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Biochemical studies on unique enzymes in the biosynthesis of butirosin

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Research aims

The aminoglycosides are a diverse class of antibacterial antibiotics including streptomycin and kanamycin as the effective anti-tuberculosis antibiotics. However, the appearance of resistant strains to the aminoglycosides interfered with their therapeutic use in the history. To counter resistant organisms, C1 position of the 2-deoxystreptamine (DOS) moiety of kanamycin A and dibekacin were semisynthetically modified with the (*S*)-4-amino-2-hydroxy butyryl (AHBA) side chain to afford amikacin and arbekacin, respectively. The AHBA was originally observed in the natural product butirosin, which inhibits a variety of aminoglycoside resistant pathogens due to the presence of the AHBA substituent on the DOS moiety. The butirosin producer *Bacillus circulans* should have the AHBA biosynthetic enzymes to introduce it onto ribostamycin. Thus, the AHBA biosynthetic enzymes were expected to modify the other DOS-containing aminoglycosides including kanamycin A and dibekacin *via* biocatalytic route.

We have investigated the biosynthetic pathway for butirosin and neomysin for the last decade, and characterized most of biosynthetic enzymes leading to ribostamycin from glucose-6-phosphate. Comparison of biosynthetic genes for several DOS containing aminoglycosides showed that BtrF, G, H, I, J, K, N, O, and U are specifically encoded in the butirosin biosynthetic gene cluster suggesting that these could be involved in the specific enzymatic reactions in the biosynthesis of butirosin including the AHBA biosynthesis and transfer reactions. Among the enzymes, Spencer group in UK reported that BtrI, J, K, O, and U are responsible for the γ-glutamyl AHBA acyl carrier protein (ACP) formation prior to the acylation of ribostamycin leading to butirosin.[1] Thus, the other remaining enzyme BtrF, G, H, and N seemed to be responsible for the AHBA transfer reaction and other specific enzymatic steps in the biosynthesis of butirosin. In this research, we aimed to characterize BtrF, G, H, and N using the recombinant enzymes expressed in *E. coli* and also by gene inactivation experiments.

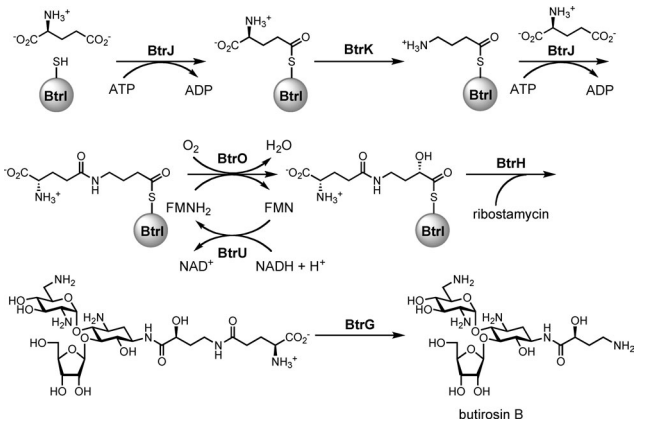
Methods

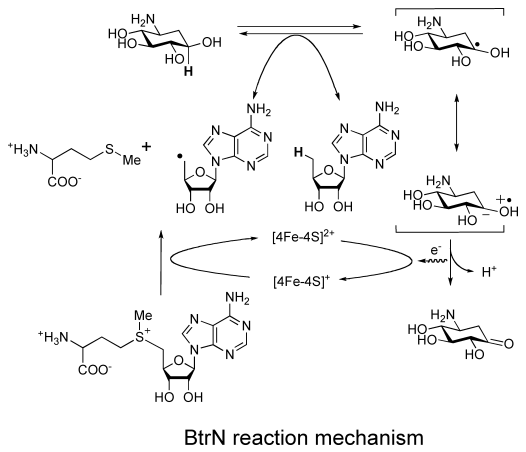
The *btrF*, *G*, *H*, and *N* genes were amplified by PCR with appropriate primers, and the amplified PCR products were digested by restriction enzymes and sub-cloned into the corresponding sites of LITMUS 28. After confirmation of the sequence, the appropriate DNA fragments were inserted into expression vectors such as pET30, pET28, and pColdI to obtain expression plasmids, which were subsequently introduced into *E. coli* BL21(DE3). *E. coli* BL21(DE3) carrying each expression plasmid were grown in LB medium containing appropriate antibiotics. The expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) and the cultivation was continued at 15～37°C for 3～18 hr. The cells were harvested by centrifugation, washed with buffer, and stored at −30°C until use. The wet cells were suspended in buffer and disrupted by sonication at 0°C to obtain cell free extracts containing recombinant enzymes.

To investigate the function of BtrN, all experiments were carried out under anaerobic conditions. The recombinant BtrN protein was purified by a standard Ni affinity chromatography. The purified BtrN was treated with dithiothreitol (DTT) at room temperature. $Fe^{II}(NH_4)_2(SO_4)_2$ and Na_2S were then added and the mixture was incubated at room temperature. The BtrN sample was then desalted by passage through HiTrap™ desalting column (Amersham Pharmacia Biotech AB). The obtained reconstituted BtrN was reduced by sodium dithionite for 1 hr at room temperature prior to use. The reduced BtrN was incubated with DOIA, SAM in 50 mM HEPES-NaOH (pH 8.0) in the presence of sodium dithionite. The reaction solution was analyzed for 3-amino-2,3-dideoxy-*scyllo*-inosose (amino-DOI) formation by HPLC after treatment with acetic anhydride and *O*-(*p*-nitrobenzyl)-hydroxylamine. To investigate the speculated hydrogen abstraction mechanism, [3-^{2}H]DOIA was prepared from amino-DOI by reduction of the keto group with NaB^2H_4. For the EPR measurements during the catalytic cycle, BtrN was incubated with SAM and DOIA in the presence of sodium dithionite at room temperature, and the reaction was freeze-quenched in liquid nitrogen at specific periods of time. EPR spectra were recorded with a JEOL JES-FA300 ESR spectrometer (9.02 GHz).

