Study of Structural Stability of Cyclophilin A by NMR and Circular Dichroism Spectra

ARTICLE *in* CHINESE JOURNAL OF CHEMISTRY · JULY 2006

Impact Factor: 1.58 · DOI: 10.1002/cjoc.200690184

4 AUTHORS, INCLUDING:

Donghai Lin
Xiamen University
144 PUBLICATIONS **1,404** CITATIONS
SEE PROFILE

Xu Shen
Shanghai Institute of Materia Medica, Shan…
257 PUBLICATIONS **4,361** CITATIONS
SEE PROFILE

Chinese Journal of Chemistry, **2006**, *24*, 1—10 **Full Paper**

Study of Structural Stability of Cyclophilin A by NMR and Circular Dichroism Spectra

SHI, Yan-Hong(施燕红) LIN, Dong-Hai*(林东海)
HUANG, Jian-Ying(黄剑英) SHEN, Xu(沈旭)

Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China

The structural stability of cyclophilin A (CypA) was investigated using H/D exchange and temperature coefficients of chemical shifts of amide protons, monitored by 2D heteronuclear NMR spectroscopy. Amide proton exchange rates were measured by H/D exchange experiments for slow-exchange protons and measured by SEA (Solvent Exposed Amides)-HSQC experiments for fast-exchange protons. Temperature coefficients of chemical shifts and hydrogen exchange rates of amide protons show reasonably good correlation with the protein structure. Totally, 44 out of 153 non-proline assigned residues still exist in 86 d of hydrogen-deuterium exchange at 4 °C, suggesting that CypA structure should be highly stable. Residues in secondary structures of α2, β1, β2, β5, β6 and β7 might constitute the hydrophobic core of the protein. The change in free energy of unfolding ($\Delta G_u^{H_2O}$) of CypA was estimated to be 92.07±6.41 kJ•mol^{-1} by circular dichroism (CD). The large free energy change is also an indicator of the high structural stability.

Keywords cyclophilin A, structural stability, hydrogen exchange, NMR, circular dichroism

Introduction

Structural stability of the protein is closely related to its biological function. Proteins often undergo conformational changes to execute their biological functions, such as an enzyme reaction or ligand binding.[1] The static three-dimensional structure can not explain all the results from functional biological assays even though it provides a profile of the ground state of the molecule.[2] Internal protein dynamics can afford a great deal of useful information to understand the mechanism of the intermolecular interaction and to design more highly potent inhibitors. Hydrogen-deuterium (H/D) exchange kinetics[3-5] and ^{15}N spin relaxation (T_1, T_2 and *NOE*),[6] monitored by nuclear magnetic resonance spectroscopy, are powerful tools to study protein dynamics in relation to protein functions. As the H/D-exchange measurements can be made in the absence of denaturants, they can avoid the uncertainties and difficulties often encountered in the estimation of thermodynamic parameters at extreme denaturing conditions.[7] Therefore, amide proton exchange measurements are usually employed to obtain valuable information on the free energy relationships on the basic unit of protein structure.[8-11]

The backbone amide protons can be exchanged with the solvent according to the following two-state scheme:[12-14]

$$\text{NH (open)} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{NH (closed)} \xrightarrow{k_{ch}} \text{ND (exchanged)}$$

where k_{op}, k_{cl} and k_{ch} are the rate of opening, closing and intrinsic chemical exchange of the protein, respectively. The observed hydrogen exchange rate (k_{ex}) can be described by the equation $k_{ex}=k_{op}k_{ch}/(k_{cl}+k_{ch})$. In the EX2 limit ($k_{cl}>>k_{ch}$), where structural reclosing is faster than the intrinsic unprotected chemical exchange rate, this equation can be simplified to be $k_{ex}=(k_{op}/k_{cl})k_{ch}$. The ratio of k_{op}/k_{cl} is the so called protection factor of the amide proton.[15] Therefore, the measurement of hydrogen exchange rate can provide site-specific information in the presence or absence of hydrogen bonded structure and the structural stability, dynamics, and other properties.[16,17]

Cyclophilin (Cyp) is a highly conserved and function-related protein family, which plays a very important role in signal transduction and immunity suppression.[18,19] As the most important number of Cyp family, CypA (165 residue, 17.8 kDa) can catalyze protein folding, assemblage and transportation, adjust the procedure of signal transduction, participate in the immunosuppressive functions of cyclosporin A (CsA).[20,21] CypA is also a very important target protein, which can bind with many different types of ligands.[22] The NMR

* E-mail: dhlin@mail.shcnc.ac.cn; Tel.: +0086-021-50806036; Fax: +0086-021-50806036
Received January 25, 2005; revised January 5, 2006; accepted March 22, 2006.
Project supported by the Hundred Talent Project of Chinese Academy of Sciences and the National Natural Science Foundation of China (Nos. 30470351, 30570352).

