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Finding of an isoleucine derivative of a recombinant protein for pharmaceutical use

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Abstract

Protein modification generally occurs by addition to the amino acid side-chains of protein at the post-translational stage, for example, by enzymatic or chemical reactions after polypeptide synthesis. Recently, the recombinant hirudin analog CX-397, a potent thrombin inhibitor, was found to contain methylated Ile residues when it was overproduced by *Escherichia coli* in the absence of amino acids in the culture medium. The Ile derivatives, deduced to be β-methylnorleucine [βMeNle; (2*S*, 3*S*)-2-amino-3-methylhexanoic acid] by systematic chromatographic analysis, do not appear to be normal post-translational modifications of the protein because Ile has no functional group in its side-chain. We, therefore, propose that βMeNle is biosynthesized by *E. coli*, activated by *E. coli* isoleucyl-tRNA synthetase (IleRS), then incorporated into the overproduced recombinant hirudin analog. The biosynthesis of βMeNle in *E. coli* is thought to occur as follows: α-ketovalerate is synthesized from α-ketobutyrate by three Leu biosynthetic enzymes, α-isopropylmalate synthase (IPMS) (EC 4.1.3.12), α-isopropylmalate isomerase (ISOM) (EC 4.2.1.33) and β-isopropylmalate dehydrogenase (IPMD) (EC 1.1.1.85), which have broad substrate specificities. α-Ketovalerate is then converted to α-keto-β-methylcaproate by three Ile and Val biosynthetic enzymes, acetohydroxy acid synthase (AS) (EC 4.1.3.18), acetohydroxy acid isomeroreductase (IR) (EC 1.1.1.86) and dihydroxy acid dehydratase (DH) (EC 4.2.1.9). Finally, this is converted to βMeNle by branched-chain amino acid transaminase (EC 2.6.1.42), one of the Ile and Val biosynthetic enzymes.

Keywords: Recombinant hirudin analog; CX-397; Thrombin; β-Methylnorleucine; Isoleucyl–tRNA synthetase; *Escherichia coli*

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1. Introduction

A number of proteins of pharmaceutical interest have been produced by recombinant DNA techniques, and the most widely used host cell culture for this purpose is *Escherichia coli*. The main advantages of using *E. coli* as the host for large-scale manufacturing of recombinant proteins are firstly that its cells will grow logarithmically in a simple medium and secondly that it is able to biosynthesize all natural amino acids within its cells; thus, amino acids do not have to be added to the culture medium as a nitrogen source, and manufacturing costs are decreased. Furthermore, although high-level expression of recombinant proteins in *E. coli* often results in the formation of insoluble and inactive aggregates known as inclusion bodies [1], recent advances in procedures for refolding inclusion body proteins have made it possible to obtain large yields of the active forms [2]. Several recombinant proteins produced by *E. coli*, such as hormones [3–5] and cytokines [6–8], which can only be obtained in trace amounts from natural sources, are used in human medicine. However, little information is available to determine whether uniformity of the product's impurity profile can be guaranteed when recombinant proteins are produced on a manufacturing scale in *E. coli* using simple, inexpensive processes as opposed to on a laboratory-scale. Impurity-profile data allow improvements in the manufacturing process to be designed; furthermore, identification of even minor impurities in products destined for therapeutic use is a significant issue for quality control. We consider that any information on impurities generated during the manufacturing scale production of recombinant proteins is important for further developments in biotechnology, and should be made widely available.

Hirudin, a potent and highly specific inhibitor of thrombin, is a single polypeptide containing 64–66 amino acid residues, and is isolated from the medicinal leech, *Hirudo medicinalis* [9,10]. Since only trace amounts of natural hirudin can be obtained from the leech, large-scale production of recombinant hirudin has been undertaken by recombinant DNA technology using *E. coli* as a host [11–14]. The naturally occurring hirudin variant-1 contains two Tyr residues at positions 3 and 63, the latter of which is sulfated [15]. This natural hirudin has been found to have 10-fold higher antithrombin activity than unsulfated recombinant hirudin [16]. Sulfation of the Tyr residue occurs due to post-translational modification of the protein by tyrosylprotein sulfotransferase, which is located in leech salivary glands [17]. We have achieved enzymatic sulfation of Tyr residues in a recombinant hirudin variant-1 analog using a sulfotransferase isolated from an anaerobic bacterium from the human intestine, *Eubacterium* A-44 [18]. Such enzymatic post-translational modifications have been shown to confer the advantage of increased antithrombin activity on recombinant hirudins [17,18].

