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Biosynthesis of a protein containing a nonprotein amino acid by *Escherichia coli*: L-2-Aminohexanoic acid at position 21 in human epidermal growth factor

(*phoA* promoter/protein engineering/protein secretion/recombinant plasmid/alloprotein)

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ABSTRACT **Endeavoring to develop a method to biosynthesize proteins substituted with nonprotein amino acids, we attempted the incorporation of L-2-aminohexanoic acid (Ahx) into human epidermal growth factor (hEGF). *Escherichia coli* YK537 strain harboring plasmid pTA1522, which has the *phoA* promoter-*phoA* signal peptide-hEGF gene, was used. Cells were cultured first in high-phosphate medium and then, for induction of the hEGF-encoding gene, transferred to low-phosphate medium containing Ahx (0.25 mg/ml). hEGF and Ahx-substituted hEGF, [Ahx^{21}]hEGF, secreted into the periplasm were recovered. After treatment with H_2O_2, [Ahx^{21}]-hEGF was clearly separated from methionine-oxidized hEGF by one-step reverse-phase HPLC. Substitution of the methionine residue of hEGF with Ahx was confirmed by the amino acid analysis of [Ahx^{21}]hEGF. The three biological activities of [Ahx^{21}]hEGF were the same as those of hEGF. From the successful production of [Ahx^{21}]hEGF, a basic strategy was established for preparing proteins substituted with nonprotein amino acid (alloprotein). Induction of the *phoA* promoter of *pho* regulon and secretion of the product to the periplasm may depress heat shock-like responses and subsequent hydrolysis of the product by cytoplasmic protease.**

Protein engineering (1) with recombinant DNA techniques has been a useful tool for elucidating the structure–function relationship of proteins as well as for furthering the production of specifically designed proteins. Site-directed mutagenesis is the major strategy in protein engineering; an amino acid residue in a protein may be replaced by any other member of the family of 20 amino acids (1). With these 20 amino acids, the number of possible amino acid sequences of proteins is enormous.

However useful protein engineering is, the variety of protein-constituting amino acids is limited. If amino acids with side chains bearing "unnatural" functional groups could be incorporated into proteins, the possibility of designing rare functions for specific proteins would be drastically expanded. Thus, the synthesis of a protein substituted with nonprotein amino acids, referred to as an "alloprotein," has much potential application in protein engineering.

Alloproteins may be synthesized in two ways—chemical synthesis and biosynthesis. In principle, any nonprotein amino acid can be chemically incorporated into peptides, provided that appropriate methods are designed for protecting the functional group. However, the molecular weights of peptides that can be chemically synthesized are usually limited and low. By contrast, for protein biosynthesis, molecular weights of the biologically synthesized proteins are practically unlimited.

Nonprotein amino acids with no cytotoxicity have been known to be incorporated into proteins. For examples, tyrosine and tryptophan residues in some proteins have been substituted with *m*-fluorotyrosine and 4-fluorotryptophan, respectively, without any effects on the protein functions, and the ^{19}F nuclei have been used as magnetic resonance probes for studying protein structures (2–5). Note that such fluorine substitution does not affect protein function, and thus substituted proteins may well be accumulated in the cell because those proteins do not disturb the physiology of the host cell. By contrast, cytotoxic nonprotein amino acids are expected to be useful for effective modifications of protein structures and functions.

Attempting to develop a method for incorporating cytotoxic amino acids into proteins, in the present study we chose L-2-aminohexanoic acid (Ahx; L-norleucine). This amino acid is similar in chemical structure to L-methionine (Fig. 1) and may be incorporated into proteins (6, 7). As the target protein for incorporation of Ahx, we chose human epidermal growth factor (hEGF) (8) because this protein has only one methionine residue, which appears important for activity (9). After surveying the conditions for Ahx administration in *Escherichia coli* cells and subsequently isolating the substituted protein, we were able to prepare Ahx-substituted hEGF ([Ahx^{21}]hEGF).

MATERIALS AND METHODS

***E. coli* Strain and Plasmid.** *E. coli* strain YK537 (F$^-$ *leuB6 thi hsdR hsdM lacY rpsL20 galK2 ara-14 xyl-5 mtl-1 supE44 endI$^-$ phoA8 recA1*) was used as the host cell, and the plasmid for the biosynthesis of hEGF and [Ahx^{21}]hEGF was pTA1522, which bears the hEGF gene with the promoter and the signal peptide sequence of *E. coli* alkaline phosphatase gene (*phoA*) (10).

