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Articles

Identification by Proton Nuclear Magnetic Resonance of the Histidines in Cytochrome b_5 Modified by Diethyl Pyrocarbonate[†]

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ABSTRACT: Diethyl pyrocarbonate (DEP) is an electrophilic reagent that is used to modify reversibly the histidine residues of proteins. Unfortunately, the lability of the acylated histidine adduct usually does not permit the isolation and identification of the modified histidine. By use of 500-MHz proton NMR spectroscopy, it has been possible to identify the C–H resonances of the nonaxial histidines of trypsin-solubilized bovine, rabbit, and porcine cytochrome b_5 and therefore observe the interaction of DEP with specific histidine residues of cytochrome b_5. In addition, the pK_a of the peripheral histidines of bovine and rabbit cytochrome b_5 have been measured in D_2O. In the bovine protein it was found that the histidines are modified sequentially with increasing DEP concentration in the order His-26 > His-15 > His-80. This order is maintained in the rabbit protein with the following additions: His-26 ≈ His-27 > His-15 ≥ His-17 > His-80. The relative reactivity of the peripheral histidines with DEP was rationalized by considering three of their characteristics: (1) the pK_a of the histidine, (2) the fraction of the side chain exposed to the solvent, and (3) the hydrogen-bond interactions of the imidazole ring.

Cytochrome b_5, which exists in both soluble and membrane-bound forms, is a protein that plays an essential role in a variety of electron-transfer reactions including lipid biosynthesis (Oshino, 1978), cytochrome P-450 reduction (Hildebrandt & Estabrook, 1971; Canova-Davis & Waskell, 1984; Pompon & Coon, 1984), and regeneration of ferrous hemoglobin in red blood cells (Hegesh et al., 1986). In the cytochrome P-450 reaction, cytochrome b_5 can provide the second of the two electrons required by the mixed-function oxidase for a complete reaction cycle. The volatile anesthetic methoxyflurane, as well as numerous other compounds, has been shown to require cytochrome b_5 for its metabolism by cytochrome P-450 (Canova-Davis & Waskell, 1984; Noshiro et al., 1979; Kuwahara & Omura, 1980; Sugiyama et al., 1979; Okita et al., 1981; Vatsis et al., 1982; Gruenke et al., 1988; Pompon, 1987; Lipka & Waskell, 1989). In studies designed to delineate the mechanism of the cytochrome b_5 requirement for the oxidation of selected substrates by cytochrome P-450, it was found that treatment of cytochrome b_5 with diethyl pyrocarbonate (DEP), a moderately selective histidine-modifying reagent (Miles, 1977), could reversibly inhibit electron transfer to cytochrome P-450 (Canova-Davis & Waskell, 1984; Konopka & Waskell, 1988a). Potentially, identification of the amino acid residues responsible for the ability of cytochrome b_5 to transfer electrons to cytochrome P-450 could provide information about why certain substrates require cytochrome b_5 for their metabolism. Thus, studies to determine which specific histidine residues were modified were undertaken (Konopka & Waskell, 1988a,b).

One of the major drawbacks of DEP is that the carbethoxy histidine derivative is unstable during the procedures conventionally used to isolate and sequence peptides. As a result, it is frequently impossible to identify modified histidine residues, although with selected proteins the modified histidine has been identified (Hegyi et al., 1974; Igarashi et al., 1985; Cooper et al., 1987; Miles, 1977). The proton NMR spectra of proteins provide detailed structural information and are typically obtained under conditions not expected to hydrolyze the carbethoxy histidine derivative of proteins. In this paper, we demonstrate that 500-MHz high-resolution ^{1}H NMR

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techniques can be used to determine which histidine residues of cytochrome b_5 are modified by DEP and that carbethoxylation of the histidines in bovine cytochrome b_5 is essentially sequential.

Materials and Methods

Materials. DEP and all deuterium-labeled chemicals, including 99.8% and 99.98% enriched D_2O, 50% NaOD, 1 M DCl, and (trimethylsilyl)propionic aid (TSP), were obtained from Aldrich. Mono- and dibasic potassium phosphate, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone, Tris-HCl, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride, and trypsin type I were obtained from Sigma. DEAE-cellulose (DE-52) was purchased from Whatman; Sephadex G-75 resin was from Pharmacia Chemicals.

Purification and Characterization of Cytochrome b_5. Bovine, rabbit, and porcine trypsin-solubilized cytochrome b_5 were purified by the method of Reid and Mauk (1982). Each preparation of ferric cytochrome b_5 had an $A_{413/280}$ ratio greater than or equal to 5.85 and showed a single band on SDS-polyacrylamide gels. Concentrations of the ferric protein were determined by using an absorption coefficient of 117 mM^{-1} at 413 nm (Strittmatter & Velick, 1956). The amino-terminal residues, alanine and aspartic acid for the bovine and low molecular weight rabbit (see below) proteins, respectively, were established by Edman degradation using a gas-phase protein sequencer (Model 470 A Applied Biosystems with an online Model 120A phenylthiohydantoin analyzer) (Hunkapiller et al., 1983) at the University of California, San Francisco Biomolecular Resource Center, and were in agreement with previous results (Mathews & Czerwinski, 1976). The carboxyl termini were determined by mass spectrometry in collaboration with Dr. Bradford Gibson using a Kratos MS-50 RF mass spectrometer equipped with a sensitive array detector (Cottrell & Evans, 1987). By determination of the molecular weight of the protonated molecular ion of trypsin-solubilized cytochrome b_5 whose amino terminus was known, the carboxyl terminus was deduced to be arginine 84 in both the calf and the rabbit protein. The details of this procedure will be published in a separate paper.

