SUMMARY

1. L-3,4-Dihydroxyphenyl alanine-extradiol cleavage is followed by intra-molecular cyclization in lincomycin biosynthesis.

The aim of the work was to assign function to the protein coded for by an *lmbB1* gene, characterize it better and confirm the assumption that 2,3-extradiol fission of the DOPA aromatic ring is the actual reaction involved in the metabolic pathway leading to lincomycin synthesis.

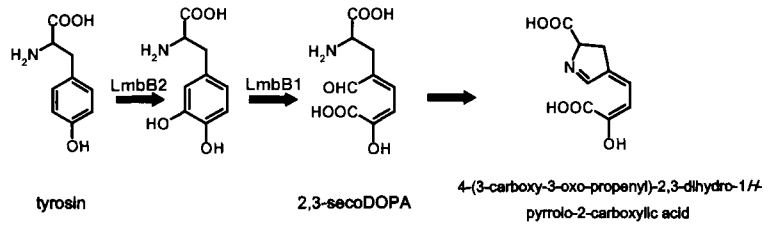

Fig. 1 Initial steps of the amino acid subpathway of the lincomycin biosynthesis.

The results of the feeding experiments with labeled intermediates and subsequent NMR analysis (BRAHME *et al.*, 1984), beared witness of the fact that the amino acid subpathway of the lincomycin biosynthesis includes 2,3-extradiol cleavage of DOPA (Fig. 1). Neusser and coworkers (NEUSSER *et al.*, 1998) showed that LmbB1 catalyzes conversion of DOPA to an unspecified yellow compound.

It appeared inapplicable to isolate the LmbB1 reaction product directly from *in vitro* reaction catalyzed by the purified LmbB1 partly as LmbB1 lost most of its activity during dialysis, most probably due to oxidation of the ferrous ion proposed as a cofactor, and partly due to the fact that many different DOPA oxidation products were produced in the system. Instead, a system similar to that applied for identification of phenazine biosynthesis intermediates (MCDONALD *et al.*, 2001) was used. The *in vivo* system was more efficient and also produced fewer DOPA oxidation products.

The LmbB1 reaction product isolated from the medium in the *in vivo* system had migration characteristics identical with those of the compound isolated from the purified LmbB1 directed reaction upon CE separation.

Combination of the high separation efficiency and detection sensitivity together with a soft ionization technique (CE-ESI-MS) used for the compound analysis allowed determination of the LmbB1 reaction product's molecular mass and also its fragmentation pattern (Fig. 2).

The purified LmbB1 reaction product isolated from the *in vitro* system and the compound isolated from that of *in vivo* showed the same migration time and provided an identical pseudomolecular ion $[M-H]^- \rightarrow$ m/z 210. The fragments of the $[M-H]^- \rightarrow$ m/z 210 were interpreted as products of decarboxylation ($[M-H]^- \rightarrow$ m/z 166), decarboxylation and dehydration ($[M-H]^- \rightarrow$ m/z 148) and both decarboxylation and decarbonylation ($[M-H]^- \rightarrow$ m/z 138).

The mass spectra were in agreement with those expected for 4-(3-carboxy-3-oxo-propenyl)-2,3-dihydro-1H-pyrrole-2-carboxylic acid predicted as an intermediate in the propylproline biosynthesis (BRAHME *et al.*, 1984).

LmbB1 contains a dioxygenase signature (NEUSSER *et al.*, 1998) and shows statistically significant similarity to conserved domain of the VOC (vicinal oxygen chelate) superfamily (superfamily involving among other type I extradiol dioxygenases cleaving aromatic rings) (VAILLANCOURT *et al.*, 2006). It can thus be concluded that LmbB1 is a 2,3-extradiol dioxygenase cleaving the DOPA aromatic ring.

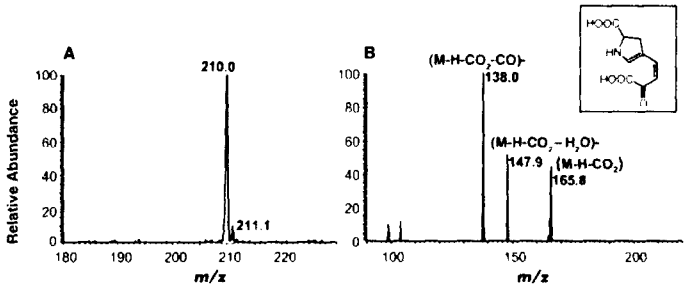

Fig. 2. CE-MS of the LmbB1 reaction product and fragmentation pattern.

