Journal of the Korean Magnetic Resonance Society **2001**, *5*, 46-55

Structural Characterization of Growth-Related Translationally Controlled Tumor Protein P23

Yoon-Hun Hong,[1] Sang-Ho Park, [1] Kyunglim Lee,[2] Bong-Jin Lee[1,*]

[1]Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Korea
[2]College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea
Received April 7, 2001

Abstract: P23, a translationally controlled tumor protein is involved in the interleukin-4 secretion from human basophils and is also known to be an IgE-dependent histamine-releasing factor. However, the precise physiological function and structure of P23 have not been elucidated. In the current study, we constructed the optimal expression and purification protocol of P23 and investigated the secondary structure and structural stability in various conditions. Circular dichroism (CD) investigation showed that the secondary structure of P23 adopts mainly a β-sheet conformation. CD spectroscopy and differential scanning calorimetry revealed that P23 is fairly stable in the pH range of neutral and mild-basic conditions and in the temperature range of 10 - 50℃. Since the thermal stability and the β-sheet content of P23 were decreased by the addition of Ca^{2+} ion, it could be suggested that Ca^{2+} ion induces structural change by partially destabilizing the structure of P23. In addition, various NMR experiments were monitored to solve the aggregation of P23. These results will provide the preliminary structural information about P23.

INTRODUCTION

The protein P23, also referred to as P21,[1,2] Q23[3,4] or translationally controlled tumor protein (TCTP),[5] was discovered independently by three groups in search of translationally regulated proteins. The mRNA of P21 has been found to be highly represented in untranslated mRNP particles of a mouse sarcoma cell line[6] and of mouse erythroleukemia cells.[2] Q23 was discovered in Swiss 3T3 cells to be one of a number of proteins whose synthesis is drastically up-regulated after serum stimulation[3,4] and, later, P23 was identified as a protein whose rate of synthesis is greatly enhanced in growing versus non-growing Ehrlich ascites tumor cells.[7,8] Investigations in different laboratories showed that P23 synthesis is regulated at the translational level.[2,3,4,8,9]

The first P23 cDNA sequences from mouse[1,5] were published a decade ago, and

*To whom : LBJ@nmr.snu.ac.kr

recent compilations of P23 sequences revealed a high degree of conservation between all eukaryotic phyla.[10] P23 is a widely expressed protein, as can be seen from the compilation of EST databases where it was identified as being expressed in 26 out of 30 human tissues.[11] The high conservation and nearly ubiquitous expression of P23 indicated that it is an important cellular protein. However, similarity searches in protein databases did not reveal a relationship of P23 to any of the known protein families, nor did they provide any other clue to the potential function of the protein.

An extracellular function of P23 has been described recently, i.e. its activity as an IgE-dependent histamine-releasing factor in connection with certain allergic diseases.[12] Other reports indicated that P23 is involved in triggering the interleukin-4 secretion from human basophils[13] and that P23 synthesis from basophils is increased after stimulation with IL-3 or anti-IgE.[14] Recently, the *Plasmodium falciparum* homologue of P23 has been described as a possible target for the anti-malarial drug artemisinin.[15] However, all these investigations did not address the question of the intracellular function of P23. The possibility that P23 has properties of a calcium-binding protein was suggested,[10] based on an earlier report on the calcium-binding activity of a similar protein from trypanosomes.[16] Various papers reported the early up-regulation of P23 synthesis after growth induction of mammalian cell lines,[3,8,17] suggesting an involvement of the protein in growth-related processes.

In the present study, we described the establishment of the expression and purification system of P23 protein. We investigated secondary structure, the thermal and pH stability of P23 by using CD, DSC, and NMR spectroscopy. In addition, the effect of calcium ion on the secondary structure and stability of P23 was elucidated.

