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Study of tyrosine-containing mutants of ribosomal protein L7/L12 from *Escherichia coli*

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Abstract

Three mutant forms of the ribosomal protein L7/L12 with replacements of Ser1, Met14 and Met26 to Tyr were studied by the methods of fluorescence spectroscopy, circular dichroism and microcalorimetry. The amino-acid residue Tyr14 in the protein L7/L12 Tyr14 is located in a region with a more organized structure than Tyr26 in protein L7/L12 Tyr26. The replacements Ser1 → Tyr1 and Met14 → Tyr14 do not affect the secondary structure of protein L7/L12. The replacement Met26 → Tyr26 stabilizes the secondary structure of protein L7/L12. A pH-induced temperature transition was observed in the pH range 5.0–7.3 in protein L7/L12 Tyr14 by tyrosine fluorescence. Analogous transitions were observed for protein L7/L12 Tyr26 by Tyr fluorescence and for the wild type protein L7/L12 by Phe fluorescence. Three pH-dependent states of protein L7/L12 and its mutant forms L7/L12 Tyr1 and L7/L12 Tyr14 were found on the microcalorimetric melting curves. The characteristics of protein L7/L12 Tyr14 are very close to the wild type protein L7/L12 and it is a suitable object for studying the structure of the N-terminal part of molecule by two-dimentional ^{1}H-NMR.

Keywords: Mutant proteins L7/L12; Fluorescent spectroscopy; Circular dichroism; Microcalorimetry; pH-titration curves; Thermostability

1. Introduction

The incorporation of native chromophores in proteins by the methods of genetic engineering allows to obtain structural analogues of proteins, to select mutants with unchanged native structure and facilitates their study by physical methods for investigation of the structure of native proteins.

We have incorporated the natural chromophore Tyr in the N-terminal part of protein L7/L12 for studying its structure by the methods of ^{1}H-NMR, fluorescence spectroscopy and circular dichroism.

Protein L7/L12 (MW 24 400) from the 50S ribosomal subunit of *Escherichia coli* exists as a non-covalent dimer in solution [1,2]. The N-terminal helical regions of two molecules L7/L12 interect by hydrophobic surfaces to form a stable dimer [3,4]. Different models of structural organization of L7/L12 dimer have been proposed: a symmetrical antiparallel model [3], a symmetrical parallel model

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[4] and a staggered parallel model [5]. The sequences in proteins L7 and L12 are almost identical with the only exception being that the amino-terminal serine group on L7 is acetylated [6]. The crystal structure of the C-terminal globular domain of protein L7/L12 (residues 53–120) was determined with 1.7 Å resolution [7,8]. The C-terminal domain is required for the binding of factors to the ribosome [9]. The residues 37–50 were found to be highly mobile by ^{1}H-NMR [10]. The N-terminal sequence of protein L7/L12 (residues 1–36) is of importance for protein dimerization, for the formation of the complex with L10 and for coupling with the ribosome [11,12].

Three mutant forms of ribosomal protein L7/L12 with Ser1, Met14 and Met26 replacements by Tyr were constructed for studying the N-terminal domain of the protein. Methods of protein purification were developed. The mutant proteins L7/L12 Tyr1, Tyr14 and Tyr26 exist as wild-type protein L7/L12 in the form of dimers in solution [13].

2. Materials and methods

The spectra of phenylalanine and tyrosine fluorescence of the wild-type protein L7/L12 and its mutant forms were measured on a spectrofluorometer described in detail elsewhere [14]. The exitation of fluorescence was done by the light of mercury lines at 265.2 nm or 280.4 nm. Measurements were done in the buffer containing 10 mM MES-TAPS, pH 7.8. The buffer system allow dimerization of the protein in solution by the data of ^{1}H-NMR.

CD spectra in the far-UV region (190–250 nm) were measured on a Jasko J-600 dichrograph in a 0.0185 cm cylindrical cell. The measurements were done at 20°C in the buffer containing 20 mM ammonium acetate buffer, pH 5.0 or 7.3. The concentration of proteins were 0.2–0.5 mg ml^{-1}. The heat denaturation curves were measured on a Jasko J-41A dichrograph under analogous conditions in a 0.049 cm cell in a thermostable cell-holder. The rate of temperature scanning was 0.9K min^{-1}. The far UV CD spectra were smoothed using the Jasco software.

