Published in final edited form as:

Magn Reson Chem. 2007 December ; 45(Suppl 1): S192–S197. doi:10.1002/mrc.2144.

Membrane orientation of the Na,K-ATPase regulatory membrane protein CHIF determined by solid-state NMR

Carla M. Franzin, Peter Teriete, and Francesca M. Marassi*

Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla CA 92037, CA, USA

Abstract

Corticosteroid hormone-induced factor (CHIF) is a major regulatory subunit of the Na,K-ATPase, and a member of an evolutionarily conserved family of membrane proteins that regulate the function of the enzyme complex in a tissue-specific and physiological-state-specific manner. Here we present the structure of CHIF oriented in the membrane, determined by solid-state NMR orientation-dependent restraints. Because CHIF adopts a similar structure in lipid micelles and bilayers, it is possible to assign the solid-state NMR spectrum measured for ^{15}N -labeled CHIF in oriented bilayers from the structure determined in micelles, to obtain the global orientation of the protein in the membrane.

Keywords

CHIF; FXYP4; Na,K-ATPase; structure; membrane protein; bilayers; solid-state NMR

INTRODUCTION

The solid-state NMR orientation-dependent frequencies measured for samples of membrane proteins in oriented lipid bilayers provide very high resolution restraints for protein structure determination and refinement.^{1–3} Measurements made in oriented lipid bilayers have the important advantage that they enable structures to be determined in an environment that closely resembles the cellular membrane and, since the alignment tensor is fixed by the sample geometry, they provide the global orientation of the protein in the membrane. For proteins in oriented lipid bilayers, the two-dimensional $^1\text{H}/^{15}\text{N}$ separated local field spectra exhibit characteristic wheel-like patterns of resonances that reflect both the protein structure and orientation.^{4–6} The direct relationship between spectrum and structure makes it possible to calculate NMR spectra from specific structural models of proteins and provides a method for structure determination.^{7,8} Here we show that this information can also be used to supplement the structural data obtained from proteins in micelles, to derive global structural information about proteins in membranes.⁹ This is illustrated for the protein corticosteroid hormone-induced factor (CHIF; FXYP4) a major regulatory subunit of the Na,K-ATPase, the principal enzyme responsible for maintaining the gradient of Na and K ion concentrations across cell membranes.

CHIF is a member of the FXYP protein family, which derives its name from a highly conserved sequence of signature amino acids (Phe-X-Tyr-Asp) preceding the single transmembrane domain (Fig. 1(A)). The FXYP proteins have been the focus of recent

*Correspondence to: Francesca M. Marassi, Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla CA 92037, CA, USA. fmarassi@burnham.org.

attention due to their ability to specifically regulate the activity of the enzyme complex in various physiological settings,^{10–13} including tumor progression^{14,15} and kidney disease.¹⁶ Their genes are expressed predominantly in tissues that are electrically excitable or that specialize in transport, and six of the seven mammalian FXYD family members interact specifically with the Na,K-ATPase, modulating the enzyme's rate constant and its affinity for intracellular Na or extracellular K. The distinct activities of the FXYD proteins have been partly ascribed to specific differences in the amino acid sequences of their transmembrane segments, which are otherwise highly homologous throughout the protein family across species. The functions are also modulated by the FXYD cytoplasmic domains and by post-translational modifications. In addition, CHIF and at least three other FXYD family members can induce ionic currents in *Xenopus oocytes* and in phospholipid bilayers; however, the direct formation of ion channels has not been demonstrated *in vivo*, and recent evidence indicates that the role of FXYD proteins in ion transport regulation is solely related to their association with the Na,K-ATPase and other ion transporters. Because the FXYD proteins play important roles in regulating mammalian tissue homeostasis, they represent important targets for functional and structural characterization.

Recently we showed that the structures of FXYD1, FXYD3, and CHIF in micelles mirror the structures of their corresponding genes, with exons corresponding to helical segments and intron–exon junctions corresponding to helix breaks or loops.¹⁷ The three-dimensional structure of FXYD1 was determined by supplementing the solution NMR restraints obtained in micelles, with information about the protein transmembrane orientation obtained from solid-state NMR experiments in oriented lipid bilayers.¹⁸ Here we show that solid-state NMR restraints can be specifically incorporated in the structure calculation to obtain the protein orientation in the membrane, and to provide a measure of cross-validation for the structural analysis.

