Salt-driven Equilibrium between Two Conformations in the HAMP Domain from *Natronomonas pharaonis*

**THE LANGUAGE OF SIGNAL TRANSFER?**

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HAMP domains (conserved in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases) perform their putative function as signal transducing units in diversified environments in a variety of protein families. Here the conformational changes induced by environmental agents, namely salt and temperature, on the structure and function of a HAMP domain of the phototransducer from *Natronomonas pharaonis* (NpHtrII) in complex with sensory rhodopsin II (NpSRRII) were investigated by site-directed spin labeling electron paramagnetic resonance. A series of spin labeled mutants were engineered in NpHtrII157, a truncated analog containing only the first HAMP domain following the transmembrane helix 2. This truncated transducer is shown to be a valid model system for a signal transduction domain anchored to the transmembrane light sensor NpSRRII. The HAMP domain is found to be engaged in a “two-state” equilibrium between a highly dynamic (dHAMP) and a more compact (cHAMP) conformation. The structural properties of the cHAMP as proven by mobility, accessibility, and intra-transducer-dimer distance data are in agreement with the four helical bundle NMR model of the HAMP domain from *Archaeoglobus fulgidus*.

Negative phototaxis in the haloarchaeum *Natronomonas pharaonis* is mediated by a transducer molecule (NpHtrII) that shares structural analogies and functional similarities with a variety of chemoreceptors reactive to attractant and repellent stimuli mediating the bacterial positive and negative taxis (1–3).

Different from chemoreceptors the repellent signal is not sensed by a periplasmic sensor domain, but by a cognate sensor protein, sensory rhodopsin II (NpSRRII), which belongs to the seven-transmembrane helices protein family. A light-induced structural rearrangement in NpSRRII (4) at the interface with the transmembrane transducer helices TM1 and TM2 causes a rotation and displacement of helix TM2, which can be considered as the starting event for signal transduction (5–7). Attractant or repellent signals are then further transmitted to the cytoplasmic signaling domain through a linker region, the latter comprising either one or two HAMP domains (8, 9).

HAMP modules are found in a wide variety of proteins connecting signal input with signal output. This diversity in the context of function and environment implies common principles of signal relay. A breakthrough in the mechanistic picture of the HAMP domain was the solution NMR structure of the HAMP domain from *Archaeoglobus fulgidus* showing a homodimeric, four helical, parallel coiled coil with an unusual interhelical packing (10). The authors suggested a model for the propagation of the signal transduction, in which “the HAMP domain alternates between the observed conformation and a canonical coiled coil” via concerted helix rotations (10). Recent results on the intact membrane-bound aspartate receptor of bacterial chemotaxis (11) and on the aerotaxis receptor Aer (12) are in agreement with the NMR structure of *A. fulgidus* (10). Despite these clear evidences for defined structural elements, indications for an intrinsic fragility of the HAMP domain are obvious. For example, the HAMP domain from NpHtrII was suggested to be in a molten-globule-like state at low salt concentration (13), whereas that from EnvZ, the osmosensing histidine kinase from *Escherichia coli*, was shown to be unable to form a stable structure by itself (14).

In the present work dynamic and structural properties of the first HAMP domain of NpHtrII are investigated in dependence of salt concentration and temperature. *N. pharaonis* is an extremely haloalkaliphilic archaean, living in salt-saturated lakes (15) at pH values around 11 (16) and temperatures of optimal growth in the 318–323 K range (17, 18). Cytoplasmic and external salt concentrations can be assumed to be similar, however, with reversed sodium to potassium ratio (19). Due to

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4 The abbreviations used are: TM, transmembrane; HAMP; domain, conserved signal transduction domain in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases; NpSRRII, *Natronomonas pharaonis* sensory rhodopsin II; NpHtrII, *Natronomonas pharaonis* halobacterial transducer of rhodopsins II; NpHtrII157, construct truncated at position 157; NpHtrII157-230, construct truncated at position 230; PML, purple membrane lipid; EPR, electron paramagnetic resonance; cw, continuous wave; DEER, double electron-electron resonance; mT, millitesla; NIEDDA, Ni(II)ethylenediamine diacetate; dHAMP, dynamic HAMP; cHAMP, compact HAMP; cp, centipoise.
HAMP Conformations in Equilibrium

effects on electrostatic and hydrophobic interactions (see, e.g., Ref. 20) the structure and dynamic properties of the HAMP domain are expected to be influenced by salt. The stability and the dynamics of proteins are also affected by temperature. In particular, an Fourier transform-infrared study performed on the NpSRII-NpHtrII159 complex presented evidence for a conformational change in the transducer HAMP domain upon lowering the temperature to 250 K (21).

In this work, it is shown that the HAMP domain of *N. pharaonis* displays an equilibrium between a dynamic/molten-globule-like conformation (dHAMP) and a more compact structure (cHAMP). The latter conformation is shown to be in agreement with the four helical bundle structure obtained by NMR for the HAMP domain from *A. fulgidus*.

**MATERIALS AND METHODS**

**Bacterial Strains**—*E. coli* XL1 was used as a host for DNA manipulation. Gene expression was carried out in *E. coli* BL21(DE3) or *E. coli* Rosetta (for NpHtrII-His) strains.

