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Direct Detection of a Histidine−Histidine Side Chain Hydrogen Bond Important for Folding of Apomyoglobin

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Abstract: Sperm whale myoglobin in which the heme group has been removed (apomyoglobin) unfolds to an equilibrium intermediate form at pH 4 and can be completely unfolded at acid pH and low salt conditions. The titration of a pair of partially buried histidine side chains, His24 and His119, is particularly important for the acid-induced formation of the intermediate form from native apomyoglobin. Modifying a recently introduced ^{1}H−^{15}N HNN-COSY nuclear magnetic resonance (NMR) experiment (Dingley, A. J.; Grzesiek, S. *J. Am. Chem. Soc.* **1998**, *120*, 8293−8297) allowed us to detect a $^{2}J_{NN}$ scalar coupling between imidazole NH nitrogen of His119 and the unsubstituted imidazole nitrogen of His24. These measurements directly verify the existence of a previously proposed side chain−side chain hydrogen bond important for the folding mechanism of apomyoglobin.

Introduction

Sperm whale myoglobin (Figure 1) in which the heme group has been removed (apomyoglobin) unfolds to an equilibrium intermediate form at pH 4 and can be completely unfolded at acid pH and low salt conditions.[1] The intermediate form (*I*) is believed to be identical to an intermediate in the kinetic folding pathway of apomyoglobin.[2] The three different equilibrium states are conveniently separated by pH change: The native state *N* at pH 6 is similar in structure to holomyoglobin except for the heme binding region which shows conformational averaging in the absence of heme.[1,3−8] The intermediate form *I* at pH 4 is a typical example of a loosely structured "molten globule" state with a compact structure, persistent nativelike secondary structure, but poorly packed side chains.[3,9−15] *I* contains a stable subdomain of N with intact A, G, and H helices[3] (Figure 1) and is stabilized by nonspecific hydrophobic interactions as well as partially formed nativelike tertiary contacts.[16,17] At acid pH and low salt apomyoglobin adopts a fully unfolded state *U*. Acid-induced unfolding of $I \rightarrow U$ can be described by a generalized charge effect rather than titration of specific groups.[18] The $N \rightarrow I$ reaction requires the uptake of about two protons, and protonating the buried and hydrogen-bonded pair of histidine residues His24•His119 is thought to be chiefly responsible.[19] Recent ^{1}H−^{15}N NMR titration data[20] confirmed the tautomeric arrangement of the His24•His119 pair in apomyoglobin as shown in Figure 1b. Tautomers and titration behavior are identical in holomyoglobin,[21−23] emphasizing the structural similarities of the two protein forms. The His24•His119 hydrogen bond that was initially proposed on the basis of X-ray[24] and neutron diffraction studies of holomyoglobin[25] should therefore likely exist in apomyoglobin also. Protonation of His119 (pK_a of 6.0 in *N*) does not affect the tautomeric state of the His24•His119 pair and does not cause unfolding of *N* to *I*.[20] His24 remains neutral until *N* unfolds to *I*. Because of its unusually low pK_a (pK_a < 4 in *N*) protonation of the fully buried histidine His24 contributes most to the free energy driving the $N \rightarrow I$ reaction.[20] Partial titration of aspartate or glutamate side chains or other histidines can account for the free energy associated with the uptake of a second proton.[20] Protonation of His119 should make its imidazole hydrogen more acidic and may effect the energetics of the His24•His119 hydrogen bond (Figure 1b). Hydrogen bonds and particularly their strength are, however, hard to detect directly. In ribonucleic acid base pairs imino-to-imino hydrogen bonds have recently been directly detected via a heteronuclear HNN-COSY experiment.[26] In this experiment $^{2}J_{NN}$ scalar couplings between hydrogen bond donating imino nitrogens and acceptor nitrogens were observed

