D_2O} it was possible to calculate values of k_1 and k_2 for various small model compounds. In order to explain the significance of the double S shaped curve in more detail, we have computed the curve for L-histidine using equation (6) and the appropriate data (see Figure 3, curve A). Curve A is the sum of curve C [which is from the first term on the right of equation (6)] and B [which is from the second term on the right of equation (6)].

At low pD the rate curve is controlled completely by reaction with $N^+D_3Im^+D$ whereas at high pD the rate curve is controlled by reaction with ND_2Im^+D. This is expected because pK_3 is three units larger than pK_1 so that the titration of the amino-group and the imidazole group occur separately. Thus, by experiments at low pD where $k_2 = 0$, it is possible to calculate k_1 by use of the first term on the right of equation (6) [or simply equation (3)], whilst using the results obtained at high pD, where $k_1 = 0$, the second term on the right of equation (6) may be used.

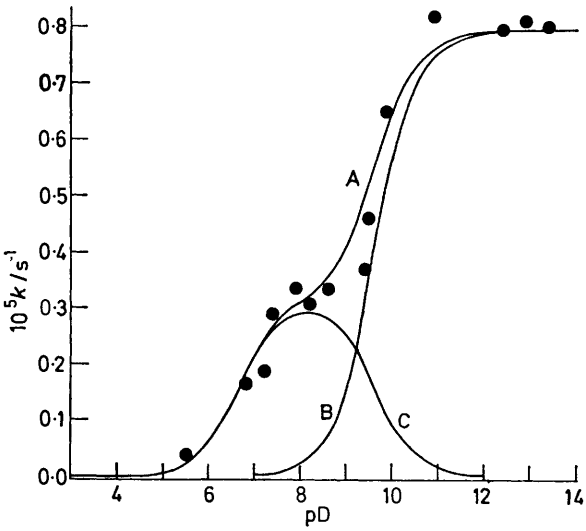

FIGURE 3 Graphs to explain the pD dependence of the H–D exchange of L-histidine. A, Computed curve using equation (6) and the following data $K_{D_2O} = 3.3 \times 10^{-15}$, p$K_1$ 6.6, pK_2 7.6, pK_3 9.6, k_1 240 dm^3 mol^{-1} s^{-1},[7] k_2 60 dm^3 mol^{-1}s^{-1} (the value of 46.7 given in ref. 7 was in error). B, Computed curve using the second term on the right of equation (6) (setting $k_1 = 0$) and the same data. C, Computed curve using the first term on the right of equation (6) (setting $k_2 = 0$) and the same data. The experimental data points obtained at 37 °C are included for reference

(i) *Determination of the* pK *of imidazole group and nearby titratable group.* As previously noted, it is possible to obtain pK_1 from the centre of the first S shaped curve. Examination of the first term on the right of equation (6) shows that this will only be accurate if $K_3 \ll K_2$. Experimentally, pK_3 is obtained from the centre of the second S shaped curve in curve A of Figure 3. Examination of the second term on the right of equation (6) shows that this also will follow only if $K_3 \ll K_2$. We have computed curves (not shown) as in Figure 3 but using pK_3 = 8.6 instead of 9.6. In this case the two S shaped curves can just be distinguished and the pK_1 and pK_3 values obtained from the centre of the curves are approximately correct. As might be expected, a computed curve with a value of pK_3 = 7.6 = pK_2 removed the inflexion altogether and a single S shaped curve resulted. In practice, the determination of pK_1 and pK_3 is possible, providing that two S shaped curves can be distinguished, which would require that p$K_3 \geq$ pK_2 + 1.