A further important aspect during the production of recombinant hirudins is their stability in solution or after lyophylization, and well-conducted stability studies are performed on such formulations. When recombinant hirudin variant-2 (Lys47) is incubated under physiological conditions, spontaneous deamidation is observed in the Asn–Gly sequence in the hirudin molecule [19,20]. Deamidation of the Asn residue leads to the formation of a succinimide (cyclic imide) intermediate within the molecule; on hydrolysis, this yields a mixture of the normal aspartyl form (Asp–Gly) and an abnormal isoaspartyl form (isoAsp–Gly). Asp–Gly, Asn–Gly or Asn–Ser sites in other recombinant proteins, including human growth hormone and human tissue plasminogen activator, have also been observed to produce the succinimide intermediate or be hydrolyzed to the isoAsp form under heat-stress after lyophilization or in aqueous solution [21–23]. The ratio of isoAsp–Gly to Asp–Gly linkages after succinimide hydrolyzation is approximately 3:1, an important consideration because the isoAsp form shows decreased biological activity [24]. Furthermore, the presence of isoAsp may increase the immunogenicity of certain proteins, leading to autoimmune reactions [25,26]. Further details of the chemical mechanisms involved in isoAsp formation at Asn and Asp residues can be obtained from reviews by Aswad et al. [24] and Clarke et al. [27].

Table 1
Isolation and structural characterization of post-translationally modified hirudins

Hirudin	Position	Formation	Origin or condition	Reference
Recombinant				
variant-1	Asp33–Gly34, Asp53–Gly54	succinimide	in acidic conditions or lyophilization	[a,b]
variant-1	Thr45	phosphorylation	in fermentation broth	[c]
variant-1	Tyr63	phosphorylation	by tyrosine kinase III	[d]
variant-1	Tyr63	sulfation	by tyrosylprotein sulfotransferase	[e]
variant-1	Tyr3, Tyr63	nitration, iodination	by tetranitromethane, sodium iodide	[f]
variant-1	Leu64, Gln65	degradation	by carboxypeptidase	[g]
variant-1 (Tyr61, 62)	Tyr62, Tyr63	sulfation	by sulfotransferase	[18]
variant-2 (Lys47)	Asn33, Asn52, Asn53	deamidation	in phosphate buffer pH 9, 37 °C	[19]
variant-2 (Lys47)	Asn33–Gly34, Asn53–Gly54	succinimide	as impurities in product	[20]
variant-2 (Lys47)	Tyr63	sulfation	by BHK cell	[h]
variant-2 (Lys47)	Tyr63	sulfation	by tyrosylprotein sulfotransferase	[17]
variant-2 (Lys47)	Tyr3, Tyr63	iodination	by chloramine-T	[i]
Naturally occurring				
variant-1	Tyr63	sulfation	*Hirudo medicinalis*	[15,28]
variant-3	Tyr63	sulfation	*Hirudo medicinalis*	[29]
P6, P18	Thr45 (P6), Thr50 (P18)	glycosylation	*Hirudinaria manillensis*	[j]

[a] H. Grossenbacher et al., Rapid Commun. Mass Spectrom. 7 (1993) 1082–1085.
[b] P. Schindler et al., J. Mass Spectrom. 24 (1996) 967–974.
[c] M. Coulot et al., J. Mol. Struct. 292 (1993) 89–104.
[d] J. Hofsteenge et al., Eur. J. Biochem. 188 (1990) 55–59.
[e] M. Suiko et al., Nucleic Acids Symp. Ser. 27 (1992) 183–184.
[f] R.C. Winant et al., Biochemistry 30 (1991) 1271–1277.
[g] J. Heim et al., Eur. J. Biochem. 226 (1994) 341–353.
[h] T. Skern et al., FEBS lett. 275 (1990) 36–38.
[i] A. Tuong et al., Anal. Biochem. 189 (1990) 186–191.
[j] V. Steiner et al., Biochemistry 31 (1992) 2294–2298.

During the synthesis of a recombinant protein, normal amino acids are assembled into the polypeptide chain in the sequence determined by the nucleotide sequence of the relevant gene, so succinimide-type modification to isoAsp at Asn–Gly sites after the synthesis of recombinant proteins can be regarded as a post-translational modification, like the sulfation of the Tyr residue described above. Based on these common modification pathways, previous reports concerning the isolation and characterization of modified hirudins are summarized in Table 1. Non-enzymatic methods may also be used to produce post-translational modifications of recombinant hirudins, for example nitration and iodination are conducted using chemical reagents (Table 1).