Results

BtrF, BtrG, BtrH, and BtrN were successfully overexpressed in *E. coli* and the soluble recombinant proteins were obtained. In addition, several co-expression systems for BtrF, BtrG, BtrH, BtrI, BtrJ, BtrK, BtrO, and BtrU were constructed to study the AHBA transfer reaction in the biosynthesis of butirosin. However, Spencer group at Cambridge University, UK has reported the γ-glutamyl AHBA (ACP): ribostamycin acyltransferase activity of BtrH and γ-





BtrN reaction mechanism

glutamyl cyclotransferase activity of BtrG during our investigation.[2,3)]

On the other hand, inactivation of the *btrN* gene caused the interruption of the biosynthetic pathway between 2-deoxy-*scyllo*-inosamine (DOIA) and 2-deoxystreptamine (DOS) indicating its involvement in the oxidation of DOIA.[4)] BtrN possesses a CXXXCXXC motif conserved within the radical *S*-adenosyl methionine (SAM) superfamily and its involvement in the oxidation was hard to be presumed. Thus, we initiated to verify the function of this unique radical SAM enzyme with its recombinant protein expressed in *E. coli*. BtrN was anaerobically purified by Ni nitriloacetic acid (NTA) affinity chromatography, since other radical SAM enzymes were reported to be sensitive to oxygen. The anaerobically purified BtrN was further treated with $Fe^{II}(NH_4)_2(SO_4)_2$ and Na_2S in the presence of dithiothreitol. The reconstituted BtrN was then reduced with 20 mM sodium dithionite at room temperature. The reduced BtrN was, then, incubated with DOIA and SAM. HPLC analysis of the oxime derivative of the reaction product revealed that amino-DOI was efficiently formed. This result clearly showed that BtrN itself catalyzes the oxidation of DOIA into amino-DOI in the presence of SAM. Further analysis of the other reaction products showed that 5′-deoxyadenosine and methionine were formed by incubation of SAM with BtrN and DOIA.

From the mechanistic point of view, the 5′-deoxyadenosyl radical generated through the reductive cleavage of SAM by the $[4Fe–4S]^+$ cluster was presumed to be involved in the abstraction of a substrate hydrogen atom to produce a radical intermediate. To investigate this hypothesis, 2-deoxy-*scyllo*-[3-^{2}H]inosamine ([3-^{2}H]DOIA) was prepared and subjected to the BtrN reaction. As a result, the ^{2}H NMR clearly showed incorporation of deuterium atom into the methyl group of 5′-deoxyadenosine. Furthermore, the reaction was carried out in a deuterium oxide buffer using non-labeled DOIA and no incorporation of the deuterium was observed in 5′-deoxyadenosine. These suggest that the BtrN reaction proceeds through direct hydrogen atom abstraction from DOIA by the 5′-deoxyadenosyl radical, not *via* the protein radical produced on a cysteine or a tyrosine residue, since such a proton is exchangeable with solvent.

Furthermore, the BtrN reaction was analyzed by EPR spectroscopy. A sharp double triplet EPR signal was observed when the EPR spectrum of the enzyme reaction mixture was recorded at 50 K. The spin coupling with protons partially disappeared by reaction with [2,2-$^2H_2$]DOIA, which unambiguously proved the observed signal to be a radical on C-3 of DOIA. On the other hand, the EPR spectrum of the 4Fe–4S cluster of BtrN during the reaction showed complex signal due to the presence of several species. Comparison of signals derived from a 4Fe-4S center of BtrN incubated with various combinations of products (5′-deoxyadenosine, L-methionine, and amino-DOI) and substrates (SAM and DOIA) indicated that the EPR signals observed during the reaction were derived from a free BtrN, a BtrN-SAM complex, and a BtrN-SAM-DOIA complex. Significant changes in the EPR signals upon binding of SAM and DOIA suggest the close interaction of both substrates with the 4Fe–4S.

Conclusion

Among several specific enzymes encoded in the butirosin biosynthetic gene cluster, we have successfully characterized BtrN as an unusual radical SAM dehydrogenase catalyzing the oxidation of the hydroxyl group by a radical mechanism.[4)] We have also observed the DOIA radical intermediate by EPR measurements, and clearly showed the radical mechanism of BtrN.[5)] Our observations should help understand the whole catalytic cycle of the BtrN and other radical SAM dehydrogenases.

During our investigation, functional analysis of γ-glutamyl AHBA acyltransferase BtrH and γ-glutamyl cyclotransferase BtrG have been reported.[2,3)] These reports demonstrated that my research proposal was reasonable and indicated potential of the enzymes for creation of modified aminoglycosides. Two enzymes BtrE and F encoded in the butirosin biosynthetic gene cluster now remain to be characterized. These enzymes seem to be involved in the unsolved epimerization step from butirosin B to A, which is also significant reaction to modify aminoglycosides. In addition to these enzymes, other unique modification enzymes such as methyltransferases and deoxygenation enzymes involved in other aminoglycoside biosynthesis will be further investigated in future and applied for creation of diverse structural aminoglycosides with improved activity against resistant strains.

References

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