Identification and pK_a Determination of the Histidine Residues of Human Low-Molecular-Weight Phosphotyrosyl Protein Phosphatases: A Convenient Approach Using an MLEV-17 Spectral Editing Scheme[†,‡]

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Received March 26, 1993; Revised Manuscript Received May 20, 1993

ABSTRACT: A useful approach using an MLEV-17 pulse sequence was developed to identify histidine $C^{\epsilon 1}H$ magnetic resonances of proteins. This technique can be readily applied to proteins dissolved directly in deuterium oxide solution and eliminates the necessity for an exhaustive exchange of NH to ND. Because of its sensitivity, this technique makes it possible to significantly extend the limitations on protein size. The utility of this spin-lock sequence is demonstrated using ribonuclease, subtilisin, and human prostatic acid phosphatase, with molecular weights ranging from 12K to 100K. With this technique, all three or four of the histidine ^1H NMR signals of two human low-molecular-weight phosphotyrosyl protein phosphatases (HCPTP-A or -B, respectively) were readily detected. Histidine peak assignments were accomplished through the use of histidine to alanine mutants of HCPTP-A and -B and a homologous bovine enzyme. Analysis of the pH titration curves of these signals provided microscopic pK_a's for the histidines in the human enzymes. A comparison of corresponding histidine pK_a values of the two isoenzymes, together with an examination of the ^1H NMR spectra of the proteins, provided evidence of significant differences in secondary structure. Titration of HCPTP-A and -B with vanadate, a strongly bound competitive inhibitor, caused the His-72 peak to appear as two signals at nearly equimolar concentrations of protein and vanadate, while the other histidine peaks were not affected. This is interpreted to mean that His-72 is at the enzyme active site.

Protein tyrosine phosphorylation is recognized as an important mechanism in regulating cell growth, proliferation, and transformation (Hunter, 1987; Fischer et al., 1991). Protein kinases and protein phosphatases act to modulate the state of this phosphorylation. In addition to membrane-associated, receptor-linked protein tyrosine phosphatases (PTPases),[1] it appears that another group of PTPases, namely, the low-molecular-weight cytoplasmic phosphotyrosyl protein phosphatases, may also act to mediate intracellular phosphotyrosyl protein dephosphorylation and thus provide intracellular signaling transduction pathways that control diverse cellular processes. The 18-kDa phosphotyrosyl protein phosphatases are widely distributed in mammalian tissues and have been isolated from human red cell (Boivin et al., 1987), human placenta (Waheed et al., 1988), rat brain (Oakada et al., 1986), and bovine liver and heart (Zhang & Van Etten, 1990; Chernoff & Li, 1985). These enzymes have been shown to readily hydrolyze substrates that include several phosphotyrosyl (but not phosphoseryl or phosphothreonyl) peptides and proteins, angiotensin, autophosphorylated tyrosine kinase P^{40}, and erythrocyte band 3 protein (Zhang & Van Etten, 1990; Waheed et al., 1988; Chernoff & Li, 1985).

Recently, we described the sequencing, cloning, and expression of two human phosphotyrosyl protein phosphatases (HCPTP-A and -B) (Wo et al., 1992a). That study concluded that the HCPTP-A and -B isoenzymes are the fast and slow forms of "red cell" acid phosphatase, respectively, and more importantly that this enzyme is not unique to the red cell but is instead expressed in all major human organs, including brain (Wo et al., 1992a). Human red cell acid phosphatase is an important genetic marker (Sokal et al, 1991). The enzyme is genetically polymorphic (Hopkinson et al., 1963) and is encoded by the ACP1 locus on chromosome 2 (Ferguson-Smith et al., 1973). However, very little is known about its intracellular substrates, physiological activity, three-dimensional structure, or genetic regulation.

Many of the kinetic and mechanistic features of the homologous bovine heart phosphotyrosyl protein phosphatase (BHPTP) have been carefully characterized (Wo et al., 1992b; Zhang & Van Etten, 1991). Early reports of chemical modification and pH dependence studies indicated that one or more cysteine and histidine residues are located at the active site and may be crucial to the catalytic function (Taga & Van Etten, 1982; Laidler et al., 1982; Waheed et al., 1988; Camici et al., 1989; Zhang, 1990). Stoichiometric trapping and study of the kinetically-competent, covalent phosphoenzyme intermediate using ^{31}P NMR established that a cysteine residue acts as an enzymatic nucleophile (Wo et al., 1992b). Alignment of the amino acid sequences of the human enzymes HCPTP-A and -B with that of BHPTP revealed extensive identities. Specifically, the sequences of HCPTP-A and -B are homologous except in the region of residues 40–73, where roughly half of the residues are different. The bovine heart enzyme contains only two histidines, whereas HCPTP-A has three histidines and HCPTP-B has four histidines. Furthermore, all eight cysteine and two histidine

[†] This work was supported by USDHHS Research Grant GM 27003 and by AIDS Research Center Core Support Grant AI 27713.

[‡] The nucleotide sequences reported in this paper have been submitted to GenBank as updates to Accession Numbers M83653 and M83654.

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[1] Abbreviations: PTPases, phosphotyrosyl protein phosphatases; HCPTP-A and -B, two isoenzymes of the low-molecular-weight, human phosphotyrosyl protein phosphatase; BHPTP, a homologous bovine phosphotyrosyl protein phosphatase; pNPP, *p*-nitrophenyl phosphate; HPAP, human prostatic acid phosphatase; DSS, dimethylsilapentanesulfonate.

0006-2960/93/0432-8479$04.00/0

residues of BHPTP are conserved in the two human isoenzymes. These two conserved histidines, i.e., His-66 and His-72, and an additional His-69 in HCPTP-B are located in the 40–73 region. Thus, the possible location of at least one histidine at the active site, plus the possibility that the various additional histidines might serve as spectral probes for studies of various regions of the isoenzymes including the unusual "variable" region (Wo et al., 1992a), all made it of interest to explore the role of the histidine residues in these related enzyme forms. Knowledge of the pK_a values of the histidine residues appeared to be important to fully understand the chemical nature of the active site of the enzyme and its catalytic mechanism.