Rabbit cytochrome b_5, purified by the method of Reid and Mauk (1982), initially consisted of two species, one whose amino terminus was blocked and one that was shortened by six amino acids with aspartic acid as its amino terminus. The latter is referred to as the low molecular weight form of rabbit cytochrome b_5 in the previous paragraph. The two forms were resolved by repeated chromatography on Sephadex G-75. It was the high molecular weight cytochrome b_5 whose amino terminus was blocked that was used in our studies. The histidines in both forms of the protein had identical pH titration behavior.

General NMR Methods. ^1H NMR spectra were taken on a 500-MHz GE-Nicolet spectrometer interfaced to a Nicolet 1280 computer. The paramagnetic form of the proteins, ferricytochrome b_5, was used for all experiments. A spectral width of 7042.25 Hz, centered on the residual H_2O peak, was used so some folding over of paramagnetically shifted peaks could be expected to occur, but these did not interfere with the analysis of the $C^{\epsilon 1}$ (C2) histidine proton. In all cases, the digital resolution was 0.43 Hz/point, and an exponential line broadening of 1 Hz was employed. All chemical shift values are corrected for the small pH dependence of the chemical shift of the internal standard (trimethylsilyl)propionic acid (TSP) (De Marco, 1977) by using

$$\delta_{corr}[\text{ppm}] = \delta_{meas}[\text{ppm}] - 0.019(1 + 10^{5.0-\text{pH}})$$

A two-dimensional double quantum filtered COSY spectrum of 7 mM bovine cytochrome b_5 in D_2O with 50 mM potassium phosphate buffer, pD 6.75, 24 °C, was taken in the phase-sensitive mode, with a spectral width of 7042.25 Hz in each dimension (Rance et al., 1983). Sixteen scans were used for each of 1024 t_1 values with 4096 complex points in the ω_2 dimension. The final data size was 1024 × 4096. A two-dimensional NOESY spectrum of the same sample was taken with a mixing time of 200 ms and a spectral width of 7042.25 Hz in each dimension (Jeener et al., 1979). A total of 512 t_1 values were recorded with 2048 complex points in the ω_2 dimension. The data were zero filled to give a final spectrum size of 1024 × 2048.

pD Titrations. Solutions of 1 mM cytochrome b_5 and 100 mM K_2HPO_4 in 0.35 mL of H_2O were prepared and lyophilized directly from 5-mm NMR tubes. The exchangeable protons were removed by resuspending the protein in 0.35 mL of 99.8% D_2O and subsequent lyophilization. This cycle was repeated twice before the cytochrome b_5 was finally redissolved in 0.35 mL of 99.8% D_2O containing 1 mM TSP. The pD was measured directly in the NMR tube at 24 °C with a narrow electrode that fits directly into the NMR tube; the pD values are uncorrected from the meter readings. The isotope effect of 0.4 pH unit on the glass electrode at the time of pH measurement is thought to be canceled by an equal and opposite deuterium isotope effect on the ionization equilibrium (Meadows, 1972).

The pD titrations were done in two stages, each starting from approximately neutral pD. In one set of experiments, the pD was increased in small steps by the addition of small amounts of 1 M NaOD, until pD 10 was reached; in the second set, the pD was decreased by the addition of small aliquots of 1 M DCl until a pD near 5 was reached. The pD was measured before and after the NMR spectrum was acquired at each step in the titration, and the values agreed to within ±0.03 pD unit. At a pD near 5, a red precipitate was observed, presumably due to the limited solubility of the protein near its isoelectric point. The precipitate was immediately removed by microcentrifugation, and the NMR spectrum was taken within 5 min. The ^1H NMR spectrum of the protein remaining in the supernatant of this solution had general features identical with those of the protein at neutral pD, indicating that the overall conformation of the protein had not changed dramatically, though the sensitivity of the experiment was diminished (but not quantitated) due to the decrease in concentration of the protein. In both experiments, the volume of the solution approximately doubled at the extremes of pD.

Modification of Cytochrome b_5 with DEP. The DEP modification experiments were conducted in D_2O at approximately 100 μM cytochrome b_5 to conserve protein. The protein solutions were buffered with 100 mM potassium phosphate at pD 7.0–7.3, and the exchangeable protons were removed by lyophilization from D_2O as in the pD titrations. The pD used for DEP modification experiments was chosen for the optimal resolution of histidine $C^{\epsilon 1}H$ resonances.

The concentration of a stock solution of DEP in anhydrous ethanol-d_6 was determined immediately before use by adding a small aliquot of the stock to a solution of known concentration of imidazole and measuring the absorbance at 240 nm on a Cary 219 spectrophotometer (Miles, 1977). Aliquots of the stock solution of DEP (≈18 mM depending on the experiment) were added to the preexchanged solutions of cytochrome b_5 in D_2O in Eppendorf tubes at 24 °C such that the final DEP to histidine ratio was equal to the values given in Figure 2. Equal volumes of ethanol-d_6 were added to give a final concentration of 0.68 M in the reaction mixture. The mixture was allowed to incubate at 24 °C for 40 min, at which time the reaction between DEP and cytochrome b_5 is complete. Histidine residue modification was then quantitated by UV difference spectroscopy and measured qualitatively by NMR spectroscopy. NMR data were acquired at 24 °C as follows: 64 scans were used for each sample, with $\pi/4$ detection pulses and a relaxation delay of 3 s. The data were processed in double precision to adequately digitize the noise. The number of *N*-carbethoxyhistidine residues was determined by UV difference spectrophotometry by adding the appropriate concentration of ethanol to the reference cuvette and using an extinction coefficient of 3200 M^{-1} cm^{-1} at 240 nm (Miles, 1977).