EXPERIMENTAL

Sample preparation

The expression and purification of CHIF, and the preparation of samples in phospholipid bilayers were as described previously.^{19,20} Briefly, 80 mg of dioleoyl-phosphatidylcholine (DOPC) and 20 mg of dioleoyl-phosphatidylglycerol (DOPG) were co-dissolved in 1 ml of CHCl₃, and added to 2–4 mg of lyophilized ¹⁵N-labeled protein dissolved in 0.5 ml of trifluoroethanol plus 50 µl of β-mercaptoethanol. After mixing, this solution was distributed on the surface of 35 glass slides with dimensions 11 × 11 × 0.06 mm (www.marienfeld-superior.com), the solvents were allowed to evaporate at ambient pressure followed by high vacuum overnight, and the slides were stacked. Oriented lipid bilayers were formed by equilibrating the stacked slides for 24 h, at 40 °C, in a chamber containing a saturated solution of ammonium phosphate, which provides an atmosphere of 93% relative humidity. The samples were wrapped in parafilm and then sealed in thin polyethylene film prior to insertion into the NMR probe. For H/D exchange, the stacked sample was incubated in a chamber where H₂O had been replaced with D₂O at 40 °C for several hours. Before insertion into the coil of the NMR probe, the stack of glass slides was wrapped in a thin layer of parafilm and then placed in thin polyethylene tubing, which was heat-sealed at both ends to maintain sample hydration during the NMR experiment. The samples contained 2–4 mg of purified ¹⁵N-labeled protein, 80 mg of DOPC, and 20 mg of DOPG.

NMR experiments

Solid-state NMR experiments were performed on a Bruker AVANCE 500 spectrometer (www.bruker-biospin.com) with a 500/89 AS Magnex magnet (www.magnex.com), using a double-resonance ¹H/¹⁵N probe with a 11 × 11 × 4 mm square coil built at the UC San

Diego NIH Resource for Molecular Imaging of Proteins (nmrresource.ucsd.edu). One-dimensional ^{15}N chemical shift spectra were obtained using single contact CPMOIST,²¹ and two-dimensional $^1\text{H}/^{15}\text{N}$ separated local field spectra were obtained using polarization inversion spin exchange at the magic angle (PISEMA),²² with ^1H irradiation field strengths of 62 kHz, a cross-polarization time of 1 ms, a recycle delay of 8 s, acquisition times of 5 ms, and continuous ^1H irradiation during acquisition to provide heteronuclear decoupling. The two-dimensional data were acquired with 512 accumulated transients and 256 complex data points, for each of 64 real t_1 values incremented by 32.7 μs . All NMR experiments were performed at 20 °C. The chemical shifts were referenced to the $^1\text{H}_2\text{O}$ resonance set to its expected position of 4.805 ppm at 20 °C.²³

Data analysis and calculations

The NMR data were processed using NMRPipe,²⁴ and the spectra were analyzed using Sparky.²⁵ Structure calculations were performed with the program XPLOR-NIH.²⁶ Solid-state NMR spectra were calculated with FORTRAN programs and tensor values as described previously.⁶ Structures were rendered in MacPymol (DeLano Scientific LLC). Analyses and calculations were performed on a 2.16 GHz Intel Core Duo MacBook Pro running OS X 10.4.8.

To obtain frequency-independent values of the root mean-square deviations (RMSDs) between the experimental and calculated dipolar coupling and chemical shift frequencies, each deviation (δ) between observed (F_o) and calculated (F_c) frequency was scaled by the resolution index (I_r),²⁷ calculated by dividing the spectral range available for each spin interaction (10 kHz; 150 ppm) by the observed linewidth (0.5 kHz; 3 ppm). This gives $\Delta_{\text{HN}} = |F_o - F_c|/I_{r,\text{HN}}$ for the dipolar coupling and $\Delta_{\text{N}} = |F_o - F_c|/I_{r,\text{N}}$ for the chemical shift, where $I_{r,\text{HN}} = 10/0.5$ and $I_{r,\text{N}} = 150/3$.