^{1}H NMR spectroscopy has long been recognized as a powerful method to investigate the ionization of histidine residues in proteins (Markley & Ibanez, 1978; Perutz et al., 1985; Forman-Kay et al., 1992). However, one of the most difficult problems in observing histidine C$^{\epsilon 1}$H resonance in the ^{1}H NMR spectra of a protein results from the severe overlap of unexchanged N–H resonances in the same region.[2] In order to minimize this problem, the protein usually must be incubated in deuterium oxide solution at high pH and/or high temperature over a long period of time. This procedure often results in significant losses of enzyme activity, especially in the case of proteases. For example, we found that overnight incubations at high pH resulted in substantial losses of enzyme activity for both HCPTP-A and HCPTP-B. Furthermore, alkaline treatment over a long period of time poses a significant danger in that the histidine C$^{\epsilon 1}$H may be exchanged to C$^{\epsilon 1}$D, resulting in a loss of some (or most) of the corresponding histidine signal from the proton NMR spectrum. In addition to exhaustive exchange procedures, the problem of overlap in the ^{1}H NMR spectra can also be minimized to some extent either by resolution-enhancement techniques using a variety of window functions (De Marco & Wüthrich, 1976; Gûeron, 1978) or by using the Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence (Carr & Purcell, 1954; Meiboom & Gill, 1958). However, without extensive incubation in D_2O, neither method is successful in applications involving large proteins (Sadler & Tucker, 1992; Jordan et al., 1985).

Here, we describe a useful approach to the detection of the histidine C$^{\epsilon 1}$H resonances of protein by using a MLEV-17 spectral editing scheme together with protein samples that either are directly dissolved in deuterium oxide solution or have the solvent exchanged merely by using a short gel filtration column. This approach eliminates the potentially severe consequences that result from the harsh treatments during extensive solvent exchange. This technique can also be readily applied to even large-sized proteins. The results of spectral studies with several different sizes of proteins are described here. This technique made it possible for us to investigate the histidine residues of HCPTP-A and -B, and to identify a probable active site histidine residue by using vanadate ion, a strong competitive inhibitor of such phosphatases (Van Etten et al., 1974).

MATERIALS AND METHODS

Materials. The pET vectors were from Novagen, and restriction enzymes were from New England Biolabs. Subtilisin Carlsberg was purchased from the Sigma Chemical Co., and ribonuclease A was obtained from United States Biochemical Co. Both proteins were used without further purification. Deuterium oxide, deuterium chloride, and sodium deuteroxide were obtained from Cambridge Isotope Laboratories. The pH measurements were done on a Corning Model 130 pH meter employing an Ingold slim-body combination electrode. The reported pH values are glass electrode readings with no correction for isotope effects (i.e., pH*).

Site-Directed Mutagenesis. Mutant proteins were generated at the DNA level by changing specific nucleotide bases. Using a pET-11d plasmid containing the sequence for wild-type HCPTP-A or HCPTP-B as a template (Wo et al., 1992a), a polymerase chain reaction (Kleppe et al., 1971) methodology was used to generate a mutagenic insert DNA (Innis et al., 1990). Because HCPTP-A and HCPTP-B contain identical nucleotide sequences in the region of interest, only two mutagenic primers were necessary to make the H157A mutants: 5′-GGAGAAGGCCGCCTGAGGC-3′ and its complement. This set of primers changes the CAC histidine codon to the GCC alanine codon. The following primers which occur at the 5′- and 3′-end of the insert DNA, respectively, were also used: 697C = 5′-GGACCATGGCGGAACAG-GCTACCAAGTCCG-3′ and 698C = 5′-GAAATGCAG-GATCCTCAGG-3′. Amplifications were performed on an Ericomp Single Block System instrument. In a total volume of 100 μL, 0.1 μg of template DNA was mixed with 100 pmol each of forward and reverse primer, 0.2 mM dNTPs, 10 μL of the (10×) buffer supplied by the manufacturer, and 2 units of Vent polymerase (New England Biolabs). Denaturation was carried out at 94 °C for 30 s, annealing at 45 °C for 1 min, and polymerization at 72 °C for 1.5 min. Altogether 25 cycles were performed, with the last polymerization step lasting 10 min. Two separate amplifications were required to generate the complete 544 bp mutagenic insert. For the first amplification, plasmid DNA containing the wild-type insert was used as the template DNA. In general, one tube contained the mutagenic forward primer plus 698C, which is the complement to the 3′-end of the insert HCPTP and contains a *Bam*H1 restriction site. A separate tube contained the reverse mutagenic primer plus 697C, which is identical to the 5′-end of the insert HCPTP and contains an *Nco*I restriction site. After amplification, the PCR products were purified by electroelution from a 1.2% agarose gel. For the second amplification, the two mutagenic fragments were combined along with the 697C and 698C primers to produce the 544 bp insert DNA. The insert DNA (0.5 μg) was next digested with *Nco*I and *Bam*H1, followed by ligation to pET-11d plasmid which had also been digested with *Nco*I and *Bam*H1. The ligation mix was then used for transformation of *Escherichia coli* DH5α. Individual colonies were selected for DNA sequencing analysis to confirm the presence of the desired mutation and absence of any others. Double-stranded DNA sequencing was performed on isolated plasmids using the Sanger dideoxy chain termination method according to the United States Biochemical protocol for Sequenase Version 2. For protein expression, the mutagenic plasmids were transformed to *E. coli* HMS174(DE3).

Preparation of Protein Samples. For the case of commercial proteins, samples were prepared for NMR experiments by simply dissolving the dry proteins in 0.6 mL of D_2O solution containing salt as subsequently described and then adjusting the pH* to the desired value with small aliquots of either 0.1 M DCl or 0.1 M NaOD. Typically protein concentrations of 1–2 mM were used in the NMR experiments.

Human prostatic acid phosphatase (HPAP) was purified to homogeneity by affinity chromatography as described previously (Van Etten & Saini, 1978; Duncan et al., 1984). The purified protein (about 12 mg) was loaded onto a G-25

[2] The C$^{\epsilon 1}$H is often termed the C-2 proton. The nomenclature is described in IUPAC–IUB Commission on Biochemical Nomenclature (1970).

size exclusion column (1.5 × 4 cm) preequilibrated with 150 mM NaCl in D_2O. Fractions with enzyme activity were combined and concentrated to about 0.6 mL using a Filtron microconcentrator. Then the pH* was adjusted to 4.8 for NMR measurements.