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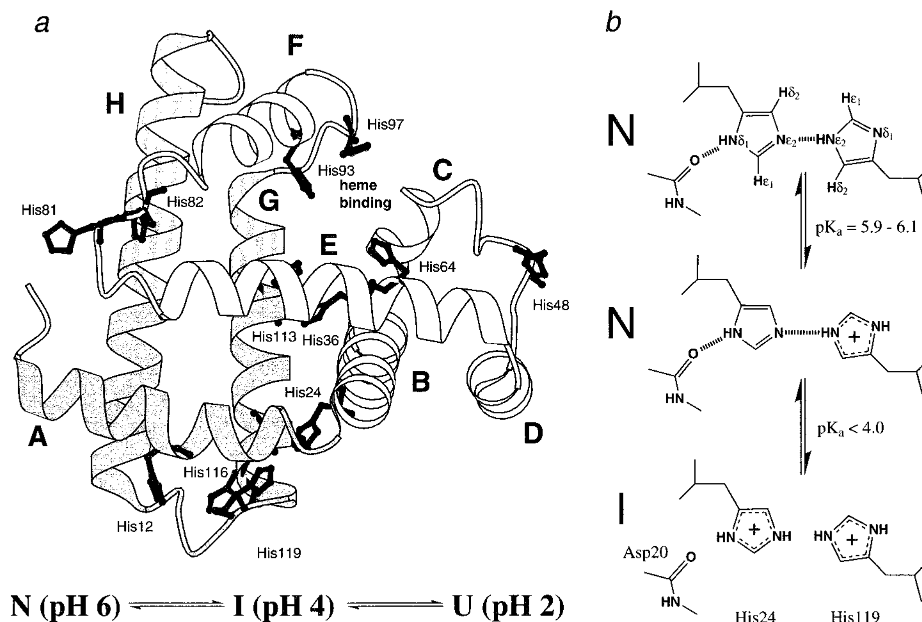

Figure 1. (a) MOLSCRIPT diagram[38] of sperm whale holomyoglobin.[36] The A, G, and H helices that are stably formed in the intermediate *I* of apomyoglobin are highlighted in gray. The ends of the helices A, G, and H are frayed, even in native apomyoglobin, as indicated by partial shading.[7] Histidine side chains are shown as ball-and-stick models. The $N \rightarrow I$ transition of apomyoglobin has a midpoint of approximately pH 4.5; while the midpoint of the $I \rightarrow U$ transition lies at approximately pH 3.2.[19,20] (b) Schematic representation of tautomeric and protonation states of the His24·His119 pair in apomyoglobin. In *N* His119 titrates with a pK_a of 5.9 to 6.0 while the pK_a of His24 is below 4. His24 and His119 are both protonated in the partiallly unfolded intermediate *I*.[20]

in RNA base pairs and more recently also in DNA.[27] Here, we modify the HNN-COSY experiment[26] to directly detect an unusual NH···N hydrogen bond in apomyoglobin and study the effects of protonating the donor side chain His119 on the size of the cross hydrogen bond $^2J_{NN}$ scalar coupling.

Experimental Section

Sample Preparation. Uniformly ^{15}N-labeled wild-type sperm whale apomyoglobin was prepared and purified as described previously.[12] Lyophilized protein was dissolved in 10 mM acetic acid-d_3 (CIL, Andover, MA) in 99.9% D_2O (Deutero, Kastellaun) or in 90%/10% H_2O/D_2O by inverting an Eppendorf tube in the cold room at 8 °C. The pH was adjusted by adding small amounts of 0.4% NaOD or 0.35% DCl (CIL, Andover, MA). Samples were stored at 4 °C. Protein concentrations were 8.9−12.2 mg/mL (sample volume 0.45 mL) corresponding to approximately 0.5−0.7 mM. All pH values are the uncorrected readings of an Aldrich Comb NMR pH electrode (Aldrich, Milwaukee, WI) at room temperature.

