Biochemistry (Moscow), Vol. 69, No. 7, 2004, pp. 806-808. Translated from Biokhimiya, Vol. 69, No. 7, 2004, pp. 989-992.

Inhibition of [^{15}N]Valine Transamination during Selective Labeling of Barstar in a T7 Polymerase System

L. V. Lopukhov, A. A. Ponomareva, and L. O. Yagodina*

Kazan Institute of Biochemistry and Biophysics, Kazan Research Center, Russian Academy of Sciences, ul. Lobachevskogo 2/31, Kazan 420111, Russia; E-mail: yagodina@mail.knc.ru

Received June 6, 2003
Revision received September 19, 2003

Abstract—Selective labeling of barstar by the stable ^{15}N isotope of the valine residue with high selectivity of the label incorporation resulting from the process of gene expression in *Escherichia coli* BL21(DE3) has been optimized. We have shown that α-aminooxyacetic acid (AOAA) significantly reduces the isotope redistribution, thus increasing the selectivity of ^{15}N incorporation into the synthesized protein, as detected by 2D-NMR. Quantitative measurements were used to determine the selectivity for the incorporation of isotope-labeled valine residue, which was 96% in the case using AOAA. Studies of the dynamics of barstar synthesis showed that no suppression of barstar yield is observed under the regulation of the T7 polymerase expression system by isopropylthio-β-D-galactoside (IPTG) and rifampicin using AOAA.

Key words: stable isotopes, NMR, transaminase, α-aminooxyacetic acid, barstar

NMR studies of the molecular dynamics of proteins selectively labeled by the stable ^{15}N isotope require large amounts of samples, which makes the use of a bacterial expression system the most feasible choice. A principal disadvantage of this technique is low selectivity of label incorporation [1] caused by amino acid catabolism; furthermore, attempts to increase the selectivity usually result in a decrease in protein yield. Protein labeling by stable isotopes in a cell-free protein-synthesizing systems can solve the problem of labeling selectivity; however, this approach is fairly expensive and technically complicated. Bacterial systems with controlled expression can be used to achieve conditions when rates of amino acid catabolism and protein synthesis are comparable and therefore a better selectivity of ^{15}N incorporation is provided. A successful example was presented by Lee [2], where it was demonstrated that at a certain time (fixed in relation to isopropylthio-β-D-galactoside (IPTG) induction) of label incorporation, the use of rifampicin in the T7 polymerase system is followed by an increase in target protein yield and noticeable decrease in label redistribution. According to our data [3], a change in the concentrations of certain amino acids (which comprise the ^{15}N exchange pool in the cell) in the growth medium can be used to increase the degree of the selectivity of [^{15}N]tryptophan incorporation during the expression of the gene of the polypeptide inhibitor of RNase barnase (barstar) in the T7 polymerase system. However, in the case of amino acids with higher catabolism rate (valine, for instance) it is not possible to completely avoid the label redistribution by this technique. As was found, the introduction of a transaminase inhibitor into the growth medium allows significant improvement of the selectivity of ^{15}N-incorporation as detected by NMR-spectra of the barstar labeled with valine residues [4]. To optimize the process of selective labeling of barstar by [^{15}N]valine in the presence of transaminase inhibitor, we attempted in this work to quantitatively characterize the influence of α-aminooxyacetic acid (AOAA) on the yield of barstar synthesized in the T7 polymerase system.

MATERIALS AND METHODS

The experiments were performed using *Escherichia coli* BL21(DE3) strain [5] transformed by the plasmid vector carrying the barstar gene under the control of T7 promoter [6]. T7 promoter is not recognized by the RNA polymerase of host *E. coli*. BL21(DE3) cells carry the gene of the necessary T7 RNA polymerase, placed under

* To whom correspondence should be addressed.

0006-2979/04/6907-0806 ©2004 MAIK "Nauka / Interperiodica"

the control of lac promoter. In this system, barstar gene expression can be induced by IPTG.

Transformation by the PGEMEX/bst plasmid (kindly provided by A. A. Shulga, Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow) was performed according to a standard procedure [7]. Colonies of *Escherichia coli* BL21(DE3) transformed by the plasmid vector were grown in LB broth containing ampicillin (120 μg/ml) at 37°C until A_{600} = 0.6. Thereafter, the cells were collected by centrifugation for 5 min at 4000*g* and transferred to SB-6 medium with ampicillin (120 μg/ml). The employed SB-6 growth medium contained standard components of M9 medium with addition of $ZnSO_4$ (0.1 mg/liter), $FeSO_4$ (0.1 mg/liter), and amino acids Glu, Asp, Ala, Gln (250 mg/liter each), and Met (100 mg/liter), pH 7.5.