Expression and purification of HCPTP-A and -B, and of the corresponding histidine mutant enzymes, were carried out by a procedure similar to that described previously (Wo et al., 1992b). The protein samples were prepared for NMR studies using the same procedure as described above for HPAP.

Phosphatase Activity Assay. The phosphatase enzyme activity was determined at room temperature by using *p*-nitrophenyl phosphate as a substrate in pH 5.0 sodium acetate buffer (Wo et al., 1992b; McTigue, 1978). The reaction was quenched with 1 mL of 1 N NaOH. The product *p*-nitrophenoxide was quantitated by measuring the absorbance at 405 nm and using a molecular extinction coefficient of 18 000 M^{-1} cm^{-1}. The protein concentration of HCPTP-A and -B was determined by the Lowry method (Lowry et al., 1951), while the protein concentration for HPAP was determined from the absorbance at 280 nm, where A_{280} is 1.4 for 1 mg/mL of pure HPAP (Davidson, 1990).

NMR Spectroscopy. ^1H NMR experiments were conducted on a Varian VXR-500 (500-MHz ^1H) NMR spectrometer at the ambient temperature of about 20 °C. The pulse sequence designed to distinguish histidine $C^{\epsilon 1}H$ resonances from residual amide proton resonances is based on a ($90°_x$–spin-lock$_y$–acq) scheme, in which the spin-lock is generated using an MLEV-17 composite-pulse sequence consisting of a large number of 90° and 180° pulses that are phase shifted relative to one another (Bax & Davis, 1985). For proteins that are larger than approximately 100 amino acids, the transverse magnetization relaxation times ($T2$) of amide protons often become extremely short as compared to those of carbon protons (Bax, 1989). The difference becomes greater as the molecular weight of the protein increases. The MLEV-17 scheme is used to keep proton transverse magnetization along the detection direction, i.e., the y axis, for a period of time before acquiring data. During this spin-lock period the magnetization of amide protons in proteins decays rapidly as a consequence of their short $T2$ values, whereas that of the histidine $C^{\epsilon 1}H$ is preserved at the end of the MLEV-17 scheme. This MLEV-17 spin-lock time can be optimized experimentally depending on protein molecular weight. Typically for proteins of 10–100 kDa, the spin-lock time is in the range of 100–10 ms.

Suppression of the residual HDO signal was achieved by presaturating the water resonance during a relaxation delay of 2.5 s. All chemical shifts were referenced externally to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0 ppm. With protein concentrations of 1–1.5 mM, excellent spectra were obtained in 64–128 transients. The free induction decay was zero-filled to 16K points and multiplied by a Gaussian weighting function (GB = 0.10) prior to Fourier transformation.

Secondary Structure Analysis of HCPTP-A and -B. The determination of the protein secondary structure content of HCPTP-A and -B was performed by the integration of one-dimensional ^1H NMR spectra using the protocol described by Wishart and co-workers (Wishart et al., 1991). Two identical samples of the corresponding protein were prepared with equal protein concentrations of 1.0 mM in 20 mM NaOAc-d_3/99.9% D_2O and 20 mM NaOAc-d_3/90% H_2O/10% D_2O solution at pH 5.0. Standard one-dimensional ^1H NMR spectra of both samples were collected using identical conditions.

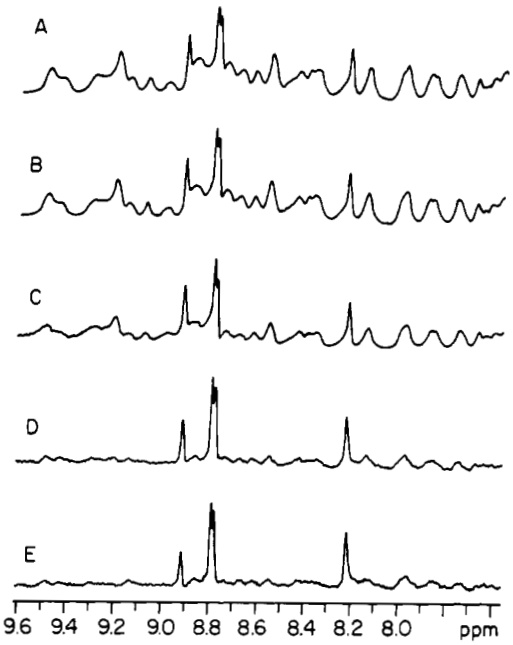

FIGURE 1: ^1H NMR spectra of 1.0 mM ribonuclease A in 150 mM NaCl/D_2O, at pH 3.2. The spectra were recorded as described in the Materials and Methods. The spin-lock times in spectra A–E were 0, 5, 20, 60, and 80 ms, respectively.

pH Titration. The pH* was measured with an Ingold slim-body combination electrode and adjusted in an Eppendorf microcentrifuge tube after removal of the sample from the NMR tube. The protein sample was titrated between pH* 5 and 10 with 2–2.5-mL aliquots of either 0.1 M DCl or 0.1 M NaOD, and NMR spectra were recorded every 0.1–0.2 pH unit. The pH* was measured before and after each NMR spectrum was acquired, and the values usually agreed to within ±0.03 pH unit. The variation of chemical shift of a histidine resonance δ_{obs} with pH was fitted to $\delta_{obs} = \delta_A + \{(\delta_{AH} - \delta_A) \times ([H^+]/(K_a + [H^+]))\}$, where K_a is the dissociation constant, δ_A is the chemical shift of the resonance of the unprotonated histidine, δ_{AH} is the chemical shift of the resonance of the protonated histidine, and $[H^+]$ is calculated from pH*. Experimental data were fitted to this equation using ENZFITTER, an nonlinear least-squares data analysis program (Leatherbarrow, 1987).

Vanadate Titration. Vanadate titration was carried out by adding 10–20-μL aliquots of 1–10 mM sodium orthovanadate (Na_3VO_4) solution directly to the protein sample in 150 mM NaOAc-d_3/D_2O buffer at pH 5.5 in the NMR tube, and then measuring ^1H NMR spectra. The pH of the vanadate solution was initially adjusted (using NaOD or DCl) to be the same as the enzyme solution. The pH of the protein samples was measured both before and after completion of the titration to ensure that any observed effects in the spectrum were not due to changes in pH.

