

Chain-Selective Isotopic Labeling for NMR Studies of Large Multimeric Proteins: Application to Hemoglobin

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ABSTRACT Multidimensional, multinuclear NMR has the potential to elucidate the mechanisms of allostery and cooperativity in multimeric proteins under near-physiological conditions. However, NMR studies of proteins made up of non-equivalent subunits face the problem of severe resonance overlap, which can prevent the unambiguous assignment of resonances, a necessary step in interpreting the spectra. We report the application of a chain-selective labeling technique, in which one type of subunit is labeled at a time, to carbonmonoxy-hemoglobin A (HbCO A). This labeling method can be used to extend previous resonance assignments of key amino acid residues, which are important to the physiological function of hemoglobin. Among these amino acid residues are the surface histidyls, which account for the majority of the Bohr effect. In the present work, we report the results of two-dimensional heteronuclear multiple quantum coherence (HMQC) experiments performed on recombinant ^{15}N -labeled HbCO A. In addition to the C2-proton ($\text{H}\epsilon_1$) chemical shifts, these spectra also reveal the corresponding C4-proton ($\text{H}\delta_2$) resonances, correlated with the $\text{N}\epsilon_2$ and $\text{N}\delta_1$ chemical shifts of all 13 surface histidines per $\alpha\beta$ dimer. The HMQC spectrum also allows the assignment of the $\text{H}\delta_1$, $\text{H}\epsilon_1$, and $\text{N}\epsilon_1$ resonances of all three tryptophan residues per $\alpha\beta$ dimer in HbCO A. These results indicate that heteronuclear NMR, used with chain-selective isotopic labeling, can provide resonance assignments of key regions in large, multimeric proteins, suggesting an approach to elucidating the solution structure of hemoglobin, a protein with molecular weight 64.5 kDa.

INTRODUCTION

Human normal adult hemoglobin (Hb A) serves as a model or paradigm for multimeric, allosteric proteins and is perhaps the most studied of all proteins. Extensive research efforts have been devoted to elucidating the relationship between the structure of Hb A and its physiologically important properties, including the cooperative binding of oxygen, and the allosteric interactions of oxygen- and hydrogen ion-binding (i.e., the Bohr effect). However, the detailed molecular basis of these properties is not fully understood and some aspects remain controversial (Ho and Lukin, 2000). The structure-function relationship of Hb in solution under near-physiological conditions can be investigated directly using NMR spectroscopy. One-dimensional (1D) ^1H -NMR spectroscopy has been used extensively to study the behavior of Hb in solution by following the response of assigned ^1H resonances to varied conditions, and to modifications of the protein provided by site-directed mutagenesis and chemical modifications (Ho, 1992, 1995; Ho and Perussi, 1994).

Although 1D ^1H -NMR spectroscopy has proven to be a valuable technique in the study of Hb, a full picture of the structure and dynamics of this protein in solution would require the assignment of multinuclear, multidimensional spectra of isotopically labeled protein samples. Labeling of Hb A with ^2H , ^{15}N , and ^{13}C has become feasible through the

development of a bacterial expression system for this protein (Shen et al., 1993, 1997). The NMR assignment of a protein as large as Hb A (64.5 kDa) presents a challenge, as its slow rate of tumbling in solution leads to rapid decay of the resonance signals, corresponding to broad NMR lines. The problem of resonance overlap is exacerbated by the high α -helical content (about 77%) of Hb, which causes the amide backbone cross-peaks to appear in a small, crowded region of the (^1H , ^{15}N) plane. We have found that this problem can be greatly reduced by performing separate NMR experiments on selectively labeled samples of tetrameric Hb A, where only one type of subunit (α or β) is isotopically labeled. In this paper, we present 2D (^1H , ^{15}N) heteronuclear single quantum coherence (HSQC) spectra of chain-selectively labeled Hb A which illustrate this technique. We also discuss heteronuclear multiple quantum coherence (HMQC) spectra, which complete the assignment of imidazole ring resonances for all 38 histidines in Hb A (i.e., 19 in each $\alpha\beta$ dimer; Dickerson and Geis, 1983).

^1H -NMR spectroscopy is ideally suited to the study of histidine residues in proteins, and in particular to investigating the contribution of these residues to the Bohr effect of Hb A (Ho and Russu, 1987; Russu et al., 1989; Busch et al., 1991; Sun et al., 1997; Fang et al., 1999). The contribution of each histidine residue to the Bohr effect can be calculated from the change in its pK value upon binding oxygen (or CO) to deoxy-Hb A. These pK values can be determined very accurately by measuring the chemical shift of the His $\text{H}\epsilon_1$ (C2 proton) resonances as a function of pH. Although the $\text{H}\epsilon_1$ resonances of histidyl residues are well separated from those of other carbon-bound protons, they overlap with the amide and aromatic regions of the protein's ^1H -NMR spectrum and are broadened in H_2O solutions.

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Thus, the $\text{H}\epsilon_1$ peaks can be resolved in the 1D ^1H -NMR spectrum of Hb only if the amide protons are exchanged by using D_2O as a solvent. The development of a bacterial expression system for unmodified Hb A (Shen et al., 1993, 1997) has enabled us to label this protein with ^{15}N , making it possible to acquire 2D- $(^1\text{H}, ^{15}\text{N})$ HSQC and HMQC spectra in either H_2O or D_2O solutions. These experiments are capable of resolving the histidine cross-peaks of a protein dissolved in H_2O , because $^{15}\text{N}\delta_1$ and $^{15}\text{N}\epsilon_2$ of His resonate at characteristic chemical shifts far removed from those of the backbone amides and other amino-acid side chains (Markley and Kainosho, 1993). These chemical shifts are indicative of the tautomeric state of each histidyl (Pelton et al., 1993), i.e., when one of the imidazole-ring nitrogens is protonated, it typically resonates at ≈ 168 ppm, while the non-protonated nitrogen resonates at ≈ 250 ppm. When both nitrogens are protonated (i.e., at pH below the pK of the histidyl), they both resonate at ≈ 178 ppm. A detailed ^{15}N -NMR study of histidine (Blomberg et al., 1977) revealed that the 2- and 3-bond couplings between the carbon-bound protons and the nitrogens of the imidazole ring range from -2 to -10 Hz. These couplings result in observable cross-peaks in the HMQC spectrum (Stockman et al., 1989; Oh et al., 1990). The expected HMQC spectra of the three possible tautomeric states of histidine are shown schematically in Fig. 1. Similarly, one- and two-bond J couplings in tryptophan result in a cross-peak between $^{15}\text{N}\epsilon_1$ and $^1\text{H}\delta_1$, as well as its directly-bonded proton.

