Abstract. Evidence is presented for the existence of a conformational equilibrium in staphylococcal nuclease, involving one out of the four histidines and none of the other aromatic residues in the polypeptide chain.

Conformational changes in proteins can be detected by a variety of physical methods. In most cases, however, it is not possible to define the nature of the change more precisely, since to do so would require separate measurements on individual amino acid residues. The preparation of selectively deuterated enzyme analogs\textsuperscript{1-4} has made it feasible to make such measurements by high resolution nuclear magnetic resonance. The spectra of the analogs are in many cases sufficiently simplified, so that spectral lines arising from chemically identical amino acid residues occupying different positions in the peptide sequence can be resolved. By this method it is therefore possible to differentiate between localized and generalized conformational changes and to specify precisely the affected segment of the polypeptide chain.

A localized conformational change affecting histidine 48 has been found in pancreatic ribonuclease.\textsuperscript{5, 6} The conformational equilibrium indicated by the data on staphylococcal nuclease (Nase) can be studied over a wider range of conditions and thus defined even more clearly.

The aromatic region of Nase spectra at four pH values is shown in Figure 1. All the changes observed in this pH range\textsuperscript{5-7} may be explained on the basis of titration shifts of His C2-H and C4-H peaks. At low pH values there is no difficulty in distinguishing the C2-H peaks since they lie well downfield of the aromatic envelope. Five peaks are resolved which shift with pH. Two of these, H2a and H2b, may be attributed to the same His residue. The evidence for this rests primarily on the following observations: (a) The individual areas of peaks H2a and H2b are smaller than those of the other His C2-H peaks and add approximately to that of a single proton peak. (b) As the pH is raised from 6 to 7, peaks H2a and H2b broaden and move together. This coalescence leads to abnormal titration curves for these peaks (Fig. 2), and suggests that these resonances are linked by exchange. (c) The addition of calcium ion at pH 7.8 causes peak H2b to grow in area at the expense peak H2a.
The relative intensities of the upfield and downfield components of H2 change with pH. At low pH the upfield peak is larger than the downfield peak; at high pH the reverse holds. This behavior is best explained by the existence of a slow conformational change affecting the environment of His residue H2. The conformational change must be a local one since the other three His residues yield single peaks and normal titration curves. At high and low pH values, relatively sharp peaks are observed for each of the two forms. Under these conditions the chemical shift between the two peaks determines a lower limit for the lifetime \( \tau \) of the His residue in each conformation:

\[
\tau \gg \frac{1}{2\pi \Delta \nu},
\]

(1)

The smallest chemical shift between the two forms (in the pH range in which the peaks are separate and sharp) is 4 Hz at pH 4.7. This gives a lower limit of 40 msec for the lifetime at this pH.

The broadening of peaks H2a and H2b and the distortions of their titration curves in the pH region 6.3–7.5 may be explained by an increase in the rate of interconversion of the two conformational forms. The condition for exchange broadening and apparent coalescence of chemical shifts is:

\[
\tau \approx \frac{1}{2\pi \Delta \nu},
\]

(2)

If the exchange rate \( (\tau^{-1}) \) changes as a function of pH, one will thus observe a transition from the slow-exchange case equation (1) to the intermediate-exchange case equation (2). This appears to be the case. However, the observed ex-
Fig. 2.—Histidine titration curves (C2-H and C4-H peaks) of Nase.
change broadening does not follow the chemical shift difference between the two conformational states exactly. At pH 4.7, where exchange is slow, $\Delta \nu$ is 4 Hz, whereas at pH 6.3, where exchange broadening and shifting begin, $\Delta \nu$ is 25 Hz. Separate peaks appear again at pH 7.6 where $\Delta \nu$ is 10 Hz. To explain these data, it is necessary to postulate that the rate of interconversion of the two conformational forms does not change monotonically with pH, but has a pH-dependent maximum somewhere between pH 6.2 and 7.4.

The equilibrium constant for the conformational transition can be defined as:

$$K = \frac{[E']}{[E]} = \frac{\text{area of peak } H2b}{\text{area of peak } H2a},$$

(3)

where $[E]$ is the concentration of the form corresponding to peak H2a and $[E']$ is that of the form corresponding to H2b. Estimates of $K$ as a function of pH may be obtained from the relative areas of peaks H2a and H2b. Values for the equilibrium constant are: 0.5 at pH 4.7; 0.7 at pH 5.0; 0.7 at pH 5.8; and 3–5 at pH 7.5. $K$ is clearly pH dependent, since $[2b] < [2a]$ at low pH and $[2b] > [2a]$ at high pH.

