CHARLES UNIVERSITY IN PRAGUE
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INSTITUTE OF INHERITED METABOLIC DISORDERS

A PATHOGENITY OF ADENYLOSUCCINATE LYASE DEFICIENCY

Abstract booklet of Ph.D. thesis

MARIE ZIKÁNOVÁ

Supervisor: Stanislav Kmoch, CSc.

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ABSTRACT

Adenylosuccinate lyase (ADSL) is an enzyme acting in two pathways of purine nucleotide metabolism. Mutations in *ADSL* gene compromising the enzyme activity lead to an inherited metabolic disease with severe neurological involvement – ADSL deficiency. Three distinct clinical phenotypes can be distinguished based on onset and severity of symptoms. The pathogenic mechanisms leading to the development of symptoms and underlying