structure of CypA (Figure 1a)[22] shows that CypA contains one short 3_{10}-helix (Glu120 to Leu122), two α-helices (α1: Pro30 to Thr41, α2: Met136 to Phe145) and eight β-strands (β1: Thr5 to Val12, β2: Glu15 to Leu24, β3: Phe53 to Ile57, β4: Met61 to Gly64, β5: Ile97 to Ala101, β6: Phe112 to Phe115, β7: Val127 to Glu134 and β8: Ile156 to Gln163). In present paper, we investigated the structural stability of CypA on the basis of amide proton exchange kinetics, temperature coefficients of amide proton chemical shifts monitored by NMR spectroscopy and the urea-induced unfolding experiments monitored by CD spectroscopy. Our results indicated the high conformational stability of the protein.

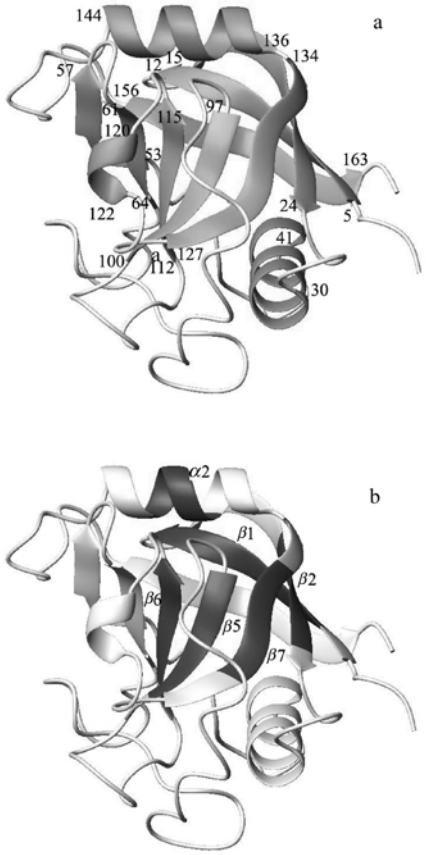

Figure 1 Ribbon diagram of the three-dimensional structure of CypA in solution solved by NMR spectroscopy, where α-helices are in green and β-strands in cyan, the N and C termini and some sequence locations are indicated (a), and the stability core of CypA was colored in red (b). Diagrams were generated using MOLMOL. [23]

Experimental

Labeled $^{15}NH_4Cl$, ^{13}C-glucose and D_2O were purchased from Cambridge Isotope Laboratory. All other chemicals used were of high-quality analytical grade, and all experiments were performed at 25 ℃.

Protein expression and purification

Uniform labeling of CypA with ^{15}N and ^{13}C was performed by growing CypA expressing *E. coli* (BL21) in M9 minimal medium with 1 g/L $^{15}NH_4Cl$ and 2 g/L ^{13}C glucose. Expression of the protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a concentration of 0.5 mmol/L when the cell density reached an optical density of 0.6—0.8 at 600 nm. The cells were harvested about 7 h after induction, and stored at −80 ℃. Frozen cells were thawed and suspended in a buffer of 20 mmol/L Tris-HCl at pH 8.0, 500 mmol/L NaCl, 10 mmol/L imidazole and 0.05 mmol/L phenylmethylsulphonylfluoride (PMSF). After sonication, centrifugation and filtration, the suspension was loaded to a column with 2 mL of Ni-NTA resin (Novagen). The column was placed in a 4 ℃ refrigerator for 30 min to be equilibrated. Then the column was washed with a buffer of 20 mmol/L Tris-HCl at pH 8.0, 500 mmol/L NaCl, 30 mmol/L imidazole. The CypA was eluted with a buffer of the same ingredients except for imidazole increased to 300 mmol/L. The protein solution was concentrated, loaded onto a Hiprep 16/60 Sephacryl S-100 column (Amersham Biosciences) equilibrated with 20 mmol/L Tris-HCl at pH 8.0, 150 mmol/L NaCl, 1 mmol/L EDTA and 2 mmol/L β-mercaptoethanol, eluted with the same buffer at a flow rate of 0.5 mL/min. The elution solution was concentrated and dialyzed against an NMR buffer of 10 mmol/L KOAc and 10 mmol/L KH_2PO_4 (pH 6.0), 5 mmol/L dithiothreitol (DTT), 0.02% NaN_3, and stored at 4 ℃. The purity of the protein was confirmed by SDS-PAGE. The unlabeled CypA sample was prepared by growing CypA expressing *E. coli* (BL21) in LB medium. The same NMR buffer was used for both unlabeled and isotopic-labeled CypA samples.

