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Synthesis of human renalase1 in *Escherichia coli* and its purification as a FAD-containing holoprotein

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ABSTRACT

Renalase is a protein ubiquitous in vertebrates, which has been proposed to modulate blood pressure and heart rate, and whose downregulation might result in hypertension. Despite its potential relevance for human health, the biochemical characterization of renalase is still lacking, possibly due to difficulties in obtaining it in recombinant form. By expressing two different gene constructs, we found that the major isoform of human renalase, renalase1, is mainly produced in *Escherichia coli* in inclusion bodies. However, by optimizing the expression conditions, significant amounts of soluble products were obtained. Both soluble renalase forms have been purified to homogeneity exploiting their N-terminal His-tag. Linking of the protein of interest to the SUMO protein did not improve solubility, but yielded untagged renalase1 after proteolytic processing of the fusion product. The two recombinant renalase forms displayed the same molecular properties. They bind equimolar amounts of FAD and appear to be correctly folded by various criteria. The procedures for the production and isolation of recombinant renalase1 here reported are expected to boost the much awaited biochemical studies on this remarkable protein.

Introduction

Renalase is a recently discovered protein, ubiquitous in vertebrates, and present in various human tissues and organs, including plasma, kidney, heart, skeletal muscle, liver, and nervous system [1,2]. Its predominant isoform, renalase1 (UniProtKB/SwissProt Q5VYX0-1), has been suggested to be secreted by the kidney into the bloodstream, where it was shown to reduce blood pressure and heart rate [1]. It was argued that the observed decrease of renalase levels in plasma of patients suffering from chronic kidney disease could represent a key factor in the increased susceptibility of these subjects to cardiovascular complications [3–10]. Thus, renalase might be a new hormone-like protein, which broadens the endocrine functions of the kidney, and provides an additional functional link between the excretory and the cardiovascular systems. Mutations within the renalase gene (*RNLS*, located on chromosome 10 at q23.31, GenBank BC005364) have been shown to be associated to essential hypertension [11]. The primary structure of renalase1, the largest of the expression products of *RNLS* generated by alternative splicing, encompasses an N-terminal signal peptide and a flavin adenine dinucleotide (FAD) binding motif, which are partially overlapped [5]. Renalase and flavoprotein-type monoamine oxidases (MAOs)[1] share a low degree of sequence identity (about 17%), concentrated in their N-terminal regions [1,12]. This similarity led to the attractive hypothesis that renalase could be a MAO-like flavin-containing enzyme. Indeed, both recombinant human renalase produced in *Escherichia coli*, and endogenous renalase isolated from human blood and urine, were reported to possess a significant, though very low, oxidase activity toward biogenic amines [1,13]. The substrate preference and the insensitivity of renalase to classical MAO inhibitors have prompted some authors to classify it as a distinct form MAO, named MAO C [12]. More recently, plasma renalase was proposed to be the main player of a feed-back

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[1] *Abbreviations used*: MAO, monoamine oxidase; FAD, flavin adenine dinucleotide; SUMO, small ubiquitin-like modifier protein; polyHis-renalase, recombinant human renalase1 fused to a N-terminal His-tag; polyHis-SUMO-renalase, recombinant human renalase1 fused to a N-terminal extansion including a His-tag and a SUMO moiety; Ser-renalase, recombinant human renalase1 carrying an extra Ser in front of the initial Met; PBS, phosphate-buffered saline; IMAC, immobilized metal affinity chromatography; IEC, ion exchange chromatography; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); MALDI-TOF, matrix assisted laser desorption ionization-time of flight; CD, circular dichroism; MAP, mean arterial pressure; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVDevP, left ventricular developed pressure; LV$dP/dt_{max}$, maximum negative rate of developed left ventricular pressure; HR, heart rate; PRI, pressure rate index.

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pathway that regulates the level of circulating catecholamines [13]. According to this hypothesis, an increase in plasma concentration of cathecolamines would either promote secretion by the kidney of a catalytically inactive renalase form (prorenalase) and activation of prorenalase to an active catecholamine-degrading enzyme [13]. Despite the evident potential importance of renalase in the physiology of the excretory and cardiovascular systems, as well as its relevance in the pathogenesis of major human diseases, no biochemical characterization of this protein has been provided so far. A major obstacle to the structural and functional study of renalase is probably the difficulty to obtain sufficient amounts of correctly folded protein by expression in heterologous hosts. We show that milligrams amounts of highly pure recombinant human renalase1 can be obtained from *E. coli*, using the expression plasmids and purification procedures here reported. The protein was found to incorporate a stoichiometric amount of non-covalently bound FAD and to possess the *in vivo* activity on blood pressure previously described for the human protein.