Culture of *E. coli*. *E. coli* YK537 strain harboring plasmid pTA1522 was cultured overnight in 2.4 liters of high-phosphate medium TG+20 (containing 640 μM KH_2PO_4) at 37°C (11). The cells were collected by centrifugation and suspended in 2.4 liters of low-phosphate medium TG+1 (containing 32 μM KH_2PO_4) (11), into which Ahx (L-norleucine; Sigma) was added. *E. coli* cells were cultured in this medium for 6 hr at 37°C. Cells were collected by centrifugation and resuspended in 600 ml of 30 mM Tris·HCl buffer, pH 8.0, containing 1 mM EDTA and 20% (wt/vol) sucrose at room temperature. After 10 min, cells were col-

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Abbreviations: EGF, epidermal growth factor; hEGF, human EGF; [Ahx^{21}]hEGF, L-2-aminohexanoic acid-substituted hEGF; Ahx, L-2-aminohexanoic acid; Ptc, phenylthiocarbamoyl.

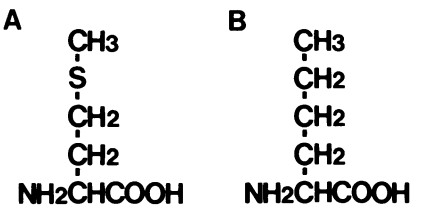

FIG. 1. Structures of methionine (*A*) and Ahx (*B*).

lected again by centrifugation, resuspended in 360 ml of cold water (osmotic shock), and retained on ice for 15 min. This suspension was subjected to centrifugation, and the supernatant containing hEGF and [Ahx^{21}]hEGF was lyophilized.

Recovery of hEGF and [Ahx^{21}]hEGF. The lyophilizate was dissolved in 24 ml of 25 mM ammonium acetate buffer, pH 5.8 (buffer A), and loaded on a column (2.6 × 90 cm) of Sephadex G-50 (particle size 50–150 μm) equilibrated with buffer A. Isocratic elution was done with buffer A at a flow rate of 0.9 ml/min. Fractions of 9 ml were collected and analyzed by the Ouchterlony double-diffusion analysis (12) with an anti-hEGF antibody. The fractions containing hEGF (and [Ahx^{21}]hEGF) were pooled and lyophilized. The lyophilizate was then dissolved in 5 ml of 20% CH_3CN/0.1% trifluoroacetate (vol/vol) and subjected to HPLC (LC-4A system; Shimadzu, Kyoto, Japan) with an ODS-120T column (0.46 × 15 cm) (Tosoh, Tokyo). Elution was done with a linear gradient of 20–40% CH_3CN at a flow rate of 1 ml/min. The peak that eluted at the same retention time as that of authentic hEGF contained partially purified hEGF and [Ahx^{21}]hEGF.

Oxidation of Methionine Residue of hEGF. This partially purified preparation of hEGF/[Ahx^{21}]hEGF (0.2 mg) was treated with 0.3% H_2O_2 for 30 min at room temperature. The oxidized preparation was then lyophilized, redissolved in 500 μl of 20% CH_3CN/0.1% trifluoroacetate, and subjected to HPLC (LC-4A system; Shimadzu) on an ODS-120T column. Elution occurred with the same linear gradient as that for the preparation before being oxidized.

Removal of Truncated [Ahx^{21}]hEGF. Ion-exchange HPLC (LC-6A system; Shimadzu) with a DEAE-5PW column (0.75 × 7.5 cm) (Tosoh) was applied for removing contamination from truncated proteins (such as [Ahx^{21},des-Leu^{52},Arg^{53}]-hEGF) in [Ahx^{21}]hEGF. Elution was done with a linear gradient of 100–400 mM ammonium acetate buffer, pH 6.0, at a flow rate of 0.5 ml/min.

Amino Acid Analysis. The purified preparation of [Ahx^{21}]hEGF or authentic hEGF was hydrolyzed in 6 M HCl for 24 hr at 115°C, and amino acid composition was analyzed (13, 14). The mixture of phenylthiocarbamoyl (Ptc) derivatives of amino acids were dissolved in 50 mM ammonium acetate buffer, pH 6.8, and the compositions of Ptc amino acids were analyzed by use of a Shimadzu LC-6A HPLC system with an ODS-80TM column (0.46 × 15 cm) (Tosoh). An appropriate gradient program was worked out (see Fig. 4), so that the elution peak of Ptc Ahx was clearly separated from those of Ptc derivatives of naturally occurring amino acids.