**Cysteine Mutants**—Cysteine-encoding mutations were introduced by using the overlap extension method as described previously (22) starting with the plasmids: pET27bmod-npsop-pII-his (23), pET27bmod-t-HtrII-His (C-terminal truncated transducer—(1–157)) (5), pET27bmod-nphtrII-1–230—C173Shis-pII-his (23), pET27bmod-t-HtrII-His (C-terminal truncated introduced by using the overlap extension method as described in Ref. 20) the structure and dynamic properties of the HAMP domain from *A. fulgidus*.

**Protein Expression, Spin Labeling, and Reconstitution into Polar Lipids**—NpSRII-His, NpHtrII157-His (variant truncated at position 157), NpHtrII1–230-His (variant truncated at position 230), and NpHtrII-His as well as their respective mutants were expressed either in *E. coli* BL21(DE3) according to Shimono et al. (25) or, in the case of NpHtrIIII-His according to Mennes et al. (24), and purified as described (23, 24). The protein expression was ligated into pET27bmod using the Ncol and HindIII restriction sites. Positively clones were verified by DNA sequencing.

**Protein Expression, Spin Labeling, and Reconstitution into Polar Lipids**—NpSRII-His, NpHtrII157-His (variant truncated at position 157), NpHtrII1–230-His (variant truncated at position 230), and NpHtrII-His as well as their respective mutants were expressed either in *E. coli* BL21(DE3) according to Shimono et al. (25) or, in the case of NpHtrIIII-His according to Mennes et al. (24), and purified as described (23, 24). The spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate (TRC, Toronto, Canada) was covalently attached to the cysteine residues of the solubilized NpSRII-His or NpHtrII-His mutants, respectively, as outlined by Pfieffer et al. (26). The corresponding side chain is abbreviated as R1 in the following.

**EPR Experiments**—Room temperature (296–299 K) continuous wave (cw) EPR spectra were recorded using a Magne-tettech Miniscope MS200 X-band spectrometer equipped with a rectangular TE102 resonator, with the microwave power set to 10 milliwatts, and B-field modulation amplitude adjusted to 0.15 mT. Sample volumes of 15 μl at final protein concentration of 15–20 mg/ml were loaded into EPR glass capillaries (0.9 mm inner diameter). For measurements under different salt concentrations, the protein-containing membranes were pelleted down at 15,700 × g, the supernatant was removed, and the sample was washed three times and finally resuspended in 10 mM Tris-HCl (pH 8) buffer containing the preferred salt concentration in the 0.15 to 3.5 M range, with addition of 40% sucrose (w/v) or 20% glycerol (v/v) when necessary.

For temperature-dependent cw EPR measurements a homemade X-band EPR spectrometer equipped with a Bruker dielectric resonator was used in combination with a liquid flow cryostat (liquid containing 30% ethylene glycol, 70% water) to adjust the sample temperature in the range 280–340 K. The microwave power was set to 1 milliwatt and the B-field modulation amplitude adjusted to 0.15 mT.

The accessibility values for paramagnetic quenchers soluble in water or lipid phases were obtained by the method of cw power saturation EPR (28, 29) and are presented as Heisenberg exchange rates, Wobs, between the spin label and the respective paramagnetic quencher (molecular oxygen or the neutral Ni(II)-ethylenediamine diacetate (NiEDDA)). The P1/2 values obtained from fitting of the power saturation curves were divided by the resonator specific proportionality factor α (1.87 MHz−1) to obtain Wobs rates according to Ref. 30. A homemade cw EPR spectrometer equipped with a loop gap resonator was used with the microwave power varied in the 0.1–65 milliwatt range. A sample volume of 5 μl was loaded into a gas-permeable TPX (Poly(methylpenten) capillary (Spintec). For reference measurements the sample was deoxygenated by a nitrogen gas flow around the capillary that was replaced by air (21% O2) for oxygen accessibility experiments. For measurements of the spin label accessibility from the water phase, a solution of NiEDDA was added to the sample to a final concentration of 20 or 3 mM and the nitrogen gas flow was restored. Prior to EPR experiments, the sample was flushed with the proper gas for 20 min. EPR spectra for inter-spin distance determination were recorded at 160 K using a homemade X-band EPR spectrometer equipped with an AEG H103 rectangular cavity. The microwave power was set to 0.2 milliwatt and the B-field modulation amplitude adjusted to 0.25 mT. A B-NM 12 B-field meter (Bruker) and a continuous flow cryostat Oxford ESR 900 allowed measurement of the magnetic field and stabilization of the sample temperature, respectively. Sample volumes of 40 μl at final protein concentrations of 15–20 mg/ml were loaded into EPR quartz capillaries (3 mm inner diameter).

