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The Membrane Mimetic Affects the Spatial Structure and Mobility of EGFR Transmembrane and Juxtamembrane Domains

Konstantin S. Mineev,* Stanislava V. Panova,† Olga V. Bocharova, Eduard V. Bocharov, and Alexander S. Arseniev

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences RAS, str. Miklukho-Maklaya 16/10, Moscow, 117997 Russian Federation

Supporting Information

ABSTRACT: The epidermal growth factor receptor (EGFR) is one of the most extensively studied receptor tyrosine kinases, as it is involved in a wide range of cellular processes and severe diseases. Recent works reveal that the single-helix transmembrane domains and cytoplasmic juxtamembrane regions play an important role in the receptor activation process. Here we present the results of our investigation of the spatial structure and mobility of the EGFR transmembrane domain and juxtamembrane regions in various membranelike environments, which shed light on the effects of the membrane physical properties and composition on the behavior of the juxtamembrane domain.

Activation of the receptor tyrosine kinases (RTKs) is of great interest in the modern scientific community. These proteins belong to the type I group of cellular receptors: they have large extracellular and intracellular domains and a single transmembrane (TM) α-helix. Among RTKs, the epidermal growth factor receptor (EGFR) from the HER family is one of the most extensively studied, as it is involved in a wide range of cellular processes and severe diseases. It is known that this receptor is active in the form of a homodimer or heterodimer with another member of the ErbB (or HER) family.[1] Nevertheless, it can form the inactive dimeric complexes on the cell membrane.[2] That is why a "coupled rotation" mechanism was suggested for the activation of the EGFR. According to that model, the protein exists in the form of a dimer in the inactive state, with interacting intracellular kinase domains (ICDs). Ligand binding causes the dimerization of the extracellular domains (ECDs), the rearrangement in both the TM domains (TMDs) and ICDs, which results in receptor activation.[3] Recent works revealed that the single-helix TMDs and cytoplasmic juxtamembrane regions (JMAs) play an important role in the described process.[4] The junction of the TM domain to the ICD was shown to increase the level of spontaneous transphosphorylation.[4] Moreover, an isolated TMD, being added to the cells, expressing the EGFR, can substantially inhibit the receptor.[5] Finally, point mutations in the TMD were found to be able to cause the ligand-independent activation of the EGFR and tumorogenic transformation of the cell culture.[6] As for the juxtamembrane regions, the presence of the JMA enhanced the catalytic activity of the isolated kinase domains of the EGFR,[4] and substitutions of R^{680} and R^{681} in the JMA of EGFR caused a substantial reduction in receptor activity.[4]

With all that in mind, it is obvious that structural data about the behavior of the TMD and JMA of EGFR in a membrane or membrane mimetic are necessary. In our recent works, we determined the three-dimensional structures of several TMD dimers from the HER family,[7−10] which provided insight into possible conformations of the EGFR TMD. The spatial structure of the isolated EGFR JM region was investigated in both water and detergent environment and appeared to be α-helical.[11] Finally, the spatial structure of the EGFR TM domain dimer in a junction with JMA domains was determined in DMPC/DHPC bicelles.[12] Analysis of the X-ray data, available for the ECDs and ICDs of EGFR,[4,13] allowed the authors to assign the obtained structure to the receptor active state.

Here we present the results of our investigation of the spatial structure and mobility of the EGFR TMD and JMA regions in various membranelike environments, which shed light on the effects of the membrane on the behavior of the JMA domain and put forward several questions regarding the spatial structure reported in the work of Endres et al.[12]

To test the effect of different membranelike media on the behavior of the JMA domain, we synthesized the construct, corresponding to residues 642−690 of human EGFR, flanked with short expression tags (EGFR-TMJMA, numeration according to UniProt entry P00533), and monitored the helicity and mobility of the JMA region within the construct under various conditions (Figure S1). DPC micelles and DMPC/DMPG/DHPC bicelles with different lipid compositions were used for the screening. NMR cross-peaks were assigned by conventional triple-resonance spectroscopy, and both secondary chemical shifts and cross-peak patterns in NOESY spectra were used to control the helicity of the JMA domain. The correlation time of rotational diffusion (τ_C) was selected to monitor the mobility of the region.[14] The ion strength, pH, temperature, lipid (detergent)/protein ratio (LPR), long-chain to short-chain lipid ratio in bicelles (q), and contents of charged lipids were varied.