Activity Assay of [Ahx^{21}]hEGF. The receptor-binding activity of [Ahx^{21}]hEGF was assayed using human KB cells (Dainippon Pharmaceutical, Osaka) and ^{125}I-labeled mouse EGF as described (15). Stimulation of DNA synthesis by [Ahx^{21}]hEGF was assayed using Swiss albino mouse 3T3 cells (Dainippon Pharmaceutical) (15). The capacity for stimulation of cell proliferation was measured using Swiss albino 3T3 cells. Cells were seeded at a density of 1.25 × 10^4 cells per well in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo) containing 5% fetal bovine serum (M. A. Bioproducts Makor Chemicals, Walkersville, MD) in 24-well dishes (1.9 cm^2, Nunc). After incubation for 18–24 hr at 37°C, the medium was replaced with fresh DMEM/0.5% fetal bovine serum containing either hEGF or [Ahx^{21}]hEGF (0.01–10 ng/ml). After incubation for 6 days at 37°C, cell numbers were determined with a hemocytometer (Erma Optical Works, Tokyo).

RESULTS

Administration of Ahx. *E. coli* YK537 strain harboring plasmid pTA1522 was cultured in the high-phosphate medium, and 15 g (wet weight) of *E. coli* cells was collected. The cells were then cultured in low-phosphate medium into which Ahx was added for the present study. To survey appropriate conditions for biosynthesis of [Ahx^{21}]hEGF, the concentration of Ahx in the medium was varied over a wide range (0–4 mg/ml). Ahx at the higher concentrations tends to reduce the amount of hEGF (and [Ahx^{21}]hEGF) finally obtained, but at a concentration range of 0.2–2 mg/ml, the final yield of [Ahx^{21}]hEGF did not vary significantly. Therefore, for larger-scale production of [Ahx^{21}]hEGF, Ahx was added to the low-phosphate medium to the concentration of 0.25 mg/ml.

E. coli cells cultured for 6 hr in low-phosphate medium containing Ahx were harvested and subjected to osmotic shock to recover hEGF and [Ahx^{21}]hEGF, which accumulated in the periplasm. Then the cell-suspension supernatant containing hEGF and [Ahx^{21}]hEGF was lyophilized and loaded on a column of Sephadex G-50. The elution profile of this lyophilizate (Fig. 2) from *E. coli* cells cultured with Ahx (0.25 mg/ml) was nearly the same as that from cells cultured without Ahx.

Isolation of hEGF and [Ahx^{21}]hEGF. The hEGF content of elution fractions from Sephadex G-50 column chromatography was assayed with anti-hEGF antibody. Because retention time of proteins on gel filtration depends primarily on molecular weight, the retention time of [Ahx^{21}]hEGF practically equals that of hEGF. Accordingly, fractions 29–36 (Fig. 2) were pooled to recover [Ahx^{21}]hEGF (and hEGF) and lyophilized. The lyophilizate was subjected to reverse-phase HPLC. Linear gradient elution was done, and only one major peak (with a shoulder) was seen (Fig. 3*A*) at the same retention time as that of authentic hEGF. This major fraction was found, by a preliminary amino acid analysis, to contain Ahx in addition to L-methionine, indicating that [Ahx^{21}]-hEGF was eluted together with hEGF.

Purification of [Ahx^{21}]hEGF. The mixture of hEGF and [Ahx^{21}]hEGF from the major fraction of reverse-phase HPLC (Fig. 3*A*, marked with a thick bar) was treated with H_2O_2 and subjected again to reverse-phase HPLC. By this treatment, the methionine residue of hEGF was oxidized, whereas the Ahx residue of [Ahx^{21}]hEGF was unaffected. Two major peaks were clearly separated from each other (Fig. 3*B*). Peak a is probably due to methionine-oxidized

