Assignment of the Histidine Proton Magnetic Resonance Peaks of Soybean Trypsin Inhibitor (Kunitz) by a Differertial Deuterium Exchange Technique†

John L. Markley* and Ikunoshin Kato

ABSTRACT: Deuterium exchange at the C(2)-H position of the two histidine residues of native soybean trypsin inhibitor (Kunitz) in ^2H_2O was followed by ^1H nuclear magnetic resonance (NMR) spectroscopy. The two histidine residues of soybean trypsin inhibitor exchange at significantly different rates at pH* 5.00, 40°. Half-times observed were: peak H1, $t_{1/2} = 61 \pm 2$ days; peak H2, $t_{1/2} = 24 \pm 2$ days. Differentially deuterated soybean trypsin inhibitor was cleaved by cyanogen bromide into two fragments each containing one histidine residue. The deuterium content of the histidine residue of each separated fragment was analyzed by ^1H NMR spectroscopy. Histidine-71 in fragment 1–114 showed approximately twice the deuterium content of His^{157} in fragment 115–181. These results lead to the assignment of ^1H NMR peak H1 to His^{157} and peak H2 to His^{71}. These assignments were extended to the histidine peaks of trypsin-modified soybean trypsin inhibitor by converting the differentially deuterated virgin soybean trypsin inhibitor to the modified form. The correlation of histidine peaks in virgin and modified soybean trypsin inhibitors was the same as proposed earlier on the basis of pK' arguments. The results demonstrate that His^{71} is the residue whose pK' value is raised from 5.27 to 5.91 on trypsin modification of soybean trypsin inhibitor [Markley, J. L., (1973), *Biochemistry 12*, 2245].

Proton magnetic resonance (^1H NMR) peaks corresponding to the C(2) ring proton of the two histidine residues of soybean trypsin inhibitor have been reported recently for both the virgin (Arg^{63}-Ile^{64} bond intact; STI)[1] and trypsin modified (Arg^{63}-Ile^{64} bond cleaved; STI*) forms of the protein. NMR titration (at 100 MHz) of the histidines revealed that the pK' obtained from histidine peak H2 at STI[1] changes from 5.27 to 5.91 on modification, whereas the pK' obtained from histidine peak H1 changes only from 7.00 to 6.82 (Markley, 1973a). The net histidine pK' change of +0.46 upon modification (due largely to residue H2) may adequately explain two puzzling features of the STI modification reaction. In order to fit the pH dependence of equilibrium data for the trypsin-catalyzed interconversion of STI and STI* to a theoretical curve, Mattis and Laskowski (1973) had to postulate the existence of a titrating group in the molecule with a pK' of 5.30 in STI and 5.86 in STI*. This group appears to be histidine H2 of the NMR studies (Markley, 1973a). Baugh and Trowbridge (1972) observed an abnormally large exothermic heat of hydrolysis for the STI → STI* reaction at pH 5.0. Their calorimetric ΔH of hydrolysis was −3.20 to −4.04 kcal/mol compared to −1.50 to −2.00 kcal/mol expected for hydrolysis of a single peptide bond. The discrepancy of −1.21 to −2.54 kcal/mol may be accounted for by the 25% fractional protonation of histidine H2 expected on modification of STI at pH 5.0, since ΔH values for the full protonation of histidine residues have been reported in the range −4.0 to −21.1 kcal/mol (Roberts et al., 1969).

As a result of these experiments the assignment of the two histidine NMR peaks H1 and H2 to His^{71} and His^{157} in the linear sequence of STI and STI* (Koide et al., 1972) became an interesting problem. Previous assignments of histidine NMR peaks in proteins have been made on the basis of chemical or enzymatic modification of the protein (Meadows et al., 1968; Kilmartin et al., 1973; Robillard and Shulman, 1972), ligand binding data (Meadows et al., 1968; Markley et al., 1970), studies of mutant proteins (Markley, 1969; Cohen and Hayes, 1974), and differential deuterium exchange of the histidine C(2)-H (Meadows et al., 1968; Bradbury and Chapman, 1972; Markley, 1975). Of these methods, differential deuterium exchange appears to be the most general and requires only a knowledge of the sequence of the protein. There are three steps in the assignment procedure: (1) a native protein sample is prepared in which the histidine C(2) hydrogens are differentially deuterated; (2) NMR spectra of the differentially deuterated protein are taken to determine the order of exchange of the histidine C(2)-H peaks; (3) the protein is analyzed to determine the level of exchange at histidines in definite residue positions.

