The Pur10 protein encoded in the gene cluster for puromycin biosynthesis of *Streptomyces alboniger* is an NAD-dependent ATP dehydrogenase

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Abstract The *pur10* gene of the puromycin (*pur*) cluster of *Streptomyces alboniger* is essential for the biosynthesis of this antibiotic. Highly purified Pur10 protein, obtained in *Escherichia coli* as a recombinant protein fused to a histidine tail, had an NAD-dependent ATP dehydrogenase activity. The K_m and V_{max} values for ATP were 0.49 mM and 14.5 nmol/min and for NAD 0.53 mM and 15.2 nmol/min, respectively. The ATP-derived product of the reaction apparently decomposed producing a triphosphorylated compound plus an adenine derivative. These and previous results suggested that Pur10 carries out the first step of the puromycin biosynthetic pathway, namely, conversion of ATP into 3′-keto-3′-deoxyATP.

Key words: *pur* cluster; Puromycin biosynthesis; Pur10; ATP dehydrogenase; *Streptomyces*

1. Introduction

The biosynthesis of the aminonucleoside antibiotic puromycin by *Streptomyces alboniger* has been studied at biochemical and genetic levels [1–3]. Adenosine has been shown to be an intermediate [1]. The biosynthetic gene cluster *pur* for puromycin biosynthesis is comprised within a 13.5-kb DNA fragment from *S. alboniger* and has been expressed in the heterologous host *S. lividans* [3,4]. It contains 10 ORFs, of which those involved in puromycin biosynthesis (9) are transcribed as a single polycistronic messenger [3]. Only the products from the *napH*, *pac*, *dmpM* and *pur8* genes, which are involved in the final steps of the pathway, have been biochemically characterized. Sequence comparisons permitted to propose specific functions for the remaining genes (*pur3*, *-4*, *-5*, *-6*, *-7* and *-10*) [3]. Thus, Pur3 would be an NMP-phosphatase, Pur4 an aminotransferase, Pur5 a methyltransferase, Pur6 a tyrosinyltransferase, Pur7 an NTP-pyrophosphohydrolase and Pur10 an ATP oxidoreductase. This allowed to propose a puromycin biosynthetic pathway with ATP as a substrate. This metabolite would be dehydrogenated by Pur10 to produce 3′-keto-3′-deoxyATP (Fig. 1) since, besides other reasons, the deduced sequence of this protein was similar to a family of putative and known NAD(P)-dependent oxidoreductases and the 3′-keto group of this intermediate would be converted into an $-NH_2$ group by the Pur4 aminotransferase [3]. Here, we show that Pur10 displays an NAD-dependent ATP dehydrogenase activity and that its coding gene (*pur10*) is essential for puromycin biosynthesis in *S. alboniger*.

2. Materials and methods

2.1. Strains, plasmids, media, DNA methodology and chemicals

S. alboniger ATCC 12461 [5], *Streptomyces lividans* 66(1326) [6], *Helminthosporium* sp. ATCC 20154 [7] and the *Escherichia coli* strains DH5α [8] and BL21(DE3)pLysS [9] were described in the indicated references. *Streptomyces* plasmids were pIJ702 [10] and pGM9 [11]. *E. coli* plasmids were pBluescript SK(−) (Stratagene) and pRSETb [12]. Plasmid DNA from *Streptomyces* and *E. coli* was prepared as described [6]. Growth of *Streptomyces* on solid medium was carried out on R5, whereas that on liquid media was on YEME, containing 34% sucrose and 5% $MgSO_4$, or puromycin-producing S medium, containing starch as a carbon source [4,6]. Solid and liquid media for *E. coli* were LB with or without 2% agar, respectively. When required, antibiotics thiostrepton (10), hygromycin B (200), neomycin B (13), ampicillin (100) and chloramphenicol (34) were added at the concentrations, in μg/ml, indicated in brackets. General DNA methodology was performed as described [13]. CHES, ATP, ADP, AMP, GTP, UTP, CTP, 3′-amino-3′-deoxyATP and cordycepin-5′-triphosphate where from Sigma. NAD, NADP, NADH, adenosine and 2′-deoxyATP where from Boehringer Mannheim. 2′-Amino-2′-deoxyATP was from USB. [α-^{32}P]ATP and [γ-^{32}P]ATP were from Amersham. 3′-Amino-3′-deoxyadenosine was obtained from *Helminthosporium* sp. as described [7], except that starch was used instead of cerellose.