Equilibrium unfolding

Urea-induced equilibrium unfolding of unlabeled CypA protein was performed in a series of NMR buffer media with different urea concentrations of 0.0—7.5 mol/L and equilibrated at room temperature for 16 h. The far-UV CD spectra were recorded from 200 to 250 nm on 0.5 mg/mL protein samples at 25 ℃, using a Jasco-810 spectropolarimeter with a cell of 0.1 cm path length. Each spectrum was the average of three consecutive scans, and background corrections were made in all the spectra.

NMR measurements

All the NMR experiments were performed on a Varian Unity Inova-600 NMR spectrometer. Protein CypA in NMR buffer was concentrated to 1.0 mmol/L by a centricon (Millipore), then 10% D_2O was added for the field-lock, and a suite of 3D heteronuclear NMR experiments, including HNCA, HNCOCA, HNCACB, CBCACONH, ^{15}N-edited NOESY-HSQC and TOCSY-HSQC, was used to confirm the resonance assignments of CypA. All the spectra were processed via the NMRPipe program[24] and analyzed via the SPARKY program.[25] Linear prediction[26] and zero-filling approaches[27] were used to obtain complex data matrixes of 1024(t3),

256($t1$), 128($t2$) before Fourier transformation to improve resolution. The Water-flip-back approach was employed in HSQC-type experiments to minimize saturation of water resonance.[28]

Measurement of temperature coefficients of chemical shifts

2D ^{15}N-^{1}H HSQC spectra were recorded at a series of temperatures from 10 to 48 ℃ at 2 ℃ interval to measure the temperature coefficients of chemical shifts for amide protons. The complex data matrix of ^{1}H-^{15}N HSQC was 1024×64 and extended to 2048×1024 by linear prediction and zero-filling approaches. Proton chemical shifts were referenced versus 2,2-dimethyl-2-silapentane-5-sulfonic acid, and ^{15}N chemical shifts were referenced with the consensus ratio of 0.101329118. [29]

Amide proton exchange experiments

A protein sample of about 270 μL in NMR buffer was dried by lyophilization. H/D exchange was initiated by dissolving dry protein in D_2O of the same volume at pD 6.6 (25 ℃). All the NMR experimental parameters were preset by a mock sample and the pH value was measured after the experiment to minimize the dead time. The first ^{1}H-^{15}N HSQC spectrum was recorded after 16 min of initiation of exchange. Spectra were recorded with 8 transients of 1024 data points and 64($t1$) increments. Linear prediction and zero-filling approaches were employed to extend the size of the complex data matrix to 2048($t2$),. 1024($t1$) before Fourier transformation. Twenty five spectra were recorded in 90 h with various time points, and other 15 spectra were recorded in 2064 h. The peak intensities in ^{15}N-^{1}H HSQC spectra were measured via the SPARKY program. To determine the hydrogen exchange rate k_{ex}, NH peak intensity I as a function of hydrogen exchange time t was fit to a single-exponential function, $I = I_0\exp(-k_{ex}t)+I_\infty$, where I_0 is the initial intensity, and I_∞ the final intensity.

SEA-HSQC measurements

SEA-HSQC experiments[30,31] were performed on ^{15}N-labeled CypA sample. Mixing time τ_m used was 10, 20, 35, 50, 75, 100, 150 and 200 ms, respectively. The data matrix of 1024($t2$) × 128($t1$) was extended to 2048($t2$)×1024($t1$) by linear prediction and zero-filling approaches. To determine hydrogen exchange rates, NH peak heights in the spectra as a function of mixing time were fit to a single-exponential function, $I = I_\infty(1-\exp(-k_{ex}\tau_m))+C$, where I_∞ is the final intensity, and C is a constant.

Results and discussion

Equilibrium unfolding of CypA

The urea-induced equilibrium unfolding of CypA was studied by CD measurement. Figure 2 displays the urea-induced unfolding transition of CypA obtained by measuring the CD changes at 222 nm, and the inset shows the far-UV CD spectra of CypA in the native (0.0 mol/L urea) and denatured (6.0 mol/L urea) states. To quantitatively access the equilibrium stability of CypA, three equilibrium unfolding parameters, *i.e.*, the free energy change of unfolding in water ($\Delta G_u^{H_2O}$), the cooperativity index of the transition (m), and the denaturant concentration at the midpoint of the unfolding transition (C_m), were calculated from the transition curve by a non linear least-square fitting. The results were obtained: $\Delta G_u^{H_2O} = 92.07 \pm 6.41$ kJ•mol^{-1}, $m = 29.60 \pm 1.91$ kJ•mol^{-1}•(mol/L)$^{-1}$, $C_m = 3.08 \pm 0.02$ mol/L. The large free energy change indicates the high structural stability of the protein.