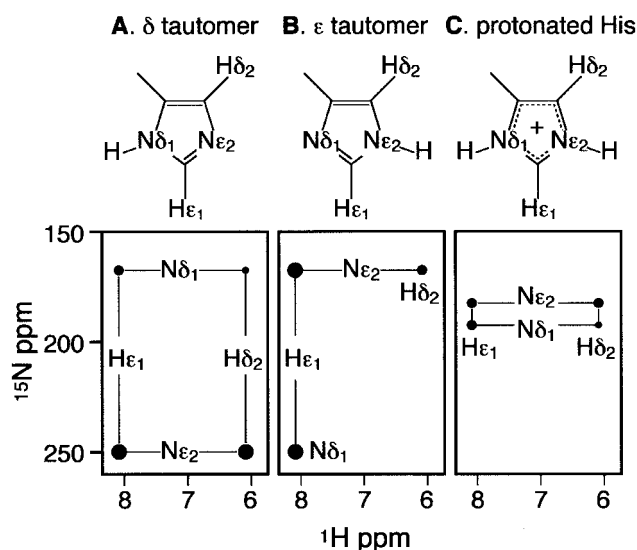


FIGURE 1 Schematic diagram adapted from Pelton et al. (1993) showing the expected $(^1\text{H}, ^{15}\text{N})$ -HMQC spectrum of the imidazole-ring for each of the three possible protonation states of a histidyl residue. Large, medium, and small black circles indicate strong, medium, and weak cross-peaks, respectively. Note that, for the charged species (C), either of the nitrogens could resonate downfield of the other; the assignment must be made based on the relative weakness of the ($\text{N}\delta_1$, $\text{H}\delta_2$) cross-peak.

MATERIALS AND METHODS

Expression of isotopically labeled Hb A

Uniformly ^{15}N -labeled, or $(^{15}\text{N}, ^2\text{H})$ -labeled, or $(^{15}\text{N}, ^2\text{H}, ^{13}\text{C})$ -labeled recombinant Hb A (rHb A) was obtained by expression from the plasmid pHE2, which contains synthetic α - and β -genes of human Hb and the *Escherichia coli* methionine aminopeptidase (MAP) gene under tac promoter control (Shen et al., 1993). *E. coli* JM109 harboring this plasmid was grown in a 2- or 5-liter Bioflo bench-top 3000 fermentor controlled by an AFS-BioCommand unit (New Brunswick Scientific, Edison, NJ). The growth medium for isotopic labeling was DM minimal medium (Looker et al., 1994) with modifications: 1 liter of medium contains 2.0 g KH_2PO_4 , 3.6 g K_2HPO_4 , 2.0 g NH_4Cl , 1.5 g $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$, 0.154 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.0 ml of trace metal solution, 10.0 g glucose, and 2.5 ml vitamin mixture (8.5 g/l NaCl, 0.1 g/l D-capantothenate, 0.1 g/l choline chloride, 0.1 g/l folic acid, 0.2 g/l myo-inositol, 0.1 g/l nicotinamide, 0.1 g/l pyridoxal-HCl, 0.01 g/l riboflavin, and 0.1 g/l thiamine-HCl). Depending on the desired isotope labeling, NH_4Cl , D-glucose, and H_2O were replaced by $^{15}\text{NH}_4\text{Cl}$ (98% ^{15}N), D-glucose- ^{13}C (99% ^{13}C), and D_2O (99.9% ^2H), respectively, from Cambridge Isotope Laboratories (Andover, MA).

The medium was inoculated with 1 liter of overnight seed culture and incubated at 30°C . The oxygen concentration was maintained above 20% with respect to air saturation by oxygen pulse purging and agitation at 400 rpm. The pH of the medium was maintained at 6.8 with a feed of 0.8 to 1.0 M Na_2HPO_4 . When the culture reached $\text{OD}_{600} = 4.5$ to 5.0, expression of rHbA and MAP was induced with 0.2 mM isopropyl 1-thio- β -D-galactopyranoside and 25 mg/l hemin. Cells were harvested 6 h after induction and stored at -80°C until further processing. rHbA was purified as previously described using Q-Sepharose chromatography and fast protein liquid chromatography (FPLC) (Shen et al., 1997).

Chain separation

α - and β -chains of Hb A were separated following the method of Bucci (1981) with slight modifications. rHbCO A in 20 mM monobasic phosphate and saturated NaCl was reacted with p-mercuribenzoate (PMB) overnight at 4°C . The next day, chain separation was checked by electrophoresis to ensure that the reaction had been completed. The product was then centrifuged to eliminate the precipitate formed during the reaction, and put through a gel-filtration column to equilibrate it for the ion exchange column (Whatman DE-52 resin). The resin was equilibrated with 10 mM sodium phosphate at pH 8.0. The α -chain (α -PMB) elutes first, the unreacted Hb A moves slowly down the column, and the β -chain (β -PMB) remains bound to the resin. After the α -PMB has been collected, the unreacted rHbCO A continues to move down the column. When the α -PMB is far enough from the β -PMB, the resin with the bound β -PMB is gently dug out into another column and quickly eluted with 50 mM sodium phosphate at pH 7.0. Purity was again checked by electrophoresis.

The PMB was removed from the SH groups by reaction with 2-mercaptoethanol. The α -PMB and β -PMB were incubated for 1–2 h at room temperature in 50 mM 2-mercaptoethanol in 0.1 M phosphate buffer at pH 7.5. After incubation, the α - or β -chain was put through a gel filtration column with the same buffer. The eluted chain was then immediately put through another gel filtration column using 0.1 M phosphate buffer at pH 7.5 only. SH titration was performed to be sure that the PMB was removed.