Conditions have been reported for the differential deuterium exchange of the histidines of native STI (Markley, 1973a; Markley and Cheung, 1973). After cyanogen bromide cleavage STI may be separated into two pieces STI[1–114] and STI[115–181] (Figure 1) each of which contains a single histidine residue (Koide and Ikenaka, 1973; Kato and Tominaga, 1970). We report here the combination of these procedures and the resulting assignment of the histi-

† From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received November 18, 1974.* A preliminary account of part of this research has been published (Markley and Cheung, 1973). This work was supported in part by Public Health Service Grants GM 19907 and GM 10831 to the Purdue Research Foundation and RR 00292 to the NMR Facility for Biomedical Studies, Carnegie-Mellon University, Pittsburgh, Pa. (where the 250-MHz spectroscopy was carried out), by the Research Corporation, and by the Pittsburgh Plate Glass Research Foundation.

[1] Abbreviations used are: STI, virgin soybean trypsin inhibitor (Kunitz); STI*, trypsin-modified soybean trypsin inhibitor (Kunitz); Hsl, homoserine lactone; pH*, glass electrode pH of a ^2H_2O solution uncorrected for the deuterium isotope effect; δ indicates chemical shift expressed in parts per million from external 5% $(CH_3)_4Si$ in CCl_4.

dine NMR peaks of STI. Differentially deuterium exchanged STI was also converted to STI* so that histidine assignments of STI could be rigorously extended to STI*.

Experimental Procedures

Materials. Soybean trypsin inhibitor (Kunitz) was obtained from Worthington Biochemical Corporation (lot SI-1AA). The inhibitor was passed through a Sephadex G-75 column (4.7 × 100 cm) equilibrated with 0.01 *M* Tris–0.10 *M* NaCl (pH 8.5) in order to remove high molecular weight contaminants and subsequently dialyzed extensively against distilled deionized water and lyophilized. Deuterium oxide (99.85% deuterium isotope) was purchased from Bio-Rad Laboratories; deuterium chloride and sodium deuterioxide were from Merck of Canada. Protein samples were lyophilized twice from ^2H_2O and then dissolved in 0.05 *M* KCl–0.050 *M* $CaCl_2$ in ^2H_2O. The inhibitor concentrations were generally 1.4 m*M*. Samples (2.0 ml) in 12 mm NMR tubes (Wilmad, 514A-5PP, flat bottom) with a Teflon insert (Thompson-Packard) were used for the 100-MHz ^1H NMR spectra. Samples (0.3 ml) in 5 mm NMR tubes (Wilmad, 526-PP) with a coaxial capillary insert (Wilmad, 520-3) were used for the 250-MHz spectra. All other chemicals used were reagent grade or the best available.

Methods. pH measurements of solutions in ^2H_2O were made at 20° using a Corning Model 112 digital pH meter with a small combination electrode (Ingold). The pH was adjusted with 1 *M* NaO^2H or 1 *M* ^2HCl in ^2H_2O added by means of a 0.2-ml micrometer syringe (Gilmont) and fine Teflon needle (Hamilton). All pH adjustments and measurements were made in either a 5- or 1-ml vial (Pierce) with stirring by a 7 × 2 mm magnetic stirring bar (Cole-Parmer) to ensure rapid and complete mixing. The notation pH* is used to denote uncorrected pH meter readings of ^2H_2O solutions made with electrodes standardized using ^1H_2O buffers. The pH* of each NMR sample was measured before and after the spectrum was taken. In the histidine titration region spectra were used only if the "before" and "after" pH* readings agreed within 0.02 unit.

