Kinetics of translation of γB crystallin and its circularly permutated variant in an in vitro cell-free system: possible relations to codon distribution and protein folding

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Abstract **Analysis of nascent γB-crystallin peptides accumulating during in vitro translation in a rabbit reticulocyte lysate cell-free system was carried out. As a consequence of the irregular distribution of rare codons along the polypeptide chain of γB-crystallin, translation of the two-domain protein is a non-uniform process characterized by specific pauses. One of the major delays occurs during the translation of the connecting peptide between the domains. Comparing the kinetics of translation of natural γB-crystallin and its circularly permutated variant (with the order of the N- and C-terminal domains exchanged) reveals that the natural N-terminal domain is translated faster than the C-terminal one. Since the N-terminal domain in natural γB-crystallin is known to be more stable and to fold faster than the C-terminal one [E.-M. Mayr et al. (1994) J. Mol. Biol. 235, 84–88], the present data suggest that the translation rates are optimized to tune the synthesis and folding of the nascent polypeptide chain. In this connection, the pause in the linker region between the domains provides a delay allowing the correct folding of the N-terminal domain and its subsequent assistance in the stabilization of the C-terminal one.**

Key words: Codon distribution; γB-crystallin; Translation, in vitro; Non-uniform translation; Protein folding; Rare codons

1. Introduction

γB-Crystallin from calf eye lens consists of two homologous domains connected by a six-residues linker peptide [1]. Both sequence homology and molecular topology of the all-β Greek key structure suggest that the protein is the product of a gene duplication. However, in spite of the extreme symmetry of the molecule, its domains show significant differences in their stability against urea denaturation. Unfolding/folding experiments reveal sequential and independent domain folding, with the C-terminal domain showing relatively low stability and enhanced unfolding compared with the N-terminal one which requires extreme denaturant conditions to unfold [2,3]. The comparison of the folding properties and stabilities of the separate domains confirms these observations: the isolated recombinant domains fold autonomously, with no tendency to form either homologous or heterologous assemblies [4]. Their urea-dependent equilibrium transitions are far apart from each other, qualitatively resembling the separate transitions of the complete molecule. The mutual stabilization of the domains is reflected by their enhanced stabilities in the full-length γB-crystallin compared to its separate domains; the corresponding $\Delta\Delta G_{stab}$ values for the N- and C-terminal domains are 9 and 15 kJ/mol, respectively. Similar differences are observed comparing the folding/unfolding kinetics [5]: The N-terminal domain folds significantly faster than the C-terminal one [2,4]. Taken together, previous findings suggest that the N-terminal domain serves as a core structure for the folding and stabilization of the C-terminal half of the molecule, thus generating the anomalously high stability of γB-crystallin.

Analysis of the codon distribution along the γB-crystallin mRNA revealed that the relative frequencies of codons in the N-terminal domain are about 2.5-fold higher than those of the C-terminal domain. As illustrated in Fig. 1, there is a boundary precisely in the linker region between the domains which consists predominantly of rare codons [6]. From these observations, one would predict that the translation of γB-crystallin proceeds in a non-uniform manner, with the N-terminal domain translated faster than the C-terminal one, and with at least one major pause in the linker peptide connecting the two domains. Since folding is a modular process [8], which is assumed to occur cotranslationally [9–14], major pauses in interdomain regions support the idea that the non-uniform rate of translation might influence the mechanism as well as the final product of folding. As a first step along this line of reasoning, the following experiments deal with the kinetics of translation of γB-crystallin and its circularly permutated variant, monitoring nascent peptides accumulating during the biosynthesis of γB-crystallin in a rabbit reticulocyte lysate cell-free system.