MATERIALS AND METHODS

Overexpression and purification of P23

The cDNA encoding P23 was cloned into the expression vector pET 22b(+), which is controlled by the T7 promoter. The *Escherichia coli* BL21(DE3) was used as a host for the expression constructs. BL21(DE3) was cultured in 5 ml of LB medium with antibiotics, ampicillin (200 ug/ml) at 37℃ overnight. M9 medium was used for preparing ^{15}N full-labeled P23. $^{15}NH_4Cl$ or $^{15}(NH_4)_2SO_4$ was used as the nitrogen source of this medium. The expression of P23 was induced by 0.5 mM isopropyl β -D-thiogalactoside at an OD_{600} of 0.6. After induction, *E. coli* was grown for an additional 5 hours before harvesting by centrifugation (10,000 g, 20 min, 4℃). The harvested cell pellet was suspended in 100 ml of buffer (20 mM Tris-HCl, 5 mM imidazole, 0.5 M NaCl, pH 7.9) and then lysed by using tip sonifier (Branson, CT, USA). The lysate was then centrifuged at 10,000 g for 20 min. Expressed P23 was insoluble and formed inclusion bodies. The inclusion bodies was solubilized in 20 mM Tris buffer (pH 7.9) with 6 M urea by stirring for 12 hours and then

centrifuged at 10,000 g for 20 mins. The supernatant was loaded to His·tag affinity chromatography and sample was then eluted with linear gradient of imidazole (60～300 mM) in 20 mM Tris buffer (pH 7.9). Fractions were analyzed for identification of presence of P23 by SDS-tricine polyacrylamide gel electrophoresis. Fractions containing P23 were dialyzed against 5 mM imidazole/0.5 M NaCl/20 mM Tris buffer (pH 7.9). The sample was loaded to His·tag affinity chromatography again. By using this method, highly purified P23 could be obtained. The purified sample was dialyzed against 10 mM potassium phosphate buffer (pH 6.5) and concentrated by Centricon-3 (Amicon).

CD experiments

CD spectra were recorded in the range 190～260 nm on a JASCO-715 spectropolarimeter at 20℃. Measurements were made using a cylindrical fuse quartz cell with 0.2 cm path length, with a 2-nm band width, 0.5 nm step resolution, 8-sec response time, and a scan speed of 50 nm/min. Four scans were added and averaged, followed by subtraction of the CD signal of the solvent. The concentration of the samples was 0.01 mM and the sample was solubilized in potassium phosphate buffer (pH 6.5). The helical content was estimated by the method of Yang *et al.*[18]

pH dependent stability was measured in the pH range from pH 1.5 to pH 12.5. Temperature-dependent denaturation was measured at a heating rate of approximately 1℃/min in the temperature range from 4℃ to 90℃ and monitored at 222 nm.

In Ca^{2+} titration experiment, $CaCl_2$ was added at the concentration of 6, 12, 24, 36, 60, and 72 mM, respectively. Temperature-dependent denaturation of sample containing 72 mM $CaCl_2$ was also measured by using the same method as described above.

DSC experiments

Calorimetric measurements were carried out on a VP-differential scanning calorimeter (VP-DSC microcalorimeter). Final concentration of sample was 1.2 mM. Data were obtained in the temperature range from 15℃ to 110℃ at a heating rate of 1℃/min.

NMR experiments

All NMR spectra were acquired by using Bruker DRX 500 MHz spectrometer equipped with a gradient unit in the College of Pharmacy, Seoul National University. Shigemi micro NMR tube was used for the experiment. The concentration of P23 was 0.2-1.2 mM. 2D ^{1}H-^{15}N HSQC was measured in 10 mM potassium phosphate buffer (pH 6.5) at 303, 313, and 323 K, respectively. To obtain the optimal condition for NMR measurement, NMR spectral resolution was monitored by the addition of various additives such as $CaCl_2$, CHAPS, glycine, leucine, arginine, $ZnSO_4$, DTT. The detailed measurement conditions were represented in Table 1.

Table 1. NMR mesurement conditions.

Additives / Concentration (mM)	Concentration of P23 (mM)	Temperature (℃)
CHAPS / 40	0.25	40, 50
Arg / 44	0.33	40, 50
Leu / 62	0.25	40
Gly / 60	0.28	40
DTT / 1	0.28	40

RESULTS AND DISCUSSION

Secondary structure of P23

The conformation of P23 was investigated by using CD spectroscopy. In Fig.1, CD spectrum of P23 showed positive maximum near 193 nm that was concluded to be π-π^* transition of helix component, and negative minimum near 208 nm was often considered as the π-π^* transition of helix component. A shoulder near 222 nm are also considered as the n-π^* transition of helix produced from exiton coupling. To determine the secondary conformation of the protein by Yang method, in which each composed of four model CD spectra representing the α-helix, β-sheet, turn and random coil, the experimental CD curve was fitted by using the JASCO secondary structure estimation program. The estimated content of α-helix, β-sheet, turn, and random coil conformation was 13.7, 46.3, 8.9, and 31.1%, respectively. From this result, it was found that P23 consists of mainly a β-sheet conformation.