It would be expected that the magnitude of the effect of the nearby charged group on the H–D exchange of the C-2 proton of the histidine residue would decrease as the distance between them increased. This is found experi-

* For convenience the adjacent charged group has been defined in terms of an α- or ε-amino group, but other possibilities are the phenolic hydroxy-group of tyrosine or an SH group of cysteine. The guanidino group of arginine, although charged, would not titrate below pH 12.

mentally as the size of the upper S shaped curve in curve A (Figure 3) decreases at the expense of the lower S shaped curve as one goes from L-histidine, to β-alanyl-L-histidine where the positively charged amino-group is moved further away from the imidazole ring.[7]

The effect of the amino group on the exchange rate in L-histidine (see Figure 3, curve A) is much larger than its effect on the chemical shifts of the H-2 and -4 resonances.[33-35] The kinetic method is therefore inherently more sensitive to the presence of a nearby charged group than the n.m.r. method, and it was hoped [7] that this advantage would have a useful application with proteins. However, when the method was applied to ribonuclease,[9] difficulty arose from the need to expose the protein to alkaline conditions for a period of days in order to obtain exchange measurable by n.m.r. techniques. The denaturation that occurred at high pH limited the application of the method to a maximum pD of 10.9. Furthermore, the accuracy of the kinetic results is less than that obtained with small molecules. Nevertheless the method may be useful if H–T methods are used [5,8,10] and with proteins that are stable to alkaline conditions.

(ii) *Temperature dependence of the rate constant of* H–D *exchange.* Rate constants for the deuteriation of L-histidine in D_2O and for the protonation in H_2O of L-histidine previously deuteriated at the C-2 position, are given in Table 2. All experiments were done at low

TABLE 2

Rate constants for exchange of C-2 protons of L-histidine at low ionic strength

pH meter reading [a]	$10^5 \times k/s^{-1}$ at 25 °C	37 °C	50 °C	50 °C [b]	50 °C [c]
5.0	<.01	0.035	0.15		0.14
7.0	0.058	0.29	1.41	1.45	1.06
9.0	0.093	0.37	2.37	2.43	1.92

[a] This is the pH meter value obtained in D_2O solution. [b] In 0.10M-NaCl. [c] Protonation in water of L-histidine in which the C-2 proton had been previously deuteriated.

ionic strength, *i.e.* in the absence of added electrolyte, except for one set in 0.10M-NaCl at 50 °C. The rate constant was not appreciably different at the higher ionic strength. The protonation of L-histidine deuteriated at the C-2 position occurred more slowly than the deuteriation reaction, presumably because of the greater ease of splitting the C(2)–H bond in the deuteriation reaction than the C(2)–D bond in the protonation reaction.

The Arrhenius energies of activation E_A calculated from the data in Table 2 at low ionic strength at pH 5, 7, and 9 are 94, 102, and 104 kJ mol^{-1}, respectively, with an error of *ca.* ±12 kJ. These are slightly less than the value of 119 kJ mol^{-1} obtained over the temperature range 35—65° for *N*-acetyl-L-histidine *N*-methylamide,[14] but much greater than the value of 46 kJ mol^{-1} obtained for imidazole over the narrow temperature range 60—70 °C.[4] This last result is unaccountably low compared with the other values. When the rate data of Vaughan *et al.*[4] at 65 °C are combined with our data on imidazole at 37 °C [6] the energy of activation obtained is 89 kJ mol^{-1}. It is concluded that the low value [4] is probably in error and the range of values is 90—120 kJ mol^{-1}.

(3) H–D *Exchange at Two Nearby Imidazole Groups Adjacent to One Charged Group with* p*K* 6—12.—The simplest model is L-histidyl-L-histidine, which has two adjacent imidazole rings and an α-amino-group that titrates at high pH. This dipeptide was previously used [36] as a model for the active site of ribonuclease, in which two histidine residues are involved. The kinetic scheme is much more complicated than in the previous section because of the fact that there are three interacting charged groups (instead of two) and two of these are subjected to H–D exchange. Nevertheless it is possible to solve the equations using the same basic procedures as before. The imidazole ring near the *N*-terminus is called ring A and that near the *C*-terminus, ring B.