In this article, we describe the finding of two novel modified hirudins, detected as impurities in the final product after overproduction of a recombinant hirudin analog by *E. coli*. Structural analysis revealed that these modifications were due to methylation of one of the two Ile residues in the hirudin molecule. The pathway leading to the methylated Ile derivatives of the protein is not thought to involve normal enzymatic or non-enzymatic post-translational modification of the protein because Ile has no functional group in its side-chain. The impurities were only observed in the absence of amino acids in the culture medium as a nitrogen source. However, although the existence of impurities in the final product when restricted cultivation conditions were used is of some concern, a scientific explanation of the modification pathway would allow appropriate improvements to the manufacturing conditions to be introduced. Therefore, we also focus on the

possible mechanisms involved in the Ile modification, and speculate on the possible biosynthetic pathway in *E. coli*.

2. Production of recombinant hirudin

The recombinant hirudin analog CX-397 is a hybrid between hirudin variant-1 [28] and variant-3 [29] in terms of its amino acid sequence, and possesses antithrombin activity greater than that of either parent hirudin [30]. The active form of CX-397 was produced by the recombinant *E. coli* strain JM109 after transformation with the expression vector pCX397, and secreted directly into the culture medium. The expression vector was constructed by introducing synthetic DNA encoding CX-397 and an *E. coli* alkaline phosphatase signal peptide into a high-level expression vector [30], which was in turn constructed from pUC18, pKK223-3 and a synthetic tryptophan promoter. As for earlier clinical studies, CX-397 was produced on a manufacturing scale using a 1000-l fermenter, and we have confirmed high productivity (about 600 mg/l) using a culture medium containing an inorganic nitrogen source (ammonium sulfate) [31]. The product was purified by three column chromatography steps, using a butyl-Sepharose, a Sephadex G-25 and a Q-Sepharose column, respectively, then filtered using an Amicon spiral cartridge and a high-output stirred cell. The experimental procedures used in the cultivation and purification of CX-397 are described in more detail in our previous report [31].

3. Existence of βMeNle in recombinant hirudin

When CX-397 was produced to excess by *E. coli* in the absence of the usual amino acids, casamino acids (casein hydrolysate) in the culture medium, the presence of two minor impurities, each accounting for 0.3% of the total absorbance peak area for the final product and showing dramatically increased retention times, was observed on reverse-phase high-performance liquid chromatography (HPLC) analysis (Fig. 1A). Neither of these absorbance peaks were observed in the final product when amino acids were added to the cultivation medium (Fig. 1B). Both impurities showed almost complete hirudin activity in a thrombin inhibition assay (Table 2), demonstrating that they were derivatives of CX-397. The characterization of the two impurities by reverse-phase HPLC and electrospray ionization mass spectra (ESI-MS) analysis, and the results of the thrombin inhibition assay, are summarized in Table 2. The increase in the molecular mass led us to speculate that these derivatives had been modified by the addition of a methylene group (mass, 14 Da). Protein alkylation generally causes an increase in hydrophobicity, and the two derivatives did indeed exhibit hydrophobic behavior, as evidenced by their retention times on reverse-phase HPLC (Table 2). Amino acid composition analysis, together with a systematic chromatographic analysis involving peptide mapping with trypsin and phenylthiohydantoin (PTH)-derivative sequencing with Edman degradation, confirmed that, compared with authentic CX-397 fragments or chemically synthesized amino acid derivatives [31], the two impurities had been modified by methylation; peak 1 resulted from methylation at Ile59 and peak 2 from methylation at Ile29. As regards their molecular structures, both derivatives were identified as β-methylnorleucine (βMeNle; 2-amino-3-methylhexanoic acid) and had an *erythro*-stereochemical configuration (Table 2). To our knowledge, there have been no previous reports concerning the modification of Ile residues

Fig. 1. Reverse-phase HPLC profile of recombinant CX-397 produced by *E. coli* in the absence (A) or presence (B) of amino acids as a nitrogen source. CX-397 solution (1300 ATU) was subjected to reverse-phase HPLC on a C-18 column using a linear gradient of acetonitrile (ranging from 19 to 24% over 50 min) in a base solvent of 0.1% trifluoroacetic acid in water, run at a flow rate of 1.0 ml/min, as described previously [31]. The fraction containing unknown peaks 1 and 2 (indicated by arrowheads) showed almost the same antithrombin activity as CX-397 itself (see Table 2).