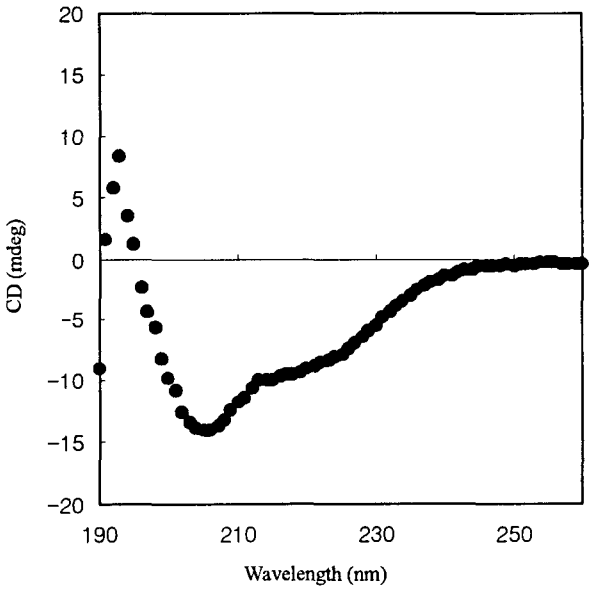

Fig. 1. CD spectrum of P23 in the far-UV region (10 mM phosphate buffer, pH 6.5).

PH-dependent stability

In order to investigate the pH-dependent stability of P23, CD experiments were conducted on the samples in the pH range from 1.5

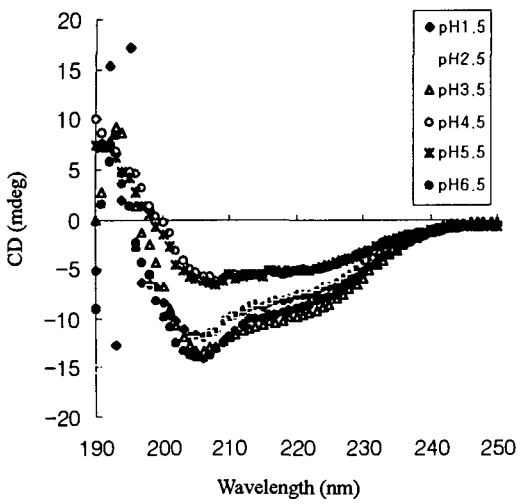

Fig. 2A. CD spectrum of P23 measured at the pH range from 1.5 to 6.5.

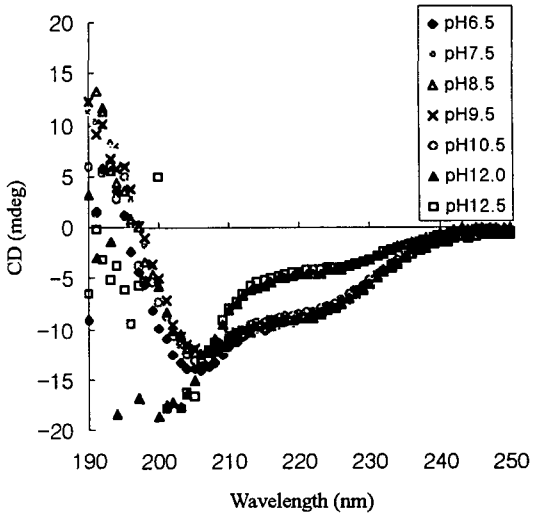

Fig. 2B. CD spectrum of P23 measured at the pH range from 6.5 to 12.5.

to 12.5 by changing the pH by 0.5. Fig.2A showed the change of CD spectra in the pH range from 1.5 to 6.5. As the pH decreased from 6.5 to 1.5, overall CD intensity decreased gradually from pH 6.5 to 4.5, but CD intensity increased from pH 4.5 to 1.5. When we consider the theoretical pI of P23 is 5.1, it could be thought that the decrease in the overall intensity of CD spectrum between 6.5 and 4.5 is due to the precipitation around the experimental value of pI.