The calorimetric studies were done with an adiabatic scanning microcalorimeter DASM-5 ('Biopribor', Russia). The volume of the measuring cells was 1 ml. The measurements were done at the heating rate 1 K min^{-1} and the specific partial volume of the proteins was 0.730 cm^3 g^{-1}. The partial heat capacity and calorimetric enthalpies for denaturation transition were obtained as in [15]. The molecular weight of the monomer (12.2 kDa) was used in the calculations. The excess heat capacity C_p^{exc} (T) was determined as described in [16]. The excess heat capacity was analysed in two stages by the procedure of deconvolution, described in [17]. The calorimetric scans were smoothed. The transition temperature values T_m have been referred to the same concentration of protein.

For each scan, the proteins were dialyzed for 18 h against 20 mM sodium phosphate buffer with corresponding pH: 5.0, 5.8, 6.8, 7.3, 7.8, and 9.2. and clarified by centrifugation for 15 min at 15 000 g. The sample concentrations did not exceed 1 mg ml^{-1}. The termodynamic parameters have been referred to the identical concentration of protein.

The absorption spectra of the proteins were measured on the Varian Cary 219 spectrophotometer. The protein concentrations were calculated in two different ways: first, by absorption at 258.5 nm ($\epsilon_{258.5}$ = 730 l mol^{-1} cm^{-1}) for the wild-type protein L7/L12 [14] and at 240 nm (ϵ_{240} = 1039 l mol^{-1} cm^{-1}) for mutant proteins L7/L12; second, by absorption at 215 nm and 225 nm [18].

3. Results and discussion

3.1. Study by methods of absorption and fluorescent spectroscopy

The unique aromatic residues in the wild type protein L7/L12 are Phe30 and Phe54.

The pH-dependences of tyrosine and phenylalanine fluorescence intensity at 308 nm are shown in Fig. 1. Ionization of tyrosine and formation of tyrosinate in the region of alkaline pH are accompaned by increasing absorption at 250 nm. It was found that the curves of spectrophotometric titration of both mutant proteins are uniprotonic, with pK_a = 10.51 ± 0.11 for Tyr14 in protein L7/L12 Tyr14, and pK_a = 10.07 ± 0.10 for Tyr26 in protein L7/L12 Tyr26. In an aqueous solution, the amino acid Tyr has pK_a = 10.07 ± 0.10. Hence, it can be assumed that Tyr14 (pK_a = 10.51 ± 0.11) is more structured

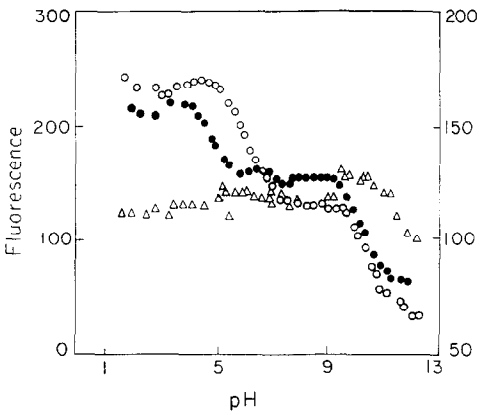

Fig. 1. pH-dependences of intensity of tyrosine and phenylalanine fluorescence. (○) L7/L12 (Tyr14), F_{308}, ex. 280.4 nm. (●) L7/L12 (Tyr26), F_{308}, ex. 280.4 nm. (△) L7/L12 w.t., F_{285}, ex. 265.3 nm (the scale on the right).

and less accessible to solution than Tyr26 (pK_a = 10.07 ± 0.10). These results agree with the data ^{1}H-nmr, according to which the amino acid Tyr14 in protein L7/L12 Tyr14 is situated in a region with a more organized structure than Tyr26 in protein L7/L12 Tyr26 [13].

An 'S'-like change in tyrosine fluorescence of protein L7/L12 Tyr14 is detected on the curves of spectrofluorometric pH-titration upon descreasing pH from 7.5–5. This transition is described by a titration curve, characterized by $pK_a = 6.06 \pm 0.06$ and n = 1.0. The transition in this pH region is connected with the titration of carboxyl groups. Glu9 and Glu21 are situated near Tyr14 in the primary sequence of protein L7/L12. The analogous transition for protein L7/L12 Tyr26 is shifted towards more acid values of pH ($pK_a = 4.9 \pm 0.05$ and $n = 1.5$). This transition is also connected with the titration of carboxyl groups. Glu27, Glu28 and Glu21 are situated near Tyr26 in the primary structure of protein L7/L12 Tyr26. Analogous transitions were also observed for the wild-type protein L7/L12 by phenylalanine fluorescence of the protein, but this effect was too small to study it quantitatively. The amino acid His is not present in protein L7/L12 and it is not considered as a possible titration group in the pH range 5–7.5.