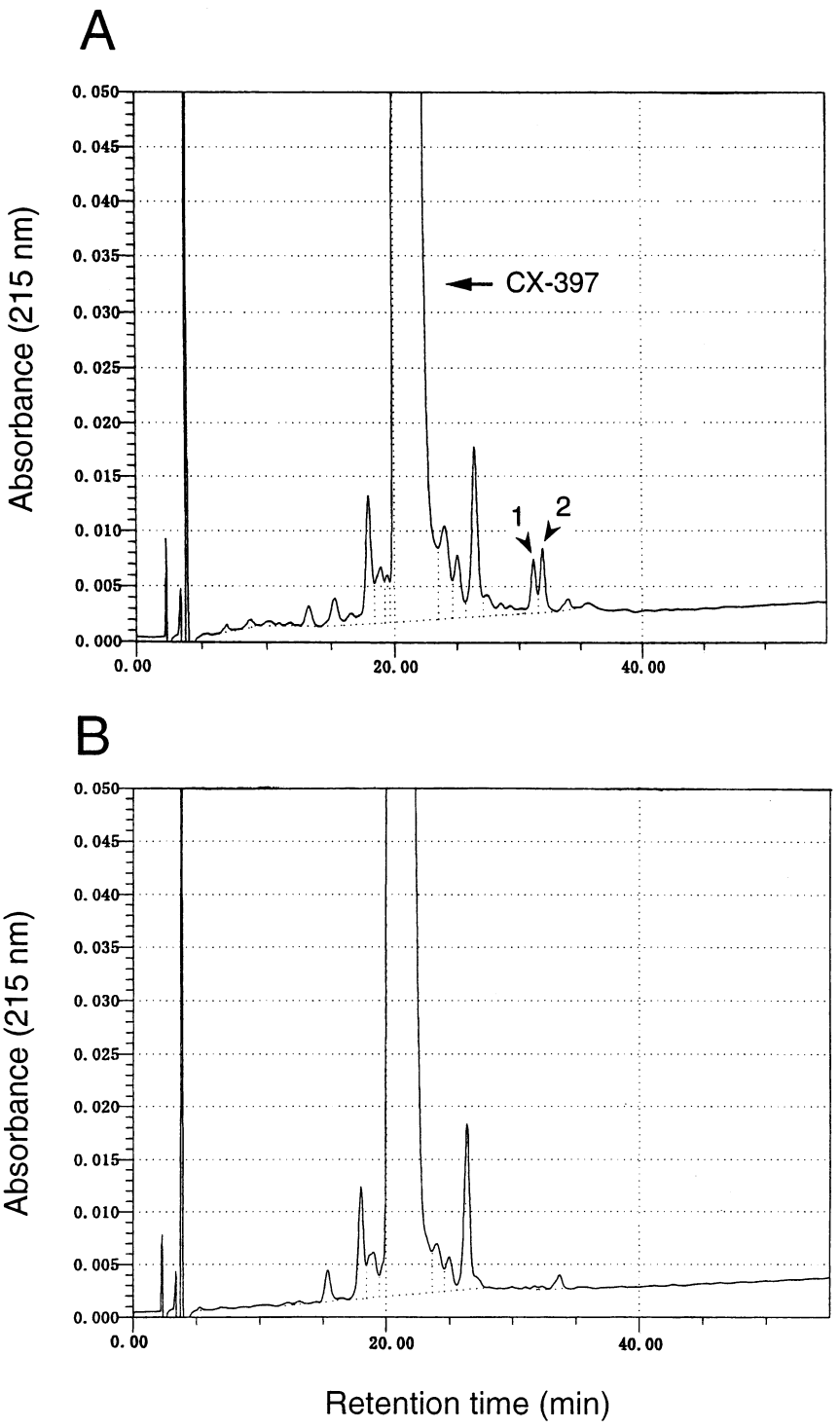
A
B
Absorbance (215 nm)
Absorbance (215 nm)
CX-397
1
2
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0.00
20.00
40.00
Retention time (min)

Fig. 1

in proteins, and it is unknown whether the presence of small amounts of βMeNle in a pharmaceutical protein would be harmful. However, based on the results of a hirudin–thrombin complex analysis [32,33], the inhibitory effects of the Ile29 and Ile59 derivatives on thrombin seem to be smaller than those of the N- and C-terminal regions of hirudin. Therefore, the presence of βMeNle in hirudin may affect the clearance of the protein rather than its activity, due to the hydrophobic nature of the methylene group.