RESULTS

Application of the MLEV-17 Scheme to the Spectral Identification of Histidine. Figure 1 demonstrates the use of the spin-lock pulse sequence to identify the histidine residues of a small (12-kDa) standard protein, ribonuclease A. Histidine assignments are known from early 2D ^1H NMR study [Rico, et al. (1989) and references cited therein]. As expected, a large number of unexchanged amide N–H's were still present in the protein after the simple solvent exchange, and the histidine peaks could not be identified unambiguously due to severely overlapping spectral signals. However, as the spin-lock time is increased, these amide proton signals decrease

rapidly as compared to those of histidine residues, and the four histidine peaks could be readily distinguished upon use of an easily optimized spin-lock time (Figure 1E).

Subtilisin Carlsberg, a 27-kDa serine protease, has five histidine residues. Earlier studies showed that it was very difficult to observe one of the five histidine peaks in the ^{1}H NMR spectrum even after extensive alkaline exchange of N–H to N–D over a 16-h period at 28 °C (Jordan et al., 1985). It was only possible to detect the missing histidine C$^{\epsilon 1}$H peak using a spin-echo pulse sequence (Bycroft & Fersht, 1988). By using the present technique, however, all five histidine resonances could be readily identified in the ^{1}H NMR spectrum using a spin-lock time of 30 ms. The sensitivity of this spin-lock technique is significantly higher than that obtained earlier with the spin-echo sequence, under otherwise similar experimental conditions. A comparison of the chemical shifts of the five histidine peaks with those observed by Jordan et al. (1985) under the same conditions suggests that histidine peak 2 in the ^{1}H NMR spectra is probably the one that was missing from the earlier published spectra. The five histidine peaks were well resolved, and pH titration experiments were easily performed from pH* 5 to pH* 8.6. The pK_a values obtained by fitting the titration data for peaks 1–5 were 7.31, 7.09, 6.97, 6.71, and 6.42, respectively, which are in excellent agreement with the values obtained by Jordan et al. (1985).[3]

Study of Human Prostatic Acid Phosphatase. Human prostatic acid phosphatase, a dimeric 100-kDa protein that has a total of 13 histidine residues per subunit (Van Etten et al., 1991), was also studied using this new technique. For large proteins such as this one, it is inevitable that some of histidine resonances have degenerate chemical shifts at certain pH conditions. However, at different pH conditions all 13 histidine peaks of human prostatic acid phosphatase could be detected. (Unfortunately, because of an apparently substantial structural change that was observed to occur in the region of pH 7, it was not possible to complete the pH titrations of the histidine residues of this protein.) One of the proton NMR spectra at pH 4.8 is shown in Figure 2A. The transverse magnetization relaxation time of amide proton becomes extremely short for large proteins of this size so that sharply improved spectra can be obtained by using very short spin-lock times, such as 5 ms in this case. The flat base line and well-resolved histidine peaks in the ^{1}H NMR spectrum made it feasible to attempt to distinguish an active site histidine residue (Van Etten et al., 1991) by using L-(+)-tartrate, a strong competitive inhibitor (K_i = 20 μM at pH 5.0). As shown in Figure 2, addition of noninhibitory D-(−)-tartrate to the enzyme solution did not significantly perturb the chemical shifts of any of the histidine peaks, whereas a subsequent addition of the inhibitory L-(+)-tartrate clearly caused one histidine peak to be shifted from 8.10 to 8.05 ppm.

Assignment and pK_a Determination of Histidine Residues of HCPTPases. Given the preceding demonstrations of the utility of the method, it was applied to a study of the histidine residues of two human cytoplasmic phosphotyrosyl protein phosphatases HCPTP-A and -B (Wo et al., 1992a). Figure 3 shows ^{1}H NMR spectra of HCPTP-A and HCPTP-B. All three of the histidine peaks of HCPTP-A, and all four of the histidine peaks of HCPTP-B, were readily detected using this spin-lock sequence, whereas in the conventional 1D spectra

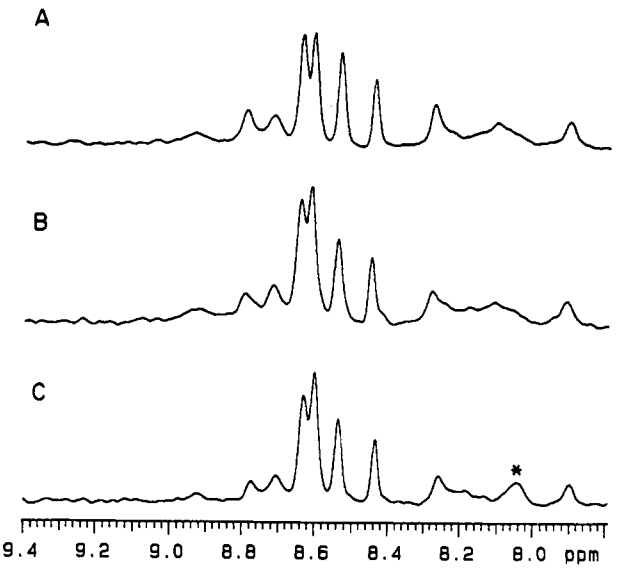

FIGURE 2: ^{1}H NMR spectra of 0.2 mM human prostatic acid phosphatase: (A) in 150 mM NaCl/D_2O at pH 4.8; (B) following the addition of 2 mM D-(−)-tartrate; (C) following the subsequent addition of 2 mM L-(+)-tartrate. The spin-lock time was 5 ms.

