Solid-State NMR Evidence for a Protonation Switch in the Binding Pocket of the H1 Receptor upon Binding of the Agonist Histamine

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Abstract: G protein coupled receptors (GPCRs) represent a major superfamily of transmembrane receptor proteins that are crucial in cellular signaling and are major pharmacological targets. While the activity of GPCRs can be modulated by agonist binding, the mechanisms that link agonist binding to G protein coupling are poorly understood. Here we present a method to accurately examine the activity of ligands in their bound state, even at low affinity, by solid-state NMR dipolar correlation spectroscopy and confront this method with the human H_1 receptor. The analysis reveals two different charge states of the bound agonist, dicationic with a charged imidazole ring and monocationic with a neutral imidazole ring, with the same overall conformation. The combination of charge difference and pronounced heterogeneity agrees with converging evidence that the active and inactive states of the GPCR represent a dynamic equilibrium of substates and that proton transfer between agonist and protein side chains can shift this equilibrium by stabilizing the active receptor population relative to the inactive one. In fact, the data suggest a global functional analogy between H_1 receptor activation and the meta I/meta II charge/discharge equilibrium in rhodopsin (GPCR). This corroborates current ideas on unifying principles in GPCR structure and function.

Information flow in the eukaryotic cell is based on a modular signaling continuum that is balanced by a large number of check posts in the plasma membrane, at the boundary between intracellular housekeeping and the extracellular environment. The G protein coupled receptor (GPCR) family plays a pivotal role in this gate-keeping activity.[1] This superfamily represents at least 500 individual membrane proteins that share a common structural motif of seven transmembrane α-helical segments and the common functional characteristic of signal mediation through GTP binding proteins (GPs). On the other hand, signaling by the various GPCR proteins is triggered by a wide variety of specific "ligands", ranging from photons, Ca^{2+} ions, and small organic molecules to complex polypeptide hormones.[2] Defining the degree of commonality in GPCR activation, which precedes the common feature of GP binding, is a major research issue.

GPCRs are key to modern drug discovery and presently are targeted by over 50% of the prescription drugs.[3] However, the industry is becoming increasingly competitive, and pharmaceutical companies must increase their drug output to sustain growth. A "rational structure-based" approach is the most promising route for the design of novel and/or more selective drugs for the diverse GPCR protein family.[4] This approach relies on highly precise information concerning spatial and electronic structures of ligands in the binding pocket, ligand–receptor communication, and the molecular mechanisms of receptor activation. Gathering such information is quite challenging for membrane proteins such as the GPCR family, whose signaling activity is also modulated by specific interactions with the complex membrane environment. Thus far, a 3D structure has been revealed through crystallography only for the visual receptor, rhodopsin, in the state blocked by an inverse agonist.[5,6] The recent technological developments in solid-state NMR (ssNMR) hold great promise in this respect.[7-9] The first information on spatial, electronic, and chemical structures of ligands, and on ligand–receptor contacts, was recently obtained through magic angle spinning (MAS) ssNMR at ultrahigh field for rhodopsin

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in a natural membrane environment.[7,10−12] More recently, a first model of the spatial structure of the neurotensin peptide bound to its target receptor was presented.[8]

Here we present an MAS ssNMR analysis with complete chemical shift assignment of the ligand bound to a nonvisual GPCR: the small agonist histamine in the binding pocket of the human H_1 receptor. A vast body of data regarding the pharmacological properties are available for this receptor, and mutagenesis studies have pinpointed several residues that can be involved in ligand recognition.[13] In addition, an elaborate homology model based upon the rhodopsin structure has been presented,[14] and a procedure for large-scale production and purification of recombinant H_1 receptor for NMR studies has recently become available.[15]

Since the K_d of histamine for the H_1 receptor is about 20 μM,[15] our results demonstrate that also small low-affinity compounds can be analyzed by ssNMR. The data reveal that the H_1 binding pocket can accommodate two different charge states of the agonist, dicationic with a protonated imidazole ring and monocationic with a neutral imidazole ring. Both states exhibit significant conformational heterogeneity, which may reflect structural heterogeneity in the frozen receptor state. This leads to the conclusion that, under physiological conditions, activation of the receptor by the ligand involves shifting a dynamic equilibrium between active and inactive receptor states. This would strongly support a generic principle in GPCR activation.[16] Such a process would alter an active and complex signal transduction network in the cell, which results in modulating the activity of the corresponding cellular signalosome.