For each of the ten test assignments, simulated annealing was performed with rigid-body internal dynamics,²⁸ at low temperature,²⁹ to orient the protein structure coordinates (PDB file 2JP3) in the membrane frame. In this step, the coordinates were grouped as a rigid body, which was allowed to undergo rotational and translational motions, and orientation restraints, derived from the dipolar couplings measured for the transmembrane Leu residues, were imposed with the XPLOR-NIH SANI potential.³⁰ During simulated annealing, the temperature was brought down from 300 to 20 K in steps of 10 K, with 7 ps of internal dynamics at each temperature. The SANI force constant was ramped from 0.00001 to 0.001 kcal Hz^{-2} .

RESULTS AND DISCUSSION

Overall features of the structure in micelles

The measurement of ^1H - ^{15}N residual dipolar couplings (RDCs) was instrumental for identifying helix breaks that map to genetic elements, and for determining the three-dimensional structures of both CHIF and the related protein FXYD1 in micelles.^{17,18} The helical structure of CHIF mirrors the structure of its gene, with four helices (H1–H4) and helix breaks mapping to distinct exons and delineating discrete structured domains. The four helices of CHIF can be traced by fitting the experimental RDCs to sinusoids with the signature α -helical periodicity of 3.6 residues, and the regularity of fit in H2 indicates that it is close to an ideal helix (Fig. 1(B)). The $^1\text{H}/^{15}\text{N}$ heteronuclear nuclear Overhauser effect (NOE) measurements indicate that all four helices have similar backbone dynamics, and that helices H1, H2, and H3 are rigidly connected (Fig. 1(C)). Helix H2 coincides with the transmembrane segment of the protein, and its amide protons exchange very slowly with

water, reflecting the strong intramolecular hydrogen bonds of the transmembrane helix in the low dielectric environment of the micelle interior (Fig. 1(D)).

Estimate of the helix tilt in lipid bilayers

Although RDCs provide very high resolution orientation-dependent restraints for structure determination and refinement of both globular and membrane proteins,^{18,31–34} they cannot determine the protein orientation in the membrane. This information, however, can be obtained by measuring anisotropic chemical shifts and dipolar couplings in samples of the protein in oriented lipid bilayers. The ¹⁵N chemical shift spectrum of CHIF in oriented bilayers has peaks near 200 ppm corresponding to backbone sites in the transmembrane helix, with NH bonds oriented nearly parallel to the magnetic field and perpendicular to the membrane plane (Fig. 2(B)).¹⁹ As observed for the protein in micelles, the amide hydrogens in this region resist exchange with D₂O, indicating that they participate in strong hydrogen bonds and confirming that the protein is folded in lipid bilayer membranes (Fig. 2(C)). The narrow chemical shift dispersion around 200 ppm indicates that CHIF crosses the membrane with a small tilt angle. Peaks near 80 ppm are from sites with NH bonds nearly parallel to the membrane surface, while several residues in the loop and terminal regions are mobile and give the peaks centered near 120 ppm, which are also seen in the spectrum obtained from unoriented bilayer vesicles (Fig. 2(A)).

To estimate the tilt of the CHIF transmembrane helix, we examined the ¹H/¹⁵N separated local field spectra of CHIF in oriented lipid bilayers. The ¹H/¹⁵N PISEMA spectra of the transmembrane region of CHIF are shown in Fig. 3(A) and (B). A comparison with the wheel-like spectra calculated for ideal α -helices ($\phi, \psi = -60^\circ, -40^\circ$) of varying transmembrane tilts indicates that CHIF adopts an approximate tilt between 15° and 20° (Fig. 3(C)). However, the RDC data and the structure of CHIF in micelles show that a break at Gly20 causes helices H1 and H2 to adopt different orientations, suggesting that the PISEMA spectrum should be fitted with two wheels corresponding to two helices with different tilts. The RDC and structural data in micelles also suggest that the fits will be distorted by deviations in torsion angle regularity at Leu17 and Leu28 (Fig. 3(C), asterisks).