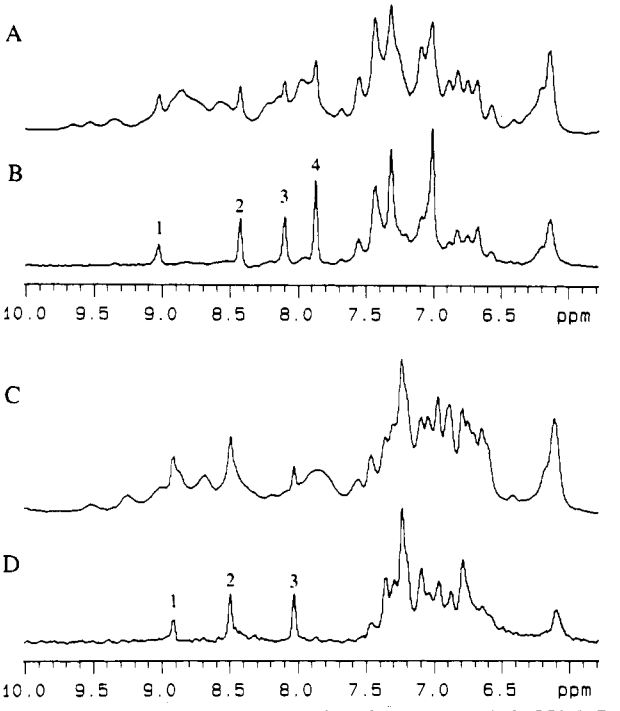

FIGURE 3: ^{1}H NMR spectra of HCPTP-A and HCPTP-B. HCPTP-B at pH 6.5 with spectra acquired by (A) a conventional single 90° pulse sequence; (B) the spin-lock pulse sequence. HCPTP-A at pH 5.5 with spectra obtained by (C) a conventional single 90° pulse sequence; (D) the spin-lock pulse sequence. Spectra were obtained in 150 mM NaCl/D_2O with a spin-lock time of 30 ms and a protein concentration of 1 mM.

many of these histidine peaks were severely overlapped by the unexchanged NH peaks.

Alignment of the amino acid sequences for HCPTP-A and HCPTP-B with that of the bovine analog BHPTP (Wo et al., 1992b) reveals extensive identities (81% and 94%, respectively) as shown in Figure 4. In particular, two histidine residues (His-66 and His-72) are conserved in all three enzymes. The secondary structure contents due to helix, coil, and β-sheet in the three enzymes were assessed by integration of selected portions of 1D ^{1}H NMR spectra of the corresponding enzymes obtained in D_2O and H_2O solution (Wishart et al., 1991). As might be expected from the sequence similarities, the results

[3] A few small peaks shown in the spectra (data not shown), also underwent pH-dependent chemical shift changes characteristic of histidine resides. These small peaks are likely due to a few peptide fragments resulting from autolysis of the protease itself, since a commercial protein preparation was used for the NMR measurements, without further purification.

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HCPTP-A   AEQATKSVLFVCLGNICRSPIAEAVFRKLVTDQNISENWR
HCPTP-B   ---A-----------------------------------V
BHPTP     ---V-------------------------------D--V

                                      66 69 72
HCPTP-A   VDSAATSGYEIGNPPDYRGQSCMKRHGIPMSHVARQITKE
HCPTP-B   I--G-V-DWNV-RS--P-AV--LRN---HTA-K---I---
BHPTP     I--G-V-DWNV-RS--P-AV--LRN---NTA-K---V---

HCPTP-A   DFATFDYILCMDESNLRDLNRKSNQVKTCKAKIELLGSYD
HCPTP-B   --A------------------------T-------------
BHPTP     --V------------------------N-R-----------

                                                 157
HCPTP-A   PQKQLIIEDPYYGNDSDFETVYQQCVRCCRAFLEKAH
HCPTP-B   ----------------------------------AH
BHPTP     --------------A-------------------VR
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FIGURE 4: Amino acid sequence alignment of HCPTP-A and -B and BHPTP (Wo et al., 1992a,b).

Table I: Secondary Structure Content Predictions of HCPTP-A and -B As Estimated by ^{1}H NMR Spectroscopy[a]

enzyme	α (%)	β (%)	coil (%)
BHPTP	51	22	27
HCPTP-B	50	22	28
HCPTP-A	36	35	29

[a] Experimental conditions: 20 mM NaOAc-d_3 buffer at pH 5.0.

indicate that HCPTP-B is almost identical to BHPTP (Table I). Consistent with this, the chemical shifts and pK_a values of peaks 1 and 2 of HCPTP-B were almost the same as those of His-72 and His-66 of BHPTP, respectively. Assignment of the His-66 and His-72 ^{1}H NMR peaks of HCPTP-B was accomplished by comparison with the corresponding histidine to alanine mutants of BHPTP (Davis and Van Etten, unpublished results). Thus, peaks 1 and 2 of HCPTP-B were assigned to His-72 and His-66, respectively.

His157Ala mutants of HCPTP-A and HCPTP-B were prepared in this study in order to make assignments of the His-157 peaks. Figure 5 shows ^{1}H NMR spectra of HCPTP-A and -B H157A mutants at pH 5.5 in comparison to those of the corresponding wild-type proteins HCPTP-A and HCPTP-B. The chemical shifts of the remaining histidine peaks in the spectra of both H157A mutants were almost identical to those of the wild-type enzymes, indicating that the three-dimensional structures of the enzymes were not disturbed by the mutation. Furthermore, kinetic measurements (K_m and V_{max}) showed that the mutation had only minor effects on the catalytic activity (Table II). Therefore, peak 3 of HCPTP-A and -B was assigned to His-157. Given the identification of His-66, His-72, and His-157 for HCPTP-B, peak 4 was then assigned to His-69 of this enzyme.

Peak 1 exhibited an essentially identical pK_a value as well as chemical shift for both HCPTP-A and -B. Assignment of His-72 of HCPTP-B was thus considered applicable to HCPTP-A. (This tentative assignment was later confirmed by the study of vanadate concentration titration with the protein.) Peak 2 in the spectrum of HCPTP-A was then assigned to His-66.

A plot of the data showing the chemical shifts as a function of pH is displayed in Figure 6 for HCPTP-A and -B, from which the pK_a values were determined as shown in Table III. In the case of His-157 of HCPTP-A, since there is a chemical shift perturbation below pH 5.5–6, the pK_a of this His-157 was estimated using the data obtained at pH 6 and above.