The detected pH-induced transition can be caused by two reasons. First, it may be determined by a pH-induced change in the quenching properties of carboxyl groups, situated near the tyrosine chromophores. Second, it may reflect the existence of pH-induced conformational transition in these proteins, and, as a result, change the environment of tyrosine chromophores. To distinguish between these two cases, we have studied the thermal stability of proteins before and after the transition by the methods of fluorescent spectroscopy, circular dichroism and microcalorimetry.

Thermal denaturation of wild type proteins L7/L12 and L7/L12 (Tyr14 and Tyr26) was studied by fluorescent spectroscopy in the temperature region 10–90°C. There were found two transitions — one low-temperature and one high-temperature — on the melting curves of all proteins (Figs. 2 and 3). The low-temperature transitions for protein L7/L12 Tyr14 coincide at pH 5.0 and pH 7.3 (Fig. 3), and the high-temperature transitions differ. At pH 5.0, the high-temperature transition is displaced to a low temperature as compared with pH 7.3, which is evidence that the stability of the protein decreases at low pH.

Both the low- and high-temperature transitions for protein L7/L12 Tyr26 are displaced to the region of lower temperatures in comparison with the transitions for the wild-type protein L7/L12 and L7/L12 Tyr14 (Fig. 2), and this corresponds to the decreased stability of the mutant protein upon heating.

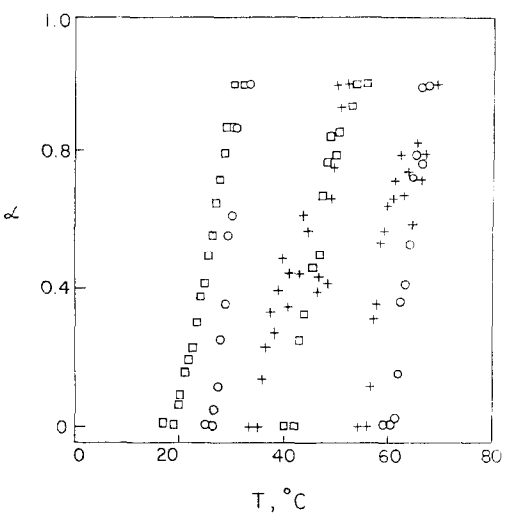

Fig. 2. Temperature transitions by fluorescence in 10 mM MES-TAPS for proteins: (○) L7/L12 Tyr14, pH 7.3. (+) L7/L12 w.t., pH 7.8. (□) L7/L12 Tyr26, pH 7.8. α-relative fluorescence.

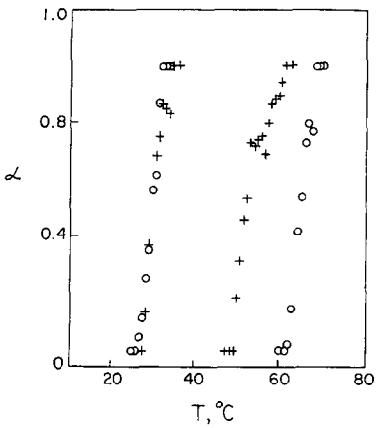

Fig. 3. Temperature transitions by fluorescence for proteins: (○) L7/L12 Tyr14, pH7.3 in 10mM MES-TAPS. (+) L7/L12 Tyr14, pH5.0 in 20mM ammonium acetate. α-relative fluorescence.