Results

When H_1 receptor proteoliposomes are stoichiometrically loaded with uniformly $^{13}C,^{15}N$ labeled histamine (i.e., both at a concentration of about 0.7 mM), the K_d of about 20 μM derived from binding assays[15] implies that at least 95% of the ligand is bound to the receptor under our experimental conditions. As shown in Figure 1, under these conditions two sets of responses are resolved in the two-dimensional MAS dipolar correlation data. Two separate correlation networks can be isolated and assigned to two distinct histamine species. Both species have an NH_3^+ functionality at the tail end and either a neutral or a cationic imidazole ring, as shown in Figure 1 in blue and red, respectively. The observed ^{13}C and ^{15}N chemical shifts are

summarized in Table 1, along with those for the free ligand as a control, measured in lipid in the presence as well as absence of H_1 receptor. Using a double-quantum-filtered (DQF) 1D analysis of the loaded receptor, where only adjacent ^{13}C labeled carbons are probed, ligand resonances could be isolated from the large natural abundance background, as shown in gray in Figure 1. These DQF data confirm that both mono- and dicationic populations are present in the bound form. Supporting evidence that these data represent bound ligand comes from experiments performed with ligand to receptor ratios of 1:3 and 20:1 using the same receptor preparations. The resulting key spectral feature is that the free ligand is dicationic with a significantly different set of chemical shifts compared to the bound dicationic form (Table 1 and Figure S1 in the Supporting Information). In particular, the C6 resonance shifts by approximately 2.2 ppm between the unbound and bound dicationic forms.

The ^{15}N chemical shifts observed from preparations with at least 95% bound histamine confirm the concept of two populations with different protonation states (Figure 2 and Table 1). The signals from the ring nitrogens in the H_1 receptor/histamine complex are within the ^{15}N reference shift range for neutral (shown in blue) as well as protonated (shown in red) imidazole nitrogens.[17] The signals of the protonated imidazole nitrogens flank the >NH signal and could not be resolved. The side chain response (35.3 ppm) is in the range expected for an NH_3^+ signal. The response around 120 ppm is from the natural abundance ^{15}N in the protein backbone amide groups. In both the 1D and 2D data sets, the signals are broad, in contrast to the relatively narrow signals observed for inverse agonists rigidly constrained in the binding pocket of rhodopsin.[7,10] This suggests additional conformational heterogeneity in the ligand for each of the two resolved H_1 receptor states.

In Figure 3 we show a comparison between the assigned ^{13}C and ^{15}N NMR peaks (see Table 1) and the computed chemical shifts for histamine species with an NH_3^+ functionality at the tail end and either a neutral (top panel) or cationic (middle panel) imidazole ring. In the two models used for the calculations the tail conformation is the same. The theoretical shift patterns are well in line with the experimental data. The difference in the computed chemical shifts between the dicationic and monocationic states (Figure 3, bottom panel) shows that a positively charged imidazole ring also has a small effect on the ^{13}C shift of the tail carbons, particularly at the C6 position. This confirms through a self-consistent approach that the global conformation of the two ligand states is similar.

Discussion

To design selective ligands for the various histamine receptor subtypes on a rational structure-based framework, it is essential to know how histamine interacts with the binding site of its target receptor in atomic detail. Pharmacological and biophysical studies have provided a significant body of indirect evidence about the local environment of the bound histamine without resolving the molecular details of the agonist binding site, the conformation of bound histamine, or the mechanisms of agonist-driven receptor activation.[18−20] Recent models for the human