The rate of deuteriation of ring A, $k_A[His]_t$, is given by equation (7) where k_A is the pseudo-first-order rate constant for deuteriation of ring A and k_1—k_4 are second-order rate constants for the deuteriation of the four possible forms in which the charged imidazole ring is adjacent to the α-amino-group. There are eight different charged forms altogether which are inter-related by twelve equilibrium constants (see Figure 4). It is

$$k_A[His]_t = [OD^-]k_1[N^+D_3Im^+DIm^+D] + k_2[ND_2Im^+DIm^+D] + k_3[N^+D_3Im^+DIm] + k_4[ND_2Im^+DIm] \quad (7)$$

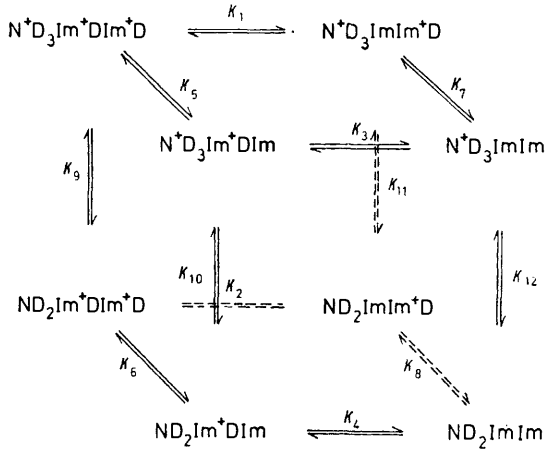

FIGURE 4 Scheme to show the microscopic dissociation constants for the two imidazole rings and the α-amino group of L-histidyl-L-histidine in D_2O

possible to obtain an equation for $[His]_t$ in terms of $[N^+D_3Im^+DIm^+D]$ and these equilibrium constants, and the concentration terms on the right side of equation (7) may be similarly substituted. Rearrangement of equation (7) then gives equation (8) for k_A. A similar equation may be derived for the rate constant k_B for deuteriation of the imidazole ring near the carboxy group, in terms of the second-order rate constants

$$k_A/K_{D_2O} = \frac{k_1[D^+]^2 + (k_2K_9 + k_3K_5)[D^+] + k_4K_5K_{10}}{[D^+]^3 + (K_1 + K_5 + K_9)[D^+]^2 + (K_5K_{10} + K_1K_{11} + K_1K_7)[D^+] + K_1K_7K_{12}} \quad (8)$$

k_5—k_8 defined by analogy with equation (7), *viz.* equation (9). The H–D exchange of the two imidazole rings of

$$k_B/K_{D_2O} = \frac{k_5[D^+]^2 + (k_6K_1 + k_7K_9)[D^+] + k_8K_1K_{11}}{[D^+]^3 + (K_1 + K_5 + K_9)[D^+]^2 + (K_5K_{10} + K_1K_{11} + K_1K_7)[D^+] + K_1K_7K_{12}} \quad (9)$$

L-histidyl-L-histidine was measured at different pD values and the values of k_A and k_B are given in Figure 5.