The spectra in Fig. 3 contain residue-specific orientation restraints that could be used for structure determination if resonance assignments were available. For example, assignment of the resonances in the spectrum from Leu ¹⁵N-labeled CHIF would give an orientation restraint for each of the seven Leu residues (17, 19, 22, 27, 28, 35, 37) in helices H1 and H2, which could be used to supplement the structural data obtained for CHIF in micelles and to determine the protein orientation in the membrane. One method to obtain resonance assignments is based on the direct relationship between structure and anisotropic frequency in the separated local field spectra of oriented proteins,^{4,5} and involves comparing the experimental spectra with the spectra back-calculated from structural models of the protein.⁷

Generation of test assignments

To assign the spectrum of the CHIF transmembrane Leu residues using this method, we combined the information from the three-dimensional structure of CHIF in micelles with the 15–20° helix tilt estimated from the solid-state NMR spectra, to generate a set of ten test assignments. Each of the ten test assignments obtained in this way was then used to generate a list of dipolar coupling restraints, which were input to the XPLOR-NIH SANI potential^{28,30} to orient the coordinates of the structure in micelles by rigid-body internal dynamics. The resulting oriented coordinates were used, in turn, to back-calculate the PISEMA spectrum, which was then evaluated for its closeness of fit to the experimental spectrum by computing the RMSDs between the observed and calculated NMR frequencies (Fig. 4).

The ten test assignments were generated based on back-calculation from two separate, but rigidly connected, ideal helices, one spanning Leu17 and 19 (H1), and the other Leu22, 27, 28, 35, and 37 (H2), each with respective tilts of 15 and 20° as indicated by the experimental spectra. Ten rotations of 36° around the long axis of helix H2, completing a full 360° circle, gave ten starting models from which ten spectra were back-calculated. The relative orientations of H1 and H2 are defined by the three-dimensional structure determined in micelles, so that each rotation around H2 also sets the orientation of H1 (Fig. 4(A)). Each rotation defines the order of peaks around the wheel-like spectra that are generated from each model (Fig. 5(A)); however, we note that the tilts of the helices are not important at this stage of the calculation, but serve to distinguish two separate helices for H1 and H2.

Analysis of test assignments

The test assignments were then obtained by comparing each back-calculated spectrum with the experimental spectrum of ¹⁵N-Leu-labeled CHIF (Fig. 5(B)). During this process the following criteria were considered: (i) experimental and back-calculated peaks were matched by giving first priority to the value of the dipolar coupling and second to that of the chemical shift, to reduce tensor-dependent uncertainty; (ii) since the torsion angles of Leu17 and Leu28 deviate from ideal helix, their frequencies were allowed to sample a wider range than those of the other leucines. This procedure yields an initial set of test assignments informed by the three-dimensional structure of the protein in micelles.

The spectra back-calculated from the coordinates obtained by simulated annealing with the orientation restraints from each test set are shown in Fig. 5(C), and the resulting RMSDs from the experimental frequencies are reported in Table 1. Of the ten test assignments, only one (Figs 5(I) and 3(B), (D)), with the lowest RMSDs, was able to reproduce the pattern of the dipolar couplings and chemical shifts observed in the experimental spectrum regardless of uncertainty in the NH bond length and chemical shift tensor, while the others yielded very different chemical shifts from those observed experimentally. For this assignment, the resulting RMSDs of 0.03 for the dipolar couplings, and 0.12 for the chemical shifts, reflect experimental errors as well as uncertainties in the tensors and in the structure coordinates. Since the chemical shifts were not included in the structural refinement, and their RMSD is analogous to the R_{free} parameter used in X-ray crystallography, they provide a measure of structural analysis cross-validation.

CONCLUSIONS

The NMR spectrum back-calculated from the structure of CHIF in micelles faithfully reproduces the experimental spectrum, providing both resonance assignments and an additional set of restraints that establish the global orientation of the protein in the membrane. The structure of CHIF oriented in the membrane in this way is shown in Fig. 6. Helix H1 (Phe10–Leu19) adopts a tilt of 32°, H2 (Gly20–Gly39) a tilt of 18°, and H3 (Lys40–Arg45) a tilt of 30°. Helix H4 (Pro49–Thr61) is curved and adopts a tilt between 80° and 60°. Although the transmembrane region of the spectrum of uniformly ¹⁵N-labeled CHIF appears to show little evidence for an ideal helix tilted at 30°, we note that while H2 has dihedral angles near the ideal values, H1, H3, as well as H4 deviate from ideality. Furthermore, the PISEMA spectra in Fig. 3 were obtained with experimental parameters optimized for observation in the transmembrane region. Finally, it is also possible that the kinks are somewhat less pronounced in bilayers than in micelles; this will have to be confirmed by measuring additional structural restraints in bilayers.