As the pH increased from 6.5 to 12.5, overall CD intensity was not changed significantly in the pH range from 6.5 to 10.5. However, the CD spectrum above pH 12.0 was significantly changed (Fig 2B). The wavelength of maximal CD intensity decreased and blue-shifted, and the CD intensity around the 220 nm decreased, which indicates the structure of P23 is completely denatured in highly basic condition. From these results, it could be concluded that P23 is fairly stable between neutral and mild-basic conditions.

Thermal stability

Fig.3 shows the temperature-dependent denaturation process monitored by the change of CD intensity at 222 nm in the temperature range from 4 to 90℃. As the temperature increased, CD intensity decreased gradually in the temperature range from 4 to 60℃ and decreased rapidly after 60℃, indicating the structural change of P23 by thermal denaturation is occurred considerably at the temperature of around 60℃. This result was different from a common pattern of temperature-dependent denaturation process that shows constant CD intensity representing denatured state after dramatic change of CD intensity by

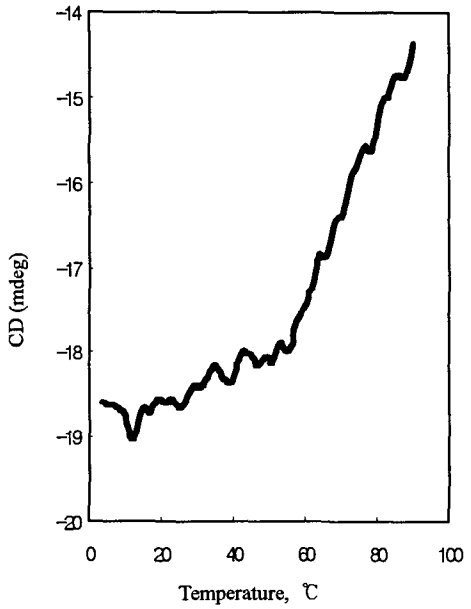

Fig. 3. Thermal denaturation profile of P23 by CD in the far-UV region of 222 nm (10 mM phosphate buffer, pH 6.5).

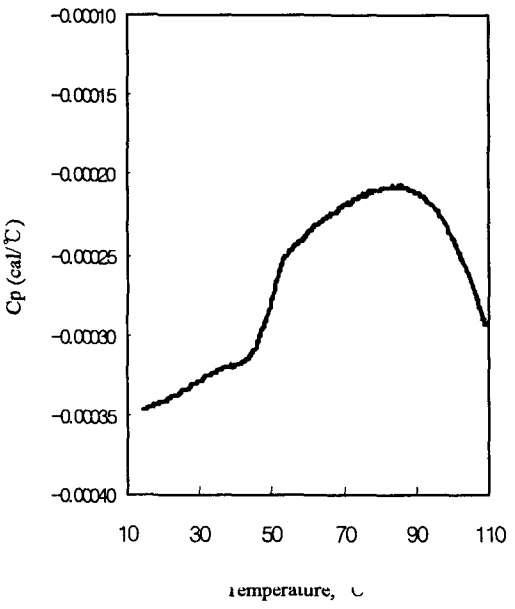

Fig. 4. Thermal denaturation profile of P23 by DSC at the temperature range from 15℃ to 110℃.

structural change. Fig. 4 shows temperature-dependent denaturation process monitored by differential scanning calorimetry (DSC). As the temperature increased, the heat capacity (Cp) of P23 increased gradually to the temperature of 50℃ and then increased rapidly after 50℃, suggesting the substantial thermal denaturation of P23 was occurred around 50℃.

The overall denaturation profile monitored by CD was similar to that of monitored by DSC. However, the transition temperature measured by CD is higher by 10 degree than that measured by DSC. The difference of transition temperature between CD and DSC experiment is probably due to difference of the sample concentration between them. In DSC experiment, the sample concentration was 1.2 mM, which is higher above 100 times than the concentration in the CD experiment. Since P23 was aggregated above the concentration of 0.25 mM, which is manifested by NMR experiments, the DSC profile may reflect the denaturation process of aggregated state of P23. In addition, the overall heat capacity of P23 during the temperature-dependent denaturation process was considerably low compared to that of common proteins, which also implies that P23 is in the aggregation state during the denaturation process monitored by DSC. From these results, it could be thought that P23 is aggregated in high concentration and the aggregation state is unstable than the monomeric or non-aggregated state of P23.