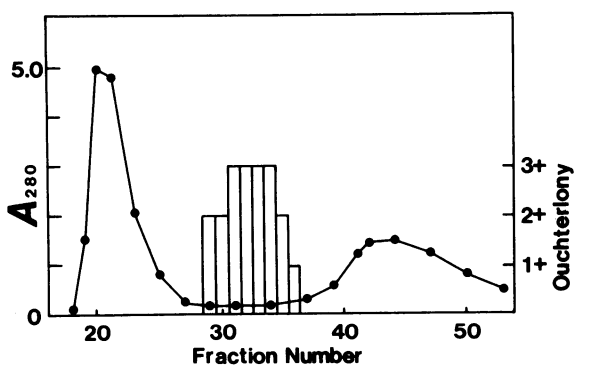

FIG. 2. Sephadex G-50 column chromatography of lyophilizate from *E. coli* periplasm. Protein content was monitored with UV absorbance at 280 nm (A_{280}), and the titer of hEGF was analyzed by Ouchterlony double-diffusion analysis (12).

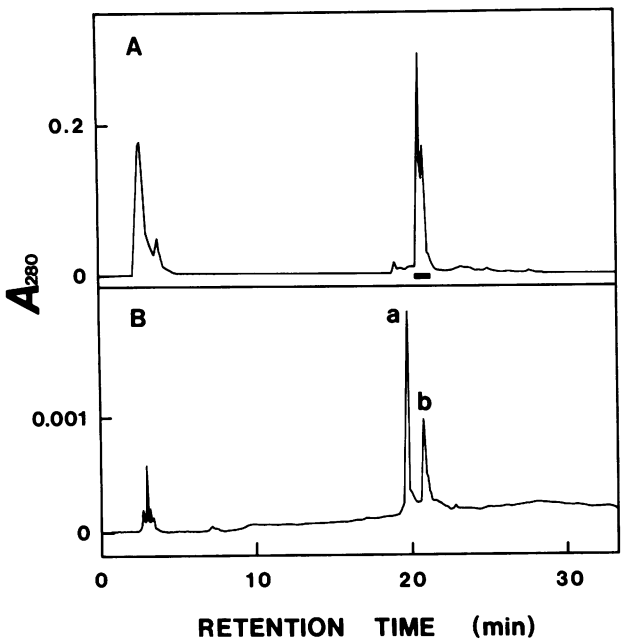

FIG. 3. Reverse-phase HPLC of partially purified hEGF and [Ahx^{21}]hEGF. (*A*) Lyophilizate containing hEGF/[Ahx^{21}]hEGF (10% of the lyophilizate was applied). (*B*) Oxidized preparation from hEGF/[Ahx^{21}]hEGF (*A*, marked with a bar) (0.2% of the oxidized preparation was applied).

hEGF because oxidation of methionine residue in a protein shortens the retention time in reverse-phase HPLC. By contrast, peak b, eluted at nearly the same retention time as that of authentic hEGF, is due to [Ahx^{21}]hEGF. Note that the area ratio of peak b ([Ahx^{21}]hEGF) and peak a (hEGF) is 25%.

The partially purified preparation of [Ahx^{21}]hEGF was then subjected to ion-exchange HPLC for removing contamination from C-terminal truncated proteins, and the first main peak containing [Ahx^{21}]hEGF was collected. Thus, finally 40 μg of purified [Ahx^{21}]hEGF was prepared from the *E. coli* cells that were cultured in 2.4 liters of low-phosphate medium containing 0.25 mg/ml of Ahx. To confirm purity, the final preparation of [Ahx^{21}]hEGF was subjected to reverse-phase HPLC, and, in fact, a single peak was seen in the elution profile.

Amino Acid Analysis of [Ahx^{21}]hEGF. Amino acid analysis of the purified preparation of putative [Ahx^{21}]hEGF (in comparison with hEGF) was done to confirm that the methionine residue of hEGF was substituted with an Ahx residue in [Ahx^{21}]hEGF. In the elution profiles of Ptc derivatives of amino acids from the hydrolysate of [Ahx^{21}]-hEGF (Fig. 4), the elution peak of Ptc Ahx was clearly seen, but the peak of Ptc methionine was missing. Area of elution peaks corresponding to 14 amino acids was measured, and amino acid composition was obtained (Table 1). Except for L-methionine and Ahx, amino acid composition of [Ahx^{21}]hEGF was practically the same as that of hEGF. In particular, the content of L-leucine, L-isoleucine, and L-valine did not decrease from hEGF to [Ahx^{21}]hEGF, discarding the possibility that these amino acids had been replaced by Ahx. All these results confirm that the methionine residue in position 21 of hEGF was substituted with Ahx by adding Ahx to the low-phosphate culture medium for *E. coli* strain YK537 harboring plasmid pTA1522.