Nuclear Magnetic Resonance Spectroscopy. Nuclear magnetic resonance (NMR) spectra were acquired at 308 K on a BRUKER DRX500 spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten) equipped with a triple-resonance, actively shielded, *z*-gradient probe. Quadrature detection in indirect nitrogen (ω_1) dimensions was achieved via the States-TPPI method.[28] In the case of the quantitative ($^2J_{HN}$)-HNN-COSY spectra 48 complex points with 1224−1600 transients each were recorded with an acquisition time of 18.4 ms for ^{15}N (ω_1). For the ^{1}H−^{15}N HMQC spectra, a total of 128 transients were signal-averaged for each of the 64 complex points with an acquisition time of 24.6 ms for ^{15}N (ω_1). Both experiments were optimized for the detection of histidine residues,[29] correlating aromatic protons and imidazole nitrogens using a 22-ms delay between the first 90° pulses at the proton and nitrogen frequency in a standard ^{1}H−^{15}N HMQC pulse sequence or a 44-ms delay for the INEPT transfer in the ($^2J_{HN}$)-HNN-COSY spectrum. Additional changes with respect to the published HNN-COSY pulse sequence[26] include a conventional INEPT-type back transfer before detection of aromatic protons in ω_2. For both experiments 2048 complex points with an acquisition time of 340.8 ms for ^{1}H (ω_2) at 500 MHz proton frequency were employed. The residual HDO line was suppressed by using a low-power presaturation pulse during the recycling delay of 1.2 s between transients. Carrier positions were set to 205.30 ppm for ^{15}N, and 4.70 ppm for ^{1}H (relative to TSP). All proton pulses are centered on the water resonance. High-power proton pulses are applied with a field strength of 26.9 kHz. ^{15}N decoupling during acquisition employs a 1-kHz GARP[30] field, while high-power nitrogen pulses are applied with a field strength of 8.3 kHz. Spectra were processed and analyzed using either Felix (Biosym/MSI, San Diego, CA) or XWIN NMR 2.1. (Bruker Analytische Messtechnik GmbH). A solvent suppression filter was used in the ω_2 dimension to eliminate distortions from residual water[31] prior to apodization with a 90°-shifted squared sinebell window function. Data sets were zero-filled twice in the direct dimension and Fourier transformed, retaining only the ^{1}H^{arom}-containing region of the spectra. The ω_2 dimensions were zero-filled to 128 complex points and multiplied by a 90°-shifted squared sinebell window function prior to Fourier transformation. Coupling constants were obtained in these quantitative *J* correlation spectra from the ratio of cross and reference peak intensities.[32] Assignment of histidine cross-peaks was achieved by transferring the assignments of Lecomte and co-workers[6,4] reported at 298 K for sperm whale apomyoglobin and as previously described.[20]

Results and Discussion

Hydrogen bonds play a dominant role in stabilizing proteins and nucleic acids. During X-ray and NMR structure refinement hydrogen bonds are indirectly inferred from the position of donor and acceptor groups. Recently a heteronuclear HNN-COSY NMR experiment[26] has been introduced to directly detect imino-to-imino hydrogen bonds in RNA base pairs via $^2J_{NN}$ scalar

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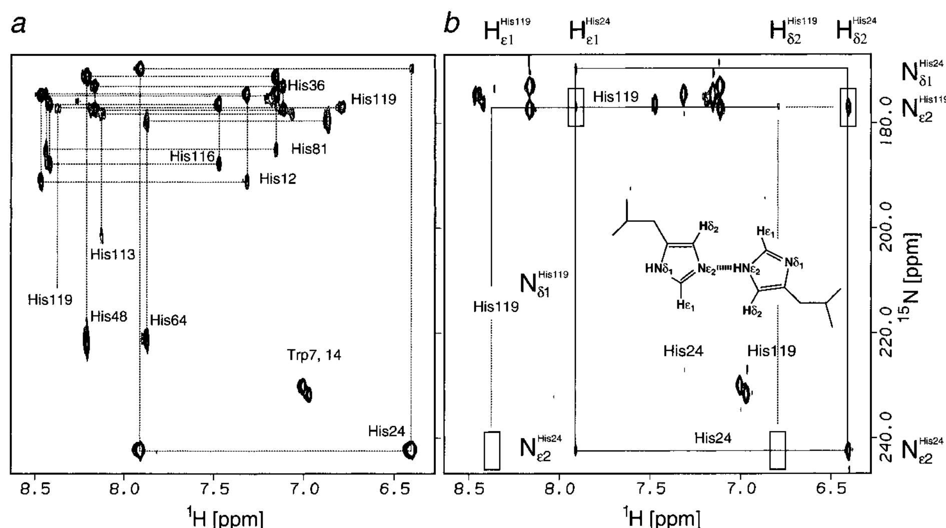