2. Materials and methods

2.1. In vitro transcription of γB-, γCP-, and γN cDNAs

Plasmids pSPT18, pET11a and pASK40 containing γB, γCP and γN cDNAs under control of the T7 promoter were used for the in vitro transcription. Transcription reactions were carried out: (i) according to [15] in 500 μl total volume of 80 mM HEPES-KOH buffer pH 7.5 containing 16 mM $MgCl_2$, 2 mM spermidine, 20 mM DTT, 3 mM ATP, 3 mM GTP, 3 mM UTP, 3 mM CTP, 375 units of RNasin (Promega), 25 μg of linearized DNA templates and 2000 units of T7 RNA polymerase (Promega); or (ii) following the 'Promega Transcription' in vitro Systems Technical Manual. The reactions were carried out at 37°C for 2.5 h and stopped by phenol/chloroform extraction. The transcripts were purified by LiCl precipitation and washed with 70% ethanol. Aqueous solutions of the transcripts were used in translation experi-

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Abbreviations: γB, γC, γCP and γN, natural γB crystallin, C-terminal domain of γB, circularly permutated γB, and N-terminal domain of γB, respectively; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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ments. The purity and integrity of the RNA was checked by PAGE under denaturing conditions (7 M urea).

2.2. Cell-free protein synthesis

Cell-free translation of wildtype γB, γCP and γN was performed at 34°C, using the rabbit reticulocyte lysate cell-free translation system described in the above 'Promega' Technical Manual and [^{35}S]methionine (Amersham). The final concentration of mRNA was ca. 60 mg/ml, the final reaction volume 25–50 μl. As a control, the reaction mixture was incubated under the same conditions, but without mRNAs. TCA-precipitable radioactivity was determined in sample aliquots spotted onto GF/C (Whatman) glass filters after NaOH hydrolysis. Production of the γ-crystallin peptides after translation of the various mRNAs, was controlled by low molecular weight electrophoresis [16].

2.3. Isolation of nascent peptides

After incubation, extracts were layered on top of 450 ml of 30% glycerol in 10 mM Tris-HCl buffer pH 7.6, containing 100 mM KCl, 10 mM $MgCl_2$, and centrifuged for 1 h at 4°C and 100,000 × *g* in a TLA-100 rotor (Beckman) in order to pellet ribosomes. The polysome pellets were suspended in a small volume (10–12 μl) of 1 mM Tris-HCl buffer pH 7.6, containing 0.5 mg/ml ribonuclease A, and incubated for 30–45 min at 37°C. In order to enhance the hydrolysis of the peptidyl-tRNA ester bond, NaOH was added to a final concentration of 10 mM, and the incubation was continued for additional 30 min.

2.4. Determination of nascent peptide lengths

The size distribution of nascent chains was analyzed using PAGE according to [16], using 16.5% T and 6% C and 6 M urea gels, with an additional spacer gel. 'Rainbow colored proteins' (M_r = 2350–46000 Da) (Amersham), as well as recombinant γB, γCP, γC and γN were used as molecular mass standards. Gels were fixed, dried in vacuo and subjected to autoradiography using a β-imager. In order to obtain relative values of radioactivity, the scanned curves were transformed with respect to the number and positions of methionine residues in peptides of different lengths. The positions of methionine residues in the case of natural γB are: 1, 44, 70, 91, 103, 137, 161, 172; those of the circularly permuted protein: 1, 5, 17, 51, 75, 86, 137, 163. The transformation of migration distances of nascent peptides into peptide lengths was based on the correlation between marker migration distances and molecular weight logarithms, using a 5-point linear polynomial regression.