Pulse EPR experiments (DEER) were accomplished at X-band frequencies (9.3–9.4 GHz) with a Bruker Elexsys 580 spectrometer equipped with a Bruker Flexedys 580 spectrometer equipped with a Bruker Flexline split-ring resonator ER 4118X-MS3 and a continuous flow helium cryostat (ESR900; Oxford Instruments) controlled by an Oxford Instruments temperature controller ITC 503S. All measurements were performed using the four-pulse DEER sequence: \[ \pi/2(\nu_{\text{obs}}) - r_1 - \pi(\nu_{\text{obs}}) - t' - \pi(\nu_{\text{pump}}) - (r_1 + r_2 - t') - \pi(\nu_{\text{obs}}) - r_2 - \text{echo} \] (31). For the DEER pulses at the observer frequency the (x)
channels were used. A two-step phase cycling \((+x, -x)\) is performed on \(\pi/2(\nu_{\text{obs}})\). Time \(t'\) is varied, whereas \(\tau_1\) and \(\tau_2\) are kept constant, and the dipolar evolution time is given by \(t = t' - \tau_1\). Data were analyzed only for \(t > 0\). The resonator was over-coupled to \(Q \sim 100\); the pump frequency \(\nu_{\text{pump}}\) was set to the center of the resonator dip and coincided with the maximum of the nitroxide EPR spectrum, whereas the observer frequency \(\nu_{\text{obs}}\) was 65 MHz higher and coincided with the low field local maximum of the spectrum. All measurements were performed at a temperature of 50 K with observer pulse lengths of 16 ns for \(\pi/2\) and 32 ns for \(\pi\) pulses and a pump pulse length of 12 ns. Proton modulation was averaged by adding traces at eight different \(\tau_1\) values, starting at \(\tau_{1,0} = 200\) ns and incrementing by \(\Delta \tau_1 = 8\) ns. In the presence of deuterated glycerol used for its effect on the spin-lattice relaxation, deuterium modulation was averaged by adding traces at eight different \(\tau_1\) values, starting at \(\tau_{1,0} = 400\) ns and incrementing by \(\Delta \tau_1 = 56\) ns. The total measurement time for each sample was between 12 and 20 h. The data analysis of the DEER traces was performed with the software DeerAnalysis 2006.1 (32).

**Fitting of Simulated EPR Spectra**—Fitting of simulated dipolar broadened EPR powder spectra to the experimental ones detected at 160 K revealed the average inter-spin distance according to the method described previously (33). To account for a range of distances expected to arise from different spin label side chain orientations, a Gaussian distribution of inter-spin distances with a distribution width of 0.2 nm is assumed. During the fitting procedure, the \(g\) tensor values, the values of \(A_{xx}\) and \(A_{yy}\) of the hyperfine tensor and the Lorentzian and Gaussian line width parameters were fixed to the values found for the corresponding reference spectra of the singly labeled species. The \(g\) tensor parameters used were determined by fitting the low temperature spectra detected at 95 GHz (34). \(A_{xx}\) and \(A_{yy}\) were fixed to 0.58 and 0.42 mT, respectively, whereas \(A_{zz}\) was variable to account for differences in the polarity of the immediate spin label environment (34, 35). The spectra were convoluted with a field-independent line shape function composed of a superposition of 29% Lorentzian and 71% Gaussian of 0.3 and 0.37 mT widths, respectively. The fitting parameters for the determination of the dipolar broadening are thus the average inter-spin distance, \(A_{zz}\) and the fraction of the singly spin labeled component. The spin labeling efficiency was in the 60–70% range for the investigated positions. Fitting of simulated EPR spectra to the experimental ones detected between 283 and 343 K was performed according to a simple Brownian model of isotropic reorientational diffusion of the nitroxide (36). The spectra were fitted with two distinct spectral components. Component 1 was characterized by longer correlation times, and due to the inherent more complex influence of the reorientational potentials in determining the real spectral shape, the simulation must be considered strongly approximated. The shape of component 2, on the other hand was typical for an almost isotropic reorientational motion of the nitroxide, thus the simulated spectral features were better representing the real spectral shape. The \(g\) and \(A\) tensor values used as input parameters were determined by low temperature spectral analysis. The reorientational correlation time \(\tau_1\) was calculated from the obtained rotational diffusion constant \((R)\) as 

\[
(6 \times R)^{-1}
\]

for the two components present in the spectra. The rotational diffusion constants and the ratio of the two spectral components were allowed to vary according to the temperature.

**RESULTS**

Spin label side chains mobility and accessibility for paramagnetic quencher molecules together with inter-spin distances (intra-transducer-dimer) have been obtained at different salt concentrations to unravel the structural changes induced on the transducer moiety. Fig. 1 shows the comparison between the HAMP models for \(N.\) pharaonis (extended representation) (13) and for \(A.\) fulgidus as derived from NMR data (10) with four of the investigated sites. The corresponding positions in the \(A.\) fulgidus HAMP model were chosen according to the primary sequence alignment for the three HAMP subdomains, namely the AS1, the connector region, and the AS2. Spin labels have been introduced at positions 78, 88–100, 106, 115, 119, 121, 122, and 148 in the transducer protein and at position 154 in the receptor moiety.