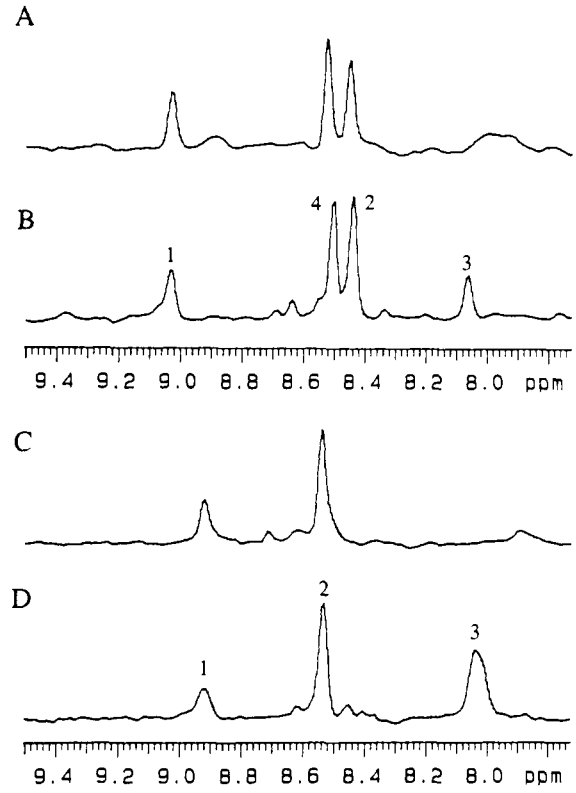

FIGURE 5: ^{1}H NMR spectra of (A) HCPTP-B_157A mutant and (B) the corresponding wild-type enzyme; (C) HCPTP-A_H157A and (D) the corresponding wild-type enzyme. Spectra were obtained in 150 mM NaCl/D_2O at pH 5.5 with a protein concentration of 1 mM and a spin-lock time of 30 ms.

Table II: Kinetic Parameters of HCPTP-A and -B Isoenzymes

enzyme	K_m[a] (mM)	V_{max}[a] [μmol/(min·mg)]	vanadata K_i[b] (μM)
HCPTP-B	0.61	95.1	36.8
HCPTP-B_H157A	0.84	103.7	
HCPTP-A	0.14	60.3	24.6
HCPTP-A_H157A	0.37	59.4	

[a] The assay was performed using *p*-nitrophenyl phosphate as a substrate in a buffer containing 100 mM sodium acetate and 1 mM EDTA at pH 5.0, 37 °C, I = 0.15. [b] The vanadate inhibition data fitted the pattern expected for competitive inhibition; assay conditions were similar to those described above except that EDTA was omitted.

Search for an Active Site Histidine. Vanadate ion is an important inhibitor of such phosphatases (Van Etten et al., 1974; Gordon, 1991). Vanadate inhibition studies with HCPTP-A and -B shows that it is a competitive inhibitor and its inhibitory effect toward HCPTP-A is somewhat higher than that of HCPTP-B (Table II). Addition of small concentrations of vanadate to solutions of HCPTP-A resulted in a typical protein–ligand titration pattern. As the vanadate concentration increased, the intensity of peak 1 decreased, while a new peak appeared 0.04 ppm downfield (Figure 7). A similar phenomenon was observed for both HCPTP-B and BHPTP (data not shown). However, the split peaks for HCPTP-B at equal concentration of protein and vanadate were not as sharp as these shown for HCPTP-A, which is consistent with their corresponding inhibition constants. This vanadate titration result indicates that the chemical shift of His-72 is directly affected by interaction with the inhibitor and that it is probably at the active site of the enzyme.

DISCUSSION

We have demonstrated a useful approach using a spin-lock MLEV-17 pulse sequence to identify histidine C$^{\epsilon 1}$H peaks of

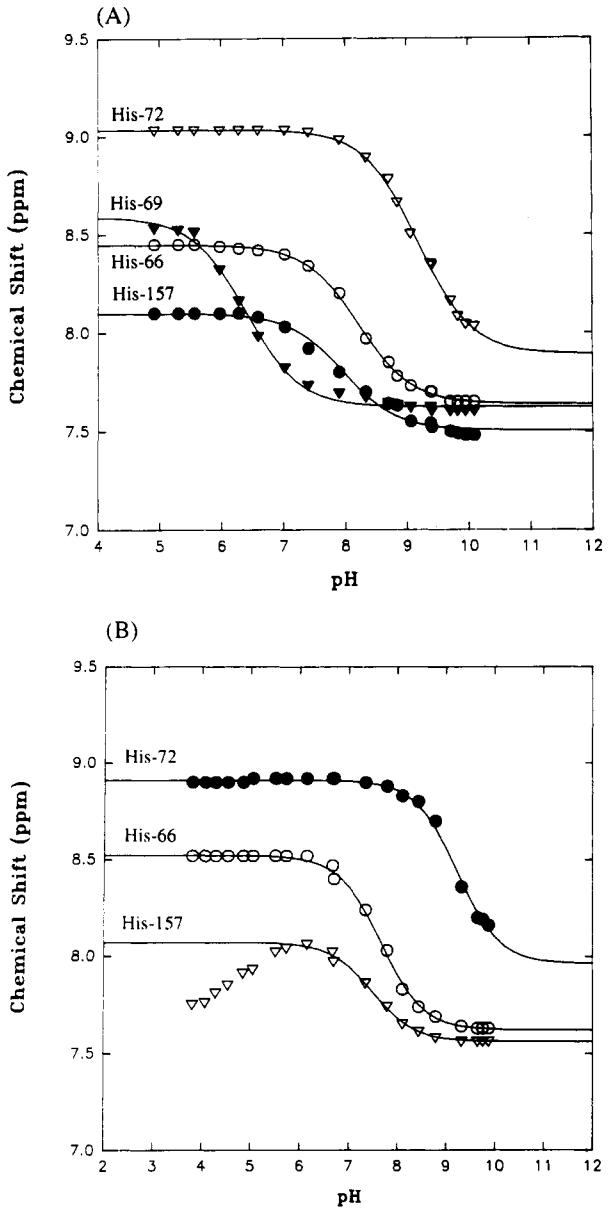

FIGURE 6: ^{1}H NMR titration curves of the histidine C$^{\epsilon 1}$H's of (A) HCPTP-B and (B) HCPTP-A. The solid lines represent the curves calculated using the parameters of Table III. The experimental points correspond to (A) HCPTP-B: (○) His-66, pK_a = 8.22; (▼) His-69, pK_a = 6.40; (▽) His-72, pK_a = 9.18; (●) His-157, pK_a = 7.72; (B) HCPTP-A: (○) His-66, pK_a = 7.67; (●) His-72, pK_a = 9.23; (▽) His-157, pK_a = 7.49.

proteins without the necessity for an exhaustive exchange of NH to ND. In proteins, protons that are either buried inside a hydrophobic core, involved in hydrogen bonding interactions, or otherwise inaccessible to the solvent generally relax much faster than those that are located on the protein surface and exposed to the solvent (Itzhaki et al., 1991; Bax, 1989). In addition, protons bound to nitrogen in a protein relax mainly through quadrupolar relaxation mechanism, while the carbon-bound protons relax by a dipolar relaxation mechanism. Thus, any unexchanged amide protons that may remain after a brief solvent exchange usually have very short relaxation times. This intrinsic difference in magnetization relaxation rates provides the basis of the present approach. Furthermore, because the difference in relaxation times between NH and C$^{\epsilon 1}$H in this spin-lock sequence is 2 times longer than that in the spin-echo sequence, the signal intensity obtained using this sequence is increased by a factor of 2. Thus, this technique also makes it possible to significantly extend the limitation on protein size.