Chain-selectively labeled hemoglobin formation

Chain-selectively labeled hybrid Hb A was prepared by mixing equal molar quantities of either isotopically labeled α -chain or β -chain with the complementary unlabeled chain. The chain-selectively labeled Hb A was then equilibrated with the appropriate buffer for NMR studies.

NMR spectroscopy

All NMR experiments were performed on a Bruker AVANCE DRX-600 spectrometer at 29°C. A 5-mm TBI triple resonance inverse probe with triple axis gradient was used, with the broad-band tuneable channel tuned to ^{15}N . Two-dimensional HMQC spectra were obtained in water (90% H_2O , 10% D_2O) using a modified version of the flip-back HMQC described by Gruschus and Ferretti (1998). An extra two-step phase cycle was added on the ^{15}N pulse for improved water suppression, and no ^{15}N decoupling was used during proton signal acquisition. Thus, all protons directly bound to a ^{15}N appear as intense, broad doublets in the proton dimension, whereas the protons coupled to ^{15}N via two or three bonds show an incompletely resolved multiplet structure.

The proton carrier frequency was set on the water resonance, and the nitrogen carrier was set at 160 ppm. Spectral widths employed were typically 22 ppm for ^1H and 240 ppm for ^{15}N . Usually 80 scans were averaged in 240 to 256 complex points in the indirect ^{15}N dimension, resulting in a total experiment time of 15 to 18 h. The ^1H time-domain acquisition size was 2K or 8K data points. The refocusing delay τ was kept short, at 5.5 ms, to preserve the signals from the ^1H directly attached to ^{15}N .

The water flip-back HMQC experiment generally provides a strong signal, but suppresses the signal of protons at chemical shifts near the water. In order to detect such signals, we performed an echo anti-echo HMQC experiment (Hurd and John, 1991; Tolman et al., 1992) which uses pulsed-field gradients for coherence selection, suppressing the solvent signal in the process without the need for selective pulses at the solvent frequency. The pulse program for this experiment was obtained by eliminating ^{15}N decoupling from the pulse program "inv4etf3gp" provided with Bruker's XWIN-NMR software, version 2.5.

Two-dimensional HSQC spectra were obtained using a pulse program adapted from the optimized 3D-NOESY-HSQC sequence of Talluri and Wagner (1996) and Briercheck et al. (1996). Single pulse carbon decoupling during ^{15}N evolution time was used for the samples triply labeled with ^{15}N , ^{13}C , and ^2H . The ^{15}N carrier frequency was set at 125 ppm, and the ^{15}N spectral width was 60 ppm. Thirty-two scans were averaged in 256 complex points acquired in the indirect ^{15}N dimension. Experimental parameters for the ^1H dimension were the same as those given above for the HMQC experiments.

Data were processed using both commercial (Bruker XWIN-NMR 2.5) and noncommercial software (nmrPipe; Delaglio et al., 1995), on either an SGI Indy 5000 or a Dell Precision Workstation 410 computer. Squared cosine windows were used in both dimensions, and a small amount of exponential line broadening was used in the proton dimension to smooth out the unresolved long-range multiplet structure. Standard one-level zero filling was used in each dimension before the Fourier transform.

Chemical shifts are reported from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for the proton dimension, using 4.76 ppm from DSS as the chemical shift of water at 29°C and a temperature coefficient of -0.01 ppm/°C. For the nitrogen dimension, the default referencing provided by the Bruker software was used. This assigns a value of 0.101329150 to the ratio Ξ of the zero-point reference frequency of ^{15}N to that of ^1H (taken as the resonance of DSS). By adding 0.32 ppm to our reported ^{15}N chemical shifts, this referencing may be brought in line with the recommendations of Wishart et al. (1995), who suggest $\Xi = 0.101329118$.

RESULTS

The 600-MHz (^1H , ^{15}N) HSQC spectra of (^2H , ^{15}N , ^{13}C) hybrid chain labeled HbCOA are shown in Fig. 2. These spectra were acquired at 29°C on samples dissolved in 90% H_2O /10% D_2O with 0.05 M phosphate at pH 6.5. Cross-peaks of tetrameric Hb A ($\alpha_2\beta_2$) with labeled α -chains and unlabeled β -chains are shown in blue; those of a sample

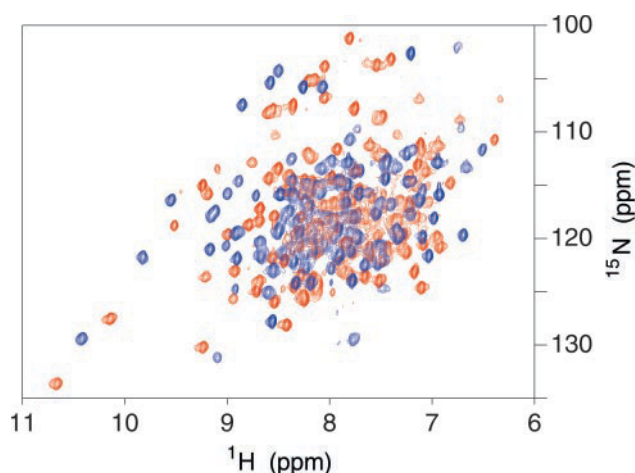


FIGURE 2 Backbone amide region of the 600-MHz (^1H , ^{15}N)-HSQC spectra of chain-selectively labeled HbCO A in water with 0.05 M phosphate at pH 6.5 and 29°C. Cross-peaks of a tetrameric Hb sample with (^2H , ^{15}N , ^{13}C)-labeled α -chains and natural-abundance β -chains are shown in blue; those of a sample with natural-abundance α -chains and (^2H , ^{15}N , ^{13}C)-labeled β -chains are shown in red.

with labeled β -chains and unlabeled α -chains are shown in red. From this figure, it is clear that the degree of overlap between these two spectra would render the assignment of the fully labeled Hb A tetramer extremely difficult if not impossible. However, by labeling one type of subunit at a time, we can resolve most of the expected cross-peaks.