2,3-extradiol fission of the DOPA aromatic ring is proposed in the biosynthesis of muscaflavin (BUGG a WINFIELD, 1998), stizolobinic acid (SAITO a KOMAMINE, 1978) and pyrrolo[1,4]-benzodiazepine antibiotics (HURLEY *et al.*, 1979) and lincomycins (BRAHME *et al.*, 1984). Although the reactions yield the same primary product, 2,3-*seco*DOPA, the next cyclization step produces different heterocyclic substances: muscaflavin, stizolobinic acid or 4-(3-carboxy-3-oxo-propenyl)-2,3-dihydro-1H-pyrrole-2-carboxylic acid (Fig. 3). While the 2,3-extradiol fission reaction is assumed to be enzymatically catalyzed in extenso in *S. hasjoo* (SAITO a KOMAMINE, 1978), a purely spontaneous 2,3-*seco*DOPA cyclization leading to muscaflavin is expected in *A. muscaria*. However, the formation of muscaflavin from the isolated 2,3-*seco*DOPA was not complementary to decay of 2,3-*seco*DOPA and an important part of 2,3-*seco*DOPA disappeared to unknown products (TERRADAS a WYLER, 1991). Consequently, the evidence of the third alternative of the 2,3-*seco*DOPA cyclization leads to a hypothesis of the employment of the discussed enzymes in the primary product cyclization. Extensive comparative studies of these reactions could be illuminating.

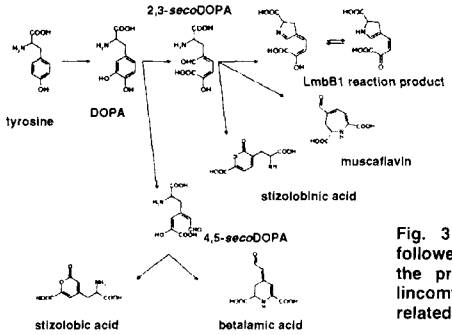

Fig. 3 DOPA aromatic ring cleavage followed by intramolecularcyclization of the product occurs in biosynthesis of lincomycin and other biosynthetically related sekundary metabolites.

2. LmbB2 - a unique heme-containing tetrahydrobiopterin (BH_4) dependent enzyme hydroxylating tyrosine aromatic ring in lincomycin biosynthesis (publication in preparation).

The aim of the work was to assign function to the LmbB2 protein, characterize it better and confirm an assumption that tyrosine hydroxylation yielding DOPA is the actual reaction involved in the metabolic pathway leading lincomycin.

The results of the feeding experiments with labeled intermediates and subsequent NMR analysis (BRAHME *et al.*, 1984), showed that the amino acid sub-pathway of the lincomycin biosynthesis includes hydroxylation of tyrosine aromatic ring to yield DOPA (Fig. 1), however, the reaction has neither been assigned to protein product of any gene of the lincomycin cluster in experiments *in vitro*, nor the respective protein has so far been characterized. Neusser and coworkers showed that if co-expressed in *E.coli* the *lmbB1* a *lmbB2* genes ensure conversion of tyrosine or DOPA to an unspecified yellow compound (NEUSSER *et al.*, 1998). Tyrosine hydroxylation activity has not been demonstrated *in vitro* and the interpretation of these data was that the LmbB2 protein either alone or in accord with LmbB1 can hydroxylate the tyrosine aromatic ring.

The LmbB2 protein was overproduced in *E. coli* at a lowered post-induction temperature and purified by affinity chromatography in its active form. Reaction product of the purified LmbB2 showing migration characteristics identical with the DOPA standard was analyzed by HR-MS. Its m/z was identical with that of DOPA standard (Fig 4). Consequently, LmbB2 is a monooxygenase catalyzing incorporation of the oxygen atom into the tyrosine aromatic ring.

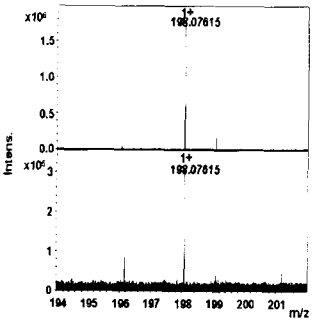

Fig. 4 HR-MS analysis of HPLC purified LmbB2 reaction product.

LmbB2 exhibits two pH optima of 8 and 9 and a temperature optimum at 55°C, however, its stability decreases after a 10-min incubation at 25°C and higher.