**Effects of Viscosity, Ionic Strength, and Salt on the HAMP Domain**—All spectra presented in this work were detected for spin labeled sites in the NpHtrII157 transducer fragment, which includes only one HAMP domain. To verify the possible influence of the truncation on the HAMP structure, cysteines were also engineered and spin labeled in the NpHtrII1230–C173S construct, carrying both HAMP domains, and in the full-length
transducer (NpHtrII–C173S). Comparison of the spectra for a selected position in the AS1 (A94R1, where R1 denotes the MTS side chain) in all three analogs are presented in Fig. 2. At low salt concentration, all spectra are characterized by the presence of the two distinct components: component 1, representing a fraction of the nitroxide population where the spin label is engaged in secondary and tertiary interactions reducing its reorientational freedom; component 2, representing a fraction of the nitroxide population where the spin label is characterized by high mobility. The term “mobility” is used in a general sense and includes effects on the spectral line shape due to motional rate, amplitude, and anisotropy of the nitroxide reorientation. These parameters characterize the motional freedom of the nitroxide in the nanosecond time scale. The ratio between the two spectral fractions was found to be slightly dependent on the truncation performed. Increasing the salt concentration led to an almost complete suppression of component 2 for all variants. This indicates that the salt effects are HAMP-specific and the influence of the downstream regions is negligible. All data presented in the following were obtained in the truncated construct NpHtrII157.

Selected room temperature EPR spectra detected under different conditions are presented in Fig. 3. All positions beyond 95 show the appearance of two distinct spectral components characterized by different mobility at low salt concentrations. The spectral component 1 can be approximately simulated with an effective isotropic reorientational correlation time of about 10 ns, whereas component 2, showing a narrow three line spectrum, is characterized by correlation times of about 1 ns. The latter fast motion exceeds the mobility observed for highly dynamic loop regions (37, 38), thus suggesting that protein backbone fluctuations must be involved in determining the extremely fast dynamics of the nitroxide.

Salt-induced spectral effects are found for all positions investigated, and become more evident for variants displaying a distinct second spectral component (e.g. see residues L105R1 and A122R1 in Fig. 3). The fraction of the dynamic component 2 is generally suppressed at high salt concentrations.

To investigate an eventual additional influence of the viscosity of the medium on the spectral changes detected, spectra were also recorded in the presence of sucrose (20% w/v, \(\eta_{sucrose} = 6.2 \text{ cp}\) and glycerol (20% v/v, \(\eta_{glycerol} = 1.7 \text{ cp}\). As a reference value, the viscosity of water at 293 K is 1.002 cp (all viscosity values are taken from Ref. 39). Although the increase in viscosity induced by 40% sucrose is much larger than that induced by high salt concentrations (\(\eta_{3M} \text{ KCl} = 0.99 \text{ cp}, \eta_{4M} \text{ KCl} = 1.02 \text{ cp,} \eta_{4M} \text{ NaCl} = 1.60 \text{ cp}\), the salt-induced spectral effects are found to be the most pronounced. Thus, we can exclude that solely viscosity-caused spectral changes take place in the HAMP domain.

To unravel the contribution of different salts on the observed spectral changes, experiments were carried out with diverse anions at the same molar concentrations (Cl\(^-\), SCN\(^-\), and SO\(_4\)\(^-\)). The suppression of the more dynamic component was observed for all anions tested. The degree of suppression was different for each salt and the dependence of the changes observed followed the nature of ions according to the Hofmeister series of neutral salts (40, 41). The most kosmotropic anion SO\(_4\)\(^-\) induced the strongest suppression of the dynamic component. Altogether, the observed spectral changes clearly suggest a conformational change in the HAMP domain induced by high salt concentrations.

Suppression of the spectral component 2 was also induced by low pH. The spectrum obtained for position 122 at pH 3.4 is presented in Fig. 3E. Although the pH is known to be one of the possible inputs for chemoreceptor signal transduction (42), the physiological pH for N. pharaonis is 11, thus the effects of low pH in the NpHtrI structure are not further investigated in this work.

Effects of Salt on TM2, NpSR11, and Purple Membrane Lipid Bilayers—As a control, the salt effect was also investigated for a spin labeled position located in transmembrane helix TM2 of NpHtrII157 (V78R1) as well as for a spin labeled position in the EF-loop of sensory rhodopsin (S154R1) located at the interface with TM2 (Fig. 4A). Increasing the salt concentration affects the spectral shapes of these spin labels, indicating that the...
transmembrane part of the transducer becomes more compact as well. Low temperature measurements performed on the singly labeled V78R1 indicate that the intra-transducer dimer distance decreases to some extent with increasing salt concentrations (data not shown.) Spin-labeled positions in the NpSRII cytoplasmic loop regions show a considerable decrease in mobility. However, the extent of the spectral changes observed (exemplified with position 154 in the EF-loop of NpSRII) are much less pronounced compared with the changes in the spectral components ratio detected in the HAMP domain, indicating that only the signal transduction domain undergoes a specific transition between two different states.