In the experiments performed under conditions of transaminase inhibition, AOAA was added until the final concentration of 1 mM. Thereafter, the culture was additionally incubated for 3 h at 37°C, and after that the cells were collected by centrifugation at 2500*g*, resuspended in 50 mM Tris, pH 8.0, containing 2 mM EDTA and 2 mM phenylmethylsulfonyl fluoride, frozen in liquid nitrogen, and stored at –20°C until protein extraction. The quantitative content of barstar compared to total cellular protein in the extract was analyzed by electrophoresis in 15% polyacrylamide gel according to Laemmli. Gels were stained with Coomassie G-250, documented using an Olympus C300 Z digital camera (Olympus, Japan) and analyzed using Scion Image software (Scion Corp., USA; www.scioncorp.com). Lane images on gels were calibrated in relation to absorption units. Production of densitograms for particular lanes and calculation of barstar content (in relation to total cellular protein) by areas of the corresponding peaks on densitograms was performed according to recommendations found in the software manual.

Barstar was extracted according to earlier described technique [6]. The concentration of extracted and purified barstar was evaluated spectrophotometrically on a Specord M-40 UV-VIS spectrophotometer (Carl Zeiss, Germany).

$^1H,^{15}N$-Spectra of barstar, selectively labeled by [15N]valine depending on AOAA concentration, were measured on a Bruker 500 MHz NMR-spectrometer (Bruker, Germany).

The chemicals used in this work were purchased from Sigma (USA), except for [15N]valine, which was produced by NPO Genetika (Russia).

RESULTS AND DISCUSSION

To decrease the redistribution of isotope during cell growth under the conditions of controlled barstar yield, which is synthesized in the T7 polymerase system, we tried to inhibit the catabolism of amino acids by using AOAA, which is known to suppress transamination [8], although its overall influence on protein synthesis in the T7 system has not been investigated. The influence of AOAA on the catabolism of labeled amino acids was evaluated by analysis of two-dimensional $^1H,^{15}N$-NMR spectra of barstar labeled with [15N]valine. For numerical processing of two-dimensional $^1H,^{15}N$-spectra, a one-dimensional projection on the ^{15}N-axis was made, corresponding to the ^{15}N-spectrum of the sample. Figure 1 presents the one-dimensional projections of the barstar spectra obtained in the presence and in the absence of AOAA. Peaks, corresponding to valine residues were determined based on the correlation with the $^1H,^{15}N$-spectrum, which was earlier obtained for completely labeled ^{15}N-labeled barstar [9]. Analysis of the peak areas of the spectra presented in Fig. 1a revealed that the content of ^{15}N in valine residues for barstar synthesized without AOAA was only 76% of its total content in the protein. Moreover, it should be noted that such relatively high percentage of ^{15}N isotope inclusion in the case of valine (which has a high rate of metabolic transformations via the Ala-Val pathway) is achieved through using a special growth medium (enriched with amino acids), which we used in all cases regardless on AOAA concentration. In the barstar spectrum obtained in the presence of [15N]Val (70 mg/liter) and 1 mM AOAA in the medium, 96% of the ^{15}N-isotope is contained in valine residues (Fig. 1b). Based on the analysis of the NMR-spectra, it can be concluded that in the absence of AOAA the exogenous [15N]Val is partially subjected to catabolism, while after the introduction of AOAA into the medium, exogenous [15N]valine catabolism is virtually not observed.

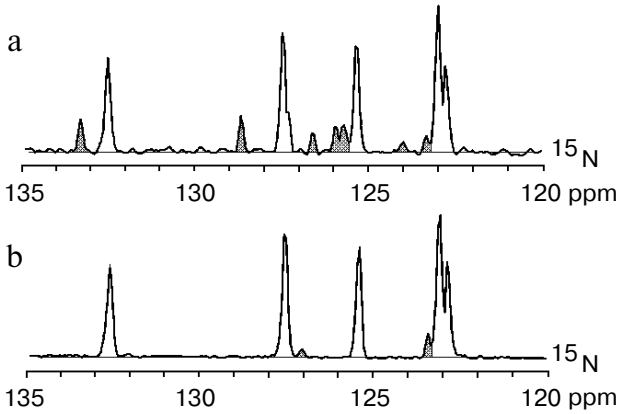

Fig. 1. a) ^{15}N-Projection of the two-dimensional spectrum of barstar labeled by [15N]Val (30 mg/liter) in SB-6 medium for 3 h. b) Projection of barstar spectrum labeled under the same conditions with addition of 1 mM AOAA. Peaks, which are not related to valine residues, are dashed.

To study the influence of AOAA on the yield of the target protein during the expression, the dependence of

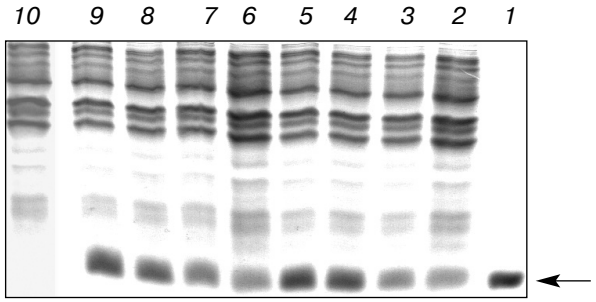

Fig. 2. Electrophoretic analysis according to Laemmli of the total cellular protein content during the barstar gene expression in *E. coli* cells BL21(DE3) depending on the AOAA content: *1*) purified barstar; *2-6*) proteins of cellular extract after IPTG induction before (*2*, *6*) and after the introduction of rifampicin in 1 (*3*), 2 (*4*), and 3 h (*5*); *7-9*) proteins of cellular extract at 1 (*7*), 2 (*8*), and 3 h (*9*) after the addition of rifampicin in the presence of 1 mM AOAA; *10*) proteins of *E. coli* BL21(DE3) cellular extract not transformed by plasmid, 3 h after IPTG induction. Barstar position is indicated by the arrow.

