Synonymous codon substitutions affect ribosome traffic and protein folding during in vitro translation

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Abstract To investigate the possible influence of the local rates of translation on protein folding, 16 consecutive rare (in *Escherichia coli*) codons in the chloramphenicol acetyltransferase (CAT) gene have been replaced by frequent ones. Site-directed silent mutagenesis reduced the pauses in translation of CAT in *E. coli* S30 extract cell-free system and led to the acceleration of the overall rate of CAT protein synthesis. At the same time, the silently mutated protein (with unaltered protein sequence) synthesized in the *E. coli* S30 extract system was shown to possess 20% lower specific activity. The data suggest that kinetics of protein translation can affect the in vivo protein-folding pathway, leading to increased levels of protein misfolding.

Key words: Chloramphenicol acetyltransferase; Codon usage; Translation; Translational pause; Cotranslational folding; Misfolding

1. Introduction

Protein folding still remains the most intriguing and puzzling problem in molecular biology. Many proteins could be successfully refolded in aqueous solutions, but their folding pathway in vivo is thought to be affected by a lot of factors, such as viscosity of cytoplasm, local pH values, concentration and activity/affinity of surrounding macromolecules and many others (for review see [1,2]). Folding of some proteins in a cell is also facilitated by chaperones, known to prevent proteins and newly synthesized nascent chains from misfolding and aggregation [1–5]. In addition, folding in vivo is apparently mainly cotranslational (recent publications [6–17]), thus a vectorial process, and as a consequence of its cotranslational and vectorial nature one might expect that ribosome itself [17–20] as well as the translation process could affect the mechanism and the pathway.

In addition to a link between the level of gene expression and codon frequency (see [21]), intriguing correlations between usage/frequencies of synonymous codons and protein structure have been reported [22–31]. A tendency of rare codons to encode turns, loops and domain linkers was also observed [22–27]. Since it is known that the ribosome traffic on a messenger is mainly modulated by both the general degeneracy of the genetic code (namely specific synonymous codon usage characteristics for a given organism) and the availability of cognate tRNAs, surrounding ribosome during translation, it might be concluded that such regions would be slowly translated [23–24]. It should be mentioned that within *Escherichia coli* and other organisms, a strong codon bias exists, and the level of cognate tRNAs was shown to be directly proportional to the frequency of codon usage [32]. Thus, in most of the cases the term rare codon would at the same time mean slowly translated codon. Since folding is a modular process [33], one might suggest that optimization of the in vivo protein folding includes the adaptation of codon selection along mRNA to a particular translation kinetics [22–31]. This could be necessary to ensure step-by-step synthesis and folding of the defined portions of the polypeptide chain growing on the ribosome from its N-terminal end. Consequently, the regions of slowed down translation might serve as interpunctuations during protein synthesis separating different folding events [22–31]. Thus, one might suggest that alteration of translation kinetics could influence the in vivo protein folding pathway. Evidences, showing that optimization of heterologous gene expression by a choice of only frequent codons often yields biologically inactive products forming insoluble aggregates, which have to be renatured artificially in order to regain similarity in structure and biological activity with native analogues [34–36] could support the idea. Since it is known that synonymous codons are translated at different rates [37,38], it is possible to change the local rates of translation by suitable selection of synonymous codons. Here, we address a question of a possible link between protein folding and protein translation by the use of chloramphenicol acetyltransferase gene with 16 synonymous rare codons replaced by frequent ones. We have used specific activity of a protein (synthesized in vitro in *E. coli* S30 extract cell-free system) as a measure of its folding state. Our results show that replacement of a set of rare codons by synonymous frequent ones at a selected place of the chloramphenicol acetyltransferase gene led to the acceleration of the ribosome traffic through the mutated region and at the same time affected the specific activity of the enzyme (in comparison with a wild-type (wt) protein), suggesting in vivo protein folding could be influenced by the rates of translation.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

The pUC18:IM3/CI-1 plasmid bearing the chloramphenicol acetyltransferase (CATIII) gene was a gift of Prof. W.V. Shaw (University of Leicester). The *Bam*HI–*Hin*dIII (1034-bp) fragment of the pUC18:IM3/CI-1 plasmid was inserted into pBluescript II KS$^-$ vector, to obtain the gene under control of the T7 promoter. Oligonucleotide directed mutagenesis was accomplished using single stranded DNA and following general procedures described in [39]. The following