Our data reveal that the chain of EGFR-TMJMA forms two separate helices (TMD and JMA) on residues 646−670 and 676−686 in DPC micelles. In bicelles, only the TM helix is formed, while the chemical shifts of the JMA residues are much

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closer to the characteristic values for the random-coil conformation (Figure 1B and Figure S2). The NOE distance

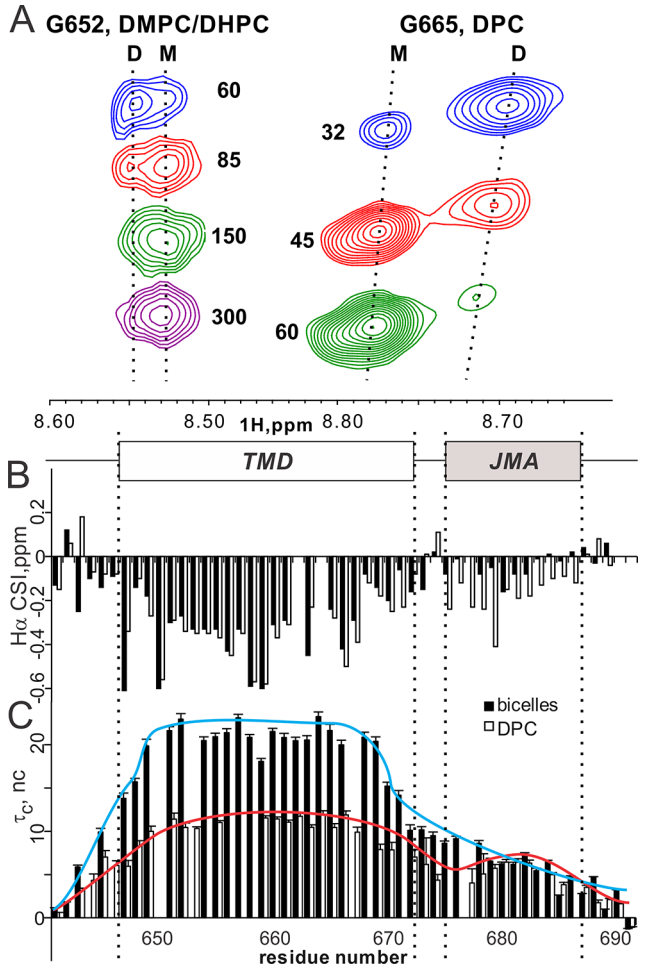

Figure 1. Effect of the membrane mimetic on the structure and mobility of the EGFR JMA region. (A) Appearance of cross-peaks, corresponding to the amide group of G652 in DMPC/DHPC bicelles (pH 6.5) and of G665 in DPC micelles (pH 5.8) in $^{1}H-^{15}N$ HSQC spectra of EGFR-TMJMA, acquired at various LPRs at 40 °C. Peaks from the monomeric (M) and dimeric (D) states of the protein and corresponding LPR values are indicated. (B and C) Hα secondary chemical shift index and correlation time of rotational diffusion for the N−H vectors plotted vs the residue number of the EGFR-TMJMA at pH 5.8 in bicelles (filled bars) and micelles (empty bars).

pattern did not reveal the presence of the helical conformation for the JMA domain in bicelles, while in DPC, several $H_{\alpha(i-3)}-H_{N(i)}$ and $H_{\alpha(i)}-H_{\beta(i+3)}$ NOEs were observed (Figure S3). This is in agreement with the work by Endres et al.;[12] they explicitly acknowledge in the supplementary data that the JMA forms a transient helix in DMPC/DHPC q = 0.25 bicelles (30% probability of helical structure and RCI of ∼0.6, according to the NMR chemical shifts). In micelles, two relatively stable regions separated by the mobile loop were detected, while in bicelles, the mobility of the C-terminal residues increased gradually with the distance from the TM domain (Figure 1C). Intermolecular lipid/protein NOE contacts reveal that in a DPC environment the JMA domain is attached to or embedded in the micelle, while in bicelles, almost no lipid/protein interaction is observed (Figure S4). In addition, neither the variation of the bicelle size (we screened the DMPC/DHPC ratio in the range of 0.3−0.8), nor the addition of 10−20% charged lipid (DMPG), nor an increase in the ionic power of solution resulted in significant changes in the JMA chemical shifts (Figures S5 and S6). Thus, we state that the JMA region is α-helical and membrane-bound in DPC micelles and mainly unstructured and water-exposed in bicelles of various sizes and compositions.