4. Predicted pathway of βMeNle incorporation into recombinant hirudin

Generally, protein methylation occurs by enzymatic addition to the amino acid side-chains of the protein at the post-translational stage, after polypeptide synthesis [34,35]. Several reviews [36–38] have described the discovery of methylated amino acids and their roles in prokaryotes and eukaryotes. As is now well-known, enzymatic methylation of the basic and acidic amino acid residues of protein molecules is brought about by amino and carboxyl methyltransferases. However, no methyated derivatives of the side-chain of Ile have been identified to date. Since the Ile residue has no functional group in its branched-chain, methylation of the Ile residues in the CX-397 molecule is not thought to be a post-translational modification. However, during the translation phase of protein biosynthesis, nonprotein amino acids are recognized by aminoacyl-tRNA synthetases which activate each of the 20 primary amino acids (amino acid + ATP → aminoacyl-AMP + pyrophosphoric acid [PPi]), then transfer them to specific tRNA molecules (aminoacyl-AMP + tRNA → aminoacyl-tRNA + AMP). For example, norleucine (Nle; 2-aminohexanoic acid) is activated by methionyl-tRNA synthetase and can be incorporated into proteins instead of Met [39–41]. Since Nle has a similar chemical structure to Met, a basic method of biosynthesizing the human epidermal growth factor protein, in which Met is substituted with Nle by *E. coli*, has been established [42]. Recently, Yokoyama's group has found that a chemically synthesized Ile derivative, diethylalanine (2-amino-3-ethylpentanoic acid), can be recognized by *E. coli* isoleucyl-tRNA synthetase (IleRS). Indeed, this mono-methylated Ile derivative was found to bind to *E. coli* IleRS almost as tightly as the true substrate, Ile (Yokoyama et al., unpublished data). Furthermore, IleRS can recognize furanomycin [α-amino (2,5-dihydro-5-methyl) furan-2-acetic acid] [43], a synthetic amino acid antibiotic, and incorporate it into β-lactamase as efficiently as Ile [44]. Although furanomycin itself does not have a similar chemical structure to Ile, the conformation of IleRS-bound furanomycin is similar to that of Ile [44].

Chibata's group has reported that βMeNle is synthesized by α-aminobutyrate-resistant mutants of *Serratia marcescens* grown in a medium containing norvaline [45]. *S. marcescens* belongs to the same family, Enterobacteriaceae, as *E. coli*. These

Table 2
Structural characterization of two impurities and their antithrombotic activities

Sample	Retention time (min)[a]	Activity (ATU/mg)[b]	Molecular mass (Da)[c]	Positions[d]		Configurations[e]	
				29	59	29	59
CX-397	20.3	17 330	7004.4 ± 0.4	Ile	Ile	2*S*, 3*S*	2*S*, 3*S*
Peak 1	31.2	15 520	7018.3 ± 0.3	Ile	βMeNle	2*S*, 3*S*	*erythro*
Peak 2	31.9	14 650	7018.1 ± 0.6	βMeNle	Ile	*erythro*	2*S*, 3*S*

[a] Retention time on reverse-phase HPLC is from Fig. 1.
[b] One antithrombin unit (ATU) neutralizes 1 National Institutes of Health unit of thrombin.
[c] Average mass on ESI-MS was measured by scanning at 750–2000 *m*/*z* in 3 s. The theoretical molecular mass of CX-397 is 7004.5 Da.
[d] Two Ile residues are located at positions 29 and 59 in the molecule.
[e] Natural L-Ile biosynthesized by *E. coli* is 2*S*, 3*S* through the application of *R*/*S* rules.

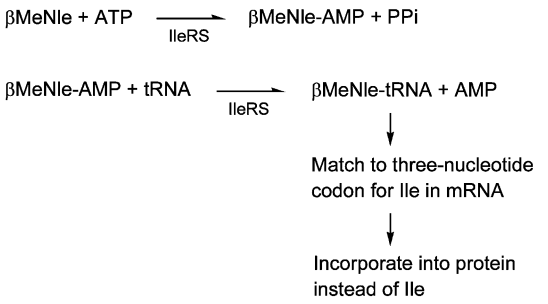

Fig. 2. Predicted pathway for the incorporation of βMeNle during protein synthesis. IleRS, isoleucyl-tRNA synthetase; PPi, pyrophosphoric acid.

findings strongly suggest that lack of amino acids in the culture medium leads *E. coli* to synthesize βMeNle, which is then activated by *E. coli* IleRS and incorporated into the overproduced recombinant protein (Fig. 2). The substrate specificity of *E. coli* IleRS may, therefore, be more ambiguous than that of the other aminoacyl-tRNA synthetases.