A total of 119 and 145 peaks were picked from the α - and β -chain labeled spectra, respectively. Each α -chain of Hb A consists of 141 amino acid residues, including 7 Pro, 4 Asn, 1 Gln, 1 Trp, and 3 non-exchangeable His (see Discussion). Thus, excluding the N-terminal backbone amide along with Lys and Arg sidechains, we expect to see a maximum of 147 cross-peaks in the (^1H , ^{15}N) spectrum of an α -chain of Hb A. Each β -chain of Hb A consists of 146 amino acid residues, including 7 Pro, 6 Asn, 3 Gln, 2 Trp, and 1 non-exchangeable His, leading to a maximum of 159 expected cross-peaks. Thus, we are able to resolve approximately 81% and 91% of the expected resonances in the 2D-HSQC spectra of hybrid α - and β -chain (^2H , ^{15}N , ^{13}C)-labeled HbCO A, respectively.

A sample of ^{15}N -labeled HbCO A grown in H_2O and then dissolved in D_2O continued to exhibit cross-peaks in HSQC spectra (results not shown) for several weeks. This indicates that several amide protons are sufficiently protected so that they exchange very slowly with the solvent. Conversely, several of the deuterated amides of the D_2O -grown sample dissolved in H_2O may not have exchanged with protons. This, and a certain degree of overlap in the HSQC spectra, could account for some of the missing number of peaks in the spectra shown in Fig. 2.

Echo anti-echo HMQC spectra were acquired separately for the two types of chain-selective ^{15}N -labeled HbCO A.

The histidine/tryptophan regions of these spectra are superimposed in Fig. 3, using the same color scheme as in Fig. 2. Because these spectra were acquired without ^{15}N decoupling, a doublet is observed at the ($^1\text{H}\delta_1$, $^{15}\text{N}\delta_1$) or ($^1\text{H}\epsilon_2$, $^{15}\text{N}\epsilon_2$) chemical shift coordinates of histidyl residues, which are not exposed to solvent. These comprise $\alpha 122$ and $\alpha 103$, which are involved in hydrogen bonds in the $\alpha_1\beta_1$ interface, and the proximal histidines $\alpha 87$ and $\beta 92$. As discussed above, this spectrum also correlates the imidazole ^{15}N chemical shifts with carbon-bound protons, $^1\text{H}\epsilon_1$ and $^1\text{H}\delta_2$, which are identified for the interfacial histidines $\alpha 122$ and $\alpha 103$, the proximal histidines $\alpha 87$ and $\beta 92$, and the distal histidines $\alpha 58$ and $\beta 63$. The assignments were aided by spectra of deoxy-Hb A as well as isolated ^{15}N -labeled α -chains of Hb A (results not shown). The upper left region of Fig. 3 reveals cross-peaks correlating $^{15}\text{N}\epsilon_1$ of each tryptophan residue with $^1\text{H}\epsilon_1$ and $^1\text{H}\delta_1$ through one- and two-bond couplings, respectively. The $^1\text{H}\epsilon_1$ resonances of

the β -chain Trp residues were assigned with the aid of HSQC spectra of deoxy-Hb A, in which the chemical shift of Trp37 $\text{H}\epsilon_1$ changes as it becomes involved in an interfacial H-bond.

For histidyl residues at the surface of the protein, the side-chain ^{15}N -bonded protons are in rapid exchange with the solvent, so they are not detected in the HMQC spectrum. However, the carbon-bound protons coupled to the nitrogen nuclei through two or three bonds yield sharp cross-peaks, which appear within the dashed rectangle in Fig. 3. Fig. 4 shows the corresponding region of the water flip-back HMQC spectrum of a different sample: fully ^{15}N -labeled HbCO A in 0.1 M phosphate buffer at pH 6.86. These conditions were chosen to closely match those of Russu et al. (1989), who reported the 300 MHz ^1H spectrum of HbCO A dissolved in D_2O . In spite of the different field strengths and solvents used, the $^1\text{H}\epsilon_1$ (C2 proton) chemical shifts of our 2D-HMQC spectrum agree very well with