The histidine exchange experiments were carried out with 20 mg/ml of STI in 0.5 *M* KCl–0.05 *M* $CaCl_2$ in ^2H_2O at pH* 5.0 and 40°. The exchange of the two histidine residues was monitored by ^1H NMR spectroscopy at 100 MHz.

The cyanogen bromide fragments from differentially exchanged STI were prepared using the method of Kato and Tominaga (1970). All operations were carried out at low pH in order to minimize the possibility of back exchange of the deuterium label. About 160 mg of lyophilized STI exchanged 28 days at pH* 5.0, 40°, was dissolved in 3 ml of H_2O and mixed with 125 ml of 88% HCOOH containing 90 mg of CNBr. The reaction was allowed to proceed for 20 hr at room temperature. At the end of the reaction the solution was diluted to 150 ml with H_2O and lyophilized. The dried material was dissolved in 6 ml of 70% HCOOH and applied to a Sephadex G-75 column (2.4 × 110 cm) equilibrated with 20% CH_3COOH. Fractions (3.5 ml) were collected. Fractions 70–85 were pooled as STI[1–114], and fractions 86–100 were pooled as STI[115–181]. After lyophilization, both fragments were rechromatographed on the same column using the same conditions.

Deuterium-exchanged STI was converted to STI* by incubation in ^2H_2O with 3 mol % of porcine trypsin (Novo) for 1 day at pH* 3.75 followed by 4 days at pH* 3.00. The solution was lyophilized and redissolved in ^2H_2O immediately before the NMR spectra were taken. The conversion to S* was 89% as monitored by disc gel electrophoresis (Mattis and Laskowski, 1973).

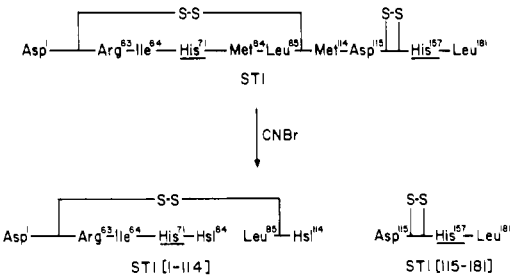

FIGURE 1: Scheme showing the cleavage of soybean trypsin inhibitor (Kunitz) by cyanogen bromide into two pieces STI[1–114] and STI[115–181] each containing one histidine residue. The Met^{84}-Leu^{86} bond of STI[1–114] is cleaved by cyanogen bromide, but the two resulting peptides are held together by a disulfide bond. Virgin soybean trypsin inhibitor (STI) is converted to modified soybean trypsin inhibitor (STI*) by trypsin hydrolysis of the reactive site peptide bond between residues Arg^{63} and Ile^{64}. [The reactive site of STI was determined by Ozawa and Laskowski (1966) and the sequence of STI by Koide et al. (1972).]

^1H NMR spectra (100 MHz) were obtained with a Varian XL 100-15 spectrometer equipped with an internal deuterium lock. The probe temperature was 32°. Spectrometer settings were as described previously (Markley, 1973a). ^1H NMR spectra (250 MHz) were obtained with the home-built spectrometer at the NMR Facility for Biomedical Research, Carnegie-Mellon University (Dadok et al., 1972). The spectrometer was operated in the correlation mode (Dadok and Sprecher, 1974) with a proton lock. The probe temperature was 31°. All chemical shifts are reported in parts per million from $(CH_3)_4Si$ in an external capillary. Spectra were stored on paper or magnetic tape. In analyzing histidine C(2)–H signal intensities, spectra were normalized to give equal areas under the envelope of aromatic resonances. It was assumed that this region contains no resonances from slowly exchangeable hydrogens. Peak intensities were determined either by cutting out and weighing or by use of the DuPont 310 curve resolver. The deuterium exchange data were fitted by a nonlinear least-squares program to first-order kinetics.