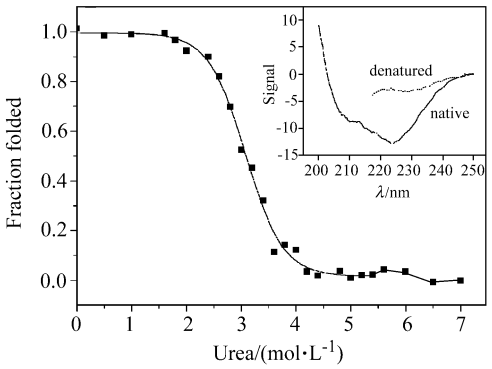

Figure 2 Urea-induced equilibrium unfolding transition of CypA at pH 6.0 and 25 ℃. The fraction unfolding values were calculated from the CD values at 222 nm, with the inset to show the CD spectra of CypA in the native and denatured states.

Resonance assignments of CypA backbone

The backbone resonance assignments of CypA were described previously.[32-34] Totally, 155 out of 159 non-proline residues were assigned with exception of four residues of Nos. 1, 2, 81 and 148. In our work, 153 out of the 155 assigned residues were confirmed except for two residues of Nos. 3 and 70, maybe due to the somewhat difference between the buffer conditions.

Identification of hydrogen bonds

The hydrogen bond network was identified from the NMR structures of CypA (protein data base: 1OCA)[22] using the MOLMOL program.[23] Hydrogen bonded amide protons were identified according to the criterion B used by Cierpicki and Otlewski,[35] namely, acceptor atom found within the distance of 0.3 nm from amide proton, angle between the donor-proton bond and the line connecting the donor and receptor heavy atoms smaller than 60 degrees. Hydrogen bond was accepted only if it was present in at least 50% of all the 20 conformers.

Relationship between temperature coefficients of the chemical shifts and structure

The sensitivity of the amide proton chemical shift to temperature, *i.e.*, the temperature coefficient of the chemical shift, $\Delta\delta/\Delta T$, has been examined to explore

whether there was hydrogen bond within the structure.[36-40] In general, $\Delta\delta/\Delta T \geqslant -4.6$ ppb/K was taken as an indicator that the amide proton might be involved in hydrogen bonding.[35,41] In the protein CypA, there are 124 out of 154 amide protons (81%) with $\Delta\delta/\Delta T$ more positive than -4.6 ppb/K (Figure 3), indicating a high structural stability. Totally, 85.1% of all those hydrogen bonded protons identified from the NMR structures, where in detail, this number is 94.1% for α-helices, 91.4% for β-strands and 50% for 3_{10}-helix, exhibited $\Delta\delta/\Delta T \geqslant -4.6$ ppb/K. The former two percentages are very high with respect to those reported in the previous article: about 70% for α-helices and 87% for β-strands.[34]

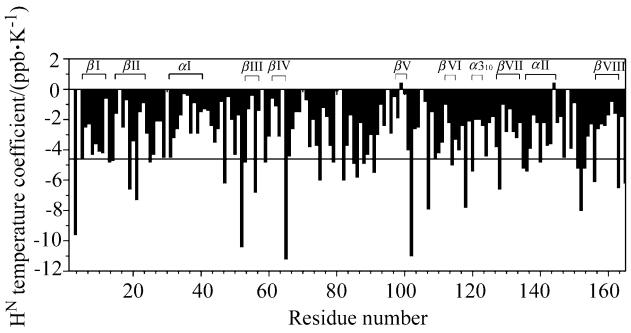

Figure 3 Temperature coefficients of amide proton chemical shifts for the CypA protein. Values of the temperature coefficients more positive than -4.6 ppb/K (solid line) are taken as indicators of hydrogen bonds.

Relationship between H/D exchange and structure

Amide proton exchange measurements can provide information in the presence or absence of hydrogen bonds in the structure and the structural stability.[11,17] The ^{15}N-^{1}H HSQC spectrum of CypA was well dispersed, and nearly all the crosspeaks have been unambiguously assigned. During the process of H/D exchange, 59 backbone amide protons shown in Table 1, were exchanged out within the 16 min of initiation of the exchange, and most of them can belong to residues located in the unstructured loop regions (Figure 4a). It was difficult to measure the exchange rates of these 59 residues by the H/D exchange experiment due to too quick exchange. In addition, 21 residues were exchanged fast, with exchange rate $k_{ex} > 1\times10^{-2}$ min^{-1}. Except for 4 residues located in regular structures: Thr5 in β1, Leu122 in 3_{10}-helix, Glu134 in β7 and Cys161 in β8, the other 17 residues (81%) are located in the unstructured loop regions. Furthermore, 29 residues were exchanged at moderate rate constant of 1×10^{-4} min$^{-1} < k_{ex} < 1\times10^{-2}$ min^{-1}, in which 18 residues (62.1%) are located in secondary structures. On the other hand, 44 residues were exchanged slowly with $k_{ex} < 1\times10^{-4}$ min^{-1}, exhibiting strong protection against H/D exchange. The NH peak intensities of the 44 residues failed to be fit to the single-exponential function due to the non-significant changes of intensities (Figure 4b). Moreover, it is interesting to note that all the 41 residues exhibit temperature coefficients of the chemical shifts more positive than -4.6 ppb/K. Except for Phe25 and Val29 located in helix-like turn, the other 42 residues (95.4%) are located in secondary structures. In these 42 residues, 26 residues are located in α2, β1, β2, β5, β6 and β7, most of which (19 residues, 73%) are hydrophobic residues and are spatially closed to each other. Thereby, it was suggested that residues including Val6-Val12 in β1, Val20-Glu23 in β2, Ile97-Met100 in β5, Phe112-Cys115 in β6, Phe129-Val132 in β7, Val139-Met142 in α2, constitute the hydrophobic stability core of CypA (Figure 1b). Interestingly, 80% of residues in CsA binding sites on CypA[42,43] (Arg55, Ile57, Phe60, Met61, Gln63, Gly72, Thr73, Ala101, Asn102, Ala103, Gln111, Phe113, Trp121, Leu122, His126) are with hydrogen-deuterium exchange rates larger than 1×10^{-4} min^{-1} indicated by Table 1. It might suggest that flexible residue in CsA binding sites favor the CypA-CsA complex formation.

