

Human cytosolic asparaginyl-tRNA synthetase: cDNA sequence, functional expression in *Escherichia coli* and characterization as human autoantigen

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ABSTRACT

The cDNA for human cytosolic asparaginyl-tRNA synthetase (hsAsnRSc) has been cloned and sequenced. The 1874 bp cDNA contains an open reading frame encoding 548 amino acids with a predicted M_r of 62 938. The protein sequence has 58 and 53% identity with the homologous enzymes from *Brugia malayi* and *Saccharomyces cerevisiae* respectively. The human enzyme was expressed in *Escherichia coli* as a fusion protein with an N-terminal 4 kDa calmodulin-binding peptide. A bacterial extract containing the fusion protein catalyzed the aminoacylation reaction of *S.cerevisiae* tRNA with [14 C]asparagine at a 20-fold efficiency level above the control value confirming that this cDNA encodes a human AsnRS. The affinity chromatography purified fusion protein efficiently aminoacylated unfractionated calf liver and yeast tRNA but not *E.coli* tRNA, suggesting that the recombinant protein is the cytosolic AsnRS. Several human anti-synthetase sera were tested for their ability to neutralize hsAsnRSc activity. A human autoimmune serum (anti-KS) neutralized hsAsnRSc activity and this reaction was confirmed by western blot analysis. The human asparaginyl-tRNA synthetase appears to be like the alanyl- and histidyl-tRNA synthetases another example of a human Class II aminoacyl-tRNA synthetase involved in autoimmune reactions.

INTRODUCTION

Aminoacyl-tRNA synthetases (aaRS) are enzymes involved in protein biosynthesis catalyzing the specific attachment of amino acids to their cognate tRNAs. Two classes of synthetases have been defined, each of 10 members, based on their primary and tertiary structures (1,2). Class II enzymes have three consensus sequence motifs; motif 1 contributes to the dimer interface, whereas motifs 2 and 3 are constituents of the catalytic site. Sub-classification can be made of the class II enzymes based on more extensive sequence and structural similarities (3). In higher eukaryotes, nine aaRS of different specificities (not including AsnRS) are associated within a multi-enzyme complex (4).

Asparaginyl-tRNA synthetase (AsnRS) is classified as a sub-class IIb enzyme together with the aspartyl- and lysyl-enzymes on the basis of similarities in their N-terminal extensions and the catalytic

domains (3,5–7). The three-dimensional structure of an AsnRS determined in this laboratory (*Thermus thermophilus* AsnRS; 8) further illustrates the strong structural homology between the three class IIb synthetases.

Autoantibodies are found in many patients with polymyositis or dermatomyositis. Some of these patients have antibodies raised against aaRS, of which anti-Jo-1, directed at histidyl-tRNA synthetase (HisRS) is by far the most common (9).

Below we describe the cDNA sequence of human cytosolic AsnRS, the bacterial expression of the recombinant enzyme and its activity assays with different sources of tRNA. Furthermore, we report its reactivity with a human autoimmune serum. The implication of the human cytoplasmic AsnRS in an autoimmune disorder is an interesting property of this enzyme.

MATERIALS AND METHODS

Restriction endonucleases, modification enzymes and unfractionated tRNAs were purchased from Boehringer Mannheim. Oligonucleotides were supplied by Genosys. Autoimmune sera (anti-HisRS, anti-AlaRS, anti-KS) were kindly provided by Dr I.Targoff (Oklahoma Research Foundation) and Dr M.Hirakata (University of Tokyo School of Medicine).

Cloning of hsAsnRSc cDNA

Molecular cloning methods were used according to Sambrook *et al.* (10). Human Expressed Sequence Tag (EST) sequences coding for peptides showing strong sequence similarities with *Brugia malayi* AsnRS were aligned. Missing 5' and 3' regions were amplified by PCR methods on human liver 5' RACE-Ready cDNA from Clontech. Thirty cycles of amplification were carried out (20 s denaturation at 94°C, 30 s annealing at 60–68°C and 5 min elongation at 68°C). The complete cDNA was amplified using the 5' RACE-Ready cDNA with the oligonucleotide primer 5'-**CCGGATCCCATATGGTGCTAGCAGAGCTGT**-3' (restriction sites are in bold and modified nucleotides are underlined) creating a *Bam*HI (and *Nde*I) restriction site for cloning the AsnRS cDNA fragment into the pCal-n expression vector (Clontech) and the oligonucleotide 5'-TCAGGTGATTTGAGATAGTTTTT-ATGG-3'.