Biological Activity of [Ahx^{21}]hEGF. To examine the effect of Ahx substitution in hEGF, cell numbers after 6-day incubation in medium containing either hEGF or [Ahx^{21}]-hEGF (0.01 to 10 ng/ml) were measured. Fig. 5 shows that [Ahx^{21}]hEGF has similar capacity for stimulation of cell

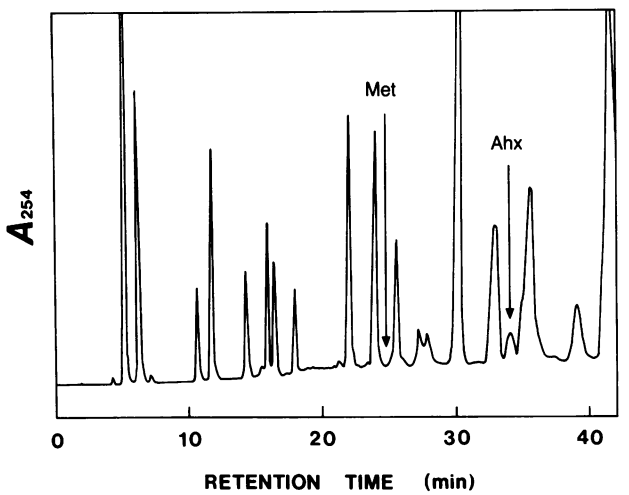

FIG. 4. Reverse-phase HPLC of Ptc derivatives of amino acids from the hydrolysate of [Ahx^{21}]hEGF. Elution was done with a linear gradient of 50 mM ammonium acetate buffer (pH 6.8) and 100 mM ammonium acetate buffer [pH 6.8, containing 80% (vol/vol) methanol], where the volume fraction of the second buffer was 5% → 25% for the first 10 min, 25% → 35% for the next 1 min, and 35% → 60% for the last 27 min. The flow rate was 1 ml/min.

proliferation as hEGF. Similarly, the receptor-binding activity and the capacity for stimulation of DNA synthesis of [Ahx^{21}]hEGF were nearly the same as those of hEGF (data not shown).

DISCUSSION

Incorporation of Nonprotein Amino Acids into Proteins. In protein biosynthesis, aminoacyl-tRNA synthetases activate cognate amino acids and attach them to cognate tRNA species. Among the family of 20 protein-constituting amino acids, discrimination of the cognate amino acid from other amino acids by an aminoacyl-tRNA synthetase is strict (16). However, the discrimination against nonprotein amino acids is not necessarily strict. In fact, some nonprotein amino acids bearing close structural similarity to protein-constituting amino acids have been found incorporated in proteins (7); incorporation of such nonprotein amino acids into the polypeptide chains will affect the biosynthesis, folding, and functions of proteins. Thus, the nonprotein amino acids that can be incorporated into proteins are useful as "inhibitors" of protein processing, secretion, and turnover (7).

Table 1. Amino acid composition, residue per mole

Amino acid	hEGF		[Ahx^{21}]hEGF, analyzed
	Predicted	Analyzed	
Ahx	0	0.0	1.3
Ala	2	2.0	2.2
Arg	3	3.4	3.1
Asx	7	6.8	6.5
Gly	4	4.0	4.1
Glx	5	5.1	5.0
His	2	2.6	2.4
Ile, Leu	7	7.0	6.8
Met	1	0.9	0.0
Phe	0	0.0	0.0
Pro	1	1.4	1.4
Ser	3	2.3	1.7
Thr	0	0.0	0.0
Val	3	2.4	3.4

Compositions of Cys, Lys, Trp, and Tyr were not determined.