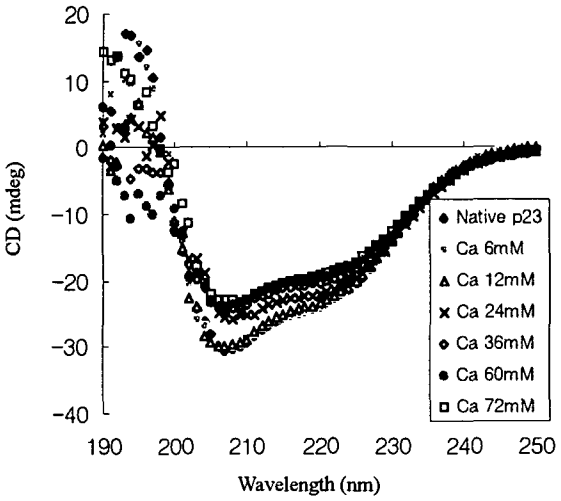

Fig. 5. Ca^{2+} titration experiment by CD. The concentrations of Ca^{2+} added to P23 were 6,12,24,36,60,72 mM, respectively.

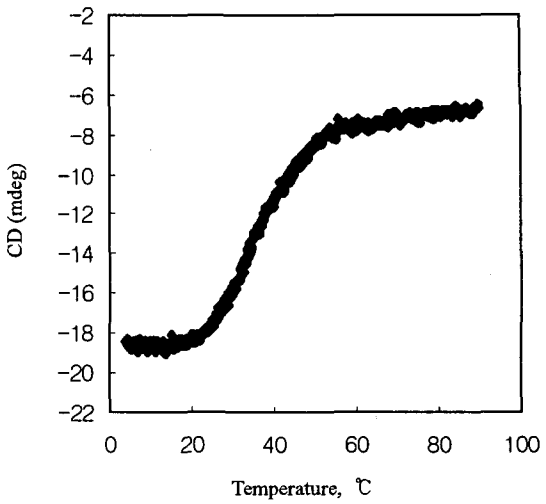

Fig. 6. Thermal denaturation of P23 containaing 72 mM Ca^{2+} followed by CD in the far-UV region of 222 nm.(10 mM phosphate buffer, pH 6.5).

Effect of Ca^{2+} binding to P23

In the previous reports, P23 was known as Ca^{2+} binding protein.[10] Ca^{2+} binding proteins have general features representing structural change by Ca^{2+} binding. Therefore, we investigated the effects of Ca^{2+} binding to P23 on the structure and thermal stability. P23 was solubilized in 10 mM phosphate buffer (pH 6.5) and its concentration was 0.01 mM. The change of CD spectrum of P23 was monitored by increasing the Ca^{2+} concentration of 6, 12, 24, 36, 60, and 72 mM respectively.

As shown in Fig.5, the overall CD intensity decreased as the concentration of Ca^{2+} increased. The most decrease was occurred at 208 nm. The secondary structure of P23 containing 72 mM Ca^{2+} was estimated. The estimated content of α-helix, β-sheet, turn, and random coil conformation was 20, 28.9, 14.8, and 36.3%, respectively. From the comparison between P23 in the presence and absence of Ca^{2+}, it was found that the secondary structure of P23 was substantially changed. The content of α-helix, turn, and random coil was slightly increased, whereas the content of β-sheet was considerably decreased. Further investigation will be needed to identify correlation between unknown function of P23 and these structural changes by Ca^{2+} binding.