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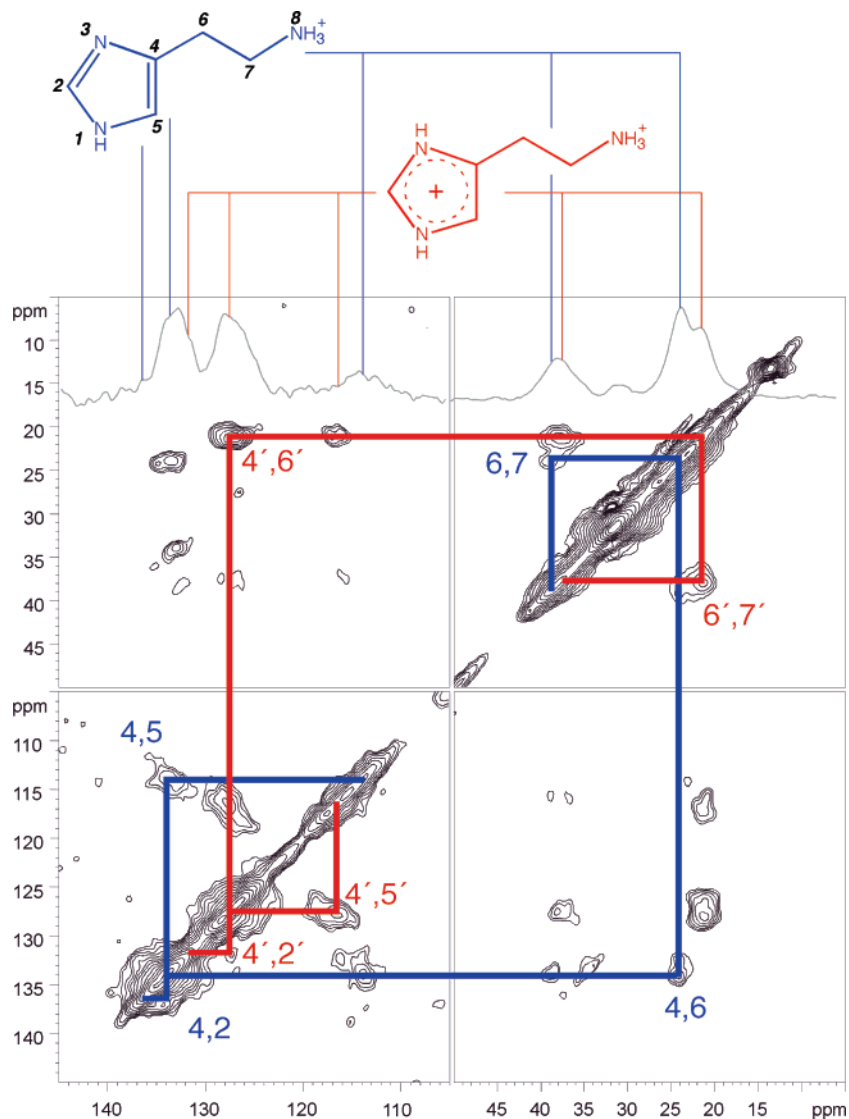
Figure 1. Olefinic and aromatic regions of a ^{13}C PDSD 2D spectrum of the histamine/H$_1$ receptor complex at about 90% ligand saturation. Assignments of the monocationic and dicationic histamine species are shown in blue and red, respectively. The top traces in gray in the upper panels represent the resonances in a DQF 1D spectrum collected from the same preparation. The DQF spectrum only presents signals from ^{13}C nuclei that have another ^{13}C as a neighbor. In this way the natural abundance background response is suppressed. The primed numbers represent the contribution from the dicationic species.

histamine H$_1$ receptor ligand binding environment are based on site-directed mutagenesis studies in combination with crystal structure data for rhodopsin.[5,14,22] However, essential questions concerning molecular and chemical requirements for ligand recognition and receptor activation remain unanswered. Recently, we have successfully overexpressed, purified, and reconstituted the human histamine H$_1$ receptor[15] and are now capable of producing sufficient quantities for NMR studies. This allows us to set up a converging effort using MAS NMR analyses and quantum chemical calculations to probe the electronic and spatial structures of the agonist histamine bound to its receptor target in a natural membrane environment.

Our data present the first complete ^{13}C and ^{15}N analysis of an agonist ligand in a GPCR binding site. First, we are able to observe and fully assign a small, low-affinity agonist in its

Table 1. Chemical Shift Assignment (ppm) for the Uniformly $^{13}C,^{15}N$ Labeled Histamine Bound to the Human H$_1$ Receptor and for the Free Ligand[a]

| position	bound ligand (monocationic)	bound ligand (dicationic)	free ligand (dicationic)
N-1	166.3	174	176
C-2	137.0	133.0	133.3
N-3	243.0	174	176
C-4	133.0	128.0	127.5
C-5	113.0	116.0	117.0
C-6	23.0	20.0	22.2
C-7	37.5	37.0	37.8
N-8	35.3	35.3	34.6

[a] The N-1 and N-3 shifts in the cationic imidazole ring of the bound ligand could not be resolved. The free ligand data are from a liposomal preparation and from a receptor proteoliposomal preparation with a large excess of ligand. Both data sets are very similar.