Solvent-Accessible Surfaces. Calculation of solvent-accessible surfaces for the nonaxial histidine residues in the crystal structure of cytochrome b_5 was done by using Connolly's implementation (Connolly, 1981; Langridge et al., 1981) of the Lee and Richards (1971) algorithm, which is part of the MIDAS molecular graphics package at the University of California, San Francisco. A sphere of 1.4 Å was used to generate the surface of the entire protein, and then areas were assigned to individual histidine side chains. Areas were then divided by the area for an exposed histidine side chain in a random coil, 159.2 Å (Rashin, 1984). Coordinates were taken from the Brookhaven Protein Data Bank (Bernstein et al., 1977), file 2b5c (Mathews et al., 1972), with the following modifications: residues 85–87 were deleted from the coordinate sets for both bovine and rabbit proteins because they were not present in our trypsin-solubilized cytochrome b_5 (Mauk et al., 1986), and the amino acid substitutions required to turn the bovine protein into the rabbit, Ala3Asp, Glx13Lys, Asn17His, Tyr27His, were modeled by using the MIDAS utility SWAPAA and interactive molecular graphics. No attempt was made to model the additional six residues found at the amino terminus of the high molecular weight rabbit protein. In the rabbit model, no change was made to the backbone dihedral angles (ϕ and ψ) found in the bovine structure. These structures were not specifically energy minimized, though gross van der Waals contacts were avoided by interactively monitoring short interatomic distances while in the MIDAS program.

Results

Chemical Shift Assignments of Bovine and Rabbit Ferricytochrome b_5. The chemical shift assignments of the three nonaxial histidine residues in oxidized trypsin-solubilized bovine cytochrome b_5 are from Reid et al. (1987) and were independently confirmed by using double quantum filtered correlated spectroscopy (Rance et al., 1983) and a two-dimensional nuclear Overhauser effect spectrum (NOESY) (Jeener et al., 1979) with a mixing time of 200 ms (data not shown). The following NOE cross peaks, indicating a spatial separation of less than 5 Å of two protons were confirmed: His-15 $C^{\epsilon1}H$ to Trp-22 ($C^{\zeta3}H$, $C^{\eta2}H$, and $C^{\zeta2}H$), His-80 $C^{\epsilon1}H$ to Tyr-6 $C^{\delta2}H$ and Tyr-6 $C^{\epsilon2}H$, and His-26 $C^{\epsilon1}H$ to Thr-55 $C^{\gamma2}H$. The chemical shifts of the axial histidines are not found in this region of the spectrum due to their close proximity to the heme, whose ring current and paramagnetic nature significantly alter the magnetic environment of their protons.

In the rabbit protein, the chemical shifts of the two additional nonaxial histidines (His-17 and -27) were assigned by comparing the one-dimensional ^1H NMR spectra of the rabbit, bovine, and porcine proteins (Figure 1). The porcine protein was examined because the only additional histidine it contains compared with the bovine protein is His-27. The three sharp peaks between 7.5 and 8.75 ppm in the bovine spectrum (Figure 1) are from the three $C^{\epsilon1}H$ histidine resonances; the four remaining broad peaks represent resonances of amide protons that partially exchange with the D_2O of the solvent when the pH is varied (Figure 3A). The broad peaks also exhibit cross peaks in the fingerprint region of the COSY spectra taken in D_2O, supporting this assignment to amide hydrogens (data not shown). Thus, the one additional sharp resonance in the porcine spectrum at 8.2 ppm has been assigned to the $C^{\epsilon1}H$ of His-27. This comparison was made at two pH values (data not shown). In the rabbit spectrum the resonances for the His-26 and -27 $C^{\epsilon1}H$ protons are consistently broader. Nonetheless, they show titration behavior characteristic of a histidine, not an amide, proton (Figure 3B). The only remaining difference in the rabbit spectrum between 7.5 and 8.75 ppm compared to the bovine and porcine spectrum is the superposition of an intense sharp peak on a broad peak at 7.57 ppm. This resonance has thus been assigned to the $C^{\epsilon1}H$ proton of His-17.

The resonance assignments for the $C^{\epsilon1}H$ protons of all nonaxial histidines are summarized in Table I. The chemical shifts of His-15, -26, and -27 of bovine and rabbit cytochrome b_5 are given at pD 6.88 and were calculated from (Bundi & Wüthrich, 1979)

$$\delta_i = \frac{\delta HA + \delta A^- \times 10^{(pH_i - pK_a)}}{1 + 10^{(pH_i - pK_a)}} \tag{1}$$

where δHA is the chemical shift of the fully protonated histidine, δA^- is the chemical shift of the $C^{\epsilon1}H$ proton of the fully deprotonated histidine, δ_i is the chemical shift at pD 6.88, and pK_a is the pD at which the histidine residue is 50% titrated. The parameters δHA, δA^-, and pK_a were obtained from the data in the titration curves in Figures 3 as described under Determination of the pK_a's of the Peripheral Histidines of Cytochrome b_5. The chemical shifts for His-17 and -80 were obtained directly from spectra observed at pD 6.78 and 6.85, respectively, and are nearly independent of pD in this range. Equation 1 could not be used to calculate the pD at 6.88 because it was impossible to obtain a full titration curve due either to low protein solubility or to the dissociation of heme