LmbB2 absorption spectrum had a maximum at 403 nm (Soret band) (Fig. 5) and its Raman spectrum was almost identical with that of protoporphyrin IX (Fig. 6) indicating that type b heme is present in the LmbB2 molecule. LmbB2 is thus a heme-containing tyrosine monooxygenase. There are many b-type heme oxygenases, members of the cytochrome P450 superfamily, hydroxylating aromatic rings in bacteria (ULLRICH a HOFRICHTER, 2007). Surprisingly, LmbB2 does not share their typical feature *i.e.* the

absorption maximum at 450 nm in their CO-reduced differential spectra, caused by the presence of cysteine ligand of their heme Fe (ULLRICH a HOFRICHTER, 2007).

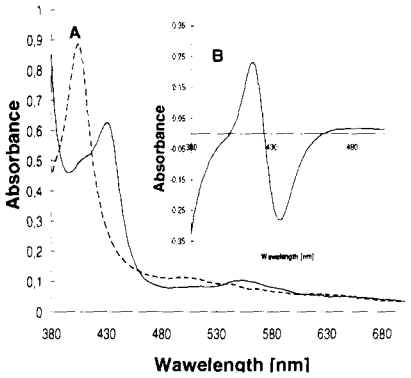

Fig. 5. (A) Absorption spectra of oxidized (full line) and reduced (dotted line) form of LmbB2. (B) Reduced CO-differential spectrum of LmbB2.

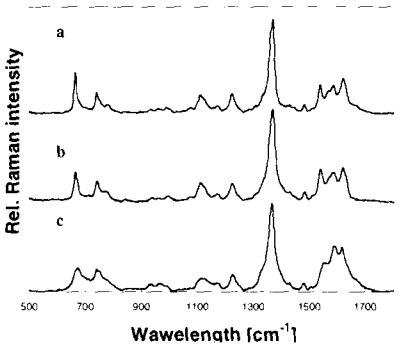

Fig. 6 Surface-enhanced resonance Raman spectra of LmbB2 (a), protoporphyrin IX (b) a hematoporphyrin (c).

Monooxygenation of tyrosine aromatic ring requires donation of redox equivalents. No detectable amount of dopaquinone was found in the LmbB2 reaction reflecting the role of DOPA as a donor found in tyrosinases. Therefore, several reduced cofactors were tested. Of them NAD(P)H rather decreased the LmbB2 activity. Nevertheless, BH_4 added to the LmbB2 reaction mixture increased its activity almost twice, *i.e.* BH_4 can reduce the heme in the enzyme. In addition, the LmbB2 reaction performed with BH_4 as a co-substrate followed Michaelis-Menten kinetics in preliminary experiments. These findings suggest that BH_4 could be a physiological reducing agent of the LmbB2 heme.

Only a small number of enzymes, including tyrosine hydroxylases, can utilize BH_4 as a cofactor and the one and only hemoprotein now known to require BH_4 is NOS, a flavo-hemoprotein which catalyzes a two-step NADPH- and O_2-dependent oxidation of arginine to generate NO and citrulline (STUEHR *et al.*, 2005). Surprisingly, no flavin was found in the LmbB2 molecule. Nevertheless, an experimental evidence for the anaerobic reduction of a group of structurally diverse hemoproteins by tetrahydrobiopterins from Fe(III) to the corresponding Fe(II) state has been reported (CAPEILLERE-BLANDIN *et al.*, 2005) confirming that the one-electron transfer from tetrahydrobiopterins to FeIII-porphyrins (obligate one-electron acceptor (WALSH, 1980) is a general reaction.

The above mentioned characteristics are in accordance with the fact, that although the LmbB2 protein catalyzes a monooxygenation of tyrosine aromatic ring, it shares no significant homology with any of the protein family known to catalyze the reaction. Moreover, with one exception (HU *et al.*, 2007) LmbB2 did not show significant similarity to any known protein. This uniqueness of the LmbB2 amino acid sequence, surprisingly, does not contrast with the fact that LmbB2 houses such a frequent prosthetic group as heme b. Heme b have been found in over 20 different folds (SCHNEIDER *et al.*, 2007). Consequently, LmbB2 is a unique heme-containing BH_4-dependent monooxygenase hydroxylating tyrosine aromatic ring.

3. Search for subunits of NDL-synthetase, the key enzyme of the lincomycin biosynthetic pathway (data for publication in preparation).

The aim of the work was to find interactions among the protein candidates for subunits of the NDL-synthetase, the key enzyme of lincomycin biosynthesis (Fig. 7).