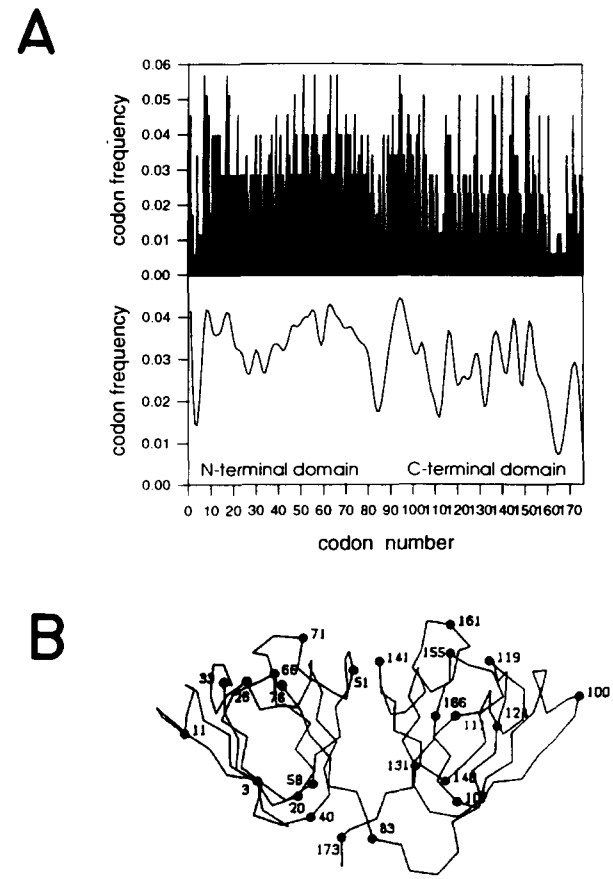

Fig. 1. Codon frequency and backbone structure of γB-crystallin. (A) Codon frequency profile for the calf (*Bos taurus*) γB-crystallin gene (full line) superimposed over codon frequency histogram according to [6,7]. (B) C^{α}-backbone of γB [1], with theoretically predicted sites of slowed- down translation denoted by residue numbers and closed circles.

3. Results and discussion

The mechanism by which the growing polypeptide chain acquires its native structure has been investigated mainly by in vitro renaturation experiments. Their results suggested protein folding to obey a simple sequential model which postulates a unique pathway with defined intermediates [17]. Recent data call this mechanism in question, for at least two reasons: (i) folding intermediates may be artifacts caused by misfolding along the pathway [18]; (ii) accessory proteins and extrinsic factors, as well as effects of the vectorial nature of in vivo folding may affect the mechanism [19,20]. In connection with the effect of cellular components, it has been shown that both chaperones and folding catalysts are involved in kinetic partitioning between proper folding and aggregation; thus, they affect the yield rather than the folding mechanism [19,20]. Reports claiming that the 'translation apparatus' plays an active role in modulating the efficiency of protein folding have been controversial in the past [19–21]. Therefore, in order to clarify codon effects, an approach has been developed that allows to quantify codon distributions and codon-usage frequencies along a given mRNA [7]. For certain multidomain proteins irregularities were detected, which seem to indicate that non-uniform translation kinetics might influence nucleation events and/or domain interactions [7,21,22]. The following experiments show that differences in codon frequencies have a significant effect on the rate of in vitro translation of γB-crystallin (cB) and its circularly permuted variant (γCP).

As has been reported [6], γB shows an irregular codon distribution with different usage frequencies along its mRNA (cf. Fig. 1). At positions along the mRNA which are characterized by low codon frequencies, the increase in residence time of the ribosome results in increased amounts of nascent peptides of sizes corresponding to the respective rare mRNA codons. The size distribution of nascent chains and the relative intensities of the particular peptide patterns for γB show that the cell-free in vitro translation is a non-uniform process with a number of pauses (Fig. 2A). Using recombinant γN and γC, as well as [^{35}S]methionine-labelled γN as molecular weight markers, it becomes clear that one of the nascent peptides coincides with the length of γN, indicating that the linker peptide must be translated more slowly compared to the other parts of the polypeptide chain. Apart from rare codons, mRNA secondary structure might also contribute to non-uniform translation; unfortunately, at present, no approaches are available to predict and quantify such effects.

Measurements of ^{35}S-incorporation into nascent peptides making use of a β-imager yielded major bands corresponding to polypeptides of 40–42, 70–75, 87–89, 99–104, 112–118, 132–