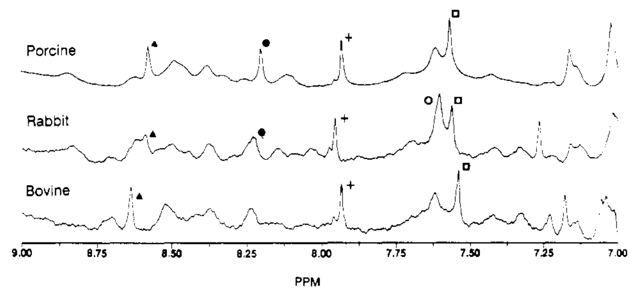


FIGURE 1: Aromatic region of the one-dimensional proton NMR spectra of bovine, rabbit, and porcine trypsin-solubilized ferricytochrome b_5. The one-dimensional spectra were obtained at pD 6.4 as described under Materials and Methods. For clarity, only the histidine $C^{\epsilon1}H$ proton of the spectra of cytochrome b_5 is illustrated. The histidines are labeled according to the numbering scheme of Mathews et al. (1972): (▲) His-26; (●) His-27; (+) His-15; (□) His-80; (○) His-17.

Table I: Proton Resonance Assignments for Ferric Cytochrome b_5

His	chemical shift (ppm) bovine	rabbit	porcine
15	7.92	7.93	7.93
17		7.57	
26	8.35	8.32	8.60
27		7.99	8.20
80	7.52	7.54	7.57

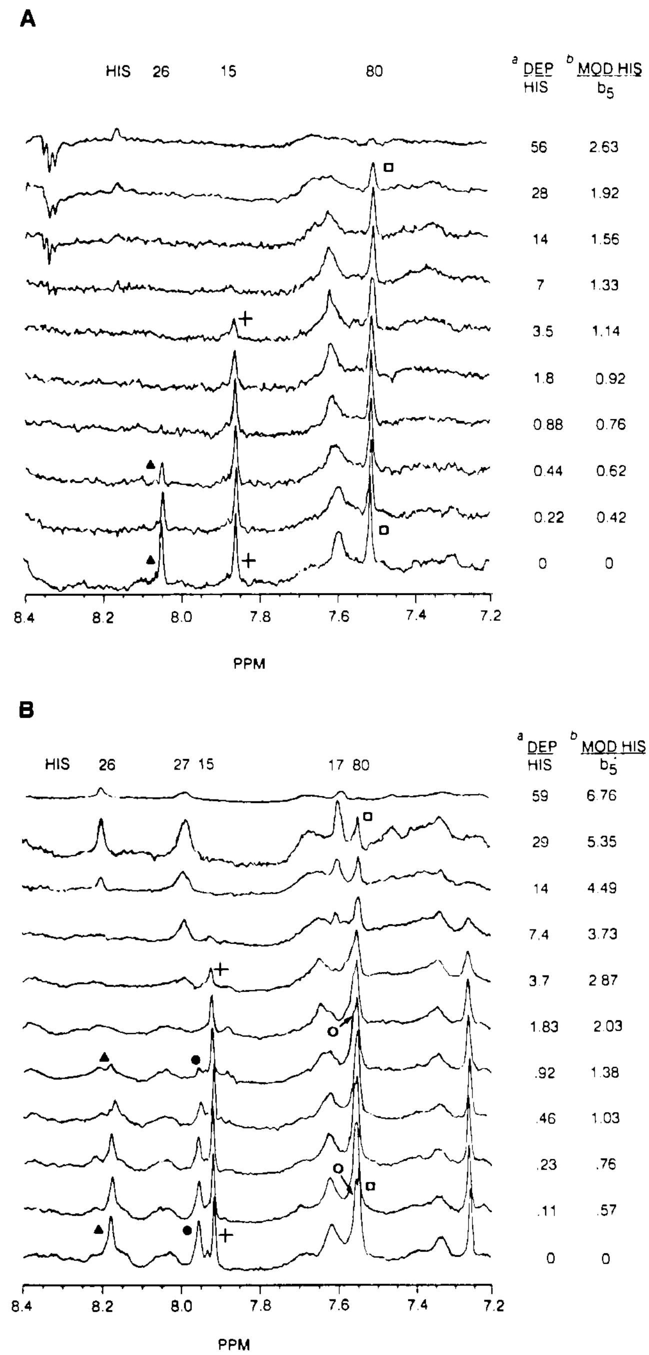


FIGURE 2: DEP modification of bovine (A) and rabbit (B) ferricytochrome b_5. The aromatic region of the proton NMR spectrum of ferricytochrome b_5 is illustrated. Cytochrome b_5 was treated with DEP, and the proton NMR spectra were obtained at pD 7.1 and 24 °C as described under Materials and Methods (a) The molar ratio of DEP to histidine that was used to modify cytochrome b_5. (b) The number of histidines modified per molecule of cytochrome b_5 was calculated from the absorbance at 240 nm by using an extinction coefficient of 3200 M^{-1} cm^{-1} (Miles, 1977). The histidines are labeled as in Figure 1.

from cytochrome b_5 at the pH's near the low pK_a's of His-17 and -80. The chemical shifts of the porcine resonances are reported at pD 6.40.