Interestingly, the ability to back-calculate the spectrum in bilayers from the structure determined in micelles indicates that the structured domains of the protein in these distinct

environments are similar, and suggests that the data obtained from these two types of samples can be used in a complementary fashion.

Acknowledgments

This work was supported by the NIH (R01CA082864). It utilized the NIH-supported Resource for NMR Molecular Imaging of Proteins (P41EB002031) and Burnham Institute NMR Facility (P30CA030199).

References

1. Cross TA, Quine JR. Concepts Magn Reson 2000;12:55.
2. Opella SJ, Marassi FM. Chem Rev 2004;104:3587. [PubMed: 15303829]
3. Quine JR, Cross TA. Concepts Magn Reson 2000;12:71.
4. Wang J, Denny J, Tian C, Kim S, Mo Y, Kovacs F, Song Z, Nishimura K, Gan Z, Fu R, Quine JR, Cross TA. J Magn Reson 2000;144:162. [PubMed: 10783287]
5. Marassi FM, Opella SJ. J Magn Reson 2000;144:150. [PubMed: 10783285]
6. Marassi FM. Biophys J 2001;80:994. [PubMed: 11159466]
7. Marassi FM, Opella SJ. Protein Sci 2003;12:403. [PubMed: 12592011]
8. Asbury T, Quine JR, Achuthan S, Hu J, Chapman MS, Cross TA, Bertram R. J Magn Reson 2006;183:87. [PubMed: 16914335]
9. Franzin CM, Teriete P, Marassi FM. J Am Chem Soc 2007;129:8078. [PubMed: 17567018]
10. Sweadner KJ, Rael E. Genomics 2000;68:41. [PubMed: 10950925]
11. Crambert G, Geering K. Sci STKE 2003;2003:RE1. [PubMed: 12538882]
12. Cornelius F, Mahmoud YA. News Physiol Sci 2003;18:119. [PubMed: 12750449]
13. Garty H, Karlsh SJ. Annu Rev Physiol 2006;68:431. [PubMed: 16460279]
14. Morrison BW, Leder P. Oncogene 1994;9:3417. [PubMed: 7970700]
15. Nam JS, Hirohashi S, Wakefield LM. Cancer Lett 2007;255:161. [PubMed: 17442482]
16. Meij IC, Koenderink JB, van Bokhoven H, Assink KF, Groenestege WT, de Pont JJ, Bindels RJ, Monnens LA, van den Heuvel LP, Knoers NV. Nat Genet 2000;26:265. [PubMed: 11062458]
17. Franzin CM, Yu J, Thai K, Choi J, Marassi FM. J Mol Biol 2005;354:743. [PubMed: 16288923]
18. Teriete P, Franzin CM, Choi J, Marassi FM. Biochemistry 2007;46:6774. [PubMed: 17511473]
19. Franzin CM, Gong XM, Thai K, Yu J, Marassi FM. Methods 2007;41:398. [PubMed: 17367712]
20. Thai K, Choi J, Franzin CM, Marassi FM. Protein Sci 2005;14:948. [PubMed: 15741345]
21. Levitt MH, Suter D, Ernst RR. J Chem Phys 1986;84:4243.
22. Wu CH, Ramamoorthy A, Opella SJ. J Magn Reson, Ser A 1994;109:270.
23. Cavanagh, J. Protein NMR Spectroscopy: Principles and Practice. Vol. xxiii. Academic Press; San Diego: 1996. p. 587
24. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. J Biomol NMR 1995;6:277. [PubMed: 8520220]
25. Goddard, TD.; Kneller, DG. SPARKY 3. University of California, San Francisco; 2004.
26. Schwieters CD, Kuszewski JJ, Tjandra N, Marius Clore G. J Magn Reson 2003;160:65. [PubMed: 12565051]
27. Marassi FM, Ramamoorthy A, Opella SJ. Proc Natl Acad Sci USA 1997;94:8551. [PubMed: 9238014]
28. Schwieters CD, Clore GM. J Magn Reson 2001;152:288. [PubMed: 11567582]
29. Chou JJ, Li S, Bax A. J Biomol NMR 2000;18:217. [PubMed: 11142512]
30. Clore GM, Gronenborn AM, Tjandra N. J Magn Reson 1998;131:159. [PubMed: 9533920]
31. Bax A, Kontaxis G, Tjandra N. Methods Enzymol 2001;339:127. [PubMed: 11462810]
32. Prestegard JH, Bougault CM, Kishore AI. Chem Rev 2004;104:3519. [PubMed: 15303825]
33. Hus JC, Marion D, Blackledge MJ. J Mol Biol 2000;298:927. [PubMed: 10801359]