Figure 2. Nuclear magnetic resonance (NMR) spectra of ^{15}N-labeled wild-type apomyoglobin acquired at 308 K (10 mM sodium acetate in 99.9% D_2O, pH 6.0) at 11.8 T on a DRX500 Bruker NMR instrument (Bruker Analytische Messtechnik GmbH, Rheinstetten). (a) $^1H-^{15}N$ HMQC spectrum correlating aromatic protons and nitrogens of histidine side chains. (b) ($^2J_{HN}$)−HNN-COSY spectrum correlating the hydrogen bonded imidazole ϵ_2 nitrogens of the His24•His119 pair. Positions of negative $^2J_{NN}$ cross-peaks are highlighted by rectangles. In principle four such negative cross-peaks should be observable, but resonances of His119 are exchange-broadened especially in the ^{15}N-dimension because of protonation/deprotonation[35] (see text for discussion). At pH 6.0 only two $^2J_{NN}$ cross-peaks are clearly visible with a third cross-peak detectable near the noise level between His119 Hδ_2 and Nϵ_2 of His24. Resonances for His24 and His119 are labeled in Figure 2b; assignments for all other histidines are shown in Figure 2a. Aliased cross-peaks Trp7, Trp14 are correlation peaks to the indole nitrogens of tryptophan 7 and 14.

couplings between hydrogen bond donating imino nitrogens and acceptor nitrogens. The cross hydrogen bond $^2J_{NN}$ coupling constants are about 6.7 Hz for U−A and 6.3 Hz for G−C base pairs.[26] Similar values have been measured also in DNA.[27] Scalar couplings are typically seen only between chemically bonded nuclei. Scalar couplings mediated by hydrogen bonds suggest significant covalent bond character and such couplings have previously been observed for NH···S−^{113}Cd and NH···S−^{199}Hg hydrogen bonds in a metalloprotein.[33,34] Here, we directly detect an unusual NH···N hydrogen bond in native apomyoglobin: The side chain−side chain hydrogen bond between His24 and His119 forms a key tertiary interaction in *N*, and titration of the His24•His119 pair has been invoked in the mechanism of acid-induced transition of *N* to the equilibrium protein folding intermediate *I*.[19,20]

Figure 2 shows a $^1H-^{15}N$ HMQC NMR spectrum (Figure 2a), optimized for the detection of correlations between aromatic protons and nitrogens in histidine side chains[29] in comparison with a quantitative ($^2J_{HN}$)-HNN-COSY experiment of an apomyoglobin sample at pH 6.0 in 99.9% D_2O (Figure 2b). In the latter experiment, in contrast to previous reports,[26,27] magnetization is transferred from the aromatic protons of histidines through two-bond couplings to the nitrogens. Nitrogen excitation is followed by a COSY-type transfer between nitrogens and back transfer. Because of the lower sensitivity only the strongest $^2J_{HN}$ correlation peaks in the $^1H-^{15}N$ HMQC spectrum are also seen in the ($^2J_{HN}$)-HNN-COSY spectrum. In addition, a strong negative peak is observed at the chemical shift of the His24 δ_2 proton and of the ϵ_2 nitrogen of His119 (Figure 2b). A second but weaker negative peak is observed at the ϵ_1 proton resonance of His24 (Figure 2b). These cross-peaks can only be explained by $^2J_{NN}$ scalar coupling between the ϵ_2 nitrogens of His24 and His119, and therefore verify the existence of the hydrogen bond as shown in Figure 1b. The intensity ratios of the negative $^2J_{NN}$ cross-peaks and of their respective reference peaks ($^2J_{HN}$ correlations between Hδ_2 and Nϵ_2, and between Hϵ_1 and Nϵ_2 of His24, respectively) scale with $I_{cross}/I_{ref} = \tan^2(\pi J_{NN}\tau)$.[26,32] With $\tau = 24$ ms a $^2J_{NN}$ coupling of 9.7 ± 0.9 Hz can be extracted for the His24•His119 hydrogen bond in apomyoglobin at pH 6.0 (Table 1).

Table 1. $^2J_{NN}$ Coupling Constants for the Histidine His24•His119 Pair in Apomyoglobin[a]

pH	4.9	5.3	5.3 (H_2O)	5.6	6.0
$^2J_{NN}$ [Hz]	11.0 (±1.0)	10.2 (±0.7)	10.2 (±1.0)	8.4 (±0.7)	9.7 (±0.9)

