# THE PRESSURE DEPENDENCE OF THE HISTIDINE RING PROTONATION CONSTANT STUDIED BY $^1$H HR-NMR

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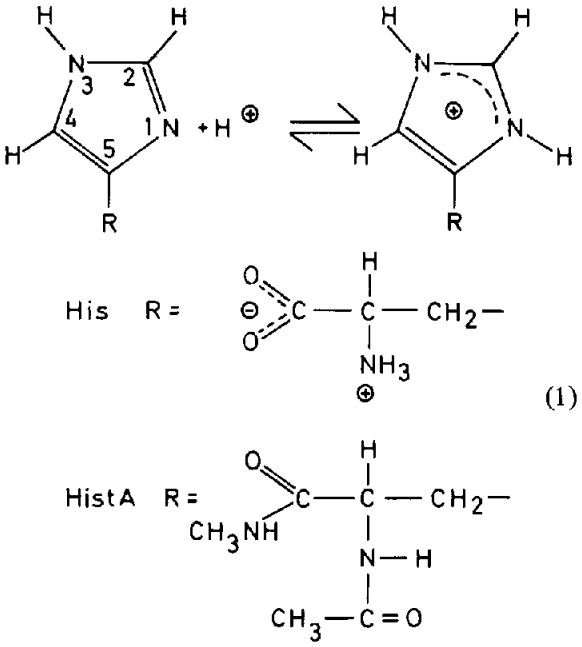
## 1. Introduction

The biological function of a variety of proteins depends on the protonation of imidazole rings in essential histidyl residues. For example, in the case of lactic dehydrogenase and chymotrypsin, proton transfer from or to histidines in the active centres of the two enzymes is involved in the mechanism of catalysis [1–4]; similarly the pH dependence of the $R/T$ equilibrium of hemoglobin is due to the protonation/deprotonation of histidine $\beta$ 146 (which forms an ionic bond to aspartate $\beta$ 94 of the same $\beta$ chain by proton uptake stabilizing the T state [5].

In the context of studying the pressure dependence of the reactivity of enzymes it appears thus indicated to analyze the influence of pressure upon the protonation constant of the histidine ring. Work on the reaction volume for this process consisted of dilatometric studies at ambient pressure [6]. Here, studies on the pressure dependence of p$K$-values (protonation/deprotonation of imidazole N1; cf. eq. (1)) are reported. Using L-histidine and $N_\alpha$-acetyl-L-histidine-$N$-methylamide it is shown by high pressure $^1$H HR-NMR spectroscopy that the small negative reaction volume of protonation ($\Delta V = -2$ ml/mol) determined at atmospheric pressure (0.1 MPa) [6] remains unchanged up to $p = 150$ MPa. This finding demonstrates that pressure-induced changes of catalytic activities or ligand binding constants of proteins [7–9] cannot be caused by p$K$ shifts of essential histidyl residues, but must involve conformational transitions of the protein.




(1)

## 2. Materials and methods

### 2.1. *Solutions*

Commercial L-histidine (His) (E. Merck, Darmstadt) and $N$-$\alpha$-acetyl-L-histidine-$N$-methylamide (HistA) (Bachem, Bubendorf) were used without further purification for the preparation of the solutions. The compounds were dissolved at 5 mM (at 293 K and 0.1 MPa) in buffers of 0.2 M ionic strength. $KH_2PO_4$/$Na_2HPO_4$, $CH_3COOH$/NaOH and Tris–HCl buffers in heavy water were prepared from the commercial products of the highest purity available (E. Merck, Darmstadt). Light water was added to the buffers to adjust the final degree of deuteration to 90%. (The

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strong water proton signal was used for the shimming of the high pressure cell.) 0.1% of trimethylsilylpropanesulfonic acid, sodium salt (E. Merck, Darmstadt) was added to the solutions as an internal standard of the chemical shift. The pD of the solutions was measured at 283, 293 and 303 K with a glass electrode. The reading of the instrument was corrected for the isotope effect [10,11]:

$$pD = pH_{inst.} + 0.35 \quad (2)$$

The pressure dependence of the pH in the buffered solutions was calculated according to [12].