5. Predicted biosynthetic pathway of βMeNle

If βMeNle is activated and transferred to tRNA due to misrecognition by IleRS during the translation phase (as shown in Fig. 2), it must first be biosynthesized within *E. coli* cells. Fig. 3 shows the predicted biosynthetic pathway of βMeNle in *E. coli*. First, L-Thr is converted to α-ketobutyrate by threonine deaminase (TD). This route is involved in the normal biosynthetic pathway of Ile in *E. coli* [46–49]. Biotransformation to βMeNle may, however, occur if α-ketobutyrate subsequently reacts with acetyl-CoA under the influence of α-isopropylmalate synthase (IPMS), one of the Leu biosynthetic enzymes. Although the usual substrate of IPMS during the biosynthesis of Leu is a α-ketoisovalerate, this enzyme can convert α-ketobutyrate to α-ethylmalate due to its broad substrate specificity [50]. Next, α-ethylmalate would be converted to α-ketovalerate by the other Leu biosynthetic enzymes, α-isopropylmalate isomerase (ISOM) and β-isopropylmalate dehydrogenase (IPMD) (Fig. 3). α-Ketovalerate would then be converted to α-aceto-α-hydroxyvalerate (which has an active acetaldehyde group derived from pyruvate) by acetohydroxy acid synthase (AS), one of the Ile and Val biosynthetic enzymes. Finally, α-aceto-α-hydroxyvalerate would be converted to βMeNle via α, β-dihydroxy-β-methylcaproate and α-keto-β-methylcaproate by three Ile and Val biosynthetic enzymes, acetohydroxy acid isomeroreductase (IR), dihydroxy acid dehydratase (DH) and branched-chain amino acid transaminase, i.e. transaminase B (TrB) [51], as shown in Fig. 3. The final stereochemical configuration of βMeNle is predicted to be 2*S*,3*S* because, as shown in Table 2, this is the configuration of the natural L-Ile biosynthesized by *E. coli*. Although a double-modified derivative in which both the Ile29 and Ile59 residues are replaced by βMeNle ought theoretically to occur as an impurity in the product if our speculations are correct, no such derivative was observed, possibly because only extremely small amounts (below the limit of detection on reverse-phase HPLC) may be produced.

6. Conclusions

The pathway by which the Ile residues of a recombinant hirudin analog are methylated to βMeNle is not thought to involve the normal process of post-translational modification. We consider that a biosynthetic pathway for βMeNle is present in *E. coli*, and that, during the translation process from gene to protein, βMeNle is activated by *E. coli* IleRS and incorporated into the protein instead of Ile. The finding of this unnatural amino acid in a recombinant protein may have significance for the quality control of minor impurities in products for pharmaceutical use, in a similar way to protein heterogeneity resulting from isoAsp formation. Feedback on the impurity profiles of final products is important in allowing improvements to be made to the manufacturing process, e.g. alteration of cultivation conditions, thereby increasing the quality of the products and decreasing the risk of side effect caused either by the impurities themselves or by their metabolites.