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GPCR binding pocket in a natural membrane environment. We consider this very important, as it implies that even relatively weakly binding ligands are amenable to structural studies by
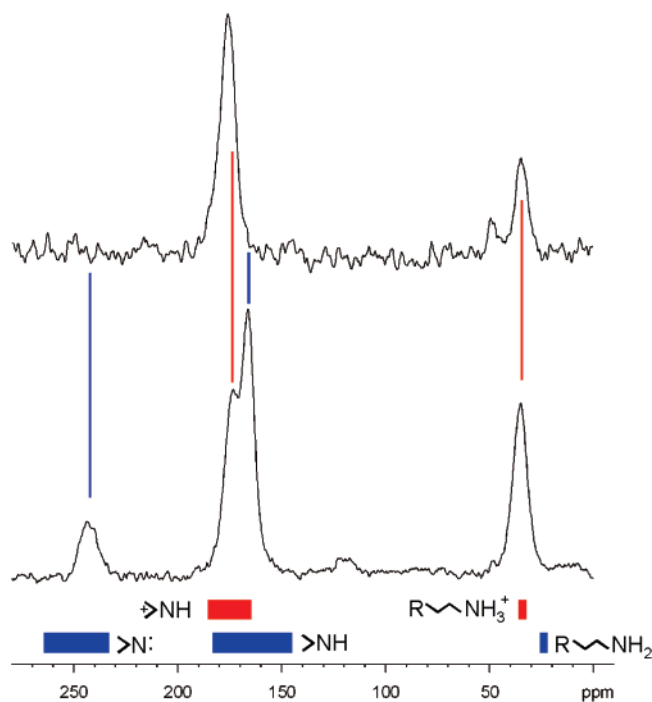
Figure 2. [15]N CPMAS 1D spectrum of the free histamine (top) and the histamine/H[1] receptor complex (bottom). The strong signals are from the imidazole ring and side chain nitrogens of the ligand. Reference chemical shift ranges for uncharged (blue bars) and positively charged (red bars) imidazole rings and tail amino groups are shown below the spectrum.[17] Note the absence of the uncharged ring signals in the free ligand and the ligation shift of the ring nitrogens in the bound ligand. The weak response around 120 ppm in the bottom spectrum represents the natural abundance ^{15}N signal of the receptor peptide bonds. This signal is relatively weak, since at temperatures in the range of 200–220 K cross-polarization may lead to nonlinear signal amplitudes due to motional time scale interference associated with the protein glass transition and the ^{1}H decoupling process.[48,49] Binding to the H[1] receptor is associated with the appearance of peaks at 243.0 and 166.3 ppm associated with a neutral imidazole ring.

ssNMR. Second, our data present a very strong indication that the bound agonist can interact with its receptor in two different charge states. Both states show shifts in their resonance positions relative to that of the free ligand, confirming ligation to the receptor (Table 1, Figure 2, and Figure S1 in the Supporting Information). It is interesting to note that the free ligand is largely present as a dication. This is observed in preparations with and without the H[1] receptor. Histamine probably accumulates at the aqueous–membrane interface where the high density of negatively charged phosphate and carboxyl moieties in the lipid headgroups promotes protonation of the imidazole ring. Asolectin membranes have a relatively high surface charge density,[22] and protonation of the imidazole group upon interaction with phospholipids has been reported previously.[23] The 2D NMR assignment of the bound ligand can only be reconciled with histamine binding to the receptor in two possible states, both containing a positively charged amino functionality with either a positively charged or a neutral imidazole ring. The 2D assignment is supported by our chemical shift calculations for two charge states of the ligand in a similar conformation based on the density functional theory modeling. Hence, the H[1] receptor binding site apparently can accommodate histamine in either a monocationic or a dicationic charge state. The