## 3. Experimental

The high-pressure $^1$H HR-NMR spectra were obtained at 100.1 MHz with a Varian XL-100-15 FT NMR spectrometer. Details of the high-pressure glass cell have been published [13,14]. 2000 transients were accumulated within 30 min for each spectrum. The low filling factor of the high pressure cell together with the low concentration of the compounds leads to a rather modest signal-to-noise ratio of the histidine ring proton spectra, and the chemical shifts determined carry an uncertainty of ±0.5 Hz. From the chemical shift of the protons H(2) and H(4), the equilibrium constant $K$ is calculated for the different sets of data via the Henderson-Hasselbalch equation:

$$\log K = pH - \log \frac{\delta\nu_1 - \delta\nu}{\delta\nu - \delta\nu_2} \quad (3)$$

$\delta\nu_1$ and $\delta\nu_2$ are the chemical shifts of the H(2) or H(4) protons of the imidazole ring in the protonated and unprotonated form, respectively and $\delta\nu$ is the chemical shift of the proton at the pH of the experiment. The experimental $\delta\nu$ for a constant temperature and pressure are plotted as a function of pH and compared visually to sets of curves calculated and plotted by computer in which $\delta\nu_1$, $\delta\nu_2$ and $K$ are treated as adjustable parameters.

Differences in p$K$ of ±0.01 and in $\delta\nu_1$ and $\delta\nu_2$ of ±1 Hz can be distinguished in the most sensitive steep part of the Henderson-Hasselbalch curves. This theoretical resolution is better than the experimental $\delta\nu$-curves. A pessimistic estimate of all possible errors influencing the reproducibility of the final p$K$ derived,

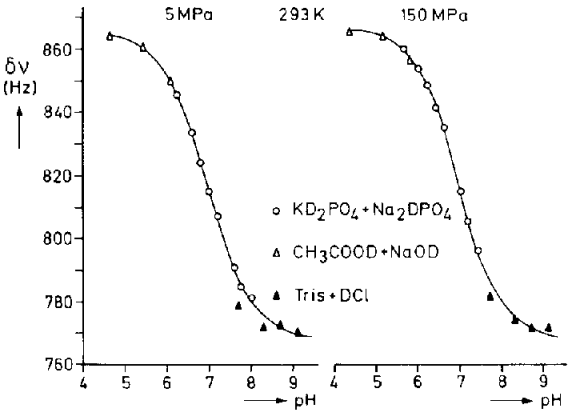


Fig.1. Pressure dependence of the imidazole ring protonation equilibrium in histidine, monitored by the chemical shift $\delta\nu$ of the H(2) proton.

which includes the uncertainty of $\delta\nu$ as well as the uncertainties in the pressure dependence of the pH of the buffers, leads to a maximal error of ±0.05 for $\Delta pK$.

The temperature was accurate to ±0.5 K, the pressures to ±0.5 MPa. Compared to the effects mentioned above, these contributions to the overall error can safely be neglected.

## 4. Results

Figures 1 and 2 illustrate some of the results obtained. The full lines correspond to the calculated best fit of the Henderson-Hasselbalch equation. In

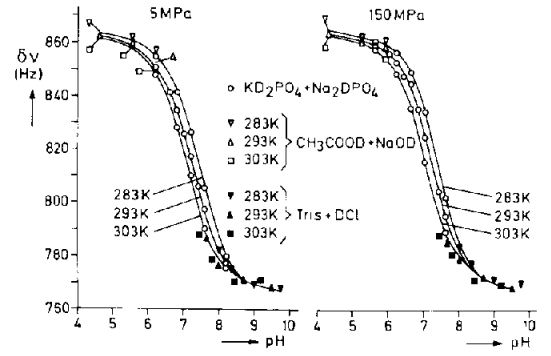


Fig.2. Pressure and temperature dependence of the imidazole ring protonation in $N_\alpha$-acetyl-L-histidine-$N$-methylamide, monitored by the chemical shift $\delta\nu$ of the H(2) proton.