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oligonucleotides were used during the additive steps of mutagenesis: 5′-TATCACTTTTATAACGTTCCATTACCGATA-3′, 5′-ATCAAAATTAACCCAAGGTAATGCTGAAAT-3′, 5′-TATCAGATTTATAACGTTCCATTACCGATA-3′, 5′-ATCAAAATTAACCCAAGGCAGCGCAGAAAT-3′, 5′-ACGTTATAAATCTGATACAAAACTGTTCCCGCAGGGCGTGACACCGGAAAACCATCTGAACATTTCTGCGCTGC-3′

2.2. In vitro transcription

The transcription reaction was carried out: (1) following the 'Promega' Transcription in vitro systems Technical Manual or (2) according to Gurevich et al. [40] in 100 μl (total volume) of 80 mM HEPES-KOH buffer, pH 7.5, containing 16 mM $MgCl_2$, 2 mM spermidine, 20 mM dithiothreitol (DTT), 3 mM ATP, 3 mM GTP, 3 mM UTP, 3 mM CTP, 2.5 μl (100 units) of RNasin (Promega), 5 μg of *Hin*dIII (pBluescript II KS$^-$ wtCATIII an its different derivatives) linearized DNA templates and 320 units of T7 RNA polymerase (Promega). The reaction was carried out at 37°C for 2.5 h and stopped by phenol/chloroform extraction. The transcript was purified by LiCl precipitation and washed with 70% ethanol [41]. The purity and integrity of the RNA molecules were checked by 5% PAGE under denaturing conditions in the presence of 7 M urea. An aqueous solution (0.8 mg/ml) of the transcripts was used in translation experiments.

2.3. Cell-free protein synthesis and nascent peptide isolation

Cell-free translation of the CATIII mRNAs was performed using the *E. coli* S30 extract in vitro translation system in the presence of [^{35}S]methionine (15 mCi/ml, Amersham) as described in the 'Promega' Technical Manual. The final concentration of CATIII mRNAs was 64 μg/ml. In some experiments the translation reactions were synchronized by adding 100 μM aurintricarboxylic acid 30 s–1 min after the addition of mRNA.

To isolate nascent chains the translation reactions were stopped by the addition of an equal volume of 20 mM Tris-HCl buffer, pH 7.6, 200 mM NH_4Cl, 20 mM $MgCl_2$, 2 mM DTT and 0.2 mM EDTA cold on ice (buffer A). Extracts were layered on the top of 150 μl of 30% glycerol in half concentrated buffer A and centrifuged for 1 h at 4°C and 100 000 rpm in 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 an additional 30 min.

2.4. Electrophoresis

Cell-free translation products were analyzed using PAGE according to [42], in either 16.5% T, 6% C or 16.5% T, 3% C (with or without 6 M urea) gels, with an additional spacer gel. The 'Rainbow ^{14}C-methylated colored proteins' (MW = 2350–46 000 Da, Amersham) were used as molecular weight markers. Gels were fixed, dried in vacuo and subjected to autoradiography using the Molecular Dynamics phosphorimager.

2.5. CATIII activity measurements

The chloramphenicol acetyltransferase activity was measured using fluorescent FAST CAT Green (Deoxy) Chloramphenicol acetyltransferase assay kit (Molecular Probes) following general procedures described in 'Molecular Probes Technical Product Manual' with minor modifications. The final concentration of the substrate in the reaction mixtures was 63 μM, which is well above the K_M of ~18 μM for this substrate. The 2.5-μl aliquots were periodically removed from the translation reactions and subjected to CAT activity assay measurements in a total 80-μl volume of the FAST CAT substrate reagent, containing 1.125 mM acetyl CoA. The assay reactions were performed at 37°C for 10–30 min and stopped by adding 1 ml of ice-cold ethyl acetate. Extracts were evaporated and redisolved in 25 μl of ethyl acetate. The reaction substrate and product were resolved by thin-layer chromatography (TLC) on silica gel 60 plates (Merck) in chloroform:methanol (85:15 v/v) solvent. The unreacted CAT substrate and the acetylated product derivative were quantitated under UV using a digital camera with the help of 'Elscript' scan and evaluation software v 1.4 (Analyse Technik Hirshman GmbH). The specific activities were calculated from quantitation of the synthesized ^{35}S-labeled protein at the same period of time of translation when samples for activity assay measurements were removed.

2.6. Miscellaneous

Molecular cloning and sequencing were performed following general procedures described in [41]. Radioactivity was monitored using a PACKARD 2200CA TRI-CARB liquid scintillation analyzer. Trichloroacetic acid precipitable radioactivity was determined in sample aliquots spotted onto GF/C (Whatman) glass filters after NaOH hydrolysis.