Experiments were also performed to address the stability of the PML bilayer at different salt concentrations to define the significance of the structural rearrangement of the HAMP domain with respect to changes in the lipid environment. Spin-labeled (5-doxylstearic acid) lipids were inserted into the native purple membrane layer and their properties investigated at different temperatures and salt concentrations. The room temperature EPR spectra are presented in Fig. 4B. Increasing the salt concentration leads to a detectable restriction in the overall motion of the spin-labeled lipids in the protein-free PML bilayer at room and high temperatures. However, in the presence of the protein complex, the dynamics of the PML layer resemble those of the protein-free PML at high salt concentration. Both the temperature-induced mobilization and the salt effects are much less pronounced (Fig. 4B), highlighting the stabilizing effect of the protein component in the membrane bilayer. The data suggest that the spectral changes observed for spin labeled positions in the HAMP domain of the complex embedded in PML cannot be caused by a major rearrangement of the membrane bilayer.

**Analysis of Side Chain Mobility and Accessibility**—A nitroxide scanning analysis in the predicted AS1 of the HAMP domain at high salt concentration was performed and compared with the previously published data obtained at low salt concentration (13) (Fig. 5A). The mobility of the nitroxides was quantified via the inverse line width of the central resonance line, $\Delta H_{pp}^{-1}$ (Fig. 5B).

At low salt concentration, a periodic pattern in the values of the mobility parameter, $\Delta H_{pp}^{-1}$, was visible only up to position 94 (Fig. 5B), suggesting the presence of an $\alpha$-helical structure, also demonstrated by high field EPR analysis (34). Starting from position 95, where the two spectral components are clearly distinguishable, the mobility data are biased toward dynamic component 2 (13). The absence of periodicity in the mobility parameter and the high mobility values found suggested a very dynamic C-terminal end of the AS1. Residues investigated in the charged connector region and in the AS2 also show a lack of rigid structure. The high dynamics observed did not enable to distinguish via EPR between a dynamic helix or a partially unfolded structure (see Fig. 5B and Ref. 13).

At high salt concentration (prevailing spectral component 1) an overall decrease in mobility in the AS1 as well as in the connector region and in AS2 is found (Fig. 5B). Moreover, periodical patterns appear in the mobility versus residue number plot, indicating the presence of secondary/tertiary interactions of the nitroxide (Fig. 5B). In agreement with this suggestion, the complex spectral shape obtained for all positions investigated at high salt concentration (Fig. 5A), shows distinct spectral contributions likely due to the presence of two spin label rotamers within a well defined secondary structure (43, 44). These facts support the presence of a dynamic domain (denoted dHAMP) at low salt concentrations and a more compact structure (denoted cHAMP) predominant at high salt concentration. The mobility data were complemented by accessibility measurements carried out to distinguish between spin label side chains exposed to the buffer, oriented toward the lipid phase or buried in the protein interior.
Results of oxygen and NiEDDA accessibility measurements performed at low and high salt concentrations are presented in Fig. 6. High exchange frequencies with NiEDDA or molecular oxygen characterize water- or lipid-exposed residues, respectively, whereas low accessibility values for both reagents are typical for positions oriented into the protein interior. The oxygen accessibilities at low salt concentration show almost constant values typical for nitroxides exposed to the bulk water (Fig. 6A). In contrast, values determined at high salt concentration exhibit a significant decrease consistent with the idea of a predominant compact HAMP structure. In agreement with the mobility data, a periodical pattern in the last part of the AS1 (beyond position 95) is clearly visible, pointing to well defined secondary and tertiary interactions in the cHAMP.

The accessibility from the water phase was measured with NiEDDA at 3 and 20 mM (see Fig. 6B). All Wex values presented were normalized to 20 mM NiEDDA concentration. For the positions investigated in the HAMP domain at low salt concentration, the scaled Wex values obtained at 3 mM NiEDDA are clearly higher than those obtained at 20 mM. The fact that the accessibility values are not proportional to the NiEDDA concentration points to the presence of two spectral components characterized by different accessibilities from the water phase.

This effect can be seen by comparison of two selected EPR spectra detected at 3 and 20 mM NiEDDA concentrations (Fig. 6C). High NiEDDA concentrations cause the suppression of spectral component 2 (associated with the dHAMP), indicating that the spin labeled positions in the dHAMP are water exposed. The small accessibility values obtained by line width analysis in the presence of 20 mM NiEDDA are then associated with the remaining component 1 (cHAMP). On the other side, at 3 mM NiEDDA concentration the central spectral line is still prevalently determined by component 2 (dHAMP). Thus, the higher accessibility values obtained at 3 mM NiEDDA reflect the water exposed character of the spin-labeled sites in the dHAMP.

The periodical pattern obtained at 20 mM NiEDDA at low salt concentrations are closely related for amplitude and trend to the values obtained at high salt concentrations, where component 1 (cHAMP) represents 90% of the spin label population. This demonstrates that spectral component 1 at low salt concentrations and the predominant spectral component at high salt concentrations represent both the cHAMP conformations. Relatively low accessibility values were obtained at high salt concentration for all R1 side chains, also for those sites expected to be surface exposed in the NMR HAMP model. This can be explained by the increased hydrophobic packing of the protein side chains at high salt concentrations. Additional measurements carried out in the presence of higher lipid content (400-fold instead of 40-fold molar excess of lipids) ruled...
out lipid-induced effects on the accessibility due to inter-complex interactions.