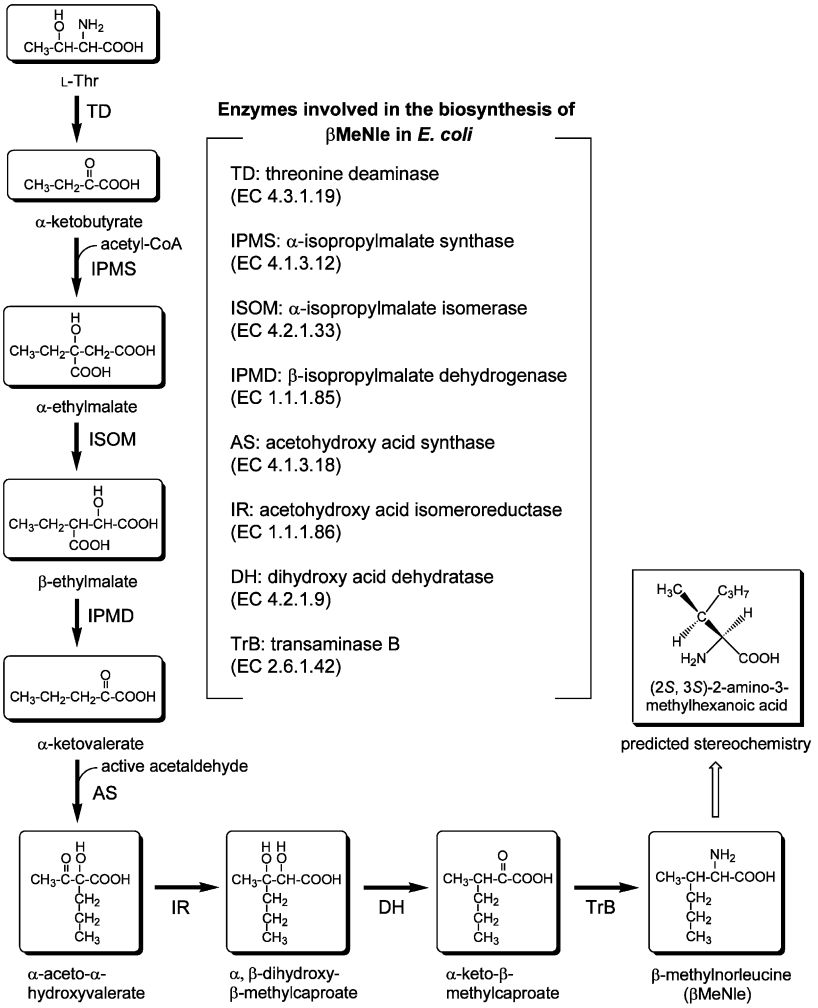

Fig. 3. Predicted biosynthetic pathway of βMeNle in *E. coli*. The biosynthetic pathway from Thr to βMeNle is indicated by the solid arrows. The *erythro*-stereochemical configuration of βMeNle, identified from its chromatographic behavior, is thought to be 2*S*, 3*S* (as indicated by the open arrow), because this is the configuration of the natural Ile biosynthesized by *E. coli*.

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References

[1] J.F. Kane, D.L. Hartley, Trends Biotechnol. 6 (1988) 95–101.
[2] S. Misawa, I. Kumagai, Biopolymers 51 (1999) 297–307.
[3] K. Itakura, T. Hirose, R. Crea, A.D. Riggs, H.L. Heyneker, F. Bolivar, H.W. Boyer, Science 198 (1977) 1056–1063.