Modification of Histidines by DEP. When DEP modification of the histidine residues of trypsin-solubilized bovine cytochrome b_5 was followed spectrophotometrically at 240 nm, the results suggested that at low DEP to histidine ratios two histidines were modified in parallel, while a third histidine appeared to be modified at a higher concentration of DEP (Konopka & Waskell, 1988b). By monitoring the $C^{\epsilon1}H$ protons of the three peripheral histidine residues of bovine cytochrome b_5, it was possible to follow the carbethoxylation of the adjacent nitrogen atom (Figure 2 and Table II). We observe that the histidines, in fact, react nearly sequentially. His-26 is the most reactive, while His-80 is the most resistant of the three peripheral histidines. It is of particular interest that the first residue to be modified, His-26, is the most reactive with the copper reagent used in a recent study by Reid et al. (1987). The order of modification of His-26, -15, and -80 in the rabbit protein is essentially identical with that in the bovine protein, indicating the overall structural similarity of both proteins. His-27 in the rabbit protein reacts in parallel with His-26. The reactivity of His-17 whose $C^{\epsilon1}H$ resonance is found as a sharp peak on the left shoulder of the resonance for His-80 is equal to or slightly less than that of His-15, making it the second most resistant of the peripheral histidines in rabbit cytochrome b_5. Unfortunately, the low signal to noise ratio prevented the accurate measurement by NMR of the number of histidines modified at a given concentration of DEP. Hence, the results obtained with UV absorption spectroscopy cannot be compared directly to our NMR results.

The unknown broad peak with a chemical shift of about 7.6 ppm in the bovine and rabbit proteins also disappears with increasing concentrations of DEP; it did not titrate between pH 5.25 and 10.05 (Figure 3) so it is not felt to be the amino-terminal proton. In the rabbit protein there is a sharp peak at about 7.26 ppm that disappears with addition of DEP but does not titrate between pD 5 and 10. This peak may be the His-17 $C^{\delta2}H$ resonance since its disappearance is concomitant with the disappearance of the His-17 $C^{\epsilon1}H$ resonance, though we have found no cross peak corresponding to it in our COSY spectrum. The Soret (413 nm) peak of cytochrome b_5 decreased by a maximum of 10% and 30% in the bovine and rabbit proteins, respectively, at the highest concentrations of DEP, indicating that the structure of the majority of the cytochrome b_5 remained intact during the titration (data not shown). This conclusion is also supported by the persistence of paramagnetically shifted peaks in their native, unmodified positions in other parts of the proton NMR spectrum (data not shown), though it is apparent in the samples containing the highest amount of DEP (Figure 2) that there is some folding over of new peaks, which are characteristically out of phase, into the histidine region at approximately 8.38 ppm, indicating some modification of the protein near the heme pocket. Ethanol, which is a product of the hydrolysis of DEP and the reaction of DEP with histidine, did not interfere with this series of experiments since its chemical shifts are upfield of the aromatic region of the spectrum of the protein.

Determination of the pK_a's of the Peripheral Histidines of Cytochrome b_5. The electrophilic reagent DEP only reacts with the nucleophilic form of deprotonated histidine; hence, the histidine with the lowest pK_a was expected to be modified first (Miles, 1977). In an attempt to understand the chemical basis of the reactivity of the histidines in cytochrome b_5, their pK_a's were measured. The proton NMR spectra obtained during the pH titrations are illustrated in Figure 3. By use of eq 1, where pH_i is the independent and δ_i is the dependent variable, the titration curves and the parameters δHA, δA^-, and pK_a (Figure 3 and Table II) were calculated from the best nonlinear least-squares fit of the plot of the upfield chemical shift of the $C^{\epsilon1}H$ peak with increasing pD. Full titration curves were obtained with His-26, -27, and -15. At pD 7.44 the $C^{\epsilon1}H$ resonances of His-27 and His-15 overlap in the rabbit protein spectra. Therefore, the His-15 resonance was assigned at pD's near this value by comparison with the bovine spectra. The

Table II: Determinants of the Reactivity of DEP with the Histidines of Cytochrome b_5

His	rel reactivity	DEP/His[a]	pK_a		solvent accessibility[b]		involvement of pyridine-like nitrogen in a hydrogen bond	
			bovine	rabbit	bovine	rabbit	bovine	rabbit
26	high	0.3	6.92	6.91	37.0	37.0	no	no
27	high	0.3	NA	6.44	NA	38.8	no	no
15	moderate	2	8.47	8.38	19.1	19.1	no	no
17	moderate	2	NA	<5	NA	40.6	yes	yes
80	low	30	<5.5	<5	33.4	33.4	yes	yes

[a] The DEP/His ratio is the molar ratio needed to modify approximately half of the specific residue, as measured by peak area in the proton NMR spectrum. [b] Percent solvent accessibility was calculated as described under Materials and Methods.

virtually identical pK_a of His-26 and -15 in the bovine and rabbit proteins suggests an identical chemical environment and structure in both proteins even though in the rabbit His-27 replaces Tyr-27 in the bovine protein.

His-80 and -17 have anomalously low pK_a's that could not be measured because they have overlapping resonances at pD 6.13 in the rabbit protein and the heme dissociates from cytochrome b_5 at acidic pD, thereby forming apocytochrome b_5. His-80 apparently begins to titrate at higher pD in the rabbit protein than in bovine cytochrome b_5 (Figure 3), though it is difficult to draw conclusions from this in the absence of a full titration curve.

Determination of the Solvent Accessibilities of the Nonaxial Histidines. In an attempt to explain the sequential reactivity of the peripheral histidines with DEP, we have calculated their solvent-accessible surfaces using the atomic coordinates from the crystal structure. The reactivity of the histidines toward DEP was expected to be influenced by its accessibility to solvent; the more exposed a residue is, the more reactive it should be. The results are shown in Table II.