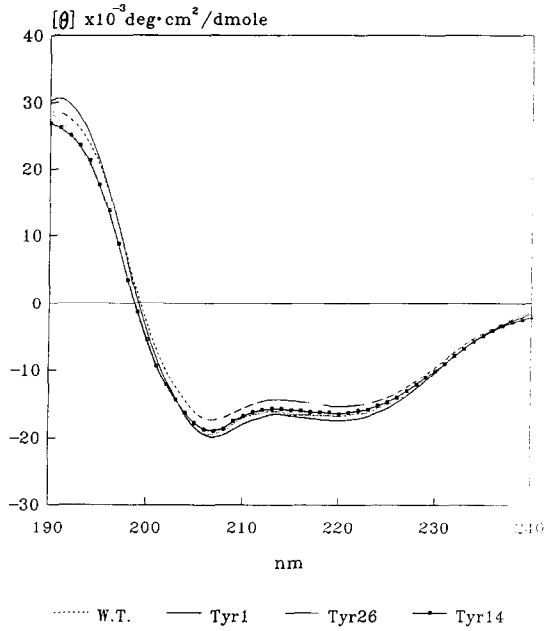

Fig. 4. Circular dichroism spectra in the far UV region for proteins (---) L7/L12 w.t., (————) L7/L12 Tyr1, (- · - · -) L7/L12 Tyr14 and (————) L7/L12 Tyr26, pH = 7.3 in 20mM ammonium acetate.

The low-temperature transitions probably evidence of the existence in protein samples of some quantity of the protein, incompletely renaturated after treating with a denaturating agent (urea) during purification of the proteins. The high-temperature transitions correspond to the melting of completely renatured proteins.

3.2. Study by method of circular dichroism

The CD spectra of wild-type L7/L12 and of three mutant forms in the far-UV region are shown in Figs. 4 and 5. Under conditions considered, the shape of the spectra of mutant proteins do not differ greatly from the wild-type protein spectra. This points to the similarity of secondary structure of different forms of L7/L12. At pH5.0, the amplitude deviation of CD spectra of the mutant forms L7/L12 Tyr14 and L7/L12 Tyr26 from the spectrum of the wild-type protein is greater than that at pH7.3. This may be the result of a lower protein stability and the tendency of the considered proteins to aggregate under such conditions. The pH range does not appear to affect the secondary structure of the proteins. The results from the CD studies are consistent with the published data from CD, crystal and NMR studies [7,10,19].

The behaviour of CD for the wild-type and mutant forms of L7/L12 was investigated in the temperature region 10–90°C. At pH close to 7, the heat denaturation is completely reversible. The temperatures of half-transition are: 63°C for L7/L12 w.t., 67°C for L7/L12 (Tyr26), 63°C for L7/L12 (Tyr1), 61°C for L7/L12 (Tyr14) at pH7.3. At pH5.0 ag-

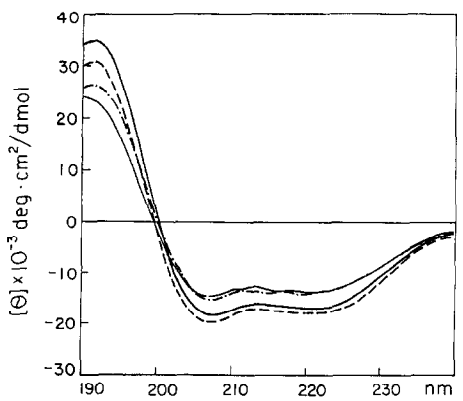

Fig. 5. Circular dichroism spectra in the far UV region for proteins (————) L7/L12 w.t., (---) L7/L12 Tyr1, (- · - · -) L7/L12 Tyr14 and (————) L7/L12 Tyr26, pH5.0 in 20mM ammonium acetate.

gregation takes place and the process is not reversible. The same value of the temperature of half-transition $T_m = 63°C$ was obtained for the wild type protein L7/L12 at pH 5.88 [19]. The T_m values, obtained for the thermal unfolding by calorimetric studies is higher than that observed by the far-UV CD studies.

The curves of heat denaturation of Tyr1 and Tyr14 mutant forms show that the thermostability of these proteins is close to that of the wild-type protein and such amino acid changes do not cause distabilisation of the structure. The data for Tyr26 mutant shows the growth of stability by 3–4°C.

3.3. Study by method of differential scanning microcalorimetry

To study the changes caused by the pH-induced transition in protein structure and registered by fluorescent spectroscopy, the calorimetric measurements were done at pH varying from 5 to 9.

At pH 5, the heat capacity curve of wild-type L7/L12 had no heat absorption peaks. From the $C_p(T)$ values, it might be suggested that the protein denatures at pH 5, that is near the isoelectric point of L7/L12 (Fig. 6).