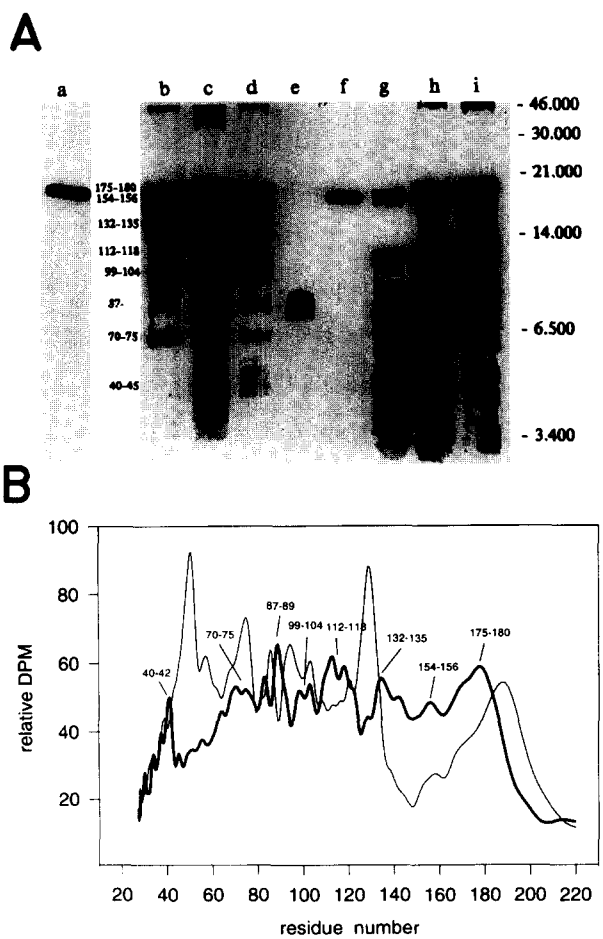

Fig. 2. PAGE of products of cell-free translation. (A) ^{35}S-Autoradiograph of PAGE of translation products. a = γB after 15 min translation; b–d = nascent peptides prepared from polyribosome fraction after 5, 10 and 15 min γB translation, respectively; e = γN after 15 min translation; f = γCP after 15 min translation; g–i = nascent peptides prepared from polyribosome fraction after 5, 10 and 15 min γCP translation, respectively. e and h represent the stage at which ^{35}S-labeling into polyribosomes reaches its maximum so that small polypeptides become detectable. In all cases, equal aliquots were subjected to electrophoresis. M_r = molecular weight markers. Major peptides are denoted with numbers according to their lengths (estimated from peptide molecular weights). (B) β-Imager scans of natural γB- (slot c in (A), thick line) and circularly permutated γCP-crystallin (slot h in (A), thin line). Numbers refer to major pauses in the translation of natural γB-crystallin.

135, 154–156 and 175–180 residues chain lengths (Fig. 2A and B). The absence of low molecular weight peptides in sample aliquots of non-fractionated cell-free extracts and the stability of the patterns of the nascent peptide over various periods of time prove specific proteolysis to be absent. Observed differences in the amount of labeled material are attributable to non-linear incorporation of radioactive label into the polyribosomal fraction, in contrast to the cell-free extract. As has been shown previously [7], in comparing the translation rates, the balance between initiation and termination is significant.

The given lengths of the nascent chains are close to those predicted from the profile of codon frequencies (Fig. 1), supporting the hypothesis that domain linkers are slowly translated regions. There are deviations from the expected locations where pauses should occur. To explain such deviations one has to consider that the specific abundance of aminoacyl-tRNA plays an important role in determining the rate of elongation in a given organism [7]. Therefore, in order to optimize the correlation of of codon usage and tRNA abundance, in the present experiments the rabbit reticulocyte lysate translation system was supplemented with excess of calf tRNA. However, the residual rabbit reticulocyte tRNA in the system may still affect the correlation between tRNA abundance and the pattern of codon usage, thus influencing the elongation rate. In the context of the present study, this cannot be of importance because of the close similarity of the codon usage of both rabbit α- and β-globin genes (which are known to code for >95% of the proteins synthesized in reticulocytes), on one hand, and the genes of calf eye-lens crystallins, on the other [23]; the quantitative comparison yields $\chi^2 = 15.21$, significance level: 0.999, degrees of freedom: 63. As it has been shown that the pattern of the codon usage correlates well with the tRNA abundance [6], it is clear that the tRNA contents of rabbit reticulocytes and calf eye lens must be comparable.

The size distribution of nascent peptides demonstrates that most of the pauses occur during the translation of the C-terminal domain of natural γB. Evidently, the relative residence time of ribosomes during translation of the 3′-part of γB mRNA is higher than that of the 5′-end of the messenger. This means that the N-terminal domain is translated faster than the C-terminal one. Independent evidence for this observation was obtained from translation experiments using the circularly permutated variant of γB. In this construct, the N-terminal 81 residues are connected via a pentaglycine linker to the 3′-end of the C-terminal domain (residues 88–174) [4]. As shown in Fig. 2A and B, in the case of γCP pausing regions are shifted to the 5′-part of the mRNA, resulting in the predominant accumulation of nascent peptides with sizes smaller than half the size of the full-length protein. Obviously, the C-terminal domain of γB is translated at a slower rate compared to the N-terminal one, independent of its position within the complete γ-crystallin sequence.