Materials and methods

Plasmid pEX-V0795-B01, based on vector pReceiver-B01a and carrying the sequence encoding the major isoform of human renalase, renalase1, fused to a sequence encoding an N-terminal polyHis extension (Fig. 1), was purchased from imaGenes GmbH (Berlin, Germany). The "Champion™ pET SUMO Protein Expression System" kit was obtained from Invitrogen (Paisley, UK). PolyHis-Senp2 protease was produced *E. coli* Rosetta(DE3) cells transformed with the plasmid described in [14] and purified according to the reported procedure. Three plasmids, based on vector pOFX, carrying a spectinomycin resistance gene and overexpressing different sets of *E. coli* chaperones, namely GroES/GroEL, DnaK/DnaJ, and DnaK/DnaJ/GrpE, under the control of the *tac* promoter, have been kindly provided by Dr. O. Fayet [15]. A polyclonal goat anti-renalase antibody was purchased from Abcam plc (Cambridge, UK). A monoclonal anti-goat/sheep IgG antibody conjugated with peroxidase was from Sigma–Aldrich. All chromatographic media and columns were obtained from GE Healthcare. Liquid chromatography was performed using an ÄKTA FPLC apparatus (GE Healthcare) equipped with a P-960 pump (GE Healthcare) for loading large sample volumes. *Crotalus durissus* phosphodiesterase was from Roche Applied Science and human recombinant MAO-A from Sigma. Protein concentration was assayed by the biuret method [16], except for purified samples in which the Bradford's method was used [17].

Plasmid construction

In order to take advantage of the restriction sites already present in pEX-V0795-B01, the following double-stranded polylinker was ligated via its A overhangs to the linear form of pET SUMO included in the kit:

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 5′-TCCATGGCTAGCGGATCCGAATTCGAGCTCAAGCTTGCGGCCGCTCGAGA-3′
3′-AAGGTACCGATCGCCTAGGCTTAAGCTCGAGTTCGAACGCCGGCGAGCTC-5′
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The resulting pET SUMO-MCS vector contains unique *Nco*I and *Xho*I restriction sites (underlined bases) at positions suitable for cloning. The sequence encoding human renalase1 was then excised from pEX-V0795-B01 as a *Nco*I/*Xho*I fragment and inserted between the same sites of pET SUMO-MCS, yielding pET-SUMO-RNLS, whose map is displayed in Fig. 1. This plasmid directs the synthesis of a fusion protein where a polyHis-tag, a "small ubiquitin-like modifier" (SUMO) unit and renalase1 are connected from N- to C-terminus. Following cleavage with the SUMO-specific Senp2 protease, polyHis-SUMO-renalase is expected to yield a renalase1 form that includes a single extra Ser in front of its N-terminal Met, which is indicated as Ser-renalase in this paper.

Optimization of expression of soluble polyHis-renalase in E. coli

Different *E. coli* strains (BL21(DE3), HMS174(DE3), JM109(DE3), C41(DE3), MC4100(DE3) AD494(DE3), Rosetta(DE3), and Origami(DE3)) were transformed with pEX-V0795-B01, cultured in 2×YT medium containing 100 mg/l ampicillin and induced with 0.1 mM IPTG under different conditions. The amount of renalase in both total cell lysates and in the soluble fraction of cell extracts was evaluated by SDS–PAGE followed by Western blotting. Blotted

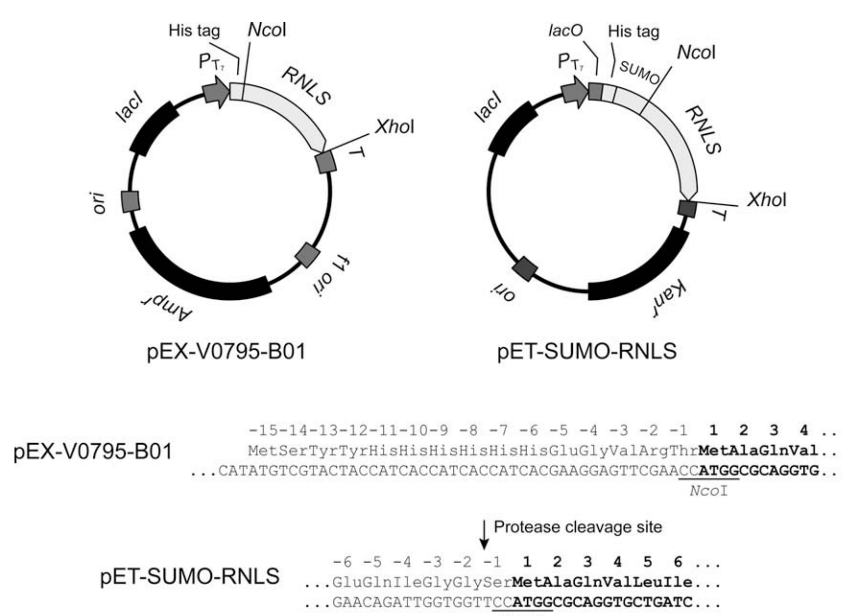

Fig. 1. Functional maps of human renalase expression plasmids. Relevant regions of pEX-V0795-B01 and pET-SUMO-RNLS are boxed. P_{T7}, T7 RNA polymerase promoter; *lac*O, *lac* operator; *T*, transcriptor terminator; *lac*I, *lac* repressor gene; *RNLS*, cDNA sequence encoding human renalase1. In the lower part of the figure, the DNA and amino acid sequences around the N-terminus of renalase are shown.