Results

The effect of deuterium exchange at pH 5.0, 40°, on the intensities of the histidine C(2)–H peaks of STI is shown in Figure 2. The top spectrum a is at zero time and the bottom spectrum b is after 28 days. The broad peak H2 clearly exchanges more rapidly than the sharper peak H1. Plots of the exchange data according to first-order kinetics (Figure 3) yield straight lines. The half-times for deuterium exchange are 61 ± 2 days for peak H1 and 24 ± 2 days for peak H2.

The sample that had been exchanged for 28 days (Figure 2b) was cleaved by cyanogen bromide and separated into two pieces, STI[1–114] and STI[115–181] (Figure 1). ^1H NMR spectra (100 MHz) of the isolated fragments dissolved in 50% deuterioacetic acid–^2H_2O are compared in Figure 4. The area of the histidine C(2)–H peak of STI[1–114] which contains His^{71} is 0.40 proton (δ −8.7) and the area of the histidine C(2)–H peak of STI[115–181] is 0.68 proton (δ −8.8). Since the respective areas of peaks H1 and H2 of the native exchanged STI sample were 0.76 proton and 0.46 proton, the histidine peak assignments are: H1, His^{157}; H2, His^{71}.

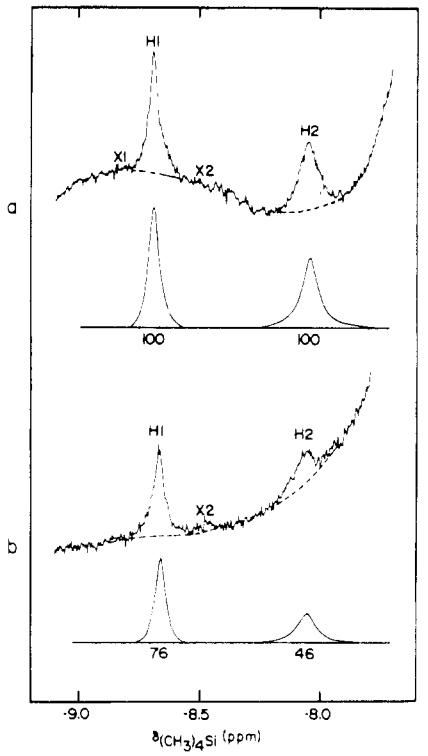

FIGURE 2: Deuterium exchange of the C(2) hydrogens of the two histidine residues of soybean trypsin inhibitor (Kunitz) as followed by ^{1}H NMR spectroscopy at 100 MHz. Exchange conditions: 1.4 m*M* STI in 0.5 *M* KCl-0.05 *M* $CaCl_2$ in ^2H_2O, pH* 5.0, 40° (spectra taken at 32°): (a) zero time; (b) after 28 days. The base-line assumptions are indicated. Spectra were fitted with peaks of constant line width. The broad N-H peak X1 exchanges more rapidly than the histidine peaks. The numbers represent percentage of original area.

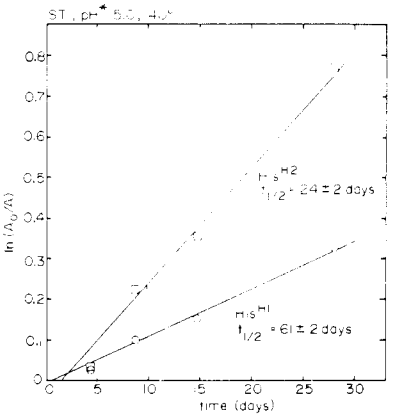

FIGURE 3: First-order plot of the histidine C(2)-H deuterium exchange data for soybean trypsin inhibitor. Exchange conditions: 1.4 m*M* STI in 0.5 *M* KCl-0.05 *M* $CaCl_2$ in ^2H_2O, pH* 5.0, 40°.