Table 1 Amide hydrogen-deuterium exchange patterns in CypA

Exchange rate range/min^{-1}	Residue
$k_{ex} < 1\times10^{-4}$	V6, F7, F8, D9, I10, A11, V12, V20, F22, E23, F25, V29, A33, E34, N35, F36, R37, A38, L39, S40, T41, I57, Q63, G64, I97, L98, S99, M100, F112, F113, I114, C115, F129, G130, K131, V132, V139, E140, A141, M142, I157, I158, D160, G162
$1\times10^{-4} < k_{ex} < 1\times10^{-2}$	E15, L17, G18, L24, A26, K31,T32, Y48, S51, F53, R55, F60, M61, C62, E86, L90, G96, A101, T116, A117, D123, H126, V127, V128, K133, I138, E143, F145, A159
$k_{ex} > 1\times10^{-2}$	T5, K28, G42, F46, G50, G65, I78, Y79, D85, N87, I89, H92, G94, T107, N108, G109, T119, L122, 134, R144, C161
	Residues disappearing in the first spectrum
	D13, G14, R19, S21, D27, E43, K44, G45, G47, K49, C52, H54, I56, G59, D66, F67, T68, R69, N71, G72, T73, G74, G75, K76, S77, G80, K82, F83, E84, F88, K91, T93, N102, A103, G104, N106, S110, Q111, K118, E120, W121, G124, K125, G135, M136, N137, G146, S147, N149, G150, K151, T152, S153, K154, K155, I156, Q163, L164, E165