[4] D.V. Goeddel, D.G. Kleid, F. Bolivar, H.L. Heyneker, D.G. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura, A.D. Riggs, Proc. Natl. Acad. Sci. USA 76 (1979) 106–110.
[5] D.V. Goeddel, H.L. Heyneker, T. Hozumi, R. Arentzen, K. Itakura, D.G. Yansura, M.J. Ross, G. Miozzari, R. Crea, P.H. Seeburg, Nature 281 (1979) 544–548.
[6] T. Staehelin, D.S. Hobbs, H. Kung, C.Y. Lai, S. Pestka, J. Biol. Chem. 256 (1981) 9750–9754.
[7] A.W. Burgess, C.G. Begley, G.R. Johnson, A.F. Lopez, D.J. Williamson, J.J. Mermod, R.J. Simpson, A. Schmitz, J.F. DeLamarter, Blood 69 (1987) 43–51.
[8] O.J. Nielsen, P. Costa-Giomi, R. Weinmann, A.J. Erslev, J. Caro, J. Immunol. Methods 111 (1988) 1–9.
[9] F. Markwardt, Methods Enzymol. 19 (1970) 924–932.
[10] M. Scharf, J. Engels, D. Tripier, FEBS Lett. 255 (1989) 105–110.
[11] R.P. Harvey, E. Degryse, L. Stefani, F. Schamber, J.-P. Cazenave, M. Courtney, P. Tolstoshev, J.-P. Lecocq, Proc. Natl. Acad. Sci. USA 83 (1986) 1084–1088.
[12] J. Dodt, T. Schmitz, T. Schäfer, C. Bergmann, FEBS Lett. 202 (1986) 373–377.
[13] C. Bergmann, J. Dodt, S. Köhler, E. Fink, H.G. Gassen, Biol. Chem. Hoppe-Seyler 367 (1986) 731–740.
[14] E. Fortkamp, M. Rieger, G. Heisterberg-Moutses, S. Schweitzer, R. Sommer, DNA 5 (1986) 511–517.
[15] D. Bagdy, E. Barabas, L. Graf, T.E. Petersen, S. Magnusson, Methods Enzymol. 45 (1976) 669–678.
[16] P.J. Braun, S. Dennis, J. Hofsteenge, S.R. Stone, Biochemistry 27 (1988) 6517–6522.
[17] C. Niehrs, W.B. Huttner, D. Carvallo, E. Degryse, J. Biol. Chem. 265 (1990) 9314–9318.
[18] R. Muramatsu, E. Nukui, A. Sukesada, S. Misawa, Y. Komatsu, T. Okayama, K. Wada, T. Morikawa, H. Hayashi, K. Kobashi, Eur. J. Biochem. 223 (1994) 243–248.
[19] A. Tuong, M. Maftouh, C. Ponthus, O. Whitechurch, C. Roitsch, C. Picard, Biochemistry 31 (1992) 8291–8299.
[20] R. Bischoff, P. Lepage, M. Jaquinod, G. Cauet, M. Acker-Klein, D. Clesse, M. Laporte, A. Bayol, A. Van Dorsselaer, C. Roitsch, Biochemistry 32 (1993) 725–734.
[21] G. Teshima, J.T. Stults, V. Ling, E. Canova-Davis, J. Biol. Chem. 266 (1991) 13544–13547.
[22] B.A. Johnson, J.M. Shirokawa, W.S. Hancock, M.W. Spellman, L.J. Basa, D.W. Aswad, J. Biol. Chem. 264 (1989) 14262–14271.
[23] M.V. Paranandi, A.W. Guzzetta, W.S. Hancock, D.W. Aswad, J. Biol. Chem. 269 (1994) 243–253.
[24] D.W. Aswad, M.V. Paranandi, B.T. Schurter, J. Pharm. Biomed. Anal. 21 (2000) 1129–1136.
[25] M.J. Mamula, Immunol. Rev. 164 (1998) 231–239.
[26] M.J. Mamula, R.J. Gee, J.I. Elliott, A. Sette, S. Southwood, P.-J. Jones, P.R. Blier, J. Biol. Chem. 274 (1999) 22321–22327.
[27] S. Clarke, R.C. Stephenson, J.D. Lowenson, in: T.J. Ahern, M.C. Manning (Eds.), Stability of Protein Pharmaceuticals, Part A: Chemical and Physical Pathways of Protein Degradation, Plenum Press, New York, 1992, pp. 1–29.
[28] J. Dodt, H.-P. Müller, U. Seemüller, J.-Y. Chang, FEBS Lett. 165 (1984) 180–184.
[29] J. Dodt, W. Machleidt, U. Seemüller, R. Maschler, H. Fritz, Biol. Chem. Hoppe-Seyler 367 (1986) 803–811.
[30] Y. Komatsu, Y. Inoue, M. Sasaki, S. Misawa, H. Takaku, H. Hayashi, Cardiovasc. Drug Rev. 18 (2000) 47–60.
[31] R. Muramatsu, T. Negishi, T. Mimoto, A. Miura, S. Misawa, H. Hayashi, J. Biotechnol. 93 (2002) 131–142.
[32] M.G. Grütter, J.P. Priestle, J. Rahuel, H. Grossenbacher, W. Bode, J. Hofsteenge, S.R. Stone, EMBO J. 9 (1990) 2361–2365.
[33] T.J. Rydel, K.G. Ravichandram, A. Tulinsky, W. Bode, R. Huber, C. Roitsch, J.W. Fenton, II, Science 249 (1990) 277–280.
[34] R. Uy, F. Wold, Science 198 (1977) 890–896.
[35] F. Wold, Annu. Rev. Biochem. 50 (1981) 783–814.
[36] W.K. Paik, S. Kim, Science 174 (1971) 114–119.
[37] M.S. Springer, M.F. Goy, J. Adler, Nature 280 (1979) 279–284.
[38] S. Clarke, Annu. Rev. Biochem. 54 (1985) 479–506.
[39] D.B. Cowie, G.N. Cohen, E.T. Bolton, H. De Robichon-Szulmajster, Biochim. Biophys. Acta 34 (1959) 39–46.
[40] C.B. Anfinsen, L.G. Corley, J. Biol. Chem. 244 (1969) 5149–5152.
[41] F. Naider, Z. Bohak, J. Yariv, Biochemistry 11 (1972) 3202–3208.
[42] H. Koide, S. Yokoyama, G. Kawai, J.-M. Ha, T. Oka, S. Kawai, T. Miyake, T. Fuwa, T. Miyazawa, Proc. Natl. Acad. Sci. USA 85 (1988) 6237–6241.
[43] K. Katagiri, K. Tori, Y. Kimura, T. Yoshida, T. Nagasaki, H. Minato, J. Med. Chem. 10 (1967) 1149–1154.
[44] T. Kohno, D. Kohda, M. Haruki, S. Yokoyama, T. Miyazawa, J. Biol. Chem. 265 (1990) 6931–6935.
[45] M. Sugiura, M. Kisumi, I. Chibata, J. Antibiot. 34 (1981) 1278–1282.
[46] H.E. Umbarger, Science 145 (1964) 674–679.
[47] H.E. Umbarger, Curr. Top. Cell. Regul. 1 (1969) 57–76.
[48] H.E. Umbarger, Annu. Rev. Biochem. 47 (1978) 533–606.
[49] H.E. Umbarger, in: K.M. Herrmann, R.L. Somerville (Eds.), Amino Acids Biosynthesis and Genetic Regulation, Addison-Wesley, Reading, MA, 1983, pp. 245–266.
[50] M. Kisumi, M. Sugiura, I. Chibata, J. Biochem. 80 (1976) 333–339.
[51] M. Sugiura, M. Kisumi, I. Chibata, J. Antibiot. 34 (1981) 1283–1289.