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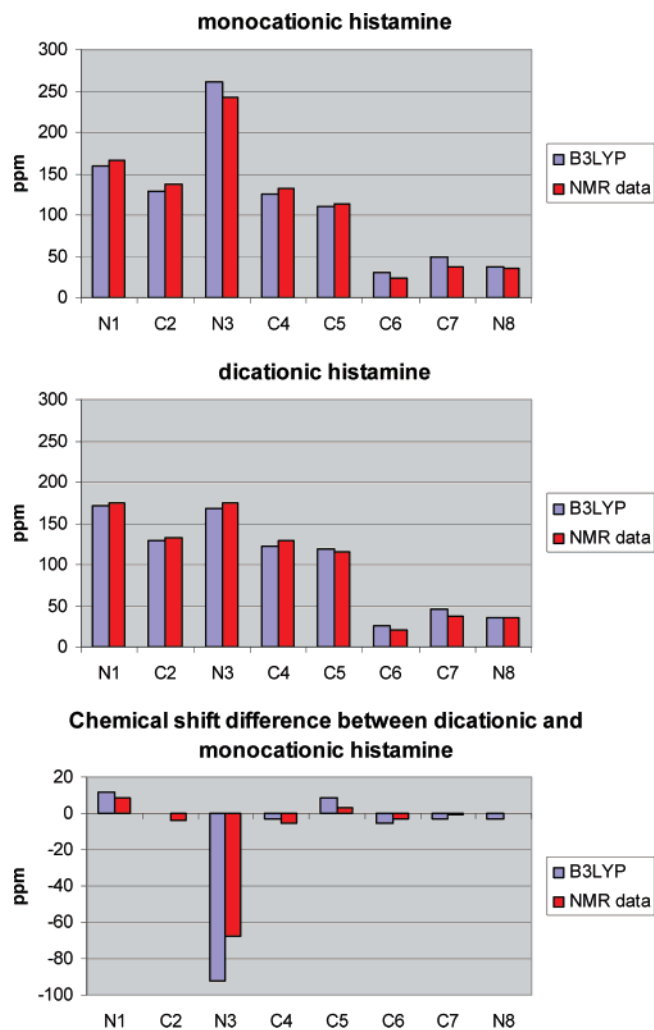
Figure 3. Comparison between the assigned (NMR data in red) and the DFT computed (B3LYP in blue) ^{13}C and ^{15}N NMR chemical shifts for the monocationic (top panel) and dicationic (middle panel) histamine species. The bottom panel shows the same comparison for the chemical shift difference between the dicationic and monocationic states.

existence of two, receptor-bound forms of histamine that differ by one unit of charge is reminiscent of the meta I to meta II discharging equilibrium in the visual GPCR rhodopsin. Here also, the ligand deprotonates while essentially retaining its conformation.[24,25] This observation of two, coexisting bound ligand states is in line with the recently proposed mechanism for β_2 adrenergic receptor activation, where agonist action also involves an equilibrium step.[16] For rhodopsin, photoisomerization of 11-*cis*-retinylidene to *all-trans*-retinylidene triggers a series of conformational changes of the opsin protein and the crucial step for activation of the G protein binding pathway is the transition from meta I to meta II involving proton transfer from the ligand to a glutamate side chain, commonly defined as the "protonation switch". This transition eliminates an ion pair and triggers rearrangement of H-bonded networks,[24–26] an essential element in propagating the activation signal to the intracellular domain. The dynamic equilibrium between the two

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states meta I and meta II depends on both temperature and pH. Under physiological conditions, this balance is shifted to the active, signaling state, meta II.[24] The rearrangement of H-bonding networks following proton transfer accommodates rotamer switches in the proline-hinge region of TM6 (Pro6.50, Tyr6.51,Ala6.52,Gly6.53, numbering according to the Ballesteros−Weinstein numbering scheme[27] further explained in the Supporting Information), and eventually, helical segments of TM3 and TM6 reposition, rearranging loop structures, leading to exposure of G protein binding sites.[24−26]

In our previous studies on inverse agonist ligands of rhodopsin, under similar experimental conditions, narrow resonances were observed (1−2 ppm), supporting the concept that these ligands are rigidly constrained in the binding site of the receptor.[7,10] This is in line with their intrinsic function to suppress the basal signaling activity of the protein. In contrast, histamine, a full agonist for the H_1 receptor, exhibits significantly broader lines (4−8 ppm) when bound to the receptor (Figures 1 and 2, Figure S1 in the Supporting Information). We interpret this as functional conformational heterogeneity, reflecting a distribution of conformational substates.