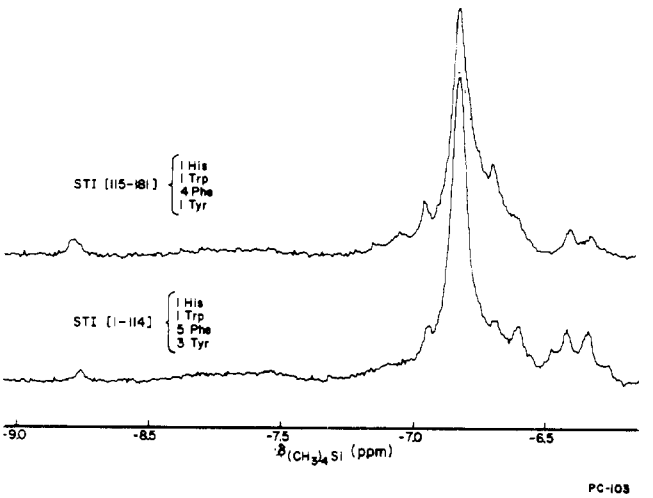

FIGURE 4: ^{1}H NMR spectra (100 MHz) of the cyanogen bromide fragments of differentially exchanged (28 days, pH* 5.0, 40°) soybean trypsin inhibitor in 50% acetic acid-d_4 in ^2H_2O: (a) STI[115-181] fragment containing His^{71}; area of the histidine C(2)-H peak (δ −8.8), 0.68 proton; (b) STI[1-114] fragment containing His^{157}; area of the histidine C(2)-H peak (δ −8.7), 0.40 proton.

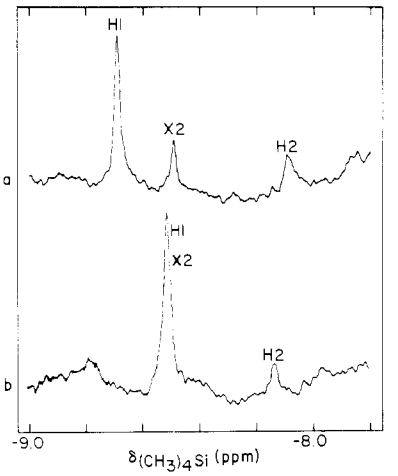

FIGURE 5: Comparison of 250-MHz ^{1}H NMR spectra at 31° of the histidine C(2)-H region of: (a) differentially deuterium exchanged virgin soybean trypsin inhibitor (STI) at pH* 5.01; (b) trypsin modified soybean trypsin inhibitor (STI*) prepared from this sample at pH* 6.05. Since peaks H1 and H2 of virgin soybean trypsin inhibitor have been assigned to His^{157} and His^{71}, respectively, the relative areas of the peaks indicate that peaks H1 and H2 of modified soybean trypsin inhibitor also correspond to His^{157} and His^{71}, respectively. Peak X2 arises from a slowly exchangeable N-H. This peak is sharp in spectra taken at 250 MHz but broad in spectra taken at 100 MHz (see Figure 2).

Spectra of a second exchanged sample of soybean trypsin inhibitor are compared in Figure 5 before and after conversion from STI to STI* in ^2H_2O. The ratios of the areas of peaks H1 to H2 before exchange and after exchange are 3.7 and 5.1. Thus, peak H1 of STI* is assigned to His^{157} and peak H2 of STI* to His^{71}. The correlation of histidine peaks in STI and STI* is in agreement with that assumed previously on the basis of pK' values (Markley, 1973a).

Discussion

Trypsin modification affects the histidine residues of soybean trypsin inhibitor in two distinct ways. First, the pK' values of both histidine residues are altered. His^{71} shows the largest change from 5.27 in STI to 5.91 in STI* whereas the pK' of His^{157} changes only from 7.00 in STI to 6.82 in STI* (Markley, 1973a). Second, increased line broadening of the histidine peaks of STI* as compared with STI at neutral pH suggests the existence of a pH-dependent slow conformational equilibrium in STI* (Markley, 1973a).