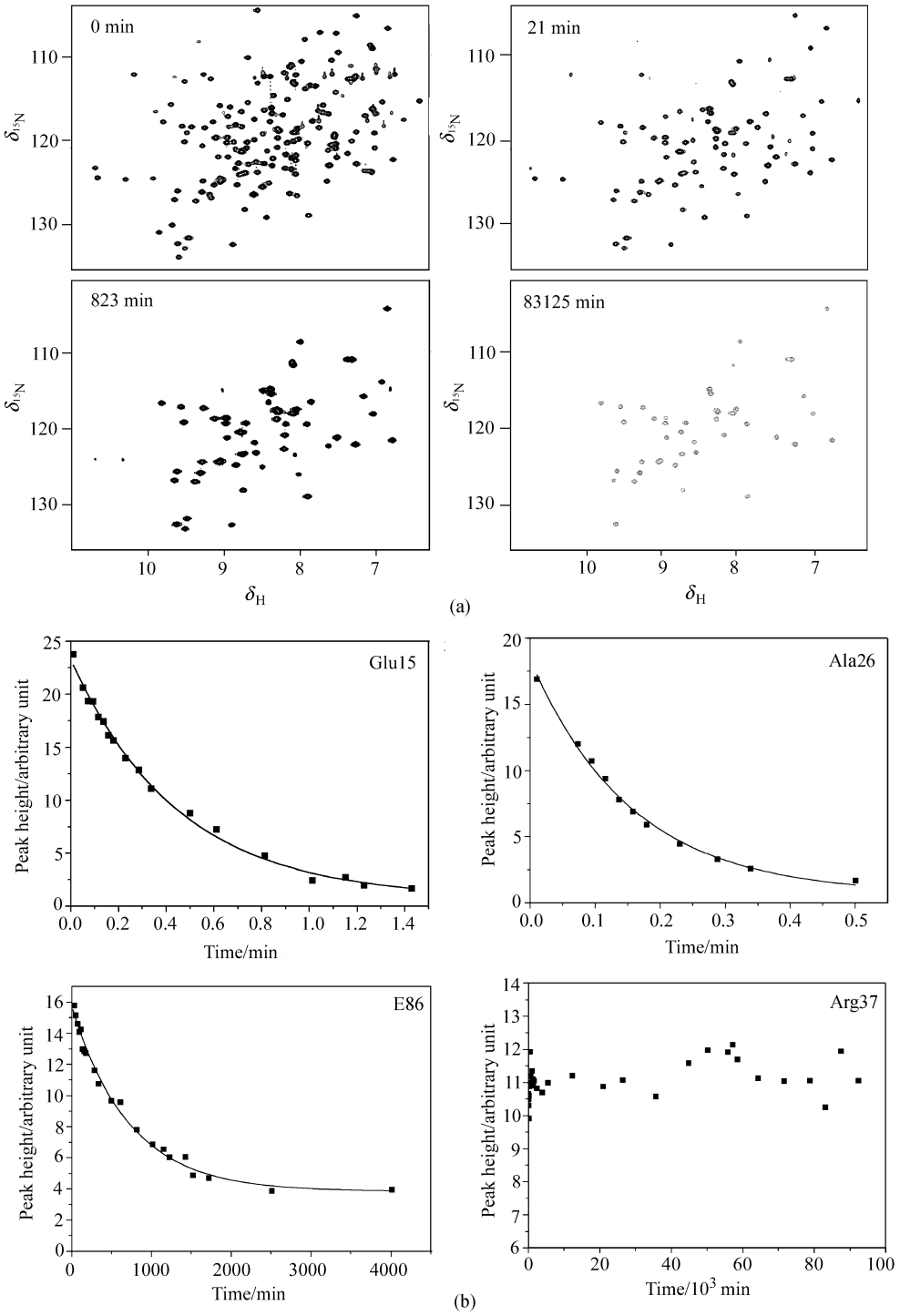

Figure 4 ^{1}H-^{15}N HSQC spectra of CypA in a few typical time periods of H/D exchange (a), and time course of H/D exchange of selected residues in CypA (b).

Measurement of fast exchange rates

The SEA-HSQC experiment was employed to measure exchange rates for rapidly exchanging amide protons in CypA ($k_{ex} > 1 \times 10^{-2}$ min^{-1}). About 48 crosspeaks, related to solvent-exposed fast exchanged amide protons, were observed in the SEA-HSQC spectra recorded even at a short mixing time of 10 ms (Figure 5a). Although most of the 57 fast exchanged amide protons can be detected in the SEA-HSQC spectra at the mixing time from 10 to 200 ms, only 24 residues can be used to quantitatively measure the exchange rates (Figure 5b, Table 2).