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Expression of hsAsnRS as a bacterial fusion protein and purification

The hsAsnRSc coding region was inserted into pCal-n vector (11) as a *Bam*HI–*Eco*RI fragment and transformed into the *Escherichia coli* strain BL21 (DE3). Cells were grown in LB at 37°C to an A_{600} of 0.6, isopropyl-1thio- β -D-galactoside was added to a final concentration of 0.2 mM and incubation at 23°C continued for a further 3 h. Cells were lysed by lysozyme and sodium deoxycholate treatment (12). The bacterial extract was applied to a 2.5 ml calmodulin resin (13).

Aminoacylation assay and kinetic parameters

The aminoacylation reaction assay was as previously described (14) in the presence of 1.12 μ M tRNA^{Asn} of unfractionated tRNA from *E. coli* MRE600, *Saccharomyces cerevisiae* or calf liver; the determination of asparagine acceptance activity in unfractionated tRNA from *E. coli* was performed with an *E. coli* protein extract, that in unfractionated *S. cerevisiae* and calf liver tRNA with hsAsnRSc fusion protein. The concentration of recombinant human AsnRS was 33 nM.

Neutralization assay

AsnRS (66 nM) was preincubated for 10 min on ice with the various sera (1:10 dilution of the sera donated by Drs Targoff and Hirakata). After preincubation the aminoacylation activity was determined using calf liver tRNA. In the aminoacylation reaction the sera are present in a 1:100 dilution.

Detection of the recombinant hsAsnRSc by western blot using autoimmune serum (anti-KS)

Protein samples were separated electrophoretically on a 12% SDS–polyacrylamide gel and transferred to a Immobilon-P membrane for western blot analysis (15). The immunological reactivity of the recombinant hsAsnRSc was tested against 5.0 μ l human anti-KS serum. [³⁵S]protein A (16.7 mM, 600 Ci/mmol; Amersham) was used to detect specific AsnRS–antibody interactions by autoradiography (Fig. 2) after 16 h exposure to Biomax film (Kodak).

RESULTS

Cloning and sequencing of the human AsnRS cDNA (EMBL database: Aj000334)

Human EST sequences coding for peptides which show strong sequence similarities with *B. malayi* AsnRS were aligned to a 1302 bp fragment. The assembled cDNA sequence comprises 1874 bp with a large predicted open reading frame of 1644 bp. This encodes a protein of 548 amino acids with a predicted M_r of 62 938. Sequence alignment of several bacterial and eukaryotic AsnRSs indicates that the human enzyme is composed of three characteristic domains; an N-terminal extension, typical for eukaryotic AsnRS, followed by a putative β -barrel domain probably involved in tRNA^{Asn} anticodon recognition and a catalytic domain containing the three Class II specific motifs (Fig. 1).

Bacterial expression and purification of the recombinant enzyme

The recombinant protein comprises an N-terminal 4 kDa Calmodulin Binding Peptide (CBP) fusion tag coupled to the AsnRS. Figure 2 shows the SDS–PAGE analysis of the AsnRS fusion protein in an unfractionated bacterial extract (lane 3) and its purified form (lane 4). The apparent molecular weight of the fusion protein is in agreement with the predicted molecular weight of AsnRS (63 + 4 kDa CBP).

Unfractionated bacterial extracts were assayed for their ability to catalyze the aminoacylation of *S. cerevisiae* tRNA with [¹⁴C]asparagine; these extracts had 20-fold greater aminoacylation activity with *S. cerevisiae* tRNA relative to *E. coli* extracts carrying only the pCal-n vector.

Bacterial extracts were loaded on a calmodulin column in the presence of calcium. EGTA eluted fractions were collected and analyzed by western blot methods for the presence of *E. coli* AsnRS contamination using a rabbit anti-*E. coli* AsnRS serum (data not shown).

Aminoacylation activity of the recombinant human AsnRS using tRNA from different origins

The purified AsnRS fusion protein was tested for its enzymatic activity with tRNA substrates of different origins i.e. *E. coli*, *S. cerevisiae* and calf liver at the same relative concentration of tRNA^{Asn}. Figure 3 shows that calf liver and *S. cerevisiae* tRNAs are both efficient substrates for the human enzyme. For both tRNAs similar plateau values are reached although the initial rate is somewhat higher for the calf liver tRNA (0.15 pmol/s⁻¹ compared to 0.09 pmol/s⁻¹ for *S. cerevisiae* tRNA).

Neutralization of AsnRS activity by a human autoimmune serum

The AsnRS fusion protein was preincubated with the different autoimmune sera (anti-KS, anti-AlaRS and anti-HisRS) and two control sera. After preincubation, residual aminoacylation activity was determined. Only the anti-KS serum neutralized the human AsnRS activity significantly with an inhibition of 98%. The other anti-synthetase sera (anti-HisRS and anti-AlaRS) did not neutralize significantly the enzyme activity (<4% of inhibition).