2.2. Preparation of a pur10 deletion mutant

From a *Mlu*I-*Eco*47III fragment of *pur* (nt 1864–4798) [3], a *Sca*I-*Bsa*AI internal fragment, comprising nt 79–910 of the *pur10* coding sequence (82%) [3], was replaced by a 1.6-kb *Sma*I fragment, which contained the promoter and lacked the transcription terminator signal of the *hyg* gene [14]. To prevent any polar deleterious effect, this gene was inserted in the same transcription orientation as the polycistronic *pur* structural genes. The resulting fragment was inserted in *Xho*I- and *Xba*I-cut pGM9, a replication thermosensitive plasmid [11]. The resulting plasmid (pSDP0.4) was electroporated into *S. alboniger* essentially as described [15]. Transformants were grown at 39°C as described [11] and neomycin B-sensitive and hygromycin B-resistant mutants were isolated. Four correct *S. alboniger* Δ*pur10* mutants were characterized by Southern blotting (data not shown).

2.3. Cloning, expression, purification and microsequencing of Pur10

To clone and express *pur10* in *S. lividans*, a 1.9-kb *Bam*HI fragment, which contained the *pur10* coding sequence (nucleotides 2611–4490; [3]), was inserted in the *Bgl*II site of pIJ702 under the *mel* promoter. The resulting construct (pSEXP0.2) was then cloned in *S. lividans*. To express *pur10* in *E. coli*, its amino terminal coding sequence was amplified by PCR by using the oligonucleotides 5′-GTGATCATATGTCCACTTATTCACAG-3′ and 5′-CGGCAC-GGTGACCATCGCGAC-3′. The first oligonucleotide created single *Nde*I and *Bcl*I sites next to the ATG initiator codon. As template, the 1.9-kb *Bam*HI fragment indicated above was used. The resulting 239-bp fragment, whose sequence was confirmed by sequencing reactions, was ligated to the remaining sequence of *pur10*, an *Nru*I-*Nae*I (nt 2938–3495) fragment [3], and cloned in the *Bam*HI-*Pvu*II sites of the expression vector pRSETb. The resulting plasmid (pECO0.3) was cloned in *E. coli* BL21(DE3)pLysS. One transformant was grown at 37°C in 1 l LB containing ampicillin and chloramphenicol up to an

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Abbreviations: aa, amino acid(s); LB, Luria-Bertani broth; nt, nucleotide(s)

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OD_{600nm} of 0.9. Culture was made up of 0.4 mM IPTG and incubation was then continued for 1.5 h. All further operations were at 4°C. Cells were spun down and then resuspended in 20 ml 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM $MgCl_2$ and 5 mM β-mercaptoethanol. Cell breaking was achieved by three cycles of freezing and thawing followed by 3× sonication for 1 min at 15 microns. Lysates were spun at 15 000×*g* for 20 min. The supernatant was saved and kept at −70°C. To purify recombinant Pur10, supernatant lysates (16 ml) were mixed with 4 ml of Ni^{2+}-NTA-agarose (Quiagen) and incubated for 1 h with gentle agitation. This mixture was placed in a 20-ml column, which was washed successively with 5×16 ml of 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM $MgCl_2$, and 5 mM β-mercaptoethanol and 5×16 ml of 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 50 mM imidazole, 1 mM $MgCl_2$ and 5 mM β-mercaptoethanol. Elution of Pur10 was achieved with 8 ml 50 mM NaH_2PO_4, pH 8.0, 300 mM $NaCl_2$, 250 mM imidazole, 1 mM $MgCl_2$ and 5 mM β-mercaptoethanol. Four 2-ml fractions were collected of which the three latter were pooled. To remove imidazole, 1.5-ml samples were passed through a PD-10 Sephadex G-25 column (Pharmacia). Elution was performed with 50 mM Tris-HCl, pH 9.4, 20 mM NaCl and 5 mM β-mercaptoethanol. A sample of the eluted protein was subjected to SDS-PAGE followed by staining with Coomassie. A main band of 42 kDa was detected, which was treated with trypsin and extracted. The resulting peptides were separated and collected using a Smart μHPLC (Pharmacia) and three of them were sequenced by tandem mass spectrometry by the 'nanospray' ionization method, using an LCQ quadrupole ion trap (Finnigan, ThermoQuest, USA).