3. Results

3.1. Replacement of synonymous codons accelerate ribosome traffic through the mutated region

In an attempt to elucidate the effect of synonymous codon substitutions on both ribosome traffic and protein folding we have replaced 16 rare synonymous codons (located at the CATIII gene ORF region 123–145) by synonymous frequent ones. The silent codon substitutions are shown in Fig. 1A.

A method of SDS-PAGE analysis of nascent chains accumulating during CAT translation has been chosen in order to monitor CAT translation kinetics. PAGE analysis of the nascent peptides (isolated from the polyribosomal fraction) showed that translation of chloramphenicol acetyltransferase in vitro is indeed a non-uniform process characterized by specific pauses (Fig. 1B). The increase in residence time of the

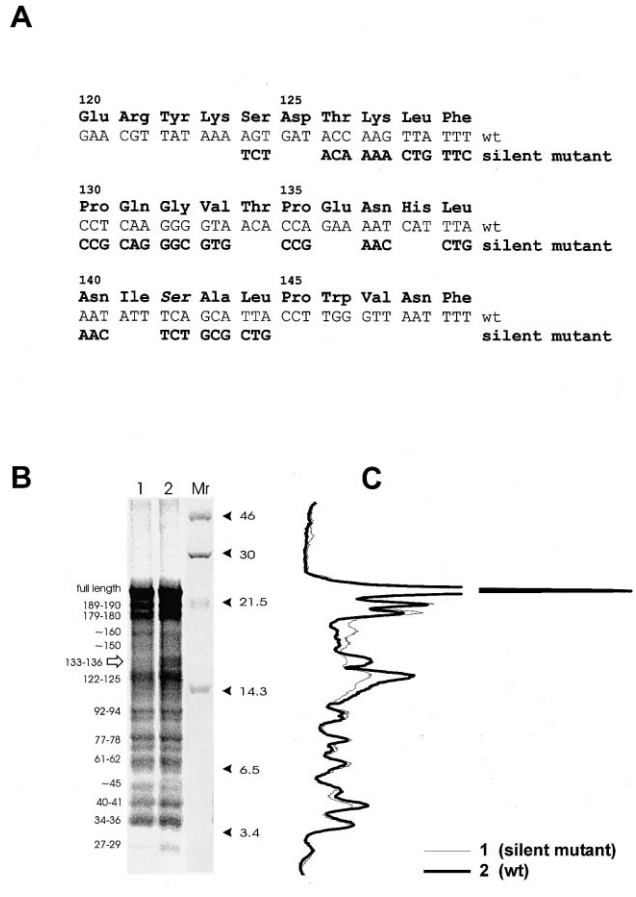

Fig. 1. A: Sequence of the CATIII open reading frame region 120–150. Silent codon substitutions are shown in bold. B: Autoradiogram of SDS gel electrophoresis of CAT nascent chains. 1: nascent peptides after 3 min of CATIII (silent codon mutant) translation; 2: nascent peptides after 3 min of CATIII (wt) translation. Arrows point the position of molecular weight markers. Molecular mass is given in kDa. C: phosphorimager scan of translation products: slots 1 and 2 in (B), respectively.

ribosome at positions along the mRNA which are characterized by low codon frequencies results in increased amounts of nascent chains of the sizes corresponding to the location of the respective rare codons. Thus, the gel electrophoresis nascent peptide band pattern reflects the non-uniform translation kinetics of the CATIII protein, allowing also to locate the pause sites from the lengths of the respective nascent chains (Fig. 1B). Translation pausing during CAT in vitro synthesis also has been recently reported by Hardesty and co-authors [43]. Using translation rates of individual codons computed from available data [44], it can be predicted that silent replacements of the 16 rare codons would result in a two-fold decrease in the time needed for a ribosome to move through the mRNA region ORF 124–145. It was observed that the amount of the respective nascent chains accumulating during translation of the silently mutated (sm) protein decreased significantly, by a factor of 2.7 (as can be concluded from phosphorimager scan data) and even one particular peptide (amino acid length 133–136) disappeared almost completely (Fig. 1B,C). This indicates that the ribosome is in fact moving faster through the mutated region.

It was also found that the total amount of the sm protein synthesized in *E. coli* S30 extract (TCA precipitated material as well as full-length protein) exceeds by 12–16% that for the wt protein (not shown). It is unlikely that this result originates from the higher level of initiation in case of sm mRNA in comparison with the wt. When the translation reactions were synchronized by the addition of aurintricarboxylic acid (inhibitor of initiation of protein synthesis [45]) essentially the same results were obtained.