To unravel the molecular details of the cHAMP conformation, a comparison of the water accessibility pattern detected by EPR at high salt concentration with the solvent accessibility of the HAMP Afi503 domain from A. fulgidus was performed (see Fig. 6D). The NiEDDA Effectivities of the AS1 and AS2 of the N. pharaonis HAMP domain are fully in agreement with the solvent accessibility of the NMR structure of the HAMP domain from A. fulgidus taken from Ref. 11. Due to the few residues investigated in the connector region, an analogous comparison could not be done.

The mobility and accessibility data show that the side chains in the cHAMP conformation, stabilized by high salt concentration, are restricted in mobility and poorly accessible from the bulk water. The cHAMP conformation is shown to be in agreement with the four helical bundle structure obtained by NMR for the HAMP domain of A. fulgidus. On the contrary, the side chains become extremely dynamic and largely accessible to water in the dHAMP conformation.

The “Two-state” Equilibrium—To investigate the origin of the two spectral components and to understand their mutual relation in terms of a two-state equilibrium, titration experiments with NiEDDA, ascorbate, and urea were performed. Additionally, the temperature dependence of the spectral component fractions was analyzed.

Titration experiments with the water soluble paramagnetic quencher NiEDDA and the reducing agent sodium ascorbate at low salt concentration are presented in Fig. 7A and B. NiEDDA induces a reversible decrease of relaxation times due to exchange interaction with the investigated spin label. Ascorbate reduces the nitroxide of spin labels irreversibly. In both cases the reaction only takes place for spin label side chains accessible from the water phase. The effect of NiEDDA is shown in Fig. 7A: the addition of 52 mM NiEDDA leads to the complete suppression of component 2 (dHAMP). Although a broadening of component 1 is not evident, it cannot be excluded. The effect of ascorbate is shown in Fig. 7B: the overall spectral intensity is decreased, leaving the ratio between the two spectral components constant (see inset of Fig. 7B superimposing the normalized spectra at time 0 and after prolonged incubation). These results prove the existence of an equilibrium between the c- and dHAMP conformations with interconversion rates smaller than the magnetic relaxation rates.

Urea titration experiments were performed with NpSRII/NpHtrII157-A122R1 to further characterize the nature of the dHAMP conformation (Fig. 7C). At both low and high salt concentrations, addition of urea was accompanied by an increase in the intensity of component 2. Strikingly, at low salt concentration, effects of urea on the spectral shape are small. This clearly indicates that the HAMP domain is already partially destabilized at low salt concentration and that the spin label associated with component 2, representing 30–40% of the molecules, is characterized by motions similar to those of a partially unfolded transducer. Conversely, at high salt concentration the urea effect on the spectral shape is pronounced. It is worth noting that 6 M urea is not sufficient to fully unfold the protein neither at low nor at high salt concentrations. For reason of comparison, the spectrum of the pure component 2 obtained by subtracting the spectrum detected at high salt concentration from the one detected at low salt concentration is presented (see Fig. 7D). The extremely dynamic nature of component 2 (dHAMP) is clearly revealed. The results associate the dHAMP dynamics to backbone fluctuations similar to those present in partially unfolded structures.

Proven that c- and dHAMP conformations exist in a two-state equilibrium, we analyzed the temperature dependence of the EPR spectra for selected positions at low and high salt concentrations to obtain the thermodynamic parameters according to the method described previously (13). The temperature dependence observed are exemplified by spectra of D106R1 in the connector region (Fig. 8). Decreasing the temperature or increasing the salt content resulted in similar spectral changes, thereby shifting the equilibrium toward the cHAMP conformation. In contrast, the cHAMP-dHAMP equilibrium was not sig-
**HAMP Conformations in Equilibrium**

FIGURE 9. Arrhenius and van’t Hoff plots for two selected positions in the HAMP domain. Measurements were performed under the following conditions: 150 mM KCl, low lipid content (filled circles); 150 mM KCl, high lipid content (open circles); 3.5 M KCl, low lipid content (open squares); 3.5 M KCl, high lipid content (open circles). A, plot of the logarithm of the reorientational correlation times $\tau_c$ (component 1, upper part of graphs; component 2, lower part of graphs) versus the inverse of temperature. B, natural logarithm of the concentration ratio of the two HAMP conformations represented by the two spectral components, $\ln(f_2/f_1)$, versus the inverse of temperature.

The variation of the component ratio with temperature at low and high salt concentrations is shown in van’t Hoff plots (Fig. 9B). At low salt concentration the $\Delta G_{298K}$ values for both positions were found to be the same within errors, $\Delta G_{298K} = 2.0 \pm 0.2$ kJ/mol, $K_{eq} = 0.5 \pm 0.4$. (The $\Delta G$ value published previously for position 91 (13) was obtained from the amplitudes of the spectral components, not from their second integral. The corrected values are: $\Delta G_{298K} = 3.0 \pm 0.4$ kJ/mol, $K_{eq} = 0.3 \pm 0.5$.) In the presence of 3.5 M KCl, the thermodynamic parameters were also found to be self-consistent, with $\Delta G_{298K}$ (S98R1) = 9.2 \pm 0.4$ kJ/mol and $\Delta G_{298K}$ (Y121R1) = 10.7 \pm 0.4$ kJ/mol. The agreement of the equilibrium constants determined for positions in different HAMP subdomains corroborates the hypothesis of a salt-dependent two-state equilibrium between cHAMP and dHAMP conformations.