2.4. Enzymic assays

To assay Pur10 from *S. lividans* (pSEXP0.2), mycelia from 10-ml YEME cultures taken at an OD_{660nm} of 3.5 were resuspended in 0.5 ml of 50 mM NaH_2PO_4, pH 8.0, sonicated and spun at full speed in a microfuge. Supernatants were saved and maintained at −70°C. To assay Pur10 activity, reaction mixtures (0.8 ml) contained (unless otherwise indicated) 50 mM Tris-HCl, pH 9.4, 20 mM NaCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM NAD, 1 mM ATP and approximately 250 μg total protein. Incubation was at 30°C at the indicated time intervals. Formation of NADH was quantitated as indicated below for recombinant Pur10. To assay recombinant Pur10, reaction mixtures (0.8 ml) contained, unless otherwise indicated, 50 mM CHES-NaOH, pH 10.2, 20 mM NaCl, 10 mM $MgCl_2$, 1 mM NAD and 1 mM ATP and 25 μl (5–10 μg purified or 250 μg crude recombinant) Pur10. These were found to be optimal conditions. Reactions took place at 30°C and were quantitated spectrophotometrically by determining the increase of absorbance, due to the conversion of NAD to NADH, at 340 nm. An NADH molar absorption coefficient of 6220 M^{-1} cm^{-1} was used. Specific activity was expressed as μmol NADH produced per min per mg protein. Protein was quantitated by using the Bio-Rad protein assay kit and bovine seroalbumin. Pac (puromycin *N*-acetyltransferase) and NapH (*N*-acetylpuromycin *N*-acetylhydrolase) were determined in *S. alboniger* Δ*pur10* cell extracts and culture filtrates as described [16], respectively. Puromycin was quantitated enzymically by a Pac assay [4].

3. Results

3.1. The pur10 gene is required for puromycin biosynthesis

To demonstrate the participation of the *pur10* product in the puromycin biosynthetic pathway of *S. alboniger*, most of the coding sequence of this gene was deleted. None of the isolated mutants produced puromycin. In contrast, they expressed both *napH* and *pac* genes, which are located upstream and downstream of *pur10*, respectively. This indicates that they still synthesized an active polycistronic transcript (data not shown). One of these mutants was transformed with the *pur10*-containing plasmid pSEXP0.2. The resulting transformants produced as much puromycin as the wild-type *S. alboniger*. In addition, these mutants when supplemented with 3′-amino-3′-deoxyadenosine produced puromycin (data not shown).

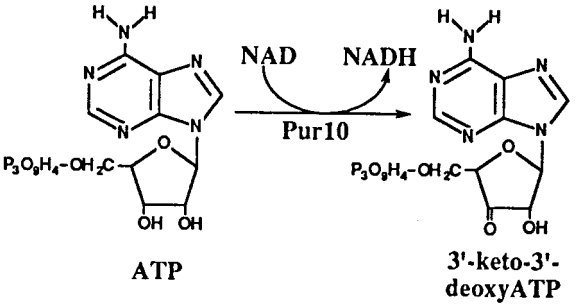

Fig. 1. Possible dehydrogenase reaction catalyzed by Pur10.

3.2. Expression in E. coli and purification of recombinant Pur10

The coding sequence of *pur10* was inserted in the *E. coli* expression vector pRSETb. The resulting plasmid (pECO0.3) was cloned in *E. coli* BL21(DE3)pLysS. Centrifuged supernatant lysates of a transformant, which was induced by IPTG, contained an extra protein of approximately 42 kDa (Fig. 2), which agrees with the addition of the molecular weights of Pur10 (38.34 kDa) and the 33 extra residues (3.79 kDa) added to its N-terminus. Although some of the fusion recombinant protein was insoluble, a sizeable fraction remained in solution (Fig. 2). The recombinant Pur10 was highly purified by means of an Ni-NTA column (Fig. 2). Trypsin peptides from the purified protein had the sequences ALMVGFAGHQGK, FTGIGEALK and VNAAFVHHYQ, which correspond to aa 15–26, 60–68 and 201–210, respectively, of the Pur10 deduced sequence [3]. Enzymic assays (see below) indicated that at average the specific activity increased from 0.06 to 1.25 μmol NADH synthesized/mg protein/min in the crude extract and purified recombinant Pur10, respectively.