At pH ranging from 5 to 7.3, the temperature dependence of the partial heat capacity for all proteins has a heat absorption peak. The reversibility of protein denaturation is partial at these conditions, increasing at the highest pH. At pH above 7.3, L7/L12 behaves like a usual globular protein, its thermograms have an apparent heat absorption peak with the maximum at 67°C (Fig. 7).

Aggregation of the wild type protein L7/L12 at pH 7.4–7.6 at hight temperatures was observed in [19] and was decreased by increasing pH and changing the buffer. The measurements in the present study were done at low protein concentrations and aggregation of proteins at pH above 7.3 was not detected.

It has been shown that the location of the peak

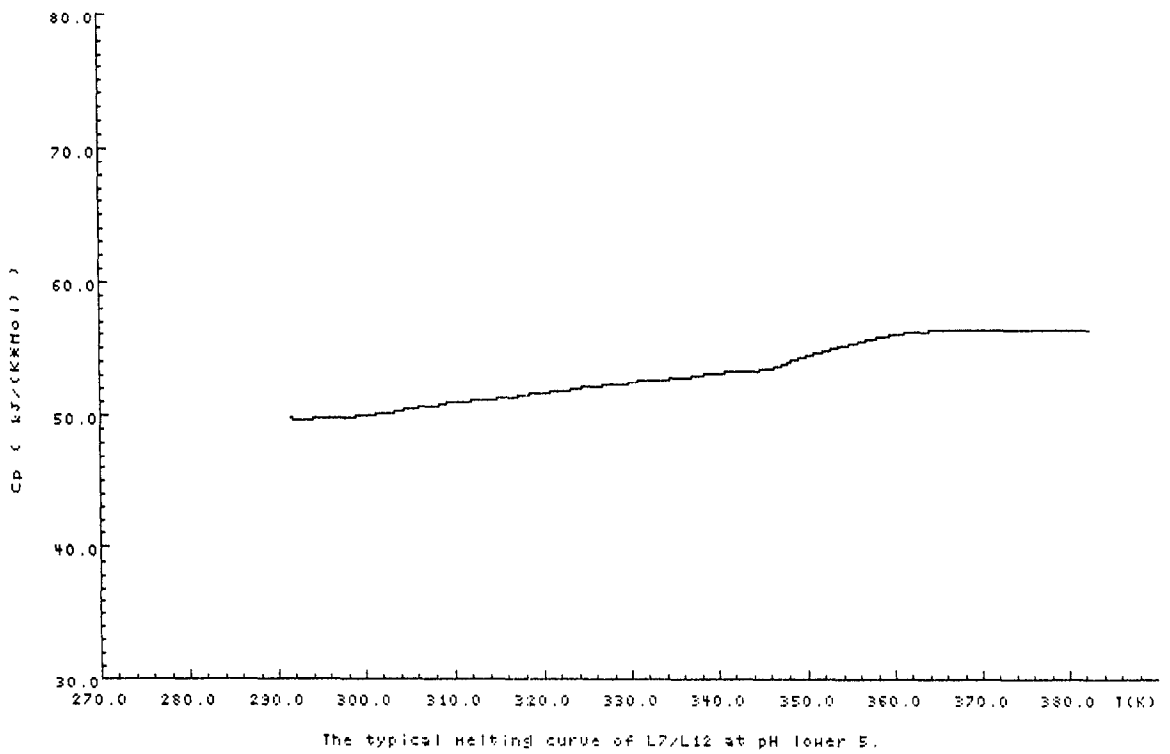

Fig. 6. Temperature dependence of the partial heat capacity for protein L7/L12 w.t., L7/L12 Tyr1 and L7/L12 Tyr14 in 20 mM sodium phosphate at pH 5.0.