**Inter-spin Distances Investigation**—The inter-spin distance analysis performed in the dimeric NpSRII-NpHtrII157 complex is presented in Fig. 10. Low temperature (160 K) cw X-band EPR spectra of singly spin labeled transducers at low and high salt concentrations were recorded (data not shown) and analyzed in terms of dipolar broadening (“Materials and Methods”). Additionally, pulse experiments (DEER) were performed on three selected R1 residues representative for the proximal end of AS1 (A88R1), the distal ends of AS1 (M100R1) and AS2 (G118R1) (Fig. 10, A–C). Cw and pulse techniques are complementary for detailed descriptions of the distance distribution function. However, as can be seen in the comparison between the cw- and pulse-detected distance for position 100 in Fig. 10C, the cw EPR obtained values are biased toward shorter distances in case the overall distribution is close to or exceeds 2 nm.

The inter-spin distances (Fig. 10D) determined at low salt concentration were already discussed previously (13). Despite the significant changes in the mobility and accessibility data observed upon salt addition at room temperature, the spectra measured at low temperature do not show relevant salt-induced changes in the intra-transducer dimer distances. This apparently ambiguous result can be explained by analyzing the temperature dependence detected for the ratio of the two components presented in Figs. 8 and 9. Decreasing the temperature leads to a shift of the equilibrium toward component 1 even at low salt concentration. Thus, lowering the temperature for distance measurements allows analysis only of the cHAMP conformation.

The distance values obtained were compared with the expected values of inter-spin distances obtained for spin-labeled residues in the NMR model of the HAMP domain from A. fulgidus with primary sequences alignment according to the HAMP domain. The expected inter-spin distances, $d_{SL}$, based on the structure of the HAMP domain from A. fulgidus (10) with primary sequences alignment according to the HAMP domain from A. fulgidus (10) were obtained by linear interpolation of log $(\tau_c$ versus $1/T$ (Fig. 9A) and found to be in agreement with previously published data(45).

FIGURE 10. Inter-spin distances obtained from pulse and cw EPR methods on singly labeled NpHtrII mutants in complex with NpSRII reconstituted in PML. Measurements were performed at low (150 mM KCl, continuous lines) and high (2 M KCl, 20% v/v glycerol, dotted lines) salt concentrations. A, normalized dipolar evolution function $V(t)$ from four-pulse DEER measurements for positions 86, 100, and 118. B, form factor $F(t)$ obtained by dividing $V(t)$ by the background factor $B(t)$ and renormalizing it at $t = 0$. C, distance distribution $P(r)$ derived from Tikhonov regularization with an $\alpha$ parameter of 100. The dashed Gaussian line shows the distance distribution obtained by fitting of a simulated spectrum to the low temperature cw EPR spectrum of M100R1 with the program “dipfit” (33). The vertical line at $r = 2$ nm is the threshold distance for accurate cw EPR shape analysis distance determination. D, inter-spin distances for selected NpHtrII positions obtained by cw EPR (this work, 2 M KCl, open squares; and 150 mM KCl, filled squares; data taken from Ref. 13, triangles) and pulse EPR techniques (stars). Arrows represent lower distance limits determined from cw EPR spectra. The gray shaded area indicates the expected inter-spin distances, $d_{SL}$, based on the structure of the HAMP domain from A. fulgidus (10) with primary sequences alignment according to the HAMP domain from A. fulgidus (10).
FIGURE 11. Models of the proposed conformations of the compact (cHAMP, left) and the dynamic (dHAMP, right) conformations of the NpHtrII HAMP domain. For modeling of the cHAMP, the NMR structure of the AF1503 HAMP domain (Protein data bank code 2ASW) was attached to the crystal structure of the NpHtrII/NpSRII transmembrane domain (code 1H2S). The dHAMP conformation was modeled based on the cHAMP model, taking into account the increased dynamics observed for this conformation.

fulgidus according to Ref. 46 (Fig. 10D, gray shaded area). The experimentally determined values fit into the simulated distance range.

DISCUSSION

In the present study we demonstrate the co-existence of a dynamic (dHAMP) and compact (cHAMP) conformation as depicted in Fig. 11. In the dHAMP conformation, spin label probes attached at different positions within the HAMP domain are characterized by high mobility, typical for nitroxides bound to C-terminal ends of proteins or to partially unfolded structures, both cases in which backbone motions play a major role in determining the overall reorientational correlation time of the R1 side chain. Moreover, high accessibility values obtained for NiEDDA indicate that each position in the dynamic conformation is exposed to the bulk water, especially for residues beyond position 95.