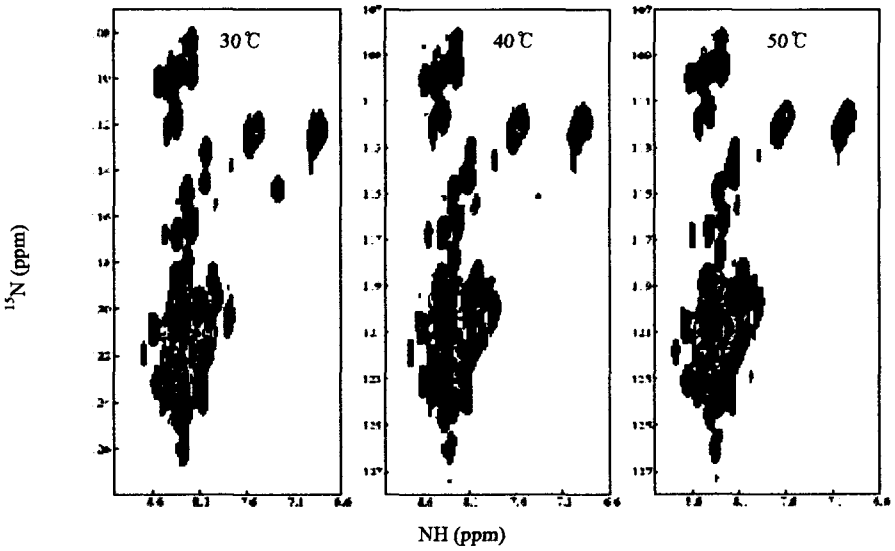

Fig. 7. ^{1}H-^{15}N HSQC spectra of P23 measured at 30℃, 40℃, 50℃, respectively.

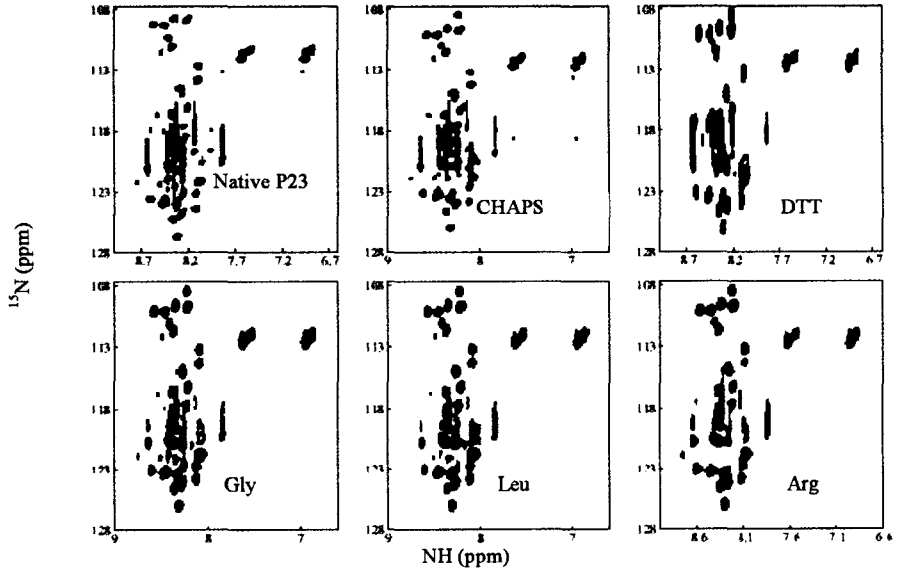

Fig. 8. ^{1}H-^{15}N HSQC spectra of P23 measured at various conditions.

To investigate the effect of Ca^{2+} on the thermal stability of P23, the experiment of temperature-dependent denaturation was performed by using CD. Sample containing 72 mM Ca^{2+} was used since the CD intensity was not changed by the addition of Ca^{2+} above 72

mM. In Fig. 6, it was revealed that the transition temperature of P23 containing Ca^{2+} decreased sharply by about 30 degree order compared to that of P23 in the absence of Ca^{2+}. From this result, it could be thought that Ca^{2+} binding to P23 destabilized the structure of P23, leading to the decrease of thermal stability.