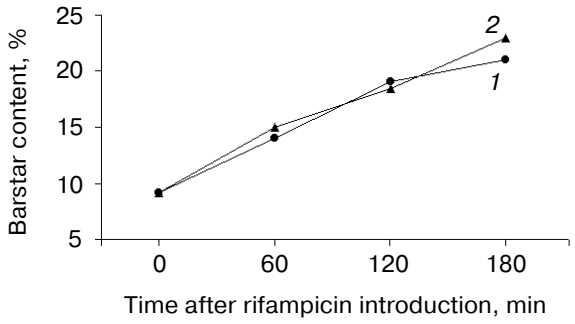

Fig. 3. Dynamics of barstar synthesis after the introduction of rifampicin in the absence (*1*) and in the presence (*2*) of 1 mM AOAA. Barstar amount is presented as percentage of total cellular protein.

barstar amount on AOAA content was determined by electrophoresis in 15% polyacrylamide gel. The expression of the barstar gene was performed at 1 mM IPTG in the presence of a complete (except for Val) set of 20 proteogenic amino acids, where rifampicin (50 mg/liter) and [^{15}N]Val (70 mg/liter) were introduced into the medium 40 min after IPTG introduction. Starting with the moment of IPTG induction of the barstar gene expression, samples were collected (as described in "Materials and Methods") for electrophoretic analysis of protein content. Figure 2 presents the changes in protein content of *E. coli* cells carrying the plasmid with the barstar gene after the introduction of rifampicin and labeled valine. As Fig. 2 illustrates, the maximal protein content in the cellular extract is observed 3 h after the introduction of these compounds. Further growth of the cells was limited by initiation of lysis. To obtain a quantitative estimation of the difference in barstar accumulation dynamics in the case using AOAA, the electrophoretic data were processed using Scion Image software. The results of mathematical processing presented in Fig. 3 demonstrate that the dynamics of barstar gene expression and total amount of synthesized barstar do not depend on AOAA at the concentration of 1 mM, which is necessary for the obtaining of barstar spectra selective in [^{15}N]Val. Analysis of the electrophoretic data in correlation with NMR-spectra showed that in the presence of AOAA the exogenous [^{15}N]Val is virtually not observed, thus indicating efficient suppression of transaminases. Since the accumulation of barstar in the bacterial cells after the introduction of rifampicin and AOAA occurred linearly for 3 h (Fig. 3), the absence of barstar (synthesized by using AOAA, other ^{15}N-containing amino acids) in the spectrum proves that the suppression of transaminase activity was achieved during the whole period. This suggests that transaminase inhibition by AOAA is irreversible as a result of the fact that *E. coli* cells lack the biochemical mechanisms for overcoming the inactivation of pyridoxal phosphate in pyridoxal phosphate-dependent enzymes.

The authors thank D. Reicher and M. Zeidel (Halle University, Germany) and also A. G. Krushelnitsky (Kazan Institute of Biochemistry and Biophysics, Russia) for their help in obtaining NMR-spectra of barstar samples.

REFERENCES

1. Wider, G., and Wuthrich, K. (1999) *Curr. Opin. Struct. Biol.*, **9**, 594-601.
2. Lee, K. M., Androphy, E. J., and Baleja, J. D. (1995) *J. Biomol. NMR*, **5**, 93-96.
3. Ponomareva, A. A., Lopukhov, L. B., and Yagodina, L. O. (2002) *Abst. VIIIth All-Russ. Conf. "Structure and Dynamics of Molecular Systems"* [in Russian], Mari State University Publishers, Yoshkar-Ola, p. 194.
4. Lopukhov, L. V., Ponomareva, A. A., and Yagodina, L. O. (2002) *BioTechniques*, **32**, 1248-1250.
5. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol.*, **189**, 113-130.
6. Krushelnitsky, A., Reichest, D., Hempel, G., Fedotov, V., Schneider, H., Yagodina, L., and Schulga, A. (1999) *J. Magn. Res.*, **138**, 244-255.
7. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N. Y., pp. 240-242.
8. Dawson, R. M. C., Elliot, C. P., Elliot, W. H., and Jones, K. M. (1986) *Data for Biochemical Research*, Clarendon Press, Oxford.
9. Lubienski, M. J., Bycroft, M., Freund, S. M. V., and Fersht, A. R. (1994) *Biochemistry*, **33**, 8866-8877.