Table III: ^{1}H NMR Chemical Shifts and pK_a Values for the Histidine Residues of HCPTP-A and -B Isoenzymes[a]

histidine	d_{HA}[b]	d_A[b]	pK_a
HCPTP-B			
His-66	8.45	7.64	8.22 ± 0.05
His-69	8.58	7.62	6.40 ± 0.05
His-72	9.04	7.89	9.18 ± 0.05
His-157	8.10	7.50	7.72 ± 0.05
HCPTP-A			
His-66	8.52	7.62	7.67 ± 0.05
His-72	8.91	7.97	9.23 ± 0.05
His-157	8.07[c]	7.56	7.49 ± 0.05

[a] Experimental conditions were 150 mM NaCl/D_2O, at ambient (~22 °C) temperature and varying pH, where pH is the direct meter reading uncorrected for D_2O. [b] Chemical shifts are for C$^{\epsilon 1}$H's. d_{HA} and d_A represent the chemical shifts for the protonated and unprotonated forms of the histidine residue, respectively. [c] Determined using titration data between pH 6 and pH 10.

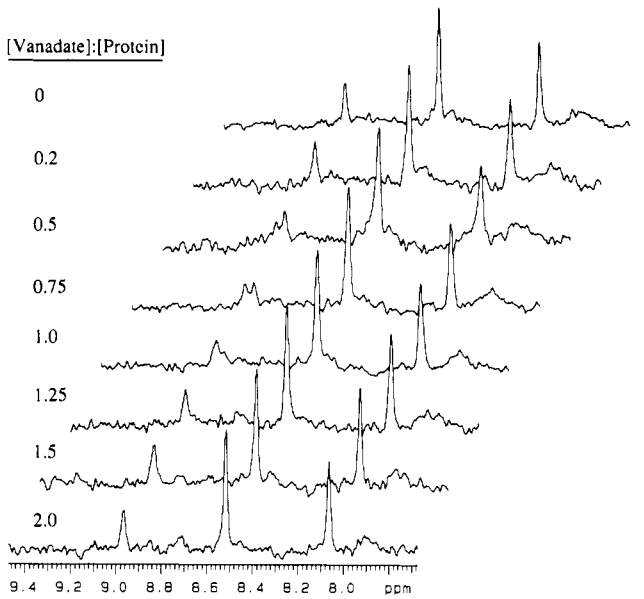

FIGURE 7: ^{1}H NMR spectra of the HCPTP-A histidine peaks shown as a function of successive additions of vanadate at pH 5.5. The spectra were obtained in 150 mM NaOAc-d_3/D_2O buffer at a protein concentration of 0.3 mM and a spin-lock time of 30 ms. The free induction decay was zero-filled to 32K points and multiplied by a Gaussian weighting function (GB = 0.10) prior to Fourier transformation.

The proton NMR spectra of ribonuclease A clearly demonstrates this new approach to detect histidine resides in proteins (Figure 1). Although this pulse sequence effectively eliminates peaks due to unexchanged amide protons, there are several reasons why the NMR spectra should still be recorded using deuterium oxide solvent. We found that a ribonuclease A sample in H_2O showed a few amide proton resonances with relaxation times that were approximately comparable to those of the histidine C$^{\epsilon 1}$H resonances (data not shown). These amide protons are likely to be ones that are located in mobile regions of the protein. Furthermore, the water signal dynamic range problem in the ^{1}H NMR spectra can also be minimized by using deuterium oxide solution.

The extreme conditions of high pH and elevated temperature used to fully exchange N–H to N–D often results in enzyme denaturation and significant losses of enzyme activity. This was the case in a study of subtilisin Carlsberg, where the recovery of the protein after extensive solvent exchange was only 20–30% (Jordan et al., 1985). Even vigorous exchange procedures cannot always guarantee complete exchange. A small percentage of remaining N–H groups in large proteins

can still cause severe overlap problems in the histidine resonance region, especially in pH titration experiments when the histidine resonances tend to broaden somewhat in the transition region of the titration. Deuterium exchange of the histidine $C^{\epsilon 1}H$ itself is usually relatively slow, with half-times of several days at pH* 5.0 and 40 °C, as was observed for two histidine residues in native soybean trypsin inhibitor (Markley & Kato, 1975). This exchange rate is also structure- and conformation-dependent. For some proteins, especially under more extreme conditions of high pH and elevated temperature, the exchange rate can be relatively rapid. This may have been the case for subtilisin Carlsberg, thus resulting in low signal intensity for one of the histidines. Such problems are virtually eliminated by the present approach.

Human prostatic acid phosphatase is a dimeric 100-kDa glycoprotein. Phosphoenzyme trapping experiments have shown that an active site histidine acts as a nucleophile (Van Etten, 1982). More recent studies on this enzyme have identified other essential active site residues, including several arginine residues (Van Etten et al., 1991). Little is known about the three-dimensional structure, including the active site. For such a large protein, it is extremely difficult to fully exchange N–H to N–D without sacrificing significant amounts of active enzyme. The MLEV-17 approach appears applicable even to such large proteins, and it can be readily applied with only minor adjustment of the spin-lock time in the pulse sequence. The new technique enabled us to detect all 13 histidine residues in the protein.