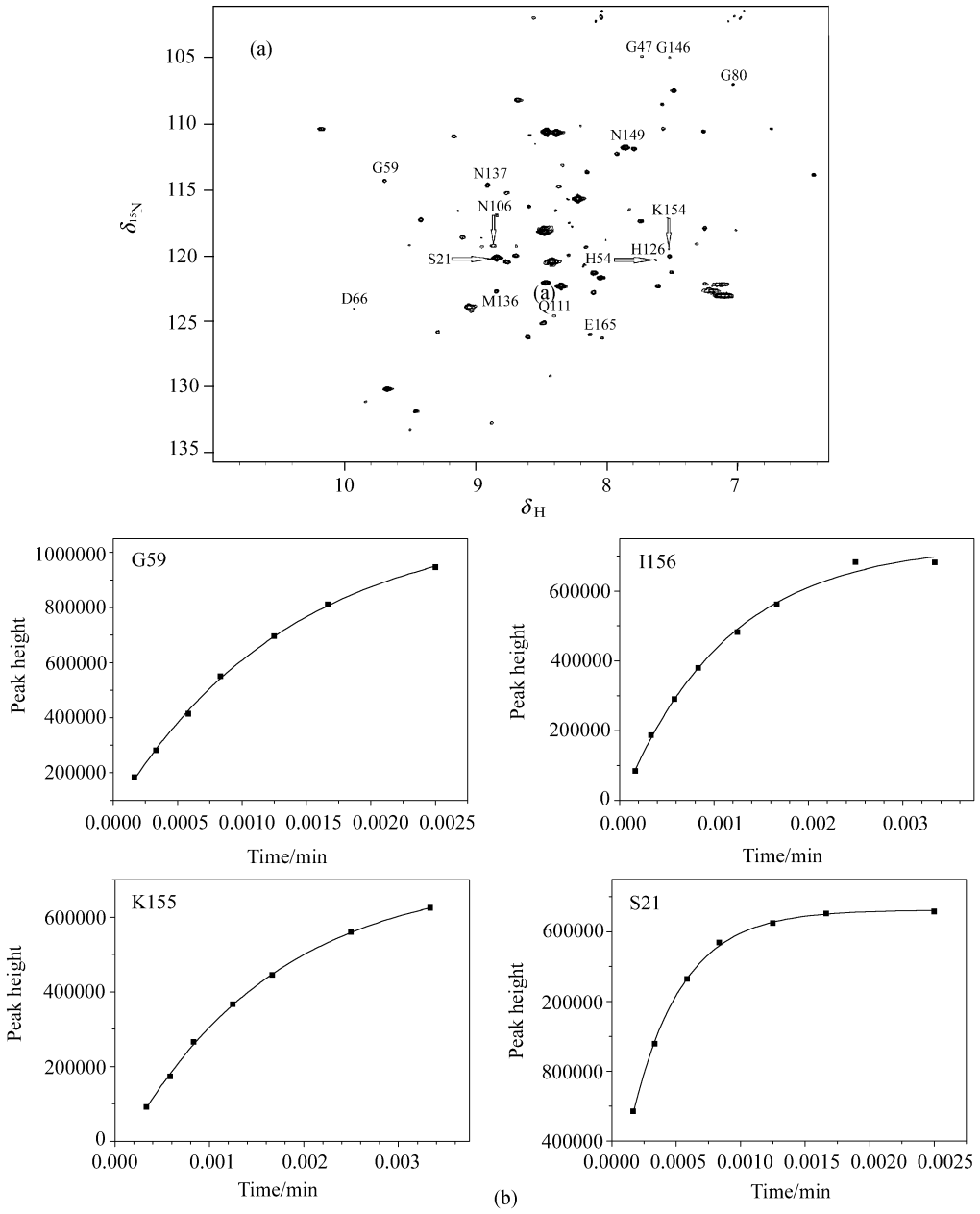

Figure 5 ^{1}H-^{15}N SEA-HSQC spectra of CypA. SEA-HSQC spectrum was acquired with a mixing time of 10 ms. Labeled cross peaks represent some fast-exchanging amide protons whose exchange rates could be reliably measured (a), and peak height versus mixing time (τ_m) plot of selected residues in CypA obtained from the SEA-HSQC experiments (b).

Table 2 Hydrogen exchange rates measured by SEA-HSQC experiments

Res	k_{ex}/min^{-1}	Res	k_{ex}/min^{-1}	Res	k_{ex}/min^{-1}
S21	2564.1	S77	917.4	N137	869.6
D27	917.4	G80	260.4	G146	313.5
G45	392.2	E84	885.0	N149	1111.1
G47	584.8	N106	751.9	G150	429.2
H54	625.3	Q111	540.5	K154	416.7
I56	561.8	E120	680.2	K155	628.9
G59	735.3	G135	277.8	I156	900.9
D66	628.9	M136	662.3	E165	540.2

Conclusion

In the present work both CD and NMR experiments were employed together to access the structural stability of CypA. The change in free energy of urea-induced unfolding ($\Delta G_u^{H_2O}$) of CypA was estimated to be significantly higher than those of the normal proteins, which is an indicator of high structural stability. Both H/D exchange and amide proton chemical shift temperature coefficient measurements demonstrate that CypA is a highly stable protein, suggesting the residues located in $\alpha 2$, $\beta 1$, $\beta 2$, $\beta 5$, $\beta 6$ and $\beta 7$ constitute a hydrophobic stability core of the protein.

Acknowledgement

The authors would like to thank Guo Hong-Xia and Cai Jian-Hua for their help in protein expression and purification.