Immunoreactivity of the anti-KS serum in a western blot experiment

Since only the anti-KS serum produced significant inhibition of AsnRS activity the interaction of this serum with recombinant protein was examined by western blot analysis. Samples of bacterial extract from the overproducing strain containing recombinant synthetase and a control strain containing only the pCal-n vector together with purified human AsnRS fusion protein were loaded on an SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to a nylon membrane and incubated with human anti-KS serum. Antigen–antibody interactions were detected using ³⁵S-labeled protein A. Figure 2 shows that the human anti-KS serum specifically interacts with the human AsnRS both in the bacterial extract and in purified form.

N-terminal extension

hsASnRS	MVLAELVYSD	REGSDA.TGD	GTKKEPKFTG	LKALMTVGSKE	PFPT...IYV	DSQKENER.W	NVLSKSQLKN	IKKMWHREQM	75
bmASnRS	.MTVYVIC.	PETGDD.GND	GSELKPLRTL	YQAMI.ITKS	SKGD...FLI	RTKKDGKQIW	EAASKTALKK	SWKHVEQEML	71
scASnRS	MSSLYLKE	ATGVDELTTA	GSQDHPFKTP	AYALFASQQK	SDATEPKLFV	FKTEDNE..Y	QEISASALKK	ARK..GCDDGL	74
ttASnRS	

hsASnRS	KSES.R....	EKKEAEDSLR	REKNLEEAKK	ITIKNDPSLP	EPKCVKIGAL	EGYRQQRVKV	FGWVHRLRRQ	GKNLMFLVLR	150
bmASnRS	KNEKVAAKML	EKDATEVGVK	.AALBEAKK	VQIELDTSLS	YITGVKIRDL	VKHRNERVCI	KGWIHRMRQ	GKSLMFFILR	149
scASnRS	KKKAVKQKEQ	ELKKQCKEAE	NAAKQLSALN	ITIKEDESLP	AAIKTRIIYDS	YSKVQQRVKV	SGWIHRLRS.	NKKVIFVVLRL	153
ttASnRSMRVFIDEI	ARHVDQEVLEL	RGWLYQRR.S	KGKIHFLLLR	37

β-barrel domain

hsASnRS	DGTGYLQCVL	ADELQCQY..	.NGVLLSTES	SVAVYGLMNL	TPKGQAPGG	HELSCDFWEL	IGLAPAG..G	ADNLINESD	225
bmASnRS	DGTGFLQVLL	MDKLCQTY..	.DALTVNTEC	TVEIYGAIKE	VPEGKEAPNG	HELIADFVKI	IGNAPPG..G	IDNVLINEAS	224
scASnRS	DGSGFIQCVL	SGDLALAQ..	.QTLDLTLES	TVTLYGTIVK	LPBGKTAPGG	VELNVDYIEV	VGLAPGGEDS	FTNKIAEGSD	230
ttASnRS	DGTGFLQATV	VQGEVPEAVF	READHLPQET	ALRVWG...R	VREDRRAPGG	FELAVRDLQV	VSR..PQ..G	EYPIGPKEHG	110

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hsASnRS	VDVQLNRRHM	MIRGENMSKI	LKARSMVTRC	FRDHFFDRGY	YEVTPPTLVQ	TQVEGGATL F	KL.....	287
bmASnRS	VDKMLDNRHL	VIRGENAAL	LRLRAAATRA	MREHFYNAGY	LEVAPPTLVQ	TQVEGGSTL F	NL.....	286
scASnRS	PSLLLDQRHL	ALRGDALSAV	MKVRAALLKS	VRRVYDEEHL	TEVTPPCM VQ	TQVEGGSTL F	KM.....	292
ttASnRS	IDFLMDHRHL	WLRHRRPFVAF	MRIRDELER	IHEFFGERGF	LRFDAPIL TP	SAVEG TTELF	EVELFD....	176