The presence of the dHAMP conformation may result from the absence of several components comprising the functional signaling arrays found in cells, such as CheA/CheW, what might destabilize the HAMP domain, thereby increasing the amount of unfolded protein in equilibrium with the cHAMP conformation. It also has to be mentioned that our investigations were carried out using either constructs lacking the methylation sites responsible for adaptation or fully demethylated transducer proteins, thereby shifting the adaptation bias toward full intrinsic deactivation or even toward a non-native attractant signaling state. However, we have shown that the dHAMP conformation exists independently from C-terminal truncations of the protein and that it is observed also with the full-length protein. In addition, the two spectral components are obvious for several positions in all regions of the HAMP domain; their presence is clearly evident from position 95 in AS1 up to position 148 beyond AS2, which rules out the possibility that they represent an artifact due to the incorporation of the spin label side chain. We therefore conclude that the dHAMP and cHAMP conformations represent different conformational states of the NpHtrII HAMP domain.

For the cHAMP conformation several findings support a parallel four helical bundle structure: (i) both the mobility and accessibility data fit to a defined pattern of periodical properties in AS1 and AS2; (ii) the periodical profile of the NiEDDA accessibility found experimentally matches perfectly with the water accessibility calculated in silico for the analogous residues in the HAMP NMR model (10); (iii) the inter-spin distances pattern observed, despite not being a clear cut evidence, is in line with the distances expected within a four helical bundle. Whereas for AS1 and AS2 an excellent correspondence was found between the experimental data and the NMR model of the HAMP domain from A. fulgidus (10), such analysis could not be performed in the connector region, where only a few positions were investigated. Thus, the four helical bundle structure obtained by NMR on an unusual archaeal membrane protein of unknown function seems to well represent the cHAMP conformation of the transducer from N. pharaonis.

Recent disulfide scanning data obtained by Swain and Falke (11) on the HAMP domain of the chemoreceptor Tar from E. coli in intact, membrane-bound chemoreceptors confirmed that the HAMP NMR model accurately depicts also the architecture of the conserved HAMP domain in the chemoreceptor. The authors found that no relevant conformational changes were induced by ligand binding. Interestingly, the agreement between the A. fulgidus intra-dimer distances and the entity of the disulfide dimer formation in the aspartate receptor is less pronounced in AS1 than in AS2, suggesting a more dynamic structure of the first region of the HAMP domain from the aspartate receptor.

A second recent in vivo cross-linking study performed by Taylor and co-workers (12) also revealed that the HAMP domain of Aer, the E. coli aerotaxis receptor, falls into the four helical bundle category, highlighting how the NMR model is indeed a prototype for HAMP domains. The authors suggested a possible mechanism of activation inducing a torque downstream to the HAMP, where the PAS domain is thought to interact. Again, evidence exists for a dynamic region in the proximal and distal ends of AS1 in the Aer HAMP domain (12).

Despite increasing knowledge on HAMP structural details, no experimental evidence clarifying the mechanism of activation neither for the negative phototaxis, nor for the positive taxis toward attractants exist. Nevertheless, the hypothesis of a “frozen-dynamic” model for the activation/deactivation of chemoreceptors put forward by Kim (47), with the signaling domains being involved in two conformations characterized by different degrees of dynamics, is now gaining acceptance in the field of research on bacterial chemotaxis. In fact, the notion that a two-state equilibrium between populations with different degrees of dynamics can be the language of the activation/deactivation of the cytoplasmic domain was again put forward by Hazelbauer et al. (48). Recently, evidence for a stabilization/destabilization mechanism for the bacterial signal transduction were also provided in vivo (49).

The peculiar aspect found for the NpHtrII HAMP domain is that two different conformations are found to co-exist, both at low and high salt concentrations, which might experimentally support the above mentioned hypothesis. These two conformations distinguished by their dynamic properties are found to be
in a two-state equilibrium, which can be shifted at least by changes of temperature or salt concentration. An equilibrium constant close to one under physiological conditions highlights the physiological relevance of this equilibrium. The HAMP domain seems to be engaged in a subtle balance between various physical interactions that leads to a small ΔG value. Shifting the cHAMP/dHAMP equilibrium would provide insight into the regulation of receptor activity.

The HAMP domains might be able to adapt their sensitivity toward a variety of attractant (shift toward the OFF state) and repellent (shift toward the ON state) inputs, generated by ligand binding to periplasmic domains, conformational changes of photoreceptors taking place in the membrane, or even, in the case of the aerotaxis receptor Aer, sensor domains being in direct spatial proximity to the HAMP domain. The temperature dependence of the equilibrium between the two HAMP conformations could also be an interpretative key to an understanding of the thermosensing function of Tar and Tsr (50, 51).

Evidence for a dynamic conformation being an intrinsic feature of the HAMP domain have already been described in literature (13, 14). Moreover, the NMR model was obtained well below the living temperature of the hyperthermophile *A. fulgidus*, which could have trapped the HAMP structure in one of its most stable conformations. Whatever the AF1503 HAMP function might be, by CD measurements the HAMP was shown to be highly destabilized at physiological temperature (close to 350 K) (10). However, chimera studies performed at 310 K showed that the HAMP was still functional. Selecting a HAMP domain from a hyperthermophile and finding it to be functional and molecular details of the transition between different functional and molecular states of the transition between different dynamic states of HAMP domains.

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