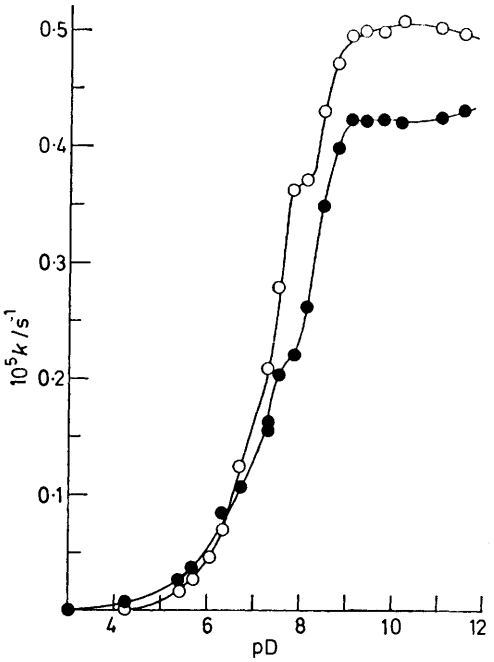

FIGURE 5 Graph of the dependence on pD of the pseudo-first-order rate constants k_A and k_B for the exchange of the H-2 protons of the imidazole rings of L-histidyl-L-histidine: k_A (ring nearest to the *N*-terminus, ●), k_B (ring nearest to the *C*-terminus, ○)

Using this data we considered the possible evaluation of the eight second-order rate constants k_1—k_8 and the seven dissociation constants involved in equations (8) and (9). We attempted to use a computer program written for the solution of a number of simultaneous equations, but this was set up in terms of an independent variable that contained no error, whereas our data for equation (8) contained errors in both k_A and pD. Furthermore, the errors in k_A were so large (*ca.* ±5%) that use of this program gave meaningless results. In principle, it is clear that using equation (8), solutions would only be possible for the various constant terms, *i.e.* k_1, $(k_2K_9 + k_3K_5)$, $k_4K_5K_{10}$, *etc.*, rather than explicit solutions for each constant. At high pD, as $[D^+] \longrightarrow 0$ and k_A becomes constant, equation (8) simplifies to (10).

$$k_A = K_{D_2O}k_4K_5K_{10}/K_1K_7K_{12} \quad (10)$$

It is not possible to extract the value of k_4 from equation (10) because of its association with five unknown equilibrium constants. It is therefore not possible to obtain second-order rate constants from the rate data.

The experimental data in Figure 5 show two S shaped curves with an inflexion near the centre as with L-histidine (Figure 3). The imidazole groups in L-histidyl-L-histidine form an interacting system with pK values in D_2O for ring A and B of *ca.* 6.2 and 7.5 respectively,[36] hence it is not possible to extract these pK values from the lower S shaped curves in Figure 5. However, as considered earlier (Figure 3), the upper S shaped curves are from the ionization of the α-amino-group, which is consistent with the occurrence of a larger upper S shaped curve for ring A (which is closer to the α-amino-group) than for ring B. Furthermore, approximate values of the pK' of the α-amino-group obtained from the centres of the upper S shaped curves are 8.05 and 8.15. The average value of 8.1 is in good agreement with the value of 7.7 obtained by Shrager *et al.*[34] which must be increased by 0.4 units to 8.1 to allow for the deuterium isotope effect.[24,25,35,36]

(4) H–D *Exchange at an Imidazole Group Adjacent to Two Titrating Charged Groups.*—Two examples have been studied experimentally, L-histidyl-L-tyrosine and L-histidyl-L-lysine, the adjacent titrating charged groups being the α-amino-group and either the phenolic hydroxy group of tyrosine or the ε-amino-group of lysine. In this case the kinetic scheme is analogous, with suitable modification, to that for the H–D exchange of ring A in section (3), which leads to equation (8), containing four

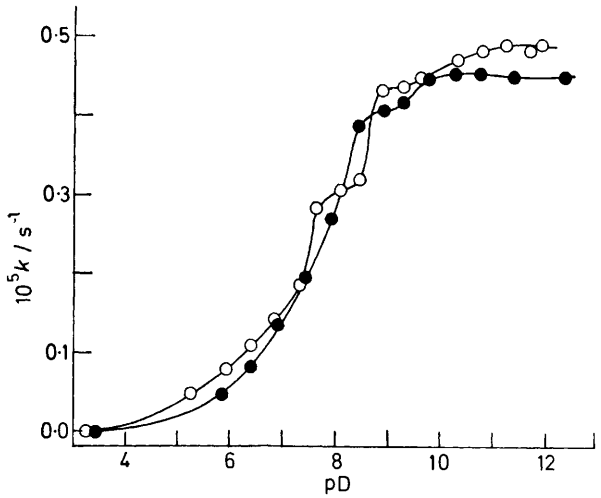

FIGURE 6 The dependence on pD of the pseudo-first-order rate constant k for H–D exchange of the C-2 proton of L-histidyl-L-tyrosine (○) and L-histidyl-L-lysine (●)

second-order rate constants and seven equilibrium constants defined in Figure 4. By analogy with section (3), it is not possible to obtain the second-order rate constants and equilibrium constants and we must consider it in terms of the treatment relevant to section (2).