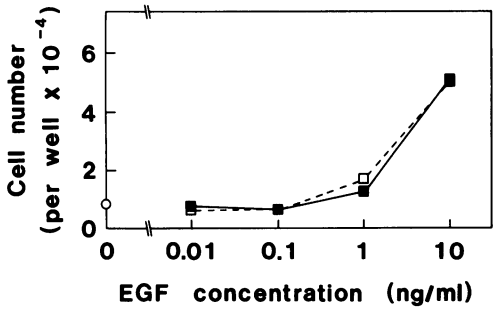

FIG. 5. Effects of [Ahx^{21}]hEGF and hEGF on the proliferation of Swiss albino mouse 3T3 cells. Each cell number (per well) was determined with the error of ≈0.3 × 10^4. ■, [Ahx^{21}]hEGF; □, hEGF; ○, control (without [Ahx^{21}]hEGF or hEGF).

Production of Proteins Substituted with Nonprotein Amino Acids. Even when a nonprotein amino acid was incorporated into cellular proteins, substituted proteins were not obtained in most cases (7). Thus, it was not possible to prepare *E. coli* aspartate transcarbamoylase substituted with the antibiotic amino acid canavanine by adding canavanine to the culture medium when the genomic gene of this protein was being induced (17). Probably this difficulty is mainly due to a response similar to the heat shock response (18).

However, Schlesinger and co-workers (19–23) succeeded in preparing *E. coli* alkaline phosphatase substituted with a variety of nonprotein amino acids, including canavanine. This success appeared to us to be primarily due to induction of the genomic gene (*phoA*) by lowering the phosphate concentration of the culture medium (24) and also to the secretion of the protein substituted with nonprotein amino acid (to be discussed later). Accordingly, in this study, we tried to develop a method for producing proteins substituted with nonprotein amino acids (alloproteins) by the use of a recombinant plasmid equipped with the *phoA* promoter and the signal peptide sequence of the alkaline phosphatase gene (10).

Biosynthesis of [Ahx^{21}]hEGF. We chose the *phoA* promoter-signal peptide system for the biosynthesis and secretion of hEGF (10). *E. coli* strain YK537 harboring plasmid pTA1522 (10) was cultured, in the first stage, in high-phosphate medium. The cells were then transferred to low-phosphate medium to induce gene expression of the fusion polypeptide, *phoA* signal peptide-hEGF, and subsequent secretion of hEGF into the periplasm (10). We thought that this procedure might also allow the secretion of hEGF substituted with nonprotein amino acid. For a nonprotein amino acid to be incorporated in hEGF, we selected Ahx, which replaces methionine residues in proteins (6, 7). hEGF contains only one methionine residue (in position 21) (8), which lies in the region of the protein structure apparently important for its activity (9).

When expression of the hEGF gene under the control of *phoA* promoter was induced by transfer of cells to the low-phosphate medium, Ahx was added to the medium. This addition of Ahx at higher concentration should raise the ratio of Ahx/L-methionine in the medium and enhance substitution of the Met-21 residue of hEGF with Ahx. However, the final yield of [Ahx^{21}]hEGF was not improved by addition of Ahx at higher concentrations tested; therefore, the concentration of added Ahx was 0.25 mg/ml, yielding a ratio of [Ahx^{21}]-hEGF to hEGF as high as 25% (Fig. 3*B*). In purifying [Ahx^{21}]hEGF, oxidation with H_2O_2 was useful for the one-step HPLC separation from hEGF. Thus, we were able to prepare [Ahx^{21}]hEGF using the *phoA* induction-secretion system. In this method for the production of alloprotein, we must have largely avoided the undesirable effects of incorporating nonprotein amino acids into cell proteins.

Effects of Incorporation of Nonprotein Amino Acids into Cell Proteins. The first difficulty in the designed biosynthesis of alloprotein is that the addition of nonprotein amino acids—in particular, cytotoxic amino acids—into the culture medium tends to depress the cell growth and, in the worst cases, to kill the cells (6). This toxicity is probably due to nonspecific substitution with nonprotein amino acids (6) in proteins that are vital to cells. Such substitution with nonprotein amino acids will, more or less, affect the protein conformation and functions, which will not favor cell physiology. For example, an antibiotic amino acid, canavanine, is probably incorporated in place of arginine residues in proteins vital to normal replication and transcription (25).