Discussion

Comparison of Experimentally Measured pK_a's with Calculated pK_a's. Our results demonstrate that bovine cytochrome b_5 contains two nonaxial histidine residues that titrate between pD 6 and 10, while rabbit cytochrome b_5 contains three histidine residues that titrate in this range (Figure 3, Table II). In recent studies Mauk et al. (1986) have calculated the p$K_{a1/2}$ values at an ionic strength of 4 mM for the peripheral histidines in bovine cytochrome b_5.

The pK_a values for ionizable amino acids on the surface of a protein can be calculated by use of the static accessibility modification of the Tanford–Kirkwood theory (Matthew & Gurd, 1986; Matthew et al., 1985). This approach considers all charged atoms in the protein and assigns a local dielectric constant to each charged group according to its solvent-accessible surface area. The greater the solvent-accessible surface area of the charged residue, the higher is the effective Coulombic shielding for the site and the less it will interact with its neighboring charges. In these computations it is assumed that for each pH and ionic strength the unique protein charge array generates an electrostatic potential at a particular site, causing the apparent pK_a to deviate from the intrinsic pK_a of the site. The experimentally determined and calculated values are compared in Table III.

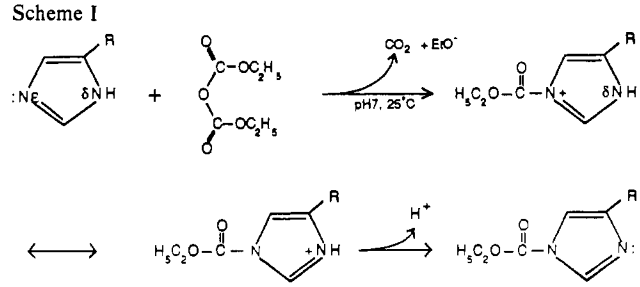
There is excellent agreement between the calculated and experimentally determined pK_a for His-26 (Table III). However, the pK_a calculated for His-15 at an ionic strength of 75 mM is 0.4 pH unit less than the experimentally determined value. The computations only consider through-space electrostatic effects and not the local effect of specific hydrogen bonds that would be expected to alter the local electrostatic

Table III: Comparison of Predicted and Experimentally Determined pK_a Values for Bovine Cytochrome b_5

residue	calcd pK_a, assumed ionic strength		exptl pK_a, av ionic strength 75 mM[c]
	4 mM[a]	75 mM[b]	
His-15	<6.5 or >8.5	8.07	8.47
His-26	7.5	6.9	6.92
His-80	6.6	6.5	<5.50

[a] These values are from Mauk et al. (1986). [b] The calculated values at 75 mM ionic strength were provided by Dr. J. Matthew (personal communication). [c] The experimentally determined values were obtained at an ionic strength that varied between 50 and 100 mM due to dilution with either DCl or NaOD during the titration.

Scheme I



potential of the ionizable groups. The X-ray crystal structure indicates that the δ^1-nitrogen of His-15 is the hydrogen donor in a hydrogen bond to the backbone carbonyl of Gln-11 (Mathews et al., 1979) which increases the electron density on the imidazole ring and consequently its basicity and pK_a.

With His-80 there is more of a discrepancy between the predicted (6.5) and experimentally (<5.5) determined values with the caveat that it was impossible to measure an exact pK_a for His-80 due to the dissociation of heme from cytochrome b_5 at low pH values. The hydrogen bond between the unprotonated δ-nitrogen of His-80 and the hydrogen of the main-chain amide of Asp-82 (Mathews et al., 1979) should decrease the electron density on the imidazole ring, thereby lowering its pK_a. The discrepancy between the measured and predicted values might arise from crystal-packing interactions, from the ionic strength of the crystallization medium (3 M phosphate buffer, pH 7.5), or because the trypsin-solubilized cytochrome b_5 used in the present studies has a different electrostatic environment at its carboxyl terminus than the lipase-solubilized cytochrome b_5 used to determine the crystal structure. The lipase-solubilized protein has two additional amino acids at the carboxyl terminus.

Effect of the Protein Environment on the Reactivity of the Histidines of Cytochrome b_5 with DEP. The neutral imidazole ring of histidine can exist in two tautomeric forms: either the ϵ-nitrogen–H tautomer or the δ-nitrogen–H tautomer. The δ-nitrogen–H tautomer is illustrated. DEP reacts with unprotonated, neutral histidine residues according to the reaction