The present technique provided a further opportunity to study the active site of human prostatic acid phosphatase. Identification of a putative active site histidine residue was achieved by using the strong competitive inhibitor L-(+)-tartrate. The only observed chemical shift perturbation of a histidine resonance is at 8.10 ppm in the spectrum, suggesting that this histidine peak may be an active site histidine that is affected by the binding of L-(+)-tartrate. However, an unequivocal identification of such histidine residues in proteins should rely not only on the detection of a ^1H NMR signal but also on its pH-dependent chemical shift change pattern. Alternatively, histidine mutants may also be used to confirm the assignment. Since the correct cDNA and protein sequences of this protein are now available, such experiments are feasible (Van Etten et al., 1991).

Because the preceding experiments provided convincing demonstrations of the utility of the method, it was applied to a new problem, the study of human phosphotyrosyl protein phosphatases HCPTP-A and -B. An initial assessment of the secondary structure using ^1H NMR techniques revealed that the structure of HCPTP-B is essentially identical to that of BHPTP, which is in a good agreement with the kinetic analysis of these two enzymes (Wo et al., 1992a,b). A significant difference was observed between the secondary structure of HCPTP-A and -B (Table I). This may reflect differences in physiological functions of the two isoenzymes, since significant differences in substrate specificity are observed for the two human isoenzymes (Pokalsky and Van Etten, unpublished results).

Vanadate and related early transition-metal oxoanions have been shown to possess broad potential as transition-state analogs of phospho-transfer reactions, including those catalyzed by acid phosphatases (Van Etten et al., 1974; Van Etten, 1982). Vanadate has found wide use as an inhibitor of protein phosphotyrosine phosphatases (Lau et al., 1989; Swarup et al., 1982; Leis & Kaplan, 1982). It was demonstrated that vanadate was able to transform NRK-1 cells (Klarlund, 1985), and the transformation of the cells by vanadate was accompanied by an increase in the phosphotyrosyl content, which might be the result of decreased phosphatase activities as well as from increased tyrosine kinase activities. Here, using ^1H NMR spectroscopy, we illustrate the specific interaction of vanadate with HCPTP-A and -B. Vanadate ions have a tendency to polymerize in aqueous solution, and this polymerization is concentration- and pH-dependent [Gordon (1991) and references therein]. However, at the dilute concentrations required for inhibition for the human isoenzymes, the polymerization of vanadate is not significant. As shown in Figure 7, the His-72 signal was split into two almost equally intense peaks when the molar ratio of vanadate to protein was only 0.75, which is also consistent with the conclusion that a monomeric form of vanadate is interacting with the enzyme active site. At saturating concentrations of vanadate, the chemical shift change observed for His-72 in the spectra of both HCPTP-A and -B is a modest downfield shift of about 0.04 ppm. Chemical shift changes of a similar magnitude were also observed for the "basic patch" histidine residues in yeast phosphoglycerate kinase upon binding of the triose substrate, 3-phosphoglycerate (Sherman et al., 1992; Fairbrother et al., 1989). In those cases, the chemical shift changes ranged from 0.03 to 0.5 ppm for several histidines in the wild-type and mutant enzymes. It has been noted that up to 75% of the shifts observed upon binding of an inhibitor are due to an increase of the corresponding histidine pK_a (Markley, 1975). In the present case, the relatively small downfield shift observed for His-72 may be related to its unusually high pK_a in both enzymes, because then the binding of vanadate to the enzyme active site would not result in any substantial increase of the histidine pK_a, but could instead just cause a minor change in the local environment. The limited nature of any such change is also consistent with the constant chemical shift of His-66 throughout the pH range of 4–6.

The observation of two His-72 signals in the vanadate titration demonstrates the relatively tight binding in the protein–inhibitor complex. The dissociation constant estimated from the NMR spectra is in the micromolar concentration range, which is consistent with kinetically-determined K_i values. The exchange rate is apparently slower than NMR time scale. These observations are all consistent with the interpretation that His-72 is at the enzyme active site. The properties of His-72 in HCPTP-A and -B in terms of chemical shifts, pK_a values, and interaction with vanadate are very similar, consistent with the expectation that the two isoenzymes possess similar catalytic mechanisms. The relatively high pK_a of His-72 suggests that this histidine residue may be associated in an interaction with another active site residue, such as a negatively charged aspartate or glutamate residue. The results also do not rule out the possibility of a His^+/Cys^- ion pair structure that was also considered on the basis of chemical modification and pH-dependence studies of BHPTP (Wo et al., 1992b).

Unlike the situation with His-72, the pK_a values of His-66 are clearly different in the two isoenzymes. Although the overall homology between the two isoenzymes is generally high, there are distinct differences in the central region where these histidines are located. Some of the corresponding amino acid residue substitutions are not conservative. This lack of sequence homology presumably accounts for the different pK_a's of His-66 in the two isoenzymes.

The additional His-69 residue in HCPTP-B shows a substantially different pK_a that is even lower than the one measured for the C-terminal His-157. In fact, this pK_a value is very close to that typically measured for free histidine. Furthermore, the proton resonance of this histidine is quite

sharp and intense as compared to that of His-72, for example. These properties of His-69 suggest that this histidine is located on the surface of the protein and is not involved in any tight interaction with other residues.

The near identity of kinetic properties and ^{1}H NMR spectra for H157A mutants of HCPTP-A and -B in comparison to the corresponding wild-type enzymes indicates that the His-157 residue does not play an important role in enzymatic catalysis or in structure stability. The pH titration curve of His-157 in HCPTP-A showed a chemical shift perturbation in the low-pH region that does not involve the ionization of the histidine residue itself, but rather seems to result from a change in an interaction between this histidine and another charged residue. An apparent pK_a of 4.8 may be estimated for this chemical shift perturbation, perhaps indicating that an aspartic acid or glutamic acid residue may form an ion pair with the protonated form of this His-157. This chemical shift perturbation is not present for HCPTP-B, further illustrating structural differences in the C-terminal region of these two enzymes.

Thus, using the MLEV-17 spin-lock pulse sequence and convenient deuterium exchange protocols, we have measured the pK_a values of the histidine residues and demonstrated structural differences between two human phosphotyrosyl phosphatase isoenzymes. This knowledge will facilitate further investigations of the catalytic function of both isoenzymes.

ACKNOWLEDGMENT

We thank Dr. Dean Carlson of the Purdue University Magnetic Resonance Laboratory and Profs. David Gorenstein and John Grutzner for advice and encouragement.

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