##### Motif 2 #####									
hsASnRS	..DYFGEEAF	LTQSSQLYLE	TCLPALGDVF	CLAQSYRA EQ	SRTRRHLEAY	THVEA ECFFL	TFDDLNRLE	DLVCDVVDRI	365
bmASnRS	..DYFGEQSF	LTQSSQLYLE	TCIPTLGDVF	HLHCSYRA EK	SRTRRHLEAY	AHVEA ECFFI	TLDDLMEK IE	ELVCDTVDRL	364
scASnRS	..NYGEEAY	LTQSSQLYLE	TCLASLGDVY	TIQESFRA EK	SHTRRHLESEY	THIEA ELAFI	TFDDLQ HIE	TLIVKSVQYV	370
ttASnRSGKAY	LSQSGQLYAE	AGALAFK VY	TFGPTFRA ER	SKTRRHLEEF	WMV EPVAFM	THEEN MALQE	ELVSVFLVARV	251

hsASnRS	L.KSP....	.AGSIVHELN	PNFQP.PKRP	FKRMVYSDAI	VWLKEHDVKK	EDGTFYEPGE	DIPEAPERLM	TDT.INEPIL	436
bmASnRS	LADEE....	.AKLLEHIN	PKFQP.PERP	FLRMEYKDAI	KWLQEHNVEN	EFGNTFTYGE	DIABAAERFM	TDT.INKPIL	436
scASnRS	LEDPI....	.AGPLVKQLN	PNFKA.PKAP	FMRLQYKDAI	TWLNEHDIKN	EGEDFKF GD	DIABAAERKM	TDT.IGVPIF	442
ttASnRS	LERRSREL..	.EMLGRDPK	A.LEPAAEGH	YPRLT YKEAV	ALVNRIAQED	PEV PLPYGE	DFGAP HEAAL	S.RRFDR PVF	325

hsASnRS	LCRFPVEIKS	FYMQRCPEDS	RLTESVDVLM	P.NVGEIVGG	SMRIFDSEEI	LAGYKREGID	PTPYWYTDQ	RKYGTCPHGG	515
bmASnRS	LNRFPSSEIK	FYMQRDAQDN	TLTESVDLLM	P.NVGEIVGG	SMRIWKFDEL	SKAFKNVEID	PKPYWYLDQ	RLYGTCPHGG	515
scASnRS	LTRFPVEIKS	FYMKRCSDDP	RVTESVDVLM	P.NVGEITGG	SMRIDDMDEL	MAGFKREGID	TDAYWYFIDQ	RKYGTCPHGG	521
ttASnRS	VERYPARIKA	FYMEPDPEDP	ELVLNDDLRL	PEGYGEITGG	QRIDHLELL	RRKIQEFGLP	BEVYDWYLDL	RRFGSVPHSG	405

##### Motif 3 #####									
hsASnRS	YGLGLERFLT	WILNRYHIRD	VCLYPRFVQR	CTP..	546				
bmASnRS	YGLGLERFIC	WLTNTNHIRD	VCLYPRFVGR	CVP..	546				
scASnRS	YGIGTERILA	WLCDRFTVRD	CSLYPRFSGR	CKP..	552				
ttASnRS	FGLGLERTVA	WICGLAHVRE	AIPFPRMYTR	MRP..	436				

Figure 1. Multiple alignment between prokaryotic and eukaryotic AsnRS sequences. The program PILEUP was used (GCG package, University of Wisconsin). The origins are (accession number in the SwissProt or EMBL data banks are indicated in parenthesis): *Homo sapiens*, hsAsnRS (AJ000334); *Brugia malayi*, bmAsnRS (P10723); *Saccharomyces cerevisiae* scAsnRS (P38707); *Thermus thermophilus*, ttAsnRS (X91009). The position where the residues are strictly conserved in this alignment are in bold type. The Class II specific motifs are indicated by #. The N-terminal extensions characteristic for eukaryotic AsnRS sequences are boxed. Dashed lines indicate the putative β-barrel domain most probably involved in tRNA anticodon recognition.

DISCUSSION

We have isolated the cDNA coding for the complete human AsnRS. This provides the first example of a mammalian AsnRS sequence. The sequence exhibits a high degree of similarity with the two other known eukaryotic AsnRSs: a 58% amino acid identity with the AsnRS from *B.malay* and a 53% identity with that from *S.cerevisiae*. Based on the following observations we conclude that the sequence we have determined is that of human cytosolic AsnRS: (i) the absence of a mitochondrial import signal, (ii) strong sequence similarities to the cytosolic AsnRSs from *B.malay* and *S.cerevisiae* and weaker similarities with bacterial enzymes, (iii) estimated molecular weight and calculated isoelectric point is typical for a cytosolic AsnRS, (iv) calf liver and *S.cerevisiae* tRNA are significantly better substrates than *E.coli* tRNA.

Despite a similar degree of overall sequence identity of human tRNA compared to tRNA from *E.coli* (62%) or from *S.cerevisiae*

(65%), *E.coli* tRNA is poorly aminoacylated by the hsAsnRS fusion protein in contrast to its *S.cerevisiae* counterpart. This could be due to one base insertion into the D-loop of the eukaryotic tRNA^{Asn} at position 21 (16).