3.2. Silently mutated protein shows reduced specific activity

The major question we were addressing in this study was whether the alterations of translation kinetics could affect chloramphenicol acetyltransferase folding during translation in vitro. Since a link between structure and function of proteins is a well established fact, one can use specific activity of a given protein as measure of its folding state. It was therefore of interest to compare the specific activities of CATs translated from the natural and the sm mRNAs. We have used equal amounts of mRNAs for both wt and sm protein produced in a separate set of translation experiments, which were run simultaneously under equal conditions. Periodically sample aliquots were removed and both the total amount of the synthesized protein (as well as full-length polypeptide) and the chloramphenicol acetyltransferase activity were measured. Surprisingly, we found that CATIII produced from the sm gene displayed reduced specific activity in comparison with that for the wt protein. The overall difference in specific activity (ratio of activity over protein amount) was found to be 20% (Fig. 2).

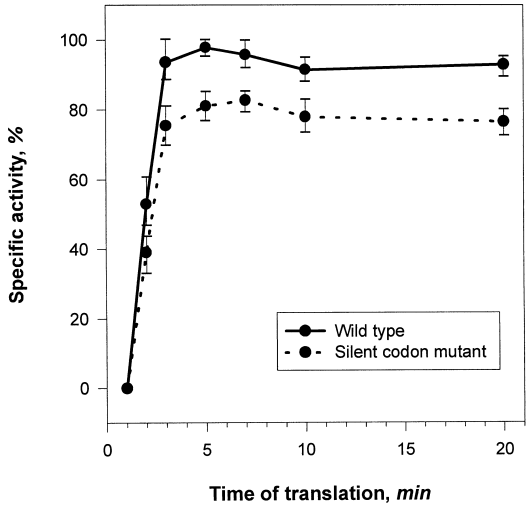

Fig. 2. Specific activities of the chloramphenicol acetyltransferase variants synthesized in *E. coli* S30 extract cell-free system. Maximum value of the specific CAT activity in each independent translation experiment was set to 100%. Average values of five experiments are shown. Specific activity was determined as a ratio of the percentage of substrate conversion (to acetylated product) to the total amount of the ^{35}S-labeled protein synthesized during the defined period of times.

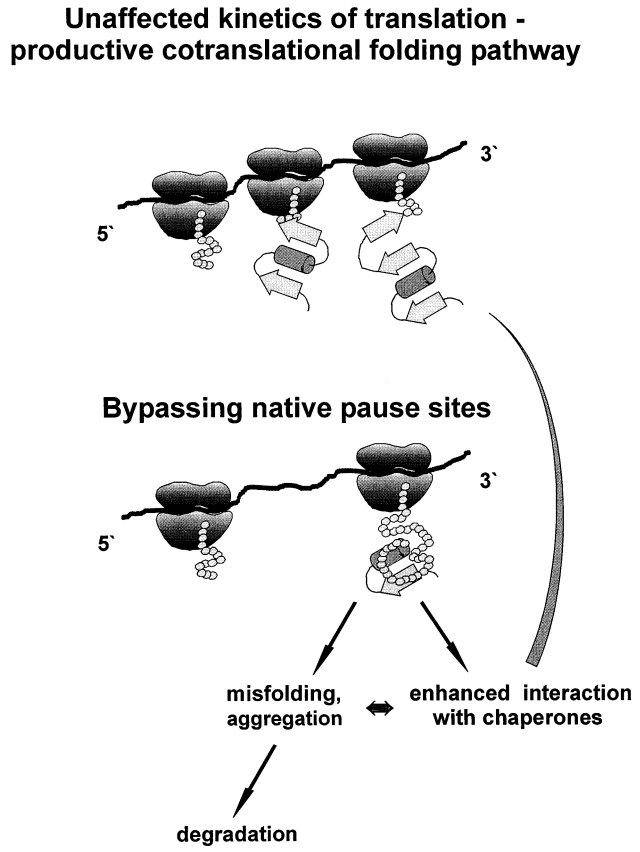

Fig. 3. Schematic representation of possible influence of translation pausing on cotranslational protein folding and misfolding.