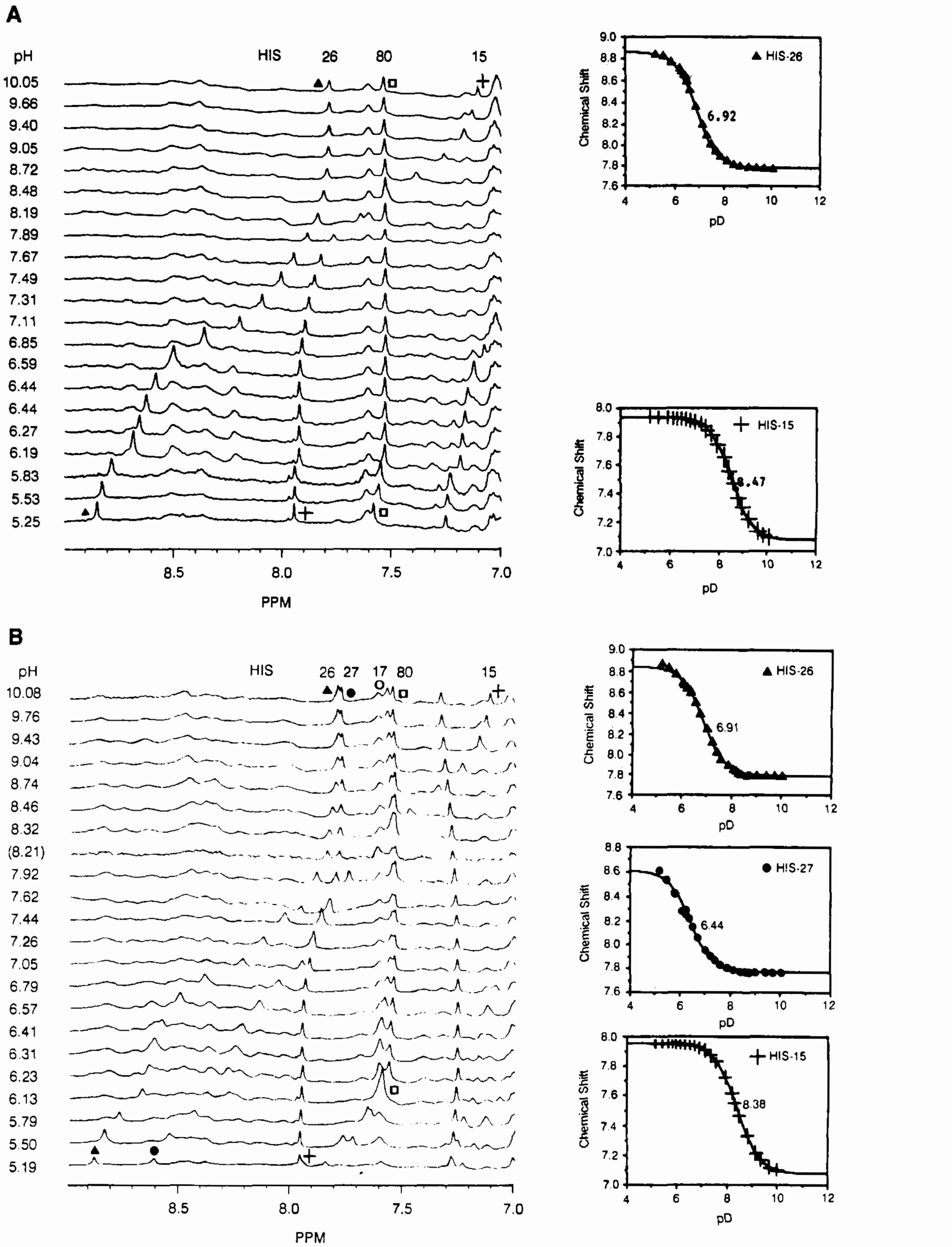


FIGURE 3: pD titration of the $C^{\epsilon 1}H$ protons of bovine (A) and rabbit (B) cytochrome b_5. The titrations were conducted as described under Materials and Methods. The peaks are labeled as in Figure 1.

(Miles, 1977) shown in Scheme I.

The reaction occurs by an initial attack of the electrophilic carbon of DEP at the basic "pyridine-like" nitrogen, i.e., the nitrogen without the hydrogen, which may be either the ϵ- or δ-nitrogen depending on the tautomer undergoing the reaction. Subsequent electron shifts in the imidazole ring are followed by the expulsion of a proton from the "pyrrole-like" nitrogen and decomposition of DEP (Barnard & Stein, 1958). The existence of two tautomeric forms of histidine results in the formation of two nonidentical products, one that is acylated at the ϵ-nitrogen and the second that is acylated at the δ-nitrogen. This may be part of the explanation of the fact that the extinction coefficient of the DEP–histidine adduct varies with the protein (Miles, 1977). This reaction mechanism suggests that the reactivity of the histidines in a protein with DEP will be influenced by (1) the hydrogen-bonding interactions of the histidine, (2) the steric accessibility of the histidine to the solvent, and (3) the pK_a of the histidine, which in fact is determined to a great extent by these latter two factors.