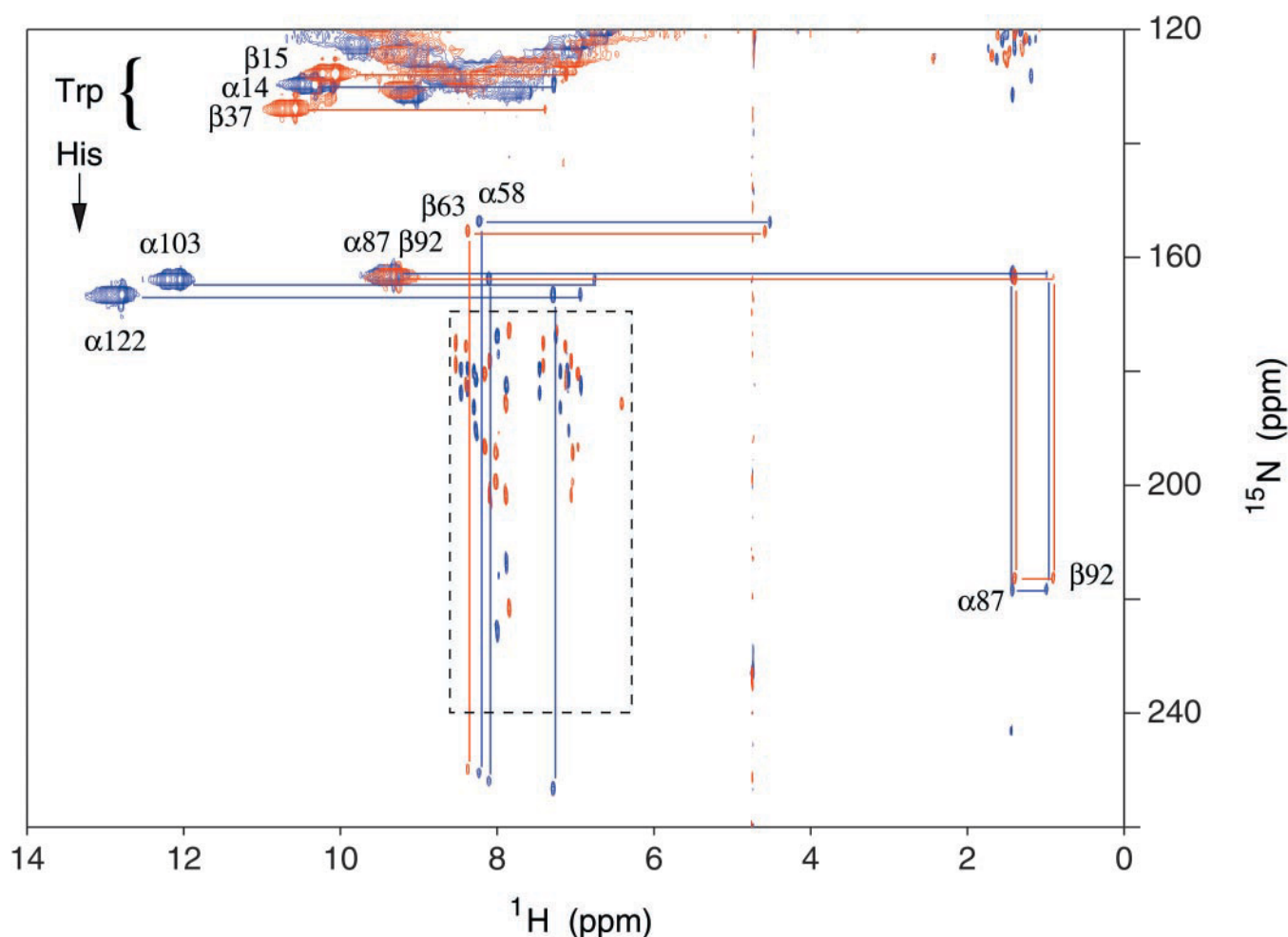


FIGURE 3 Histidine region of the 600-MHz (^1H , ^{15}N) echo anti-echo HMQC spectra of chain-selectively labeled HbCO A in water with 0.05 M phosphate at pH 6.5 and 29°C. Cross-peaks of a tetrameric Hb sample with ^{15}N -labeled α -chains and natural-abundance β -chains are shown in blue; those of a sample with natural-abundance α -chains and ^{15}N -labeled β -chains are shown in red. Cross-peaks originating from the same residue are connected by lines. The area within the *dashed rectangle* contains cross-peaks originating from surface (solvent-exposed) histidyls.

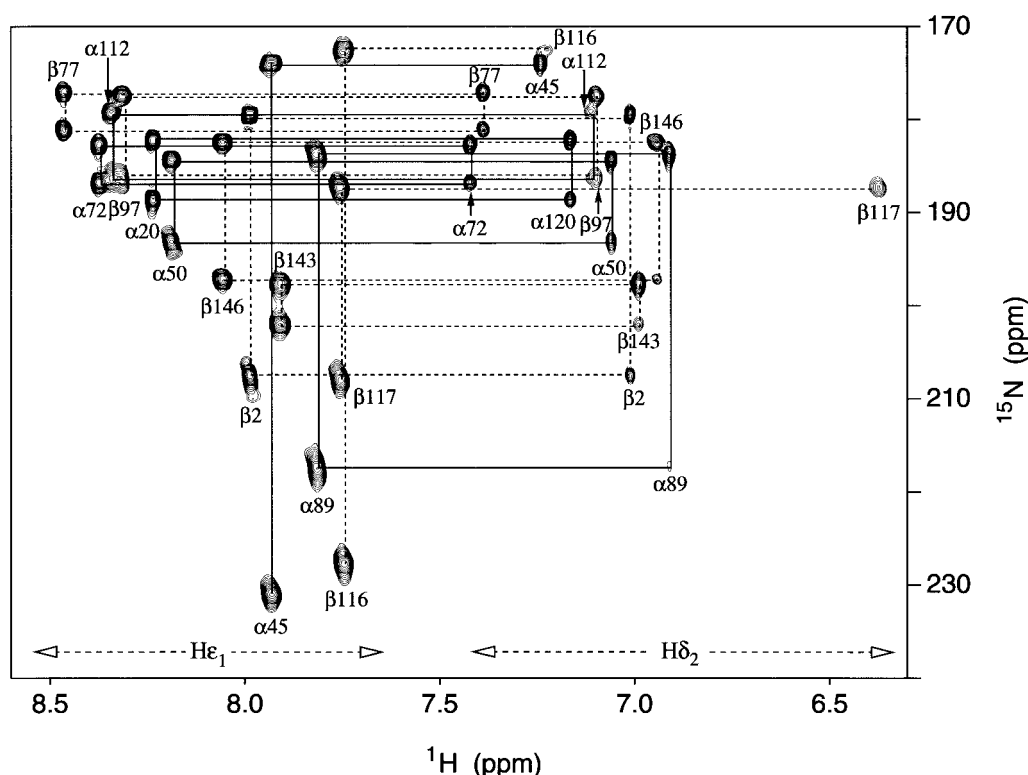


FIGURE 4 Surface histidines region of the 600-MHz (^1H , ^{15}N)-HMQC spectrum of HbCO A in water with 0.1 M phosphate at pH 6.86 and 29°C. Lines connect cross-peaks originating from the same residue; *solid* and *dashed* lines are used for α - and β -chain residues, respectively.

those of the 1D spectrum of Russu et al. (1989); see Fig. 5. The assignment of these resonances, traditionally labeled A, B, . . . , L, were obtained earlier from ^1H -NMR studies (see Sun et al., 1997, Fang et al., 1999, and references therein), except for resonance H. The 2D-NMR spectrum shown in Fig. 4 renders obvious the assignment of each $^1\text{H}\delta_2$ (C4) resonance by its correlation with the $^1\text{H}\epsilon_1$ chemical shift of the same histidyl residue. Here, $^1\text{H}\delta_2$ and $^1\text{H}\epsilon_1$ resonances are identified for six α -chain and seven β -chain surface histidyls. Together with the two proximal, two distal, and two interface residues discussed above, they account for all 19 histidyls per $\alpha\beta$ dimer of the hemoglobin molecule (Dickerson and Geis, 1983). The assignments of all histidine and tryptophan side-chain ^1H and ^{15}N chemical shifts observed in HbCO A are listed in Tables 1 and 2.