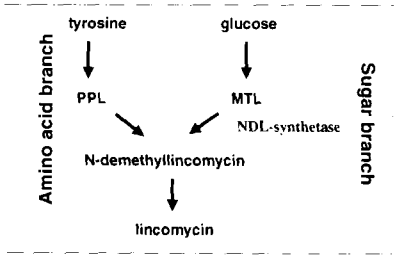

Fig. 7 Scheme of the lincomycin biosynthesis.

NDL-synthetase catalyzing condensation of the key intermediates of the lincomycin biosynthesis (PPL a MTL; (CHUNG *et al.*, 1997)) is a complex of readily dissociable non-identical subunits which have not been identified by now. Sequence analysis, inactivation, Western blot and activity experiments helped to choose protein candidates of NDL-synthetase subunits or related proteins (LmbC,D,E,F,IH,Q and T).

Except for LmbC, the function of none of the candidate proteins has been demonstrated experimentally. LmbC catalyzes PPL activation, which probably precedes the condensation reaction itself. The knowledge of interactions of the candidate proteins with LmbC can bring very valuable information. In addition, since proteins usually have about 5 interaction partners (WILDOVA a RUMLOVA, 2008) it can be expected that LmbC would be a part of the net of interactions in the NDL-synthetase complex.

Reciprocal interactions among the candidate proteins were tested by means of the yeast two-hybrid system in pairwise arrangement. Full-length proteins were tested.

With a single exception protein LmbC was only involved in interactions which according to the number of positive clones (intensive growth, blue color on –Leu-glu selective medium) appear disputable (Tab. 1). The reason could be that in the yeast a substantial part of the LmbC is in insoluble form as demonstrated by Western blotting, indicating that the heterologous *lmbC* expression is apparently problematic. The LmbC level could be so low that it would be without the detection limit or LmbC interactions could be naturally very weak. However, almost all the disputable interactions were found reciprocally.

In Tab. 1 interactions detected among proteins LmbC/LmbE, LmbD/LmbE, LmbF/LmbE, LmbIH/LmbE, LmbQ/LmbE and LmbN/LmbE seem to be most important. As LmbE shows homology to a protein acting on amide bond, its multiple interaction could suggest that it is a true NDL-synthetase subunit and, moreover, a catalytic subunit directly driving the PPL and MTL condensation. From this point of view, the interaction with LmbIH, which has a regulatory function according to Western blot experiments (HOLA *et al.*, 2003) is also interesting. This interaction could mean that there is a direct control (without other intervention) of the catalytical subunit by a regulatory protein. The interaction of LmbE (possible catalytic subunit) with LmbN (showing similarity to ACP, acyl carrier proteins, which in non-ribozomal protein synthetases serve for transfer of growing chains to catalytic centers (ANSARI *et al.*, 2004) seems to be logical.

		Lmb protein – AD						
DNA-BD-Lmb protein		C	D	E	F	IH	N	Q
	C	-	+	+	+	-	-	-
	D	(+)	-	+	+	+	-	+
	E	+	+	-	+	(+)	+	+
	F	-	-	+	-	-	-	-
	IH	-	-	(+)	+	-	-	-
	N	-	-	-	-	-	-	-
	Q	-	-	-	-	-	-	-

Tab. 1 Results of pairwise testing of interactions among proteins – candidates of NDL-syntetase subunits.

-	no interaction
+	disputable interaction; 2-3 of 5 clones tested (in one/both domain combinations) on a selective medium (-Leu-glu) grew well and colonies were of blue color
(+)	interaction found; 3 of 5 clones tested (in one/both domain combinations) on a selective medium (-Leu-glu) grew well and colonies were of blue color
+	interaction found; 4-5 of 5 clones tested (in one/both domain combinations) on a selective medium (-Leu-glu) grew well and colonies were of blue color

In summary, proteins encoded by the *lmbD, lmbE, lmb*F and *lmbN* genes can be considered very important candidates of NDL-synthetase subunits. LmbD interacts with LmbC whose relation to the condensation reaction has been already determined experimetally and which is apparently a part of the net of interactions formed by the LmbE, LmbF, and LmbN proteins. Homologs of the proteins (KOBĚRSKÁ *et al.*, 2005) can also be found in the gene cluster controlling biosynthesis of biosynthetically related celesticetin. It can be considered that the proteins could control condensation of the amino acid and sugar parts of the antibiotics. Roles of the LmbF and LmbD proteins is hardly predictable at present. LmbE could play a role of the central catalytic subunit, LmbC role of the PPL activating protein, and LmbN role of ACP. The whole complex could be controlled by LmbQ and LmbIH.

Nevertheless, the yeast two-hybrid system is an artificial system and the detected interactions should also be confirmed by other methods.