Stimulation of Transcription of Heat Shock Genes by Nonprotein Amino Acids. The second difficulty is that nonprotein amino acids, in particular cytotoxic amino acids, induce transcription of heat shock genes (18). Such a heat shock-like response is triggered by the accumulation in the cytoplasm of conformationally aberrant polypeptides, resulting from the use of nonprotein amino acids in protein biosynthesis (18). In the heat shock response, expression of heat shock genes with the "heat shock promoter" is remarkably enhanced, whereas expression of other genes with normal promoters is depressed (26). One such heat shock protein, protease La (27), is responsible for the hydrolysis of aberrant proteins synthesized in abnormal environments (26). Because of this heat shock induction of protease La, the rate of protein degradation is significantly raised (28–30). Thus, the heat shock-like response by nonprotein amino acids probably depresses the transcription of the gene in the plasmid and enhances the degradation of translation products.

Stimulation of Transcription of *pho* Regulon Genes. These two difficulties must be overcome in any *in vivo* synthesis of proteins substituted with nonprotein amino acids. In our study plasmid pTA1522, which has the *phoA* promoter-*phoA* signal peptide-hEGF gene (10), was used. Transfer of *E. coli* cells (after being cultured in the high-phosphate medium) to the low-phosphate medium specifically induces the transcription of a set of genes of "*pho* regulon" that code for periplasm and outer-membrane proteins involved in the essential adaptation to the low-phosphate medium (31, 32). The *phoA* gene coding for alkaline phosphatase is one gene of the *phoA* regulon (31, 32). By contrast, the biosynthesis from non-*pho* regulon genes is depressed, and cell growth is significantly slow (24).

***phoA* Promoter in Plasmid Is Useful.** Under these circumstances, the major difficulties in biosynthesis of proteins substituted with nonprotein amino acids can be overcome simultaneously, provided that the gene of the protein for the incorporation is under the control of the *phoA* promoter in the plasmid. The biosynthesis of vital proteins for replication and transcription is not active in this condition, so that toxicity of the nonprotein amino acid is not serious. Thus, the amount of aberrant polypeptides in the cytoplasm is probably kept small, even in the presence of nonprotein amino acid. This situation avoids the onset of the heat shock-like response, which induces the transcription of heat shock genes but depresses the transcription of genes (hEGF gene in this study) in the plasmid.

Degradation of Alloprotein Is Avoided. Because the heat shock-like response by nonprotein amino acids was not serious under our conditions for alloprotein synthesis, protease La, which was primarily responsible for the hydrolysis of aberrant proteins (28–30), was not significantly induced. Therefore, degradation of alloprotein was avoided. Furthermore, in this method, the alloprotein [Ahx^{21}]hEGF is secreted into the periplasm. Consequently, [Ahx^{21}]hEGF was successfully kept from degradation by cytoplasmic proteases. Thus, for the recovery of alloproteins, the present

system, including the *pho* regulon induction and the secretion to the periplasm, is useful.

Ahx Substitution of hEGF. hEGF has an antiparallel β-sheet structure bearing the methionine residue in position 21 (33). Substitution of the methionine residue with Ahx (replacement of a sulfur atom by a methylene group) could affect EGF conformation. However, in this study, the biological activities of [Ahx^{21}]hEGF were the same as those of hEGF, suggesting that the Met → Ahx substitution did not significantly affect hEGF conformation. A Met → Ahx substitution should be useful for stabilizing the conformation of a protein, the activity of which is lost upon oxidation of a key methionine residue.

Concluding Remarks. The basic strategy for the biosynthesis of alloprotein was accomplished. Thus, secretion to the periplasm proves useful for depressing the heat shock-like response and for avoiding the degradation of alloprotein. Success of the designed biosynthesis of [Ahx^{21}]hEGF has encouraged us in our attempt to develop practical methods for the synthesis of alloproteins. The final yield of [Ahx^{21}]-hEGF was appreciably lower than the yield of hEGF synthesized without Ahx. No doubt a more efficient method for the biosynthesis of alloproteins would be desirable. Note also that hEGF has only one methionine residue, which was replaced by Ahx in these experiments. A more general method for the site-specific incorporation of nonprotein amino acids is a challenging subject for further study.

The design of specific nonprotein amino acids will be important to the development of useful alloproteins. For efficient biosynthesis, such nonprotein amino acids should be recognized and efficiently attached to tRNA species by aminoacyl-tRNA synthetase. We have already found, for *E. coli* isoleucyl-tRNA synthetase, that the conformation of an amino acid bound to the synthetase can be elucidated by the analysis of transferred nuclear Overhauser effect (34). Such an analysis has application for studying which moieties of amino acids are recognized by the synthetase and can provide a guide in the design of nonprotein amino acids for alloproteins.

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