3.3. Substrate specificity and kinetic parameters of the reaction catalyzed by recombinant Pur10

Initial experiments with supernatant from *E. coli* (pECO0.3) indicated that it contained an NAD-dependent

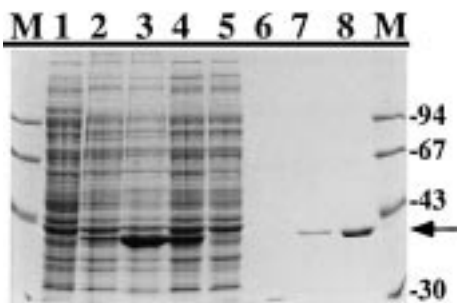

Fig. 2. Purification of recombinant Pur10. A Coomassie stained gel of SDS-PAGE (9%) is shown. Lane 1: Crude extract of BL21(DE3)pLysS(pRSETb); lanes 2 and 3: crude extracts of BL21(DE3)pLysS(pECO0.3) prior to or 1.5 h after addition of IPTG, respectively; lane 4: soluble supernatant fraction; lane 5: first eluate from the Ni-NTA-agarose column; lane 6: last eluate with 20 mM imidazole-containing buffer; lane 7: last eluate with 50 mM imidazole-containing buffer; lane 8: pooled fractions eluted with 250 mM imidazole-containing buffer; lane M: molecular weight markers (sizes are given). The arrow indicates recombinant Pur10. For details see Section 2.

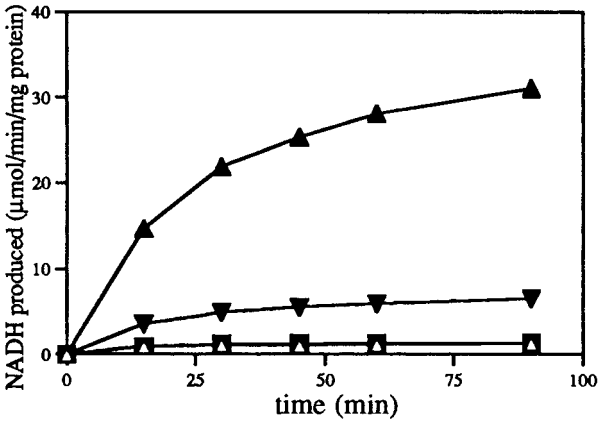

Fig. 3. Reaction catalyzed by Pur10. The NAD-dependent ATP dehydrogenase activity of purified recombinant Pur10 was performed as indicated in Section 2. (▲) Complete reactions; (△) in the absence of ATP or in the presence of either AMP or adenosine instead of ATP; (■) in the absence of NAD; (▼) in the presence of ADP instead of ATP.

ATP dehydrogenase activity, which was absent in the *E. coli* (pRSETb) control. This activity was also present in the purified recombinant protein (Fig. 3). The optimal reaction conditions are indicated in Section 2. Most striking was its high optimal pH (10.2; Fig. 4). Mn^{2+} partially replaced Mg^{2+} (up to 80% of NADH production). In contrast, Zn^{2+} inhibited the reaction. NADP was not a cosubstrate for Pur10. Of all other nucleotides/nucleosides tested in the presence of NAD (ADP, AMP, CTP, GTP, UTP, 2′-deoxyATP, 3′-deoxyATP (cordycepin-5′-triphosphate), 2′-amino-2′-deoxyATP, 3′-amino-3′-deoxyATP and adenosine) only ADP allowed the reaction to take place, although at lower rates than ATP (Fig. 3). The Lineweaver-Burk plot of the initial rates (up to 20 min) against the substrate concentrations were all linear. The calculated K_m and V_{max} for ATP were 0.49 mM and 14.5 nmol/min, respectively, and for NAD, 0.53 mM and 15.2 nmol/min, respectively, whereas the K_m for ADP was 3.13 mM.

3.4. Studies on the Pur10 reaction products

The Pur10 reaction products were examined by a variety of thin layer chromatographic assays. Three compounds were detected. One carried an adenine-related moiety as shown by UV absorption, whereas the other contained the three phosphate groups, which were detected by using either [α-^{32}P]ATP or [γ-^{32}P]ATP. The third one was NADH (data not shown). These results suggested that, under the experimental conditions used, the ATP-derivative product was unstable and, therefore, could not be isolated.