4. Conclusions

The translation of proteins has been shown to be a non-uniform process depending on both the general degeneracy of the genetic code and the specific codon usage characteristic for a given organism. Assuming that folding of the growing polypeptide proceeds during or immediately after synthesis, pauses may serve as interpunctuations during translation, leading to the temporal separation of folding events. The present in vitro translation experiments give clear evidence that the modular structure of γB-crystallin is reflected by non-uniform translation kinetics, with pauses in the subdomain and domain regions of the mRNA. The overall rate of translation of the N-terminal domain exceeds the translation of the C-terminal domain, to the effect that the N-terminal half of the molecule may serve to stabilize the marginally stable C-terminal half [5].

In vitro denaturation/renaturation experiments show similar characteristics: the N-terminal domain folds faster than the C-terminal one, again allowing complete refolding of the complex two-domain protein. Evidently, non-uniform translation and sequential domain folding represent two different mechanisms to optimize the modular selfassembly of domain proteins.

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References

[1] Wistow, G., Turnell, B., Summers, L., Slingsby, C., Moss, D., Miller, L., Lindley, P. and Blundell, T.L. (1983) J. Mol. Biol. 170, 175–202.
[2] Rudolph, R., Siebendritt, R., Neßlauer, G., Sharma, A.K. and Jaenicke, R. (1990) Proc. Natl. Acad. Sci. USA 87, 4625–4629.
[3] Sharma, A.K., Minke-Gogl, V., Gohl, P., Siebendritt, R. and Jaenicke, R. (1990) Eur. J. Biochem. 194, 603–609.
[4] Mayr, E.-M. (1994) Dissertation, Regensburg University.
[5] Mayr, E.-M., Jaenicke,R. and Glockshuber, R. (1994) J. Mol. Biol. 235, 84–88.
[6] Krasheninnikov, I.A., Komar, A.A. and Adzhubei, I.A. (1990) in: Actual Problems of Biochemistry of Eukaryotes and Prokaryotes, Pushchino, pp. 146–159.
[7] Krasheninnikov, I.A., Komar, A.A. and Adzhubei, I.A. (1991) J. Prot. Chem. 10, 445–454.
[8] Wetlaufer, D.B. (1981) Adv. Prot. Chem. 34, 61–92.
[9] Cowie, D.B., Spiegelman, S., Roberts, R.B. and Duerksen, J.D. (1961) Proc. Natl. Acad. Sci. USA 47, 114–122.
[10] Zipser, D. and Perrin, D. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 533–537.
[11] Kiho, Y. and Rich, A. (1964) Proc. Natl. Acad. Sci. USA 51, 111–118.
[12] Hamlin, J. and Zabin, I. (1972) Proc. Natl. Acad. Sci. USA 69, 412–416.
[13] Bergman, L.W. and Kuehl, W.M. (1979) J. Supramol. Struct. 11, 9–24.
[14] Peters, T. and Davidson, L.K. (1982) J. Biol. Chem. 257, 8847–8853.
[15] Gurevich, V.V., Pokrovskaya, I.D., Obukhova, T.A. and Zozula, S.A. (1991) Anal. Biochem. 195, 207–213.
[16] Schagger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[17] Jaenicke, R. (1987) Progr. Biophys. Mol. Biol. 49, 117–237.
[18] Baldwin, R.L. (1995) J. Biomol. NMR 5, 103–109.
[19] Jaenicke, R. and Buchner, J. (1993) Chemtracts: Biochem. Mol. Biol. 4, 1–21.
[20] Jaenicke, R. (1993) Curr. Opin. Struct. Biol. 3, 104–112.
[21] Crombie, T., Boyle, J., Coggins, J.R. and Brown, A.J.P. (1994) Eur. J. Biochem. 226, 657–664.
[22] Krasheninnikov, I.A., Komar, A.A. and Adzhubei, I.A. (1989) Dokl. Nauk. SSSR 305, 1006–1012.