Some eukaryotic synthetases are involved in pathological conditions (9). Patients with systemic autoimmune diseases make specific autoantibodies that are directed against self structures. According to one hypothesis, these autoantibodies arise through an immune response to foreign antigens such as infectious agents that share, by molecular mimicry, common structures with host proteins. Autoantibodies are found in most patients with polymyositis or dermatomyositis and 35–40% of these patients have myositis-specific antibodies. 25–30% of these patients have antibodies raised against aminoacyl-tRNA synthetases, of which anti-Jo-1, directed at histidyl-tRNA synthetase (HisRS) is by far the most common (9).

Of the several autoimmune sera tested for their capacity to neutralize the hsAsnRS activity, only the anti-KS auto immune

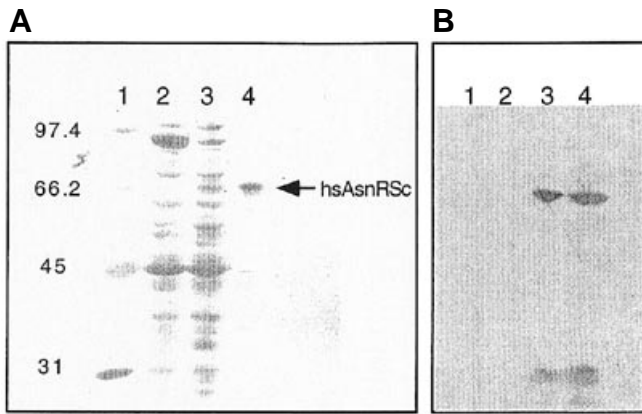


Figure 2. Expression in *E. coli*, purification and immunological reactivity with the anti-KS serum of the recombinant hsAsnRSc. (A) Coomassie Brilliant Blue stained SDS gel (12% polyacrylamide). Lane 1, molecular mass marker; lane 2, bacterial control extract; lane 3, bacterial extract containing the recombinant CBP tagged protein; lane 4, purified hsAsnRSc. (B) Autoradiography of the western blot performed with the SDS gel showing the immunoreactivity of hsAsnRSc with anti-KS serum.

serum isolated by Dr M.Hirakata was able to neutralize the activity of the recombinant hsAsnRSc. The other anti-synthetase sera (anti-AlaRS and anti-HisRS) did not show any significant inhibition. Besides its neutralizing activity, the anti-KS serum was also able to recognize the recombinant AsnRS fusion protein on an immunoblot. It has been shown that anti-Jo-1 antibodies recognize multiple conformation-dependent and independent epitopes on human HisRS and that auto-epitopes vary among different myositis patients (17). Furthermore, it has been demonstrated that the substrates ATP and histidine act as competitive inhibitors for the formation of the synthetase-anti-Jo-1 antibody complex, whereas the tRNA acts in a non-competitive way (18). The human AsnRS has yet to be characterized for this complex formation.

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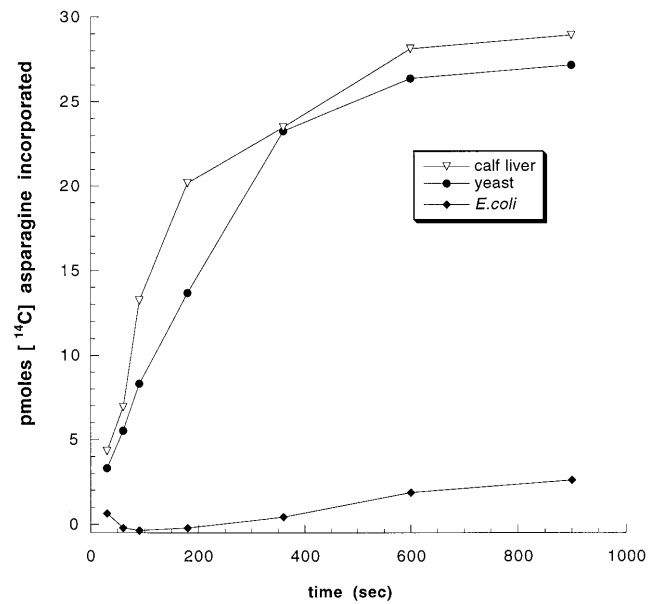


Figure 3. Asparaginyl-tRNA synthetase activity of the recombinant human enzyme with tRNA from different sources. Incorporation by the purified enzyme (33 nM) of [¹⁴C]asparagine (pmol) into unfractionated tRNA from *E. coli* (◆), from yeast (●) and from calf liver (▽).

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