DISCUSSION

Isotope labeling strategy for hemoglobin

As is shown in Fig. 2, the degree of overlap in the HN HSQC spectra of the α - and β -chains would make any multinuclear, multidimensional study of hemoglobin extremely difficult, if not impossible, on a Hb sample fully labeled in both α - and β -chains. These spectra were obtained on samples partially (about 80%) deuterated, which makes the lines narrower in both H and N dimensions. If no deuteration is employed, the resolution is significantly

poorer, making the use of nondeuterated samples impossible for a fully labeled protein, save for a few resonances separated from the main amide region. The spectral resolution can be further improved using recently developed transverse relaxation optimized spectroscopy (TROSY; Pervushin et al., 1997), especially for deuterated rHb. In a sensitivity-enhanced TROSY-HSQC spectrum (Pervushin et al., 1998; and results not shown) of (^{15}N , ^2H) α -labeled HbCO A, the ^1H and ^{15}N linewidths are reduced to approximately 60% and 50% of their respective values in the conventional HSQC spectrum.

The idea of separating the α - and β -chains and recombining them is not new, as demonstrated by the fact that well established procedures for chain separation have been perfected; see, e.g., Bucci (1981) and references therein. Here, we demonstrate the power of this approach in simplifying the crowded NMR spectra of multimeric proteins. As another illustration of the usefulness of this approach, we report the completion of proton and nitrogen assignments of the imidazole side-chain resonances for all histidine residues in HbA, as well as of key indole side-chain resonances of all tryptophan residues.

Interfacial histidines

The x-ray structure of hemoglobin shows that there are two histidines in the $\alpha_1\beta_1$ interface, $\alpha 103\text{His}$ and $\alpha 122\text{His}$

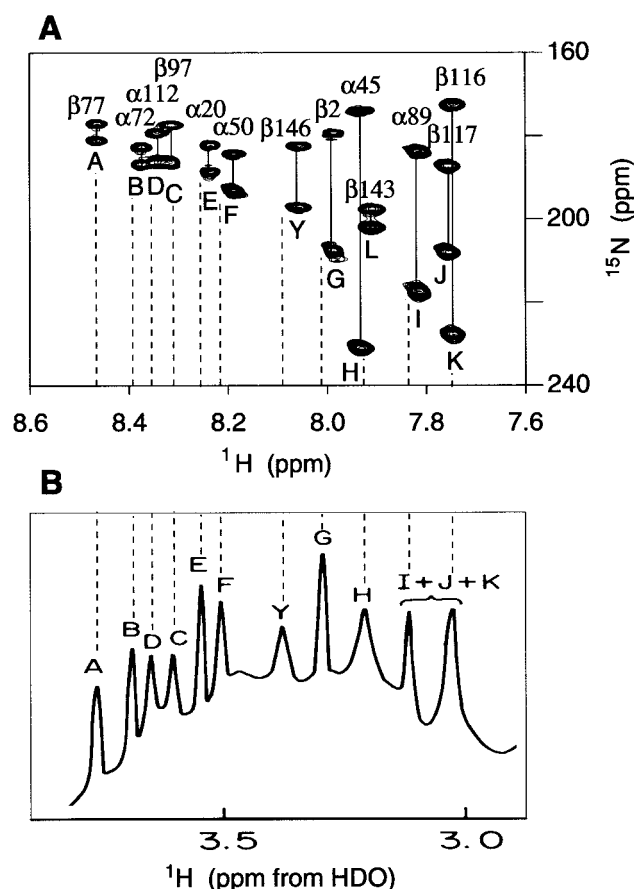


FIGURE 5 (A) Surface histidines He_1 region of the 600-MHz (^1H , ^{15}N)-HMQC spectrum of HbCO A in water with 0.1 M phosphate at pH 6.86 and 29°C. (B) 300-MHz ^1H -NMR spectrum of HbCO A in D_2O with 0.1 M phosphate at pH 6.86 and 29°C, scanned from the original figure in Russu et al. (1989).

(Dickerson and Geis, 1983). The HSQC and HMQC spectra show clearly that the exchangeable proton resonances at 12.1 ppm and 12.9 ppm belong to imidazole He_2 and Ne_2 resonances of histidines from α -chains (see Figs. 1 and 3). The resonance at 12.1 ppm from DSS (7.4 ppm from H_2O) was assigned to a H-bond between $\alpha 103\text{His}$ and $\beta 108\text{Asp}$ (Russu et al., 1987) based on NMR results and the available x-ray structures of Hb. However, a recent high-resolution x-ray structure of deoxy-Hb obtained by Tame and Vallone (1998) differs from the earlier structure (Fermi et al., 1984) by a 180° rotation of $\alpha 103\text{His}$, which suggests that Ne_2 forms a H-bond with the side-chain oxygen of $\beta 131\text{Gln}$. Further evidence for the involvement of $\beta 131\text{Gln}$ is provided by ^1H -NMR of mutant rHb (βQ131E) (C.-K. Chang and C. Ho, unpublished results).