Trypsin modification of soybean trypsin inhibitor involves the cleavage of a single peptide bond between Arg^{63} and Ile^{64} at the reactive site of STI (Ozawa and Laskowski, 1966). Since modification changes the chemical environment of both histidine residues, it is of interest to know if they both are located near the reactive site where they could be affected directly by the cleavage or are influenced indi-

rectly as a result of a change in the conformation of STI on modification. Recent X-ray data for the STI-porcine trypsin complex reveal that His^{71} is located near the reactive site of STI and that His^{157} is located on the side of the STI molecule farthest removed from the reactive site (Sweet et al., 1974). The simplest explanation for the 0.64 unit increase in the pK' of His^{71} would be a direct electrostatic interaction between the histidine ring and the negatively charged newly formed carboxyl-terminal -Arg^{63} of STI*. The His^{71} ring would have to lie significantly closer to the carboxyl of Arg^{63} than to the new amino-terminal Ile^{64}- for the pK' to shift upward. This simple model requires that the distance between the Arg^{63} carboxyl and His^{71} ring be no greater than 5 Å. [This calculation is based on the work of Tanford and Roxby (1972).] In the STI-porcine trypsin complex the ring of His^{71} is 6 Å distant from the Arg^{63}-Ile^{64} bond.

The simple mechanism proposed above for the pK' shift of His^{71} of STI on modification may be an oversimplification since it is known that STI undergoes a conformational change on modification that affects the environment of a tyrosine residue (McKee and Laskowski, personal communication) and the mobility of a tyrosine residue at δ −6.88 and a phenylalanine residue at δ −7.00 (Markley, 1973b). Indeed some kind of conformational change on modification is required to explain the pK' shift of His^{157}. The question of a simple direct interaction between Arg^{63} and His^{71} in STI* can be investigated further by obtaining the pK' value of His^{71} in des-Arg^{63} STI* which can be made from STI* by carboxypeptidase B cleavage (Ozawa and Laskowski, 1966).

References

Baugh, R. J., and Trowbridge, C. G. (1972), *J. Biol. Chem. 247,* 7498.

Bradbury, J. H., and Chapman, G. E. (1972), *Biochem. Biophys. Res. Commun. 49,* 891.

Cohen, J. S., and Hayes, M. B. (1974), *J. Biol. Chem. 249,* 5472.

Dadok, J., and Sprecher, R. (1974), *J. Magn. Reson. 13,* 243.

Dadok, J., Sprecher, R., and Bothner-By, A. A. (1972), 13th Experimental NMR Conference, Pacific Grove, Calif.

Kato, I., and Tominaga, N. (1970), *FEBS Lett. 10,* 313.

Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., and Ho, C. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1246.

Koide, T., and Ikenaka, T. (1973), *Eur. J. Biochem. 32,* 401.

Koide, T., Ikenaka, T., Ikeda, K., and Hamaguchi, K. (1974), *J. Biochem. 75,* 805.

Koide, T., Tsunasawa, S., and Ikenaka, T. (1972), *J. Biochem. 71,* 165.

Markley, J. L. (1969), Ph.D. Thesis, Harvard University, Cambridge, Mass.

Markley, J. L. (1973a), *Biochemistry 12,* 2245.

Markley, J. L. (1973b), *Ann. N.Y. Acad. Sci. 222,* 347.

Markley, J. L. (1975), *Acc. Chem. Res. 8,* 70.

Markley, J. L., and Cheung, S.-M. (1973), *Proc. Int. Conf. Stable Isot. Chem. Biol. Med., 1st, 1973,* 103.

Markley, J. L., Williams, M. N., and Jardetzky, O. (1970), *Proc. Natl. Acad. Sci. U.S.A. 65,* 645.

Mattis, J. A., and Laskowski, M., Jr. (1973), *Biochemistry 12,* 2239.

Meadows, D. H., Jardetzky, O., Epand, R. M., Rüterjans, H. H., and Scheraga, H. A. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 766.

Ozawa, K., and Laskowski, M., Jr. (1966), *J. Biol. Chem. 241,* 3955.

Roberts, G. C. K., Meadows, D. H., and Jardetzky, O. (1969), *Biochemistry 8,* 2053.

Robillard, G., and Shulman, R. G. (1972), *J. Mol. Biol. 71,* 507.

Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., and Blow, D. M. (1974), *Biochemistry 13,* 4212.

Tanford, C., and Roxby, R. (1972), *Biochemistry 11,* 2192.