4. Discussion

One of the major open questions in molecular biology is to what extent protein folding models developed using in vitro approaches reflect the in vivo situation. Many attempts to achieve in vitro 100% refolding of the denatured proteins were only partially successful. This indicates that apparently additional information supplementing the amino acid sequence and additional factors should probably be considered. It is now known that chaperones and folding catalysts appear to be mainly involved in kinetic partitioning between proper folding and aggregation; thus they affect the yield rather than the folding mechanism [46,47]. It is therefore likely that some

additional factors, and in particular kinetics of protein translation should be taken into account [22–31]. It was therefore of interest to investigate the direct effect of the changes of the rates and kinetics of translation on protein folding. It should be mentioned that conditions of in vitro translation closely resemble that of the in vivo protein synthesis [48,49]. In addition the in vitro system possesses several advantages, allowing e.g. easily to control the amount of the mRNA added and the protein synthesized.

4.1. CAT translation and folding in E. coli S30 extract system

We have replaced 16 rare codons by frequent ones at a selected place of CATIII gene and compared the translation and folding (as measured by the specific CAT activity) of the wt and sm protein. Our data show that the silent codon exchanges affect ribosome traffic at a mutated region of CATIII mRNA and abolish particular translational pause. A lot of experimental data provide evidence that rates of chain elongation during translation of proteins are not uniform (see [23]). Non-uniform character of distribution of codons with different usage frequencies along mRNA is assumed to be a main factor which modulates the translation kinetics. Intensive studies have been carried out previously on the determination of the translation rates of certain individual codons [37,38] as well as on the determination of the effect of synonymous codon usage on the overall level of gene expression [50–53]. However, there are at present only few data directly showing that replacement of certain synonymous codons could lead to the alteration of ribosome traffic on mRNA at a selected place [54]. Our data clearly corroborate this observation.

As a next step we have compared the specific activities of the sm and wt protein. We found sm protein to possess about 20% lower specific activity. Since specific activity of a given protein could be considered as a measure of its proper folding one might conclude that CATIII folding was affected. It can be suggested that accelerated rates of translation of a selected region allowed the particular part of the polypeptide chain to appear earlier in time during translation in comparison with the wt protein. This could lead to an affected interaction of this polypeptide region with the preceding one, which (one may speculate) was not yet properly folded (due to a lack of time). Such misfolded nascent chains can further undergo enhanced interaction with chaperones (due to an altered chaperone affinity) and this could shift the equilibrium back to the productive folding pathway. Alternatively, such misfolded nascent chains could be trapped and finally degrade (Fig. 3). The phenomenon of cotranslational ubiquitination (and degradation) of proteins [55–57] clearly supports the idea that misfolding of proteins could start during protein translation. Cotranslational folding is assumed to be a modular process proceeding by parts and thus one can conclude that the formation of the normal intermediate(s) on a cotranslational CAT folding pathway was probably affected. Codon substitutions were introduced in a region (ORF 123–145) represented in a CAT three-dimensional structure by a long loop and a beta-sheet [58,59]. This region bears the Ser residue (at position 142), which is known to be of catalytic importance and absolutely conserved among all CATs [60,61]. Also this region is in part involved in subunit interactions.

The observed drop in a specific activity in case of sm protein could be, however, explained by a number of reasons. Since CATIII is active only in a form of a trimer [60,61] one can suggest that polypeptides produced from the sm mRNA:

be only in part able to form trimers (80% of the wt), as those unable to form trimers bear some folding 'defects' which does not allow trimer formation and e.g. represent more efficient target for chaperones, in particular DnaJ, DnaK and GroEL;

could form trimers, among which only 80% are active, again due to some folding 'defects' which leave 20% of the trimers inactive;

could form trimers which possess only 80% of specific activity, compared to the wt protein due to the incorrect architecture of the active site.

Further experiments will be required to explore these suggestions.

The fact that the difference in specific activity remains unchanged during the time of translation and even (20 min) after the moment, when CATIII translation is stopped by puromycin (results not shown) indicates that improper folding CATIII 'variants' are trapped and stable.

Taking together all the data mentioned above we argue that folding of CAT can be affected by the rates of translation and location of specific pauses.

It was reported previously that replacement of ten consecutive non-preferred codons in the indoleglycerol-phosphate synthase region of the TRP3 gene with synonymous preferred codons causes a 1.5-fold reduction in relative indoleglycerol-phosphate synthase activity observed during expression of the protein in yeast cells [62]. Likewise the authors concluded that translational pausing promotes the correct intracellular folding of the TRP3 protein. The importance of pausing was also demonstrated in case of signal recognition particle receptor alpha-subunit cotranslational assembly and membrane binding (in a rabbit reticulocyte lysate cell-free translation system) [54].

One might suggest that both in eukaryotic and prokaryotic cells specific features of non-uniform translation could be necessary to ensure proper in vivo protein folding.

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