The ^1H resonance at 12.9 ppm from DSS (8.2 ppm from H_2O) was previously assigned to a H-bonded proton between $\alpha 126\text{Asp}$ and $\beta 35\text{Tyr}$ in the $\alpha_1\beta_1$ interface (Asakura et al., 1976). This assignment was based on the invariance of this resonance to Hb oxygenation, and its absence in Hb

TABLE 1 Histidyl imidazole ring ^1H and ^{15}N chemical shift assignments for HbCO A in H_2O , 0.1 M sodium phosphate at pH 6.86 and 29°C

Residue	$\text{N}\delta_1$	$\text{N}\epsilon_2$	$\text{H}\delta_2$	$\text{H}\epsilon_1$	$\text{H}\delta_1$	$\text{H}\epsilon_2$
α 20	188.57	182.19	7.165	8.236		
α 45	231.03	173.95	7.241	7.933		
α 50	193.13	184.30	7.060	8.189		
α 58	250.55	153.78	4.512	8.231		
α 72	186.77	182.69	7.422	8.373		
α 87	163.23	218.24	0.998	1.425	9.411	
α 89	217.31	183.76	6.911	7.817		
α 103	251.84	163.75	6.763	8.118		12.125
α 112	186.26	179.18	7.113	8.343		
α 122	253.24	166.49	6.943	7.293		12.877
β 2	207.52	179.45	7.011	7.989		
β 63	249.99	155.23	4.576	8.378		
β 77	181.10	177.07	7.388	8.464		
β 92	163.42	216.21	0.903	1.396	9.344	
β 97	186.28	177.44	7.098	8.316		
β 116	227.75	172.58	7.226	7.747		
β 117	208.02	187.30	6.374	7.756		
β 143	202.02	197.58	6.990	7.910		
β 146	197.16	182.45	6.944	8.056		

Philly ($\beta 35\text{Tyr} \rightarrow \text{Phe}$). This assignment appeared to be consistent with 300-MHz ^1H nuclear Overhauser (NOE) spectra (Russu et al., 1987). However, a recent study (Nakatsukasa et al., 1998) of rHb ($\beta 35\text{Tyr} \rightarrow \text{Phe}$) obtained by site-directed mutagenesis showed that this mutant Hb has a ^1H -NMR peak at approximately 13 ppm, and exhibits oxygen-binding cooperativity and affinity inconsistent with those reported for Hb Philly. These results cast doubt on the supposed amino-acid substitution in Hb Philly as well as the assignment of the 12.9 ppm ^1H -NMR peak. The 2D- (^1H , ^{15}N)-HMQC spectrum shown in Fig. 3 clearly demonstrates that this resonance originates from the He_2 proton of a histidine (see Fig. 1B). The presence of this cross-peak indicates that the proton is protected from exchange with solvent, while its absence from the spectrum of a sample with natural abundance α -chains and ^{15}N -labeled β -chains shows that it comes from a histidine in an α -chain. The only possible candidate is $\alpha 122\text{His}$. This residue is in the $\alpha_1\beta_1$ interface, within 6 Å of $\alpha 126\text{Asp}$ and $\beta 35\text{Tyr}$ (Baldwin, 1980); thus, the arguments from NOE results supporting the previous assignment of the 12.9 ppm peak do not contradict its assignment to $\alpha 122\text{His}$. The proximity to the aromatic ring of $\beta 35\text{Tyr}$ would explain the pronounced upfield shift of the carbon-bound He_1 proton at 7.29 ppm. Furthermore,

TABLE 2 Tryptophan ^1H and ^{15}N chemical shift assignments for HbCO A in H_2O , 0.1 M sodium phosphate at pH 6.86 and 29°C

Residue	$\text{N}\epsilon_1$	$\text{H}\epsilon_1$	$\text{H}\delta_1$
α 14	129.53	10.40	7.28
β 15	127.66	10.14	7.12
β 37	133.81	10.66	7.39

as N δ_1 of α 122His is packed tightly against β 30Arg (Baldwin, 1980), it cannot be protonated without causing a steric clash; thus, the observed cross-peak must originate from H ϵ_2 .

Heme pocket histidines

The resonances of the proximal and distal histidines were unambiguously assigned in this work using hybrid samples of HbCO A labeled with ^{15}N at either the α - or β -chains. These cross-peaks were absent in the spectra of deoxy-Hb A (results not shown), because of the proximity of these histidines to the paramagnetic iron. The NH single-bond cross-peaks at 9.4/163.4 ppm belong to an imidazole N δ , as the complete pattern looks like Fig. 1 A. This pattern and the extreme upfield shift of H ϵ_1 and H δ_2 are consistent with the proximal histidines, whose N ϵ_1 is bound to the heme iron. The assignment of the H ϵ_1 of the distal histidine in isolated α -chains in the CO form was first carried out by Dalvit and Ho (1985) by means of truncated-driven NOE measurements. By using phase-sensitive 2D- ^1H -NMR techniques, Dalvit and Wright (1987) extended the assignments of the distal histidine and assigned resonances in the vicinity of the heme, including those of the imidazole proton resonances of the proximal histidine in isolated α -chains of Hb A in the CO form at 36°C and pH 5.3. Here, we confirm those assignments and add the assignment of the imidazole protons of the distal and proximal histidines of the β -chains of HbCO A. Our ^1H assignments of the proximal and distal histidines in the α -subunits agree to within 0.1 ppm with those reported by both Dalvit and Wright (1987) and by Martineau and Craescu (1992) for isolated α -chains of HbCO A under slightly different buffer and temperature conditions. It is noteworthy that, although the N ϵ_2 chemical shift of the distal histidines indicates a protonated state, no single-bond NH can be detected for either α - or β -chains, presumably due to exchange with a water molecule trapped in the distal heme pocket.

Surface histidines

Previous work from our laboratory has assigned the H ϵ_1 (C2) resonances for all surface histidine residues, except α 45. Although resonance H was tentatively assigned to α 45 (Ho and Russu, 1985), later it was thought that it might be one of the distal histidines. The present study unequivocally shows that resonance H is not a distal histidine and thus, assigns resonance H to α 45His, completing the assignment of the imidazole side chain of all histidyl residues in HbCO A.