Our observation of two different protonation states for the bound histamine imidazole ring, resolved by MAS NMR, and of concomitant line broadening indicates a mechanism for the H_1 receptor similar to that established for rhodopsin: two states switching between the two protonation states of the ring, with one state triggering further activation of the receptor in a nonlinear response mechanism. At physiological temperature these states probably are in rapid, dynamic equilibrium and would not be resolved by MAS NMR. For rhodopsin the dynamics is suppressed in the resting state by the retinylidene chromophore ligand, which operates as an inverse agonist and stabilizes an ion pair by forming a protonated Schiff base. The meta I/meta II transition in this system is triggered after the ligand is photoisomerized into its agonist conformation. In contrast, the H_1 GPCR does not contain an intrinsic ligand, and the dynamic proton-transfer equilibrium is an intrinsic property of the receptor that gives rise to its constitutive activity.[14] Under our experimental conditions we have frozen this equilibrium by lowering the temperature to 203 K. This clarifies why the separate states are resolved in our NMR analyses. Under physiological conditions, the receptor would participate in a continuous complex signaling network, and the agonist histamine would influence this network by stabilizing the active receptor state relative to the inactive state. Stabilization would occur through a nonlinear response to the shift in the proton-transfer equilibrium. Conversely, an inverse agonist would stabilize the inactive state and quench the effect of the receptor in the network. In this way one receptor species can make an apparent contribution to several signal transduction processes in parallel.

How do our present data compare with current models of the H_1 receptor? Recent molecular modeling studies based on the rhodopsin structure[13,14] indicate that the binding site of the H_1 receptor presents a relatively acidic microenvironment. Furthermore, several mutagenesis studies agree that the hista-mine tail nitrogen binds through a charge-pair interaction with the carboxylic group of Asp3.32 in TM3.[21,28−30] This is fully supported by our finding that histamine retains a protonated amino group in both receptor states. Asp3.32 is well conserved among biogenic amine receptors and may be a common motif in binding a variety of H_1 receptor agonists and antagonists.[29,31] In addition, studies with H_1 receptor mutants provide evidence for a direct contribution of Asn5.46 and Lys5.39 in the upper part of TM5 to settling the τ and π nitrogens of the imidazole ring, respectively.[21,30,32−34] Modeling studies[13] further suggest a role for Tyr3.33 (TM3) and Thr5.42 (TM5) in stabilizing these interactions through H-bonding to Lys5.39 and Asn5.46, respectively. In addition, Tyr3.33 and Phe6.55 (TM6) may be involved in $\pi-\pi$ interaction with the imidazole ring. In a controlled protein environment, a lysine ϵ-amino group and an imidazole ring may team up in a reversible proton-transfer pair. Additional experiments will be required to specify the interactions between histamine and its contact residues in the receptor binding pocket. For instance, the recently developed SIDY technique[35] allows mapping of contact sites between a labeled ligand and unlabeled protein residues. Such an approach will include other ligand classes and will be a major tool in revealing specific binding interactions. The observation that the ligand is dicationic in the free state suggests that the dicationic form of the ligand represents the state in which it first binds to the receptor, upon which receptor activation is stabilized by proton transfer, in analogy to rhodopsin. Further ssNMR studies using partial agonists, antagonists, and inverse agonists will help to clarify this essential element in GPCR activation.

High-field solid-state 1D and 2D heteronuclear MAS NMR dipolar correlation spectroscopy on uniformly labeled histamine bound to the active site of the human histamine H_1 receptor provides a complete ^{13}C and ^{15}N chemical shift assignment of the NMR response of the ligand bound to the receptor. Our data, complemented by DFT chemical shift calculations, reveal two receptor states, containing either a monocationic or a dicationic histamine, and supplement converging evidence that a protonation switch governing the transition between these two states is involved in receptor activation.

It is generally accepted that polar or charged groups in the protein interior participate in specific ionic interactions and/or H-bonded networks that stabilize the GPCR conformation.[14,25,36,37] In fact, strong evidence has been presented that conserved ion pairs are important for thermal stability of the ground-state structure of rhodopsin,[25,36,38] while receptor activation is thought

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to involve redistribution of ion pairs and rearrangement of H-bonded networks. Studies involving different H_1 antihistamines, such as cetirizine, loratadine, and epinastine, corroborate the notion that the histamine H_1 receptor exists as an equilibrium between inactive and active substates, with agonists promoting an active conformation of the receptor and inverse agonists stabilizing an inactive state.[39] We propose that these conformational substates interconvert through an equilibrium step, involving a protonation switch. The close analogies with both the activating meta I ↔ meta II equilibrium in rhodopsin and the proposed activating cascade in the β_2-adrenergic receptor suggest that this is a generic and essential element in GPCR activation.