[a] $^2J_{NN}$ coupling constants were calculated from the intensities of $^2J_{NN}$ cross and reference peaks according to $I_{cross}/I_{ref} = \tan^2(\pi J_{NN}\tau)$[26,32] for $\tau = 24$ ms. In principle four pairs of such cross and reference peaks should be observable. $^2J_{NN}$ coupling constants were derived from the $^2J_{NN}$ cross and reference peak with the highest signal-to-noise: For pH 5.3, 5.6, and 6.0, the His24 Hδ_2 to His24 Nϵ_2 reference peak and the respective $^2J_{NN}$ cross-peak detected at the chemical shift of His24 Hδ_2 and His119 Nϵ_2 were used; in the pH 4.9 spectrum, the His119 Hδ_2/Nϵ_2 reference peak and its $^2J_{NN}$ cross-peak are most intense and were used to calculate $^2J_{NN}$. At pH 4.9 His119 is fully protonated, and its resonances are therefore not broadened because of protonation/deprotonation, as is the case at other pH values. At pH 4.9 peak intensities of native state histidines are decreased because of a significant population of molecules in the intermediate form. For pH 5.3, 5.6, and 6.0 the low signal-to-noise reference peaks, His119 Hδ_2/Nϵ_2 and His24 Hϵ_1/His24 Nϵ_2, and associated $^2J_{NN}$ cross-peaks were used to estimate the uncertainty of $^2J_{NN}$ in form of a standard deviation ($n = 3$). For pH 4.9 only one additional pair of cross and reference peak, the His24 Hϵ_1/Nϵ_2 reference peak and its respective $^2J_{NN}$ cross-peak could be analyzed yielding $^2J_{NN} = 10.1$ Hz. The uncertainty must be at least as large as the largest variation estimated for other pH values. In the pH 5.3 spectrum in 90%/10% H_2O/D_2O only His24 Hδ_2/Nϵ_2 reference and respective $^2J_{NN}$ cross-peak were detected. The reference peak between His119 Hϵ_1 and His119 Nϵ_2 and the associated $^2J_{NN}$ cross-peak was not observed under any conditions studied. H_2O = measured in 90%/10% H_2O/D_2O. All other values are from spectra in 99.9% D_2O.

To test whether protonation of His119 (pK_a = 6.0) has an effect on the size of the $^2J_{NN}$ coupling constant across the His24•His119 hydrogen bond ($^2J_{HN}$)-HNN-COSY spectra were recorded at various pH values. To take advantage of the higher transfer efficiency in the INEPT transfer between the imidazole

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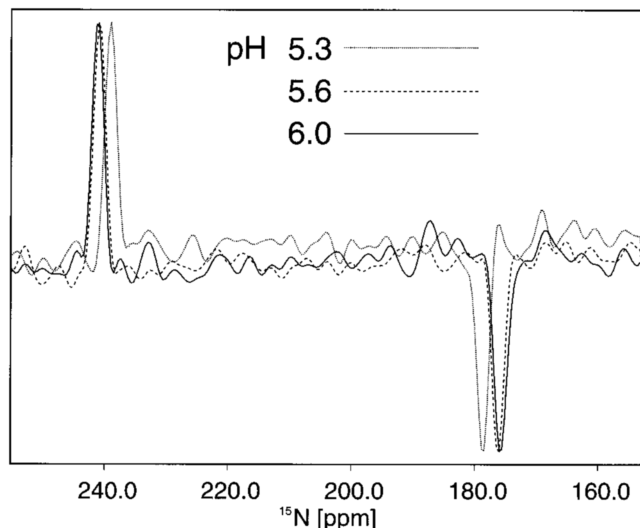

Figure 3. Analysis of ($^2J_{HN}$)−HNN COSY spectra of ^{15}N-labeled wild-type apomyoglobin acquired at 308 K (10 mM sodium acetate in 99.9% D_2O) at 11.8 T on a DMX500 Bruker NMR instrument. Traces from ($^2J_{HN}$)−HNN COSY spectra at pH 6.0, 5.6, and 5.3 along the proton frequency of the His24 δ_2 proton (zero-filled to 1024 complex points). $^2J_{NN}$ cross-peaks to the ϵ_2 nitrogen of His119 (right) are negative with respect to the reference peak (left). The traces are plotted with Felix (Biosym/MSI, San Diego, CA) in the "normalized" display mode.