NMR study

Although the appropriate condition for NMR experiments was searched by using CD experiments, it was very difficult to analyze NMR spectra due to the severe peak broadening and overlap. This result may be caused by the aggregation of P23 in much higher concentration for NMR experiments than for CD experiments. Therefore, we conducted the various NMR experiments to improve the spectral resolution by resolving the aggregation of P23. First, we measured the 2D ^{1}H-^{15}N HSQC spectra at the temperature of 303, 313, and 323K, respectively (Fig. 7). However, the spectral resolution was not improved significantly by changing the temperature. Second, we monitored the spectral resolution by addition of various additives. Additives such as Gly, Leu, Arg, CHAPS, DTT, $ZnSO_4$, and $CaCl_2$ were used to inhibit aggregation of P23 and improve spectral resolution. It was known that CHAPS and Leu inhibit aggregation by hydrophobic interaction, Arg inhibits aggregation by electrostatic interaction, DTT inhibits aggregation occurred due to disulfide bond formation, and Gly stabilizes protein of monomer. Despite of these various experiments, neither NMR peak broadening nor the spectral resolution were improved, indicating that such additives could not inhibit the aggregation of P23 (Fig. 8). In addition, the sample was precipitated by addition of $ZnSO_4$ and $CaCl_2$. From these results, it could be suggested that the aggregation of P23 is induced by complex mechanism or concentration-dependent manner. Therefore, mutations of specific amino acids or deletion of specific region which may be involved in solubility or aggregation of P23 would be more efficient method to improve the solubility of P23.

Acknowledgements

This work was supported by a grant (1998-001-F00778) from The Ministry of Education, Korea, and in part by 2001 BK21 project for Medicine, Dentistry, and Pharmacy.

REFERENCES

1. Chitpatima S.T., Makrides S., Bandyopadhyay, R., and Brawerman G. *Nucleic Acid Res.*, **16**, 2350 (1988).
2. Yenofsky, R., Bergmann, I. and Brawerman, G. *Natl. Acac. Sci. USA.*, **79**, 5876-5880 (1982).
3. Thomas G., Thoams G., and Luther H. *Proc. Natl. Acad. Sci.*, **78**, 5712-5716 (1981).
4. Thomas G. and Thomas G. *J. Cell Biol.*, 2137-2144 (1986).
5. Gross, B., Gaestel, M., Bohm, H. and Bielka, H. *Nucleic Acids Res.*, **17**, 8367 (1989).

6. Yenofsky, R., Cereghini, S., Krowczynska, A. and Brawerman, G. *Mol. Cell. Biol.*, **3**, 1197-1203 (1983).
7. Benndorf, R., Nurnberg, P. and Bielka, H. *Exp. Cell Res.*, **174**, 130-138 (1988).
8. Bohm H., Benndorf R., Gaestel M., Gross B., Nurnberg P., Kraft R., Otto A., and Bielka H.*Biochem. Int.*, **19**, 277-286 (1989).
9. Bohm H., Gross B., Gaestel M., Bommer, U. A., Ryffel, G. and Bielka H. *Biomed. Biochim. Acta.*, **50**, 1193-1203 (1991).
10. Sanchez, J. C., Schaller, D., Ravier, F., Golaz, O., Jaccoud, S., Belet, M., Wilkins, M.R., James, R., Deshusses, J. and Hochstrasser, D. F. *Electrophoresis*, **18**, 150-157 (1997).
11. Adams, M. D., Kerlavage, A. R., Fleischmann, R. D., Fuldner, R, A., Bult, C. J., Lee, N. H., Kirkness, E. F., Weinstock, K. G., Gocayne, J. D., White, O., et al. *Nature*, **377** (6547S), 3-174 (1995).
12. MacDonald, S. M., Rafnar, T., Langdon, J. and Lichtenstein, L. M. *Science*, **269**, 688-690 (1995).
13. Schroeder, J. T., Lichtenstein, L. M. and MacDonald, S. M. *J. Exp. Med.*, **183**, 1265-1270 (1996).
14. Nielsen, H. V., Johnsen, A. H., Sanchez, J. C., Hochstrasser, D. F. and Schiotz, P. O. *Allergy*, **53**, 642-652 (1998).
15. Bhisutthibhan, J., Pan, X. Q., Hossler, P. A., Walker, D. J., Yowell, C. A., Carlton, J., Dame, J. B. and Meshnick, S. R. *J. Biol. Chem.*, **273**, 16192-16198 (1998).
16. Haghighat, N. G. and Ruben, L. *Mol. Biochem. Parasitol.*, **51**, 99-110 (1992).
17. Bommer, U. A., Lazaris-Karatzas, A., De Benedetti, A., Nurnberg, P., Benndorf, R., Bielka, H. and Sonenberg, N. *Cell. Mol. Biol. Res.*, **40**, 633-641 (1994).
18. Yang, J. Y., Wu, C.-S.C., and Martinez, H.Z. *Methods Enzymol.*, **130**, 208-269 (1986).