With L-histidyl-L-tyrosine, there appear to be three S shaped curves in Figure 6, the centres of which have pD values of *ca.* 6.8, 8.6, and 10.1. The pK values for the imidazole, α-amino and phenolic hydroxy groups, corrected for the deuterium isotope effect by adding 0.4, are 6.15, 7.88, and 10.48, respectively.[35] The agreement between these two sets of data is rather poor. For L-histidyl-L-lysine there are only two S shaped curves,

the first very broad one, appears to embrace an interacting system with two titrating groups with closely spaced pK values as with L-histidyl-L-histidine. The centres of the two S shaped curves occur at pD values of 7.4 and 9.2 and the pK values of the imidazole, α-amino-, and ε-amino-groups (corrected for the deuterium isotope effect by adding 0.4) are 6.25, 7.77, and 10.75, respectively.[35] The value of 7.4 falls between the pK values of the imidazole and α-amino-groups as expected from the above analysis, but the value 9.2 cannot be considered to represent the ε-amino-group of pK 10.75. The reason for this very considerable discrepancy is not clear and taken together with the rather poor agreement obtained in the case of L-histidyl-L-tyrosine, leads to the conclusion that it is not safe to extract pK data from curves, when there is more than one adjacent titrating charged group.

(5) *General Discussion.*—The kinetic equations have been described for the deuterium exchange of the proton at the C-2 position of an imidazole ring for four different cases: an isolated imidazole,[4] an imidazole with one adjacent charged group of pK 6—12,[7] two adjacent imidazole rings with one adjacent charged group of pK 6—12, and an imidazole with two adjacent charged groups of pK 6—12. In the simplest case, the general kinetic scheme shown in Figure 1,[4] can be simplified at temperatures <40 °C, because reaction (2) is so slow that it may be neglected; however, it must be considered at $\geqslant 60$ °C. The kinetic schemes for the more complex cases are developed for the low temperature case in which reaction (2) is neglected. The first two cases have been examined in considerable detail and the second-order rate constants and equilibrium constants may be evaluated explicitly from experimental data. However, this is not the case for the two more complex systems that involve numerous second-order rate constants and equilibrium constants.

If there is a nearby charged group with a pK about two units or more greater than that of the histidine residue, it will result in a double S shaped curve as shown in Figure 3A, from which one can also obtain the pK of the adjacent charged group. Furthermore, the magnitude of the second step in the curve is related to the proximity of the adjacent charged group to the histidine ring. Unfortunately, more difficult cases such as L-histidyl-L-histidine and L-histidyl-L-tyrosine gave experimental results in Figures 5 and 6 that could not be readily interpreted in terms of the simple treatment.

By measurement of the rate of H–D exchange as a function of pD for a histidine residue in a protein, it is possible to determine both k_2 and the pK of the histidine. Using the qualitative arguments summarised in Table 1, the histidine H–D exchange date for (i) lysozyme, (ii) the distal histidine residues of carbon monoxide myoglobin and leghaemoglobin, and (iii) the histidines in the active site of ribonuclease, has been rationalised in terms of the known environments of these histidine residues as obtained from their three dimensional structures. It is anticipated that this approach may be useful for other proteins, although its application to complex cases that involve more than one type of environment (*e.g.* a non-polar environment as well as a charged group) would be fraught with difficulty.

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