Table 1
Pressure dependence of the p$K$ for the imidazole ring protonation in $N_\alpha$-acetyl-L-histidine-$N$-methylamide (HistA) and L-histidine (His)

| HistA $\delta\nu$[a] | $T$ (K) | $p$ (MPa) | | | |
|---|---|---|---|---|---|
| | | 5 | 50 | 100 | 150 |
| H(2) | 283 | 7.43 | 7.40 | 7.39 | 7.36 |
| H(4) | | 7.30 | 7.34 | 7.36 | 7.30 |
| H(2) | 293 | 7.22 | 7.24 | 7.23 | 7.17 |
| H(4) | | 7.20 | 7.16 | 7.17 | 7.14 |
| H(2) | 303 | 7.08 | 7.08 | 7.05 | 7.01 |
| H(4) | | 7.08 | 7.04 | 7.04 | 7.00 |
| His | | | | | |
| HC2 | 293 | 6.94 | 6.92 | 6.93 | 6.92 |
| HC4 | | 6.90 | 6.88 | 6.90 | 6.88 |

[a] The $\delta\nu$ as function of $p_H$ have been analyzed for H(2) and H(4) separately with the Henderson-Hasselbalch equation

the pH-region where the data obtained in various buffers overlap, the scatter between the $\delta\nu$ obtained in the different system amounts to ~±0.5 Hz and ~±0.02 pH. This shows, that the composition of the buffering solutions influences $\delta\nu$ only marginally. All p$K$-values derived from the analysis of the data are compiled in table 1. From these data a reaction volume $\Delta V = -1 \pm 1$ cm$^3$ . mol$^{-1}$ is derived. No temperature or pressure dependence for $\Delta V$ can be seen in the data.

Comparison of the given data with results published for the protonation constant of the imidazole ring of His in light and heavy water respectively [15] is only possible by combining the data from different sources [16], which determine the p$K$ in light water at an ionic strength and His-concentration comparable to the conditions of these experiments [17], or measure the p$K$ in light and heavy water [18]. The best estimates lead at 293 K to p$K$ = 6.8 ± 0.1 which compares well with the p$K$ in table 1, considering especially the fact, that activity coefficients have to be neglected in the analysis of the data.

## 5. Discussion

The dilatometric studies by Weber [6] showed that the reaction volume at atmospheric pressure for the imidazole ring protonation in His is very small: $\Delta V = -2$ cm$^3$ . mol$^{-1}$.

The high pressure $^1$H HR-NMR results given here prove this to hold also for pressures up to 150 MPa, and thus for all pressures encountered by living organisms in the biosphere of the earth. From this we conclude, that the protonation/deprotonation of the imidazole ring yields at most a very weak pressure dependence; the corresponding pressure derivative (d$pK$/d$p$) shows a behaviour similar to the dissociation of other weak acids and bases [19]. At the pH of our studies, the amino acid moiety of His exists in the zwitterionic form. The removal of the two charges in the neutral HistA (eq. (1)) does not affect d$pK$/d$p$. The only measurable effects is a pressure-independent shift of the p$K$ by ~0.3 units.

Thus it can be concluded safely that the p$K$ of histidyl residues in a random coil protein should be rather independent of pressure. Therefore any pressure influence upon the catalytic activity of enzymes with essential histidines, or upon the ligand binding properties of a protein is expected to result only from changes in the three-dimensional structure of the protein. These conformational changes can either influence the biological function of the protein directly, or indirectly via changes in the microenvironment of histidyl residues altering the intrinsic p$K$-value of these groups and thus shifting the pH-optimum of catalytic activity. $^1$H HR-NMR studies on ribonuclease [20], as well as dilatometric studies on ovalbumin, lysozyme, bovine serum albumin, and ribonuclease [21,22] gave evidence for the influence of the microenvironment upon the p$K$ of essential histidyl groups revealing significant reaction volumes for the protonation/deprotonation equilibria of the respective proteins.

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