34. Tolman JR, Al-Hashimi HM, Kay LE, Prestegard JH. J Am Chem Soc 2001;123:1416. [PubMed: 11456715]

Figure 1.

Secondary structural features of CHIF in micelles. Residue numbering begins at 1 after the signal sequence. Leu in H1 and H2 are in red. Leu22 and Leu35 were introduced by mutations of native Met. (A) Amino acid sequence of CHIF (NCBI accession: NP_068 702). (B) Values of ^1H - ^{15}N residual dipolar couplings (RDCs) with the characteristic periodicity in the α -helical regions of the proteins fitted sinusoids. (C) ^1H / ^{15}N heteronuclear NOEs. Positions left blank correspond to Pro (3, 9, 49, 53, 58, 62) or overlapped peaks. (D) Amide hydrogen/deuterium exchange profile, with exchange rates classified as rapid (less than 1 h), short (less than 3 h), medium (less than 12 h), or long (more than 24 h).

Figure 2. ^{15}N chemical shift solid-state NMR spectra of uniformly ^{15}N -labeled CHIF in phospholipid bilayers: (A) Unoriented bilayer vesicles; (B), (C) Bilayers oriented with the normal parallel to the magnetic field, in (B) H_2O or (C) D_2O .

Figure 3.

Solid-state NMR $^1\text{H}/^{15}\text{N}$ PISEMA spectra of CHIF in oriented phospholipid bilayers, showing peaks from residues in the transmembrane region for (A) uniformly ^{15}N -labeled CHIF or (B) Leu ^{15}N -labeled CHIF. The red circles mark the positions of the peaks corresponding to transmembrane Leu residues. The Leu peaks were assigned by comparison with the spectrum in panel C, predicted for ideal helices. (C) Leu spectrum calculated for two distinct ideal α -helices with transmembrane tilt of 15° (thick line) or 20° (thin line), and specific rotations around the helix long axes. White asterisks mark the peaks from Leu17 and Leu28, whose dihedral angles are expected to deviate from ideal helix. (D) Leu

spectrum back-calculated from the structure of CHIF after refinement with the dipolar couplings assigned in panel B.

Figure 4.

Procedure for generating and analyzing the test assignments. (A) Helices H1 and H2 viewed down the long axis of H2 from the *N*-terminus. Rotations of 36° around H2 also set the rotation of the rigidly connected H1. (B) Flow chart outlining the procedure.

Figure 5.

Generation of ten test assignments for the spectrum of Leu ^{15}N -labeled CHIF, and resulting spectra obtained by back-calculation from the structures refined with the test dipolar couplings. (I–X) Results obtained for 10 different test assignments obtained by rotating around the helix long axis. (A) Spectra predicted for ideal helices. (B) Experimental spectra with test assignments. (C) Spectra back-calculated from the structure of CHIF after refinement with each set of assigned dipolar couplings.

Figure 6.

Structure of CHIF determined in micelles and oriented in the membrane using the data from oriented lipid bilayers. Z is the axis perpendicular to the membrane surface, and parallel to the magnetic field in the solid-state NMR experiments. The seven transmembrane Leu residues are in red.

Table 1

RMSDs between experimental and back-calculated values of the ^1H – ^{15}N dipolar couplings and ^{15}N chemical shifts obtained for each of the ten test assignments

Test assignment	^1H – ^{15}N dipolar coupling	^{15}N chemical shift	Total
I	0.031	0.12	0.13
II	0.023	0.26	0.26
III	0.029	0.20	0.20
IV	0.028	0.19	0.20
V	0.030	0.28	0.28
VI	0.022	0.32	0.32
VII	0.131	1.12	1.13
VIII	0.020	0.35	0.35
IX	0.205	0.67	0.70
X	0.355	0.75	0.83