The MAS NMR data are compatible with site-directed mutagenesis studies on the H_1 receptor and with direct involvement of Asp3.32 of TM3 in binding the agonist histamine. Our study paves the way for 3D conformational analysis of histamine and other ligands in the H_1 receptor binding site, as well as for studies aiming to identify closely interacting protein side chains. The availability of such structural information will enable rational drug design by screening of lead compounds acting on GPCRs and optimization of bioinformatic predictive methodologies.

Methods

Sample Preparation. Uniformly $^{13}C,^{15}N$ labeled histamine was prepared from uniformly labeled histidine (Cambridge Isotope Laboratories, 98% ^{13}C, 96−99% ^{15}N) in a good overall yield of 70% by means of enzymatic conversion using L-histidine decarboxylase type III-S from *Lacto bacillus 30*.[40] His-tagged human histamine H_1 receptor was overexpressed in Sf9 cells as described.[15] Levels of 5−7 mg of functional receptor/L were produced, purified, and reconstituted into asolectin lipid bilayers. Functionality was established by ligand binding assays.[15] Loading with uniformly labeled histamine was achieved by suspending ca. 20 mg of reconstituted receptor (based upon functional protein) in ca. 500 μL of PBS, pH 7.4, followed by incubation at 30 °C for 30 min with labeled histamine. Subsequently, the ligand charged receptor proteoliposomes were transferred into a 4 mm boron nitride MAS rotor to a total volume of about 100 μL by centrifugation in a swing-out rotor (40000*g*, 15 min, 4 °C) to ensure dense packing. Excess buffer was then removed, and the rotor was closed with a boron nitride or Kel-F cap.

NMR Experiments. MAS NMR analyses were performed at −70 °C using a 750 MHz wide-bore Avance NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a variable-temperature probe tuned to ^1H and ^{13}C or ^{15}N. Cross-polarization was achieved with a 2 ms $^1H-^{13}C$ contact time or a 2.5 ms $^1H-^{15}N$ contact time. In both cases a 64-step ramp from 100% to 70% of full ^1H power was applied, corresponding to an approximately 50 kHz nutation frequency.[41] Two-pulse phase-modulated (TPPM) decoupling was applied during acquisition and free precession periods.[42] The TPPM phase shift was set to 15°, and the pulse length was optimized separately for each sample. DQF spectra were obtained with post-C7 ^{13}C irradiation[43] with a spin rate of 6250 Hz using 120 kHz of CW ^1H decoupling during double quantum excitation and reconversion periods. Generally, 1024 points were collected in the directly detected dimension with a dwell time of 5.5 μs and an interexperiment delay of 1 s. All 1D spectra were processed with 80 Hz line broadening. The carboxyl response of a tyrosine·HCl sample was used as an external ^{13}C shift reference, and the lipid methyl signal at 13.45 ppm was used as an internal reference to correct for spectrometer drift during the course of the measurements. *N*-Acetylvaline was used as an external reference for ^{15}N chemical shifts. 2D C−C correlation spectra were obtained with proton-driven spin diffusion (PDSD) techniques.[7] The spectra were Fourier transformed and baseline corrected in both dimensions. The directly detected dimension contained 1024 points and was transformed with 150 Hz of line broadening. In the indirectly detected dimension, 200 points were collected and processed by linear prediction with 55 coefficients to 300 points before zero filling to 1024 points and multiplication by a shifted sine bell window function. The 2D data were collected in a series of 10 full 2D experiments, each consisting of 400 averages, which were processed separately and summed to improve the signal-to-noise ratio.

DFT Chemical Shift Calculations. ^{13}C and ^{15}N chemical shift calculations were performed for histamine in the monocationic as well as the dicationic charge state within the density functional theory framework. We used the B3LYP functional[44,45] with a 6-31G(d,p) Gaussian basis set both for the geometry optimization and for the NMR shielding tensor calculation employing the gauge-independent atomic orbital (GIAO) method.[46] The isotropic ^{13}C and ^{15}N chemical shifts are quoted in parts per million relative to the ^{13}C chemical shift of tetramethylsilane (TMS) and the ^{15}N chemical shift of NH_3, respectively, calculated within the same level of theory. All calculations have been performed with the Gaussian98 program.[47]

Supporting Information Available: Complete ref 47, explanation of the GPCR residue numbering scheme, and Figure S1 showing differences in the chemical shifts and line widths in the bound and the free ligand. This material is available free of charge via the Internet at http://pubs.acs.org.

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