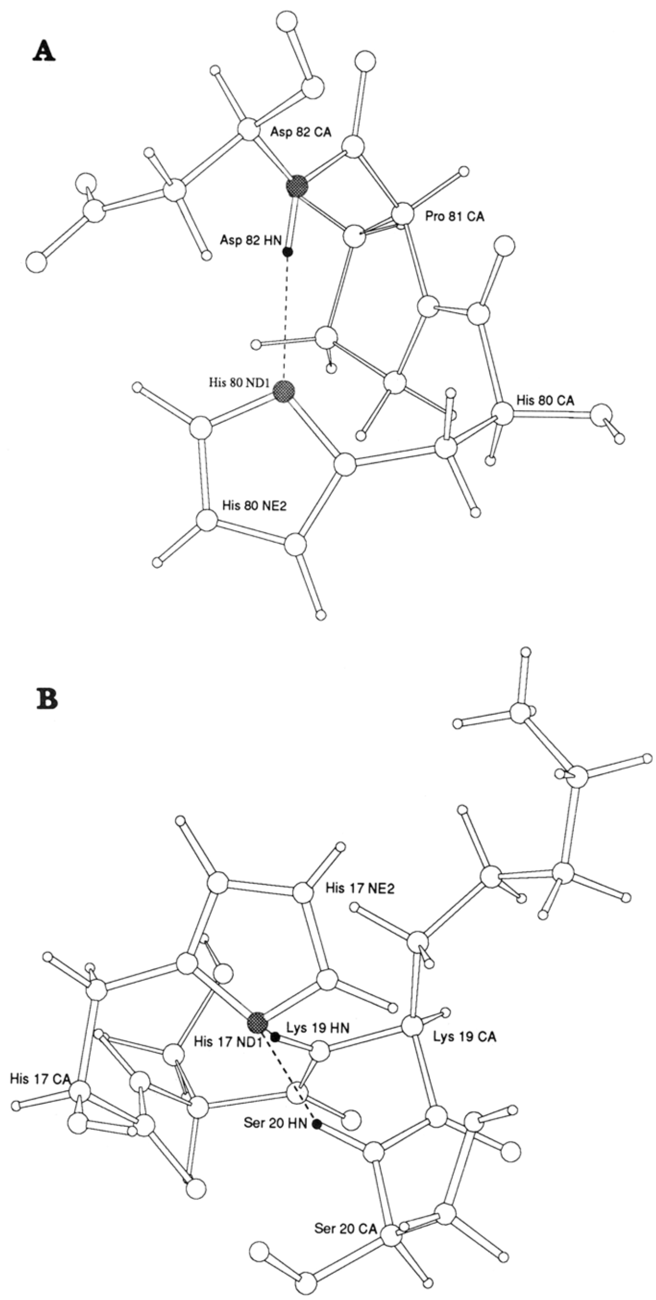


FIGURE 4: Local environment and hydrogen-bonding interactions of His-80 (A) and His-17 (B). (A) The hydrogen bond between the δ-nitrogen of His-80 and the hydrogen of the main-chain amide of Asp-82, which is estimated to be 2.22 Å in length, is illustrated. The distance between the δ-nitrogen of His-80 and the backbone amide nitrogen of Asp-82 is 3.09 Å. The His-80 δ-nitrogen–hydrogen-Asp-82 amide nitrogen bond angle is 152.4°. (B) Interactive computer graphics model of the His-17 replacement for Asn-17. The oxygen of the Asn side chain is isosteric with the δ-nitrogen of histidine. The distances between the δ-nitrogen of His-17 and the protons of the main-chain amides of Ser-20 and Lys-19 are estimated to be 1.93 and 1.94 Å, respectively. The analogous nitrogen to nitrogen distances are 2.73 and 2.75 Å. The bond angles formed by the Ser-20 and Lys-19 amide nitrogen, hydrogen, and δ-nitrogen of histidine are 140.9° and 141.8°, respectively.

These three considerations appear to explain semiquantitatively the observed reactivity of the nonaxial histidines of cytochrome b_5 with DEP. His-26 and -27 are very reactive, which is expected in view of their pK_a of 6.9, extensive solvent accessibility (Chothia, 1976), and lack of hydrogen-bonding interactions with other amino acids in the protein.

His-15 is moderately reactive with DEP (Figure 2). Its pK_a is approximately 8.4 and thus will be more extensively protonated at pD 7 than His-26 and -27; its solvent accessibility is approximately half that of His-26 and -27, and examination of the crystal structure reveals the δ-nitrogen of the imidazole ring is the hydrogen donor in a hydrogen bond to the backbone carbonyl of Gln-11 (Mathews et al., 1979). So although the tautomer with the pyridine-like nitrogen is available, it will be extensively protonated at neutral pD, thus moderating its reaction with DEP. His-80 has low reactivity (Figure 2) with DEP. Its pK_a is less than 5, and its solvent accessibility is high at 33.4%; His-80 was thus expected to be very reactive with DEP. However, the crystal structure reveals that the δ-nitrogen of His-80 is the hydrogen acceptor in a hydrogen bond with the nitrogen of the main-chain amide of Asp-82 (Figure 4A) (Mathews et al., 1979). This means the only nitrogen available to react with DEP at pD near neutrality is the pyrrole-like ε-nitrogen, i.e., the nitrogen with the hydrogen, which is unreactive.

His-17 is like His-80 in having a low pK_a and an extensive amount (40%) of its surface area accessible to solvent. It should therefore be very reactive with DEP. However, it is merely moderately reactive with DEP, although this is somewhat difficult to observe in Figure 2B due to the partial overlap of the peaks for the $C^{\epsilon 1}H$ protons of His-80 and -17. In the crystal structure of the bovine protein, the side-chain oxygen of Asn-17 is the hydrogen acceptor in a hydrogen bond with the backbone amide of Ser-20 (Mathews et al., 1979). However, in the rabbit protein a histidine residue occupies position 17. When a histidine residue is superimposed on Asn-17 by modeling using interactive molecular graphics, the δ-nitrogen of His-17 is observed to be in a position analogous to that of the oxygen of the Asn-17 side chain (Figure 4B). Hydrogen bonding of the $N^{\delta 1}$ atom of a histidine to a proton donor forces the histidine tautomer to have the hydrogen atom at the $N^{\epsilon 2}$ position. Thus, only the unreactive pyrrole-like nitrogen will be exposed to DEP. The fact that His-17 is more reactive with DEP than His-80 suggests that the His-17–Ser-20 hydrogen bond may be weaker than the His-80–Asp-82 hydrogen bond.

The feasibility of observing the modification by DEP of histidine residues in proteins has been previously demonstrated and reconfirmed herein by using proton NMR (Smith & Mildvan, 1981). However, our report is the first detailed NMR and molecular graphics analysis of the reaction of DEP with a protein whose X-ray crystal structure is known (Argos & Mathews, 1975). It has provided unprecedented insight into the chemical basis of the reactivity of specific histidines in a protein with DEP.

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