proton and the directly bonded nitrogen and to measure $^1J_{HN}$ couplings across the hydrogen bond,[27] we initially attemptedto measure ^{1}H−^{15}N correlation spectra in 90%/10% H_2O/D_2O. The imidazole protons were however line broadened to such an extent that signal intensities were too low to make the data useful. Additional quantitative correlation spectra in 99.9% D_2O at pH 5.6, pH 5.3 and pH 4.9 were therefore recorded by nitrogen excitation via two-bond couplings as described for the pH 6.0 experiment. Little variations in the $^2J_{NN}$ coupling constant with pH are seen as His119 becomes fully protonated at pH 4.9 (Figure 3 and Table 1). Interestingly, a $^2J_{NN}$ cross-peak was observed at the chemical shift of the His119 δ_2 proton and the His24 ϵ_2 nitrogen at pH 5.6 or below (data not shown) presumably because line broadening for the His119 resonances caused by protonation/deprotonation[35] is reduced as His119 becomes fully protonated. In contrast, $^2J_{NN}$ cross and reference peaks arising from the Hϵ_1/Nϵ_2 $^2J_{HN}$ correlation of His119 were not observed at any pH.

In general the resonances of histidine side chains appear to be strongly affected by chemical exchange because of protonation/deprotonation which should cause line broadening particularly near the pK_a of the titrating group.[35] Additional line broadening could be caused by chemical exchange between the two tautomeric forms of histidine side chains and differences in relaxation may affect the signal intensities also. In the ^{1}H−^{15}N NMR experiments transfer efficiencies for the different correlations vary because of different $^2J_{HN}$ and $^3J_{HN}$ coupling constants in the histidine side chains as discussed in detail by Pelton et al.[29] Overall for histidine side chains the signal intensities of ^{1}H−^{15}N correlation cross-peaks appear to be influenced most strongly by the protonation/deprotonation and tautomer equilibria because the two histidines that are either fully protonated (His36) or fully neutral (His24) and are fixed in their tautomeric state because of local interactions in holomyoglobin (His36 and His24) consistently show the strongest signals in the ($^2J_{HN}$)-HNN-COSY spectra (Figure 2b).

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To measure the potential isotope effect on the $^2J_{NN}$ coupling in NH···N and ND···N hydrogen bonds respectively, we also acquired a ($^2J_{HN}$)-HNN-COSY spectrum at pH 5.3 in 90%/10% H_2O/D_2O. The resulting $^2J_{NN}$ coupling constant of 10.2 ± 1.0 Hz was identical, within experimental error, to the value obtained in 99.9% D_2O (Table 1).

The $^2J_{NN}$ coupling constant for the histidine pair in apomyoglobin is larger than the coupling constant measured for U−A (6.7 ± 0.5 Hz) and G−C base pairs (6.3 ± 0.2 Hz) in RNA[26] and T−A (6.7 ± 0.3 Hz) and G−C base pairs (6.3 ± 0.3 Hz) in DNA.[27] The nitrogen−nitrogen distance in the X-ray structure in holomyoglobin[36] is 2.75 Å (deoxymyoglobin, pH 8.4, PDB code 1MBD) compared to 2.82 and 2.95 Å in U−A and G−C base pairs, respectively.[37] Although uncertainties in the quoted distances are larger than 0.1 Å and therefore overlapping, this limited data set suggests that $^2J_{NN}$ coupling constants appear to inversely correlate with the nitrogen−nitrogen distance. The hydrogen isotope effect, however, is small. The data also suggest that changing the protonation state of His119 in the His24·His119 histidine pair of native apomyoglobin from half protonated (pH 6.0) to fully protonated (pH 4.9) does not affect the nitrogen−nitrogen distance significantly enough to cause measurable effects on the $^2J_{NN}$ coupling constants.

Using a modified HNN-COSY NMR experiment introduced for RNA studies,[26] we directly detected a NH···N hydrogen bond in proteins. Performing a quantitative ($^2J_{HN}$)-HNN-COSY experiment by exciting the nitrogens via two-bond couplings starting from nonexchanging aromatic protons with favorable relaxation properties may prove to be a general approach for the detection of hydrogen bonds especially in nucleic acids, even when the proton involved in hydrogen bonding is not detectable because of solvent exchange. In apomyoglobin the experiment has allowed us to directly detect a side chain−side chain hydrogen bond that is a key tertiary interaction at the interface of the structural core of apomyoglobin and elements of secondary structure that become unfolded in the intermediate state *I* at pH 4.

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Note Added in Proof. After submission of this manuscript the detection of the more common hydrogen bonds in proteins involving NH and CO groups via heteronuclear coupling constants was reported.[39]

Supporting Information Available: Pulse sequence and table describing the analysis of ($^2J_{HN}$)−HNN COSY cross-peaks (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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