The spatial structure of the EGFR-TMJMA in DPC micelles was calculated on the basis of chemical shifts, J couplings, and NOE restraints (see Table S1). As expected, the protein formed two α-helices, located at ∼90° to each other (Figure 2A),

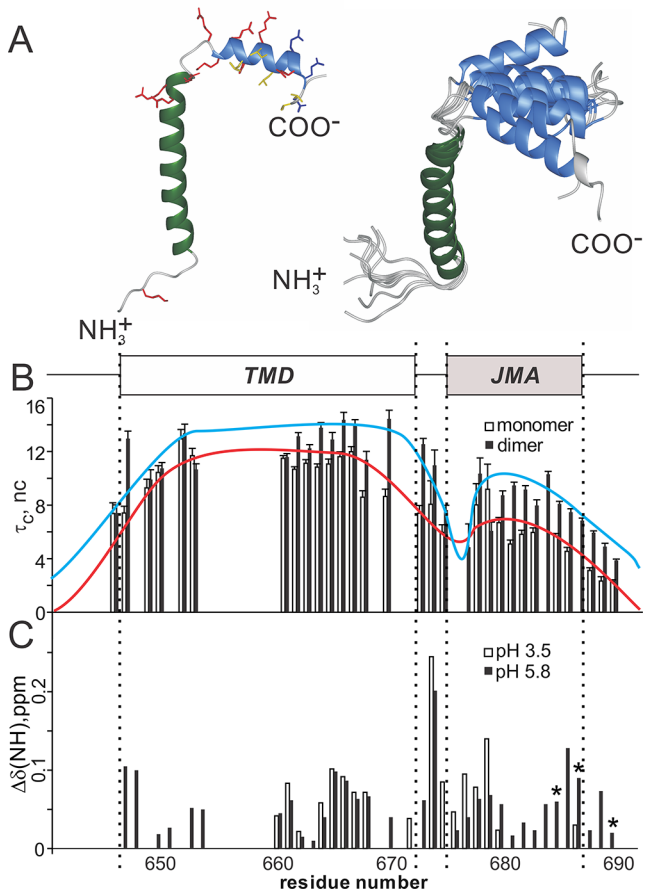

Figure 2. (A) Spatial structure of the EGFR-TMJMA in DPC micelles at pH 5.8 in ribbon representation. Hydrophobic, positively charged, and negatively charged side chains are shown as yellow, red, and blue sticks, respectively. A set of 10 NMR structures, superimposed by the backbone atoms of the TMD helix, is shown at right. The structure is deposited in the Protein Data Bank as entry 2N5S. (B) Correlation time of rotational diffusion for the N−H vectors of the EGFR-TMJMA in monomeric (empty bars) and dimeric (filled bars) states at pH 5.8 plotted vs residue number. (C) Changes in amide cross-peak positions in $^{1}H-^{15}N$ HSQC spectra of EGFR-TMJMA (expressed in units of generalized chemical shift), observed upon the dimer−monomer transition in DPC micelles at pH 3.5 (empty bars) and pH 5.8 (filled bars). Positions of Glu residues are denoted with asterisks.

implying that the JMA domain lies on the micelle surface. The bulky side chains of nonpolar residues of the JMA domain are directed inside the micelle. There are no reliable data, such as long-range NOE contacts or residual dipolar couplings, to support the found orientation of the JMA helix, but it is in agreement with intermolecular lipid/protein contacts observed for the JMA domain in a micelle environment. The TM helix

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includes residues T646−R671, which is consistent with both the length and position of the EGFR TMD, observed in the EGFR/ErbB2 heterodimer[8] and in the EGFR TMD-JMA construct homodimer in bicelles.[12] The JMA helix includes residues K676−R686, with the TALOS+-predicted propensity of helical structure being ∼90%. The length and stability of the JMA helix in a DPC environment are essentially different from those observed in bicelles (30% propensity for residues L679−Q684[12]). Cα secondary shifts demonstrate that the helicity of the JMA region in micelles does not change with the ambient pH (Figure S2).