The use of ^{15}N labeling provides new means for assigning the imidazole protons of the surface histidines. First, because it can label only one type of chain at a time, the NMR spectra can be edited to contain only the resonances from either the α -chain or the β -chain. Second, it is very easy to

correlate the H ϵ_1 and H δ_2 resonances via coupled HMQC. Such a correlation is capable, for example, of directly solving the problem of assigning the resonances J and K, which until recently were lumped together as β 116His or β 117His. Fang et al. (1999) mutated β 116His into Gln, and thus assigned resonance J to β 117His. In the assignments reported in that work, extensive use was made of correlation between C2(H ϵ_1) and C4(H δ_2) protons by means of TOCSY experiments. Although such experiments work, the spectra take a long time to acquire, the cross-peaks are weak, and there is always the problem of overlap with the resonances of the aromatic residues. It is actually quite remarkable that cross-peaks were obtained for a protein of this size (64.5 kDa), for side-chain protons coupled via 4 bonds! The HMQC spectrum in Fig. 4 shows right away that there is a C4(H δ_2) proton shifted unusually far upfield, to about 6.4 ppm, coupled to peak J. In the amino acid sequence of Hb, β 117His is followed by a Phe, whose ring current would create an upfield shift of an imidazole proton of β 117His and therefore peak J must belong to β 117His.

Further information is obtainable from the ^{15}N chemical shifts. As shown schematically in Fig. 1, the chemical shift separation between the $^{15}\text{N}\delta_1$ and $^{15}\text{N}\epsilon_2$ resonances of histidines is expected to be large (up to 80 ppm) for residues with pK values much lower than the pH of the NMR sample. This separation should decrease for histidyls with higher pK, finally resulting in degenerate chemical shifts when the pK is much higher than the sample pH and both imidazole nitrogens are protonated. This expected trend is followed quite well by 12 of the 13 distinct surface histidyls of Hb A, as seen by correlating the cross-peaks observed at pH 6.86 (Fig. 4) with the measured pK values (Ho and Russu, 1987; Russu et al., 1989; Fang et al., 1999). For example, β 77His exhibits a ^{15}N separation of 4.0 ppm, and has a pK of 7.81 in 0.1 M phosphate (Ho and Russu, 1987), whereas α 45His shows a ^{15}N separation of 57.1 ppm, and has a pK of 6.09 in 0.1 M HEPES plus 0.1 M chloride (Fang et al., 1999). However, β 143His is a striking exception to this trend. With a pK of 5.73 in HbCO A and the corresponding value of 4.70 for deoxy-Hb A in 0.1 M HEPES plus 0.1 M chloride (Fang et al., 1999), β 143His is expected to exhibit a ^{15}N chemical shift separation larger than that of α 45His, but the observed separation is only 4.4 ppm. This anomalous behavior is a clear indication that β 143His experiences a very unique environment as reflected by unusually low pK values for both deoxy and CO forms and by perturbed nitrogen chemical shifts. Located in the lining of the central cavity of Hb A, this residue is exposed to solvent (Dickerson and Geis, 1983). For a discussion of the environment and properties of β 143His, see Fang et al. (1999).

Tryptophan side chains

The HMQC spectra of chain-selectively ^{15}N -labeled Hb A have also provided partial side-chain assignments for

α 14Trp, β 15Trp, and β 37Trp. The assignment of α 14Trp is straightforward. The HN cross-peak at 10.4/129.5 ppm shows a clear correlation with a sharp peak at 7.28/129.5 ppm, which must, thus, be the H δ_1 proton. A strip at 10.4/129.5 ppm from a 3D-NOESY-HSQC experiment performed at 34°C (results not shown) contains cross-peaks at proton frequencies of 7.28 and 7.53 ppm, identifying thus the H ζ_2 proton at 7.53 ppm. The assignments for the indole proton resonances of α 14Trp in isolated α -chains of Hb A in the CO form at pH 5.3 and 36°C were first published by Dalvit and Wright (1987), and nearly identical values were reported later by Martineau and Craescu (1992) under very similar sample conditions. Our assignments under somewhat different conditions are very close to theirs. As distinct from both previous reports, we see the H δ_1 proton at a chemical shift of 7.28 ppm, not 7.32. As we detect a through-bond connectivity, we are confident that our identification of H δ_1 of α 14Trp is correct.

The β -chains possess two tryptophans, whose indole NH resonances are well resolved from each other and from the bulk of the amide resonances. The HMQC spectrum has proved the identity of these NH resonances as indole by the correlation with an aromatic proton through a long-range coupling to the nitrogen. The differential assignment was made by using an Hb sample in the deoxy form, where the β 37Trp indole NH becomes involved in an interfacial hydrogen bond and its resonance is shifted downfield (results not shown). The chemical shifts of the three Trp residues are listed in Table 2.

The assignment of the Trp residues is very important for understanding the structure-function relationship in hemoglobin. β 37Trp is of particular interest, because it lies within the $\alpha_1\beta_2$ interface, a region essential to the cooperative oxygenation of Hb A. The environment of β 37Trp is significantly different in the various conformations of Hb A (for details, see Ho and Lukin, 2000). Recently, using the pHE2 plasmid provided by our laboratory, Hu and Spiro (1997) have assigned UV resonance Raman bands due to α 14Trp and β 37Trp. Thus, another application of our ability to observe Trp residues in our NMR spectra is to make a direct comparison between NMR and resonance Raman results on structural changes associated the cooperative oxygenation of Hb A and other mutant hemoglobins.

CONCLUSION

Two-dimensional heteronuclear NMR spectroscopy is seen to be capable of revealing the resonances of individual amino acids in proteins as large as hemoglobin, with a molecular weight of 64.5 kDa. Chain-selective isotopic labeling reduces spectral overlap and provides unambiguous residue-specific information. Here, the HMQC spectrum has helped, completing the assignment of all histidines of HbCO A by confirming and extending the previous assignments. The surface histidine residues have previously been

shown to be responsible for 86% of the alkaline Bohr effect at pH 7.4 and 55% of the acid Bohr effect at pH 5.1 (Sun et al., 1997; Fang et al., 1999). The assignments were aided by the use of chain-selective isotopically labeled samples, obtained by separating the α - and β -chains of uniformly ^{15}N -labeled Hb A and recombining them with the complementary chains of the natural abundance protein. This technique promises to be useful in the study of this and other multimeric, allosteric proteins and regulatory enzymes.

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