References

1 Kay, L. E. *Nat. Struct. Biol.* **1998**, *5*, 513.
2 Frauenfelder, H.; Sligar, S. G.; Wolynes, P. G. *Science* **1991**, *254*, 1598.
3 Englander, S. W.; Lallenbach, N. R. *Q. Rev. Biophys.* **1983**, *16*, 521.
4 Englander, S. W.; Mayne, L. *Annu. Rev. Biophys. Biomol. Struct.* **1992**, *21*, 243.
5 Baldwin, R. L. *Curr. Opin. Struct. Biol.* **1993**, *3*, 84.
6 Palmer, A. G. *Curr. Opin. Biotechnol.* **1997**, *7*, 732.
7 Englander, S. W.; Mayne, L.; Bai, Y.; Sosnick, T. R. *Protein Sci.* **1997**, *6*, 1101.
8 Clarke, J.; Itzaki, L. S. *Curr. Opin. Struct. Biol.* **1998**, *8*, 112.
9 Arrington, C. B.; Robertson, A. D. *J. Mol. Biol.* **2000**, *296*, 1307.
10 Bai, Y.; Sosnick, T. R.; Mayne, L.; Englander, S. W. *Science* **1995**, *269*, 192.
11 Li, R.; Woodward, C. *Protein Sci.* **1999**, *8*, 1571.
12 Hvidt, A.; Nielsen, S. O. *Adv. Protein Chem.* **1966**, *21*, 287.
13 Kim, K. S.; Woodward, C. *Biochemistry* **1993**, *32*, 9609.
14 Englander, S. W.; Sosnick, T. R.; Englander, J. J.; Mayne, L. *Curr. Opin. Struct. Biol.* **1996**, *6*, 18.
15 Bai. Y.; Milne, J. S.; Mayne, L.; Englander, S. W. *Proteins* **1994**, *20*, 4.
16 Bai. Y.; Englander, J. J.; Mayne, L.; Milne, J. S.; Englander, S. W. *Methods Enzymol.* **1995**, *259*, 344.
17 Sivaraman, T.; Arrington, C. B.; Robertson, A. D. *Nat. Struct. Biol.* **2001**, *8*, 331.
18 Handschumacher, R. E.; Harding, M. W.; Rice, J.; Drugge, R. J.; Speicher, D. W. *Science* **1984**, *226*, 544.
19 Borel, J. F. *Phamacol. Rev.* **1989**, *41*, 259.
20 Takahashi, N.; Hayano, T.; Suzuki, M. *Nature* **1989**, *337*, 473.
21 Fisher, G.; Wittmann-liebold, B.; Lang, K.; Kiefhaber, T.; Schmid, F. X. *Nature* **1989**, *337*, 476.
22 Ottiger, M.; Zerbe, O.; Güntert, P.; Wüthrich, K. *J. Mol. Biol.* **1997**, *272*, 64.
23 Koradi, R.; Billeter, M.; Wüthrich, K. *J. Mol. Graphics* **1996**, *14*, 51.
24 Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu G, Gfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277.
25 Goddard, T. D.; Kneller, D. G. *SPARKY 3*, Program, University of California, San Francisco,
26 Zhu, G..; Bax, A. *J. Magn. Reson.* **1992**, *100*, 202.
27 Ernst, R. R.; Bodenhausen, G.; Wokaun, A. *Principles of NMR in One and Two Dimensions*, Clarendon Press, Oxford, **1987**, p. 106.
28 Grzesiek, S.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 12593.
29 Wishart, D. S.; Bigam, C. G.; Yao, J.; Abildgaard, F.; Dyson, H. J.; Oldfield, E.; Markley, J. L.; Sykes, B. D. *J. Biomol. NMR* **1995**, *6*, 135.
30 Lin, D. H.; Sze, K. H.; Cui, Y. F.; Zhu, G. *J. Biomol. NMR* **2002**, *23*, 317.
31 Lin, D. H. *Chin. J. Chem.* **2004**, *22*, 1395.
32 Wüthrich, K.; Spitzfaden, C.; Memmert, K.; Widmer, H.; Wider, G. *FEBS Lett.* **1991**, *285*, 237.
33 Neri, P.; meadows, R.; Gemmecker, G.; Olejniczak, E.; Nettesheim, D.; Logan, T.; Simmer, R.; Helfrich, R.; Holzman, T.; Severin, J.; Fesik, S. *FEBS Lett.* **1991**, *294*, 81.
34 Spitzfaden, C.; Weber, H. P.; Braun, W.; Kallen, J.; Wider, G.; Widmer, H.; Walkinshaw, M. D.; Wüthrich, K. *FEBS Lett.* **1992**, *300*, 291.
35 Cierpicki, T.; Otlewski, J. *J. Biomol. NMR* **2001**, *21*, 249.
36 Jiménez, M. A.; Nieto, J. L.; Rico, M.; Santoro, J.; Herranz, J.; Bermejo, F. J. *J. Mol. Struct.* **1986**, *143*, 435.
37 Dyson, H. J.; Rance, M.; Houghten, R. A.; Lerner, R. A.; Wright, P. E. *J. Mol. Biol.* **1988**, *201*, 161.
38 Andersen, N. H.; Chen, C.; Marschner, T. M.; Krystek, S. R. Jr.; Bassolino, K. A. *Biochemistry* **1992**, *31*, 1280.
39 Skalicky, J. J.; Selsted, M. E.; Pardi, A. *Proteins* **1994**, *20*, 52.
40 Andersen, N. H.; Neidigh, J. W.; Harris, S. M.; Lee, G. M.; Liu, Z.; Tong, H. *J. Am. Chem. Soc.* **1997**, *119*, 8547.
41 Baxter, N. J.; Williamson, M. P. *J. Biomol. NMR* **1997**, *9*, 359.
42 Theriault, Y.; Logan, T. M.; Meadows, R.; Yu, L.; Olejniczak, E. T.; Holzman, T. F.; Simmer, R. L.; Fesik, S. W. *Nature* **1993**, *361*, 88.
43 Pflugl, G.; Kallen, J.; Schirmer, T.; Jansonius, J. N.; Zurini, M. G.; Walkinshaw, M. D. *Nature* **1993**, *361*, 91.

(E0501254 SONG, J. P.)