Besides the spatial structure, the dimerization of the EGFR-TMJMA was investigated. The dilution experiments showed that there are two possible states of the protein in DPC micelles, and the populations of states are dependent on the LPR (Figure 1A). On the basis of rotational diffusion, we assigned the states to the monomer and dimer of EGFR-TMJMA (Figure 2B). The rotational diffusion of the TM helix is on average slower by 2 ns in the dimeric state than in the monomeric state (∼12 ns). In turn, the motions of the JMA helix become ∼3 ns slower upon dimerization, while the τ_C for this region in the monomeric state of the EGFR-TMJMA is only 6 ns, which corresponds to the 50% deceleration. This effect is statistically much greater than the deceleration of the TM domains (17%). In other words, in the dimeric form, motions of the JM regions are more strongly coupled to the motions of the TM helix than in the monomeric form. It may indicate the involvement of the JMA in the dimerization process of the EGFR-TMJMA construct.

The exchange between two forms of the protein is slow on the NMR chemical shift time scale, allowing the measurement of the dimerization constant and investigation of the structural difference between the monomeric and dimeric states of EGFR-TMJMA (Figure S7). The free energy of dimerization was measured using our recently published NMR-based approach[15] in a set of detergent-dilution experiments. The experiments demonstrated clearly that DPC is not an "ideal solvent" for the dimerization of EGFR-TMJMA in terms of the kinetics of the process (γ = 0.24 ± 0.02), indicating that the substantial rearrangement of micelles is required for the dimerization and that the obtained free energy would be irrelevant (Figure S8).

A previous study suggested that intermolecular salt bridges are formed between the Arg and Glu side chains of JMA domains in the active state of the EGFR.[4] Thus, we studied the effect of dimerization on the spatial structure of EGFR-TMJMA, monitoring the changes in the generalized chemical shifts of the backbone N−H groups ($\Delta\delta$N/6 + $\Delta\delta$H) upon the dimer−monomer transition at pH 3.5 and 5.8, below and above the pK_a of Glu side chains, respectively. The profile of chemical shift changes (Figure 2C) demonstrates that the backbone of the TMD similarly "senses" the dimerization under both conditions, suggesting that regardless of the pH, the spatial structure of the TMD dimer is conserved. The different picture is observed for the JMA domain. At low pH, amide chemical shifts in the JMA helix are unchanged upon dimerization, while at high pH, they are rather sensitive to the oligomeric state of the EGFR-TMJMA. Therefore, at least when Glu side chains are charged, the JMA helix is somehow involved in the dimerization process. On the other hand, no large changes in the chemical shifts of the backbone or side-chain amide groups were detected upon the dimerization of EGFR-TMJMA, suggesting that salt bridges are not formed in the JMA regions in the dimeric state, which is in agreement with the recently reported model of the dimeric structure in bicelles.[12]

The variation of LPR in the range of 60−300 in q = 0.4 bicelles also revealed the slow dimer−monomer transition, occurring at LPR 60−80 (Figure 1A). Unlike the process in micelles, it is not accompanied by large changes in the amide chemical shifts: we managed to detect only five signals with pronounced splitting of NMR cross-peaks at pH 6.5 and an LPR 80: G652, G663, G665, M668, and R671. None of the found peaks belongs to the JMA region, and none of the observed chemical shift changes is larger than 0.02 ppm. Because of the small spacing between the NMR cross-peaks, corresponding to the two states, we managed only to roughly estimate the free energy of dimerization as −1.5 ± 0.5 kcal/mol, according to the equations previously derived for bicelles.[10] Thus, we can conclude that EGFR-TMJMA forms a weak dimer in DMPC/DHPC bicelles, and the dimerization of the protein does not cause the changes in the local structure of the TMD helix or stabilization of the JMA conformation. Apparently, in bicelles, the JMA domain does not participate in the protein−protein interaction.