The titration curves in Figure 2 yield the following pK values for the five His species: H1, 6.50; H2a, 6.55; H2b, 5.80; H3, 5.75; H4, 5.55. The maximum error in the pK values is ±0.10 unit. All titration curves have normal shapes except H2a, which has an unusually steep slope.

Titration curves constructed from data on the selectively deuterated analogs Nase-D1–5 (Putter, Markley, and Jardetzky) agree very well with those of the protonated Nase. The only significant difference is in peak H2a which is detected only at low pH as in Nase-P1 (cf. ref. 4). The position of the Tyr peaks Y1–Y7 are constant over the range pH 5.5–8.0 and so is the position of the tryptophan peak W. Slight deviations are observed below pH 5.5 and above pH 8.0. These may reflect minor conformational differences attributable to an isotope effect. The important finding resulting from this series of experiments is that no evidence of a conformational equilibrium can be found in the spectrum of any aromatic residue, except the peak labeled H2. The conformational transition must therefore be highly localized.

Of great interest for the further identification of the observed conformational transition is the behavior of a Nase derivative, Nase-CT. Nase-CT consists of two, nonevalently bonded, peptide fragments of Nase produced by trypsin cleavage. The N-terminal pentapeptide of Nase, which does not contain aromatic amino acids, is absent in this derivative, and so is Lys 49 which is adjacent to the position of trypsin cleavage. The proton composition of the aromatic region of Nase-CT is identical to that of Nase.

Titration curves of His C2-H peaks of Nase-CT are compared with those of Nase in Figure 3. Conversion of Nase to Nase-CT has only a slight effect on the titration curves of H1, H3, and H4. The pK values of H1 and H4 are unchanged, and the pK value of H3 is increased slightly from 5.75 to 5.80. The resonances of H2, on the other hand, are affected substantially by formation of Nase-CT. Conformational form $E$ predominates over the entire titration region, not just above pH 7 as in Nase. Small peaks attributed to the other con-
formational form $E'$ are detected at pH 5.8–6.0 and 7.3. The region of intermediate exchange between conformations $E$ and $E'$ is much more extensive in the partially cleaved derivative than in native Nase, extending from pH 6.4 to 7.0. The lifetime of the conformational forms in this region is approximately 20 msec. Because of the intermediate exchange rate, only one, averaged titration curve is observed for H2a and H2b, with an apparent pK of 6.40. Its slope is abnormally steep as is that of H2a of Nase. These findings lead us to suggest that the residue involved in the conformational transition is histidine 46. The evidence is admittedly indirect and must be confirmed by an unequivocal assignment of peak 2 to this residue.

A very interesting shift of the equilibrium can be observed on addition of Ca$^{++}$. The pH dependence of Ca$^{++}$ binding to 4.6 mM Nase was studied at a ratio of [Ca$^{++}$]/[Nase] = 5. The histidine titration curves are shown in Figure 4. The two H2 peaks are relatively sharp throughout the titration region in the presence of Ca$^{++}$, indicating that Ca$^{++}$ binding increases the lifetimes of the two
conformational states of the enzyme. The region around pH 7 is not obscured by exchange effects, and it is clear that the two H2 titration curves crossover. The pK of H2b is lowered from 5.80 to 5.50 suggesting that Ca^{2+} binds directly to the corresponding histidine residue in the conformation (E'). The pK values of all other peaks are unaffected by Ca^{2+}. The conformational equilibrium is shifted toward the form (E'). Peak H2a becomes smaller as the pH is raised and disappears at pH 8.5 and above.

Addition of the inhibitor 3'5' thymidine diphosphate (pdTp) also produces a slight shift in the equilibrium in favor of (E'), although form (E) still predomi-
phosphate anion of pdTp. The combined addition of Ca$^{++}$ and pdTp leads to a complicated pattern of shifts which will be discussed more fully at another time. The selective effect of both Ca$^{++}$ and pdTp on the equilibrium involving H2 does suggest, however, that the corresponding histidine residue lies in the vicinity of the active site.

3 Putter, I., A. Barreto, J. L. Markley, and O. Jardetzky, these PROCEEDINGS, 64, 1396 (1969).
7 Taniuchi, H., C. B. Anfinsen, and A. Sodja, these PROCEEDINGS, 58, 1235 (1967).