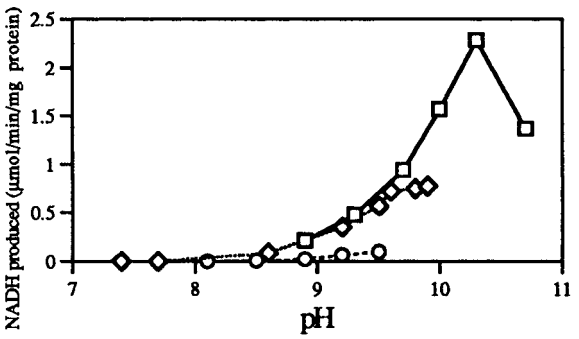

Fig. 4. Effect of pH on the Pur10 reaction. Assays were performed as described in Section 2, except that the pH of the reactions was changed as indicated. Buffers used were: (○) NaH_2PO_4; (◇) Tris-HCl; (□) CHES-NaOH, each at 50 mM.

3.5. Expression of Pur10 activity in S. lividans

The Pur10 activity of *S. alboniger* extracts during different stages of growth were practically undetectable, even in the periods prior to and during puromycin production. This could result from low production levels, high inactivation rates, or both, of Pur10. Despite this, Pur10 activity, assayed as indicated in Section 2, was shown to be present in cell extracts from *S. lividans* (pSEXP0.2) with kinetics similar to that of purified recombinant Pur10. In contrast, no activity was observed in lysates from *S. lividans* (pIJ702). As in the case with purified recombinant Pur10, ADP was also a substrate, although less efficient than ATP, whereas AMP and adenosine were not (data not shown).

4. Discussion

The results presented here indicate that the *pur10* gene, a member of the *pur* cluster of *S. alboniger*, is an essential gene for puromycin biosynthesis. Its product was synthesized in *E. coli* as a fusion, recombinant, protein which was highly purified and enzymatically characterized. Previous amino acid sequence comparisons suggested that Pur10 was an NAD(P)-dependent ATP dehydrogenase [3]. Indeed, this has been proved in the present work, which shows in addition that Pur10 uses NAD as a cofactor but not NADP. Within the family of oxidoreductases to which Pur10 pertains, there are several deduced proteins which seem to be implicated in the biosynthesis of antibiotics: RdmF (anthracyclines [17]), LmbZ (lincomycin [18]), ORF7 (ansamitocins, GenBank accession number U33059) and RifL (rifamycin [19]). Therefore, and to the best of our knowledge, Pur10 is the first characterized oxidoreductase of this family which is implicated in antibiotic biosynthesis in bacteria and fungi. Purified recombinant Pur10 has an optimal high pH (10.2), which is not unusual in other members of that oxidoreductase family. Examples are the rat biliverdin reductase (pH 8.7) [20], and the D-galactose dehydrogenase of *Pseudomonas fluorescens* (pH 9.5) [21]. In relation to the crude native enzyme obtained from *S. lividans* (pSEXP0.2), the 33 extra residues of the recombinant Pur10 do not seem to affect drastically its catalytic properties with respect to the substrate, co-substrate and reaction products. However, the optimal pH and Mg^{2+} concentration of purified Pur10 are higher than those of the crude enzyme from *S. lividans* (pSEXP0.2). Although ADP is also a substrate for Pur10, it is less efficient than ATP.

Although the ATP-derived product produced by Pur10 could not be identified, several data provide hints on its chemical structure. Firstly, 3′-amino-3′-deoxyadenosine complements the puromycin synthetic ability of *pur10* deletion mutants, which indicates that the product of Pur10 must be a precursor of this intermediate. In principle, such a precursor should carry a 3′-keto group, to allow an aminotransferase (Pur4) to introduce a 3′-NH_2 group ([3,22]; for a review see [23]). Particularly, a ketosugar (*scyllo*-inosose) is the substrate of the StsC aminotransferase, an enzyme highly similar to Pur4, in the streptomycin biosynthetic pathway of *S. griseus*

[22]. Secondly, two compounds were detected as products of the Pur10 reaction. One contained three phosphate groups and the other was an adenine derivative. It is known that ketosugars are unstable at alkaline pHs and decompose by a mechanism for which a reverse aldol addition has been proposed (see for instance [24]). This might explain why the putative ATP-keto derivative decomposes. Finally, within a variety of nucleotides assayed, only ATP and ADP were substrates for Pur10, and for the reaction to take place, both -OH groups at 2′ and 3′ of the ribofuranose moiety of ATP were required. Logically, dehydrogenation at 2′ should not be required for the biosynthesis of puromycin. This leaves the 3′ position as the site of dehydrogenation. Therefore, the reaction catalyzed by Pur10 should most likely be that indicated in Fig. 1, which, as previously proposed [3], would form the first step of the puromycin biosynthetic pathway.

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