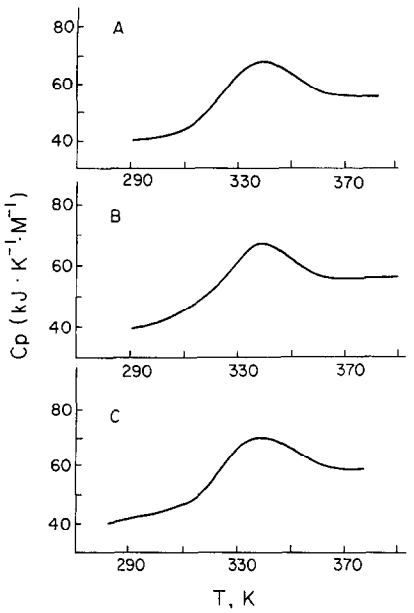

Fig. 7. Temperature dependence of the partial heat capacity in 20 mM sodium phosphate at pH 7.3–9.2 for proteins: (A) L7/L12 Tyr1; (B) L7/L12 Tyr14; (C) L7/L12 w.t.

maximum of all L7/L12 forms does not practically depend on pH in this pH region (pH 7.3–9.2). The protein denaturation is reversible completely; the Cp values measured at pre- and post-transition temperatures (25°C and 90°C) are very close. This means that the mutations L7/L12 Tyr1 and L7/L12 Tyr14 do not affect the denaturational heat capacity increment and, consequently, the hydrophobic core design.

The experimental enthalpy values of the wild-type L7/L12 and mutant protcins L7/L12 Tyr1 and L7/L12 Tyr14 differ only negligibly (within the experimental error, $\Delta \bar{H}_{cal} = 293 \pm 11$ kJ mol^{-1}), where the enthalpy values are given per mole of protein L7/L12 with molecular mass of 12 200 g mol^{-1}. This values agree with the calorimetric data, obtained in [20], where $\Delta H_{cal} = 286$ kJ mol^{-1} and the transition temperature is 68–70°C.

The process of thermal denaturation can be analysed by comparing the values of calorimetric and van't-Hoff enthalpies [21]. The calorimetric enthalpy exceeds the van't-Hoff enthalpy evaluated within a simple two-state model ($R = 1.2$). This value of R means that the L7/L12 molecule is not a single cooperative block, but has a complicated domain structure. It was reported [3,4,7,8], that at least two domains are determined within L7/L12 molecule structure and that an unstructured hingle region exists in the protein [10].

Thus, from calorimetric studies it has been determined that the mutations Ser1 → Tyr1 and Met14 → Tyr14 do not affect free energies of protein structure formation and their distribution over cooperative units of protein molecules.

4. Conclusion

As a result of studying wild-type protein L7/L12 and mutant proteins L7/L12 (Tyr1, Tyr14 and Tyr26), it has been found that the amino acid residue Tyr14 is situated in a more organized part of the L7/L12 Tyr14 molecule than Tyr26 in protein L7/L12 Tyr26. The replacements Ser1 → Tyr1 and Met14 → Tyr14 do not affect the secondary structure of protein L7/L12. The replacement Met26 → Tyr26 stabilizes the secondary structure of protein L7/L12 Tyr26.

The pH-induced conformational transition in protein L7/L12 Tyr14 at pH from 7.5 to 5.0 is caused by the change in the ionic state of carboxyl groups. The state of the protein before and after the transition differs in thermostability and the environment of Tyr residues. Analogous transitions were found in other mutant proteins and in wild-type protein L7/L12. The position of the transition on the pH scale depends on the location of Tyr in the N-terminal domain of the molecule.

The analysis of the microcalorymetric melting curves of protein L7/L12 and its mutant forms shows the existence of transitions, probably reflecting the independent melting of some structural cooperative regions of the molecule. This may be the melting of the dimer to monomers, the independent melting of the N-terminal and the C-terminal globular domains of the molecule. The cooperative regions in the C-terminal parts of the subunits in the dimer unfold independently. The increasing value of ΔH_{cal} for the dimer in comparison with the oxidised protein is evidence of the ordered structure of N-terminal region of molecule [20]. From the ^{1}H-nmr [13]

and fluorescent studies of mutant proteins L7/L12 Tyr14 and L7/L12 Tyr26 was found that Tyr14 and Tyr26 are situated in structural ordered regions in the N-terminal domain of the molecule. The thermal unfolding leads in the first place to the desruption of the tertiary structure of the intact protein L7/L12 [20]. The desruption of many of the electrostatic interactions by acid and thermal denaturation destroy only a part of the native secondary structure [19]. The amino-terminal domain dissosiates into monomers in the presence of hight concentrations of guanidinium chloride [19].

The characteristics of the mutant protein L7/L12 Tyr14 are very similar to those of the native protein L7/L12, and the mutant is a suitable object for further, more profunded studying of the N-terminal domain of the protein by two-dimensional ^{1}H-NMR spectroscopy.

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