Two major aspects need to be discussed, with respect to the results, reported here. First, our data raise several questions, regarding the previously published dimeric structure of the EGFR TMD-JMA construct.[12] This structure was characterized by the N-terminal location of the TMD dimerization interface, relatively high magnitude of the helix crossing angle, and antiparallel mode of JMA-JMA interaction. Here we show that in bicelles (1) the EGFR-TMJMA dimer−monomer transition occurs at LPR 60−80, (2) JMA domains are mainly unstructured with the possibility of a transient helical conformation, and (3) NMR cross-peaks of JMA residues are not affected by the dimerization of TMD. All three issues seem to contradict the work by Endres et al.[12] If JMA domains are flexible and almost unstructured (which is acknowledged by the authors), how can that happen, that there is a contact between the JMA helices in the dimer stable enough to observe intermolecular NOE cross-peaks? Moreover, if JMA helices are in a contact within the dimer, that should result in the NMR chemical shift changes of JMA cross-peaks upon dimerization. Finally, authors worked at LPR 150, and no data about the dimer−monomer transition are present; we show that under such conditions the protein is predominantly in the monomeric state. However, we need to admit that Endres et al. worked with a different construct of EGFR that is longer by seven residues and contains additional KLWS sequence at the C-terminus, which may affect the free energy of dimerization, and the dimer could be observed at LPR 150, but there was no proof for that given.

Altogether, listed points suggest that the reported dimeric structure is possibly the result of the misinterpretation of the NMR data. In the course of structure determination, authors relied on either $^{13}C/^{15}N$-filtered or $^{13}C/^{2}H$-filtered NOESY experiments, allowing the direct detection of intermolecular contacts in the mixtures of isotope-labeled and unlabeled proteins. However, according to our experience, all isotope-labeled proteins contain 1−2% unlabeled nuclei, while unlabeled species have ∼1% of ^{13}C at natural abundance. Being combined, the two effects give rise to the residual peaks in filtered spectra, with the intensities being ∼3% compared to those of conventional nonfiltered NOESY. Because the cross-peaks in NOESY spectra behave as the reversed sixth power of the distance between the nuclei, "strong" intramolecular peaks

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at 2.4 Å, which leaked through the filter due to the imperfect isotope labeling and natural abundance, will have intensities comparable to those of the intermolecular peaks at 3.5−4 Å, additionally reduced 2-fold due to 50% population of the dimers with different isotope labeling of the subunits. For that reason, in our studies, we record the reference-filtered NOESY spectra in the absence of the unlabeled protein[7,10] to identify the residual intramolecular cross-peaks. Of course, all this information does not rule out any of the other findings, reported by Endres et al., and the proposed model of the EGFR TMD-JMA construct seems reasonable and is confirmed by the results of computer modeling. Nevertheless, we think that the structure determination of the EGFR TMD in the presence of the receptor JMA regions needs to be revised.

Second, we show that the properties of the membranelike environment affect the helicity and stability of JMA in the context of the EGFR-TMJMA construct. In DPC micelles, where no planar bilayer is present, and the surface is loose, the JMA domains are helical and surface-bound and are involved in the protein dimerization process. In bicelles, where the long-chain lipids form a patch of a planar bilayer[16] and the surface is relatively rigid, the JMA domains are disordered, and their motions are uncoupled from the motions of the EGFR TMDs. Therefore, the question of which conformation of JMA is biologically relevant is raised. Bicelles are believed to provide a more native environment for membrane proteins, especially for those containing the juxtamembrane regions, interacting with the cellular membrane.[17] However, in this particular case, we claim that micelles allow the formation of a nativelike structure for the EGFR-TMJMA, providing some interactions, necessary to support the helical structure of the JMA region. JMA regions of EGFR are shown to be helical by the number of studies,[4,12,18] and the conformation of the JMA-JMA dimer was even found to be dependent on the type of EGFR ligand.[19] Besides, we suppose that the observed ability of the membrane environment to govern the helicity and membrane binding propensity of the EGFR JMA domains may indicate that cellular membranes can play an active role in the EGFR activation process.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00851.

Supplementary tables, figures, and experimental details (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mineev@nmr.ru.

Present Address

†S.V.P.: Manchester Institute of Biotechnology, University of Manchester, 131 Princess St., Manchester M1 7DN, U.K.

Author Contributions

K.S.M. performed NMR experiments, analyzed the data, and wrote the paper. S.V.P. conducted some NMR experiments and analyzed the data. O.V.B. produced and purified the protein. E.V.B. took part in the writing of the paper. A.S.A. supervised the project.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

NMR, nuclear magnetic resonance; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; ICD, intracellular domain; ECD, extracellular domain; JMA, cytoplasmic juxtamembrane region; TM, transmembrane; TMD, transmembrane domain; DPC, dodecylphosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; LPR, lipid-to-protein rato; HSQC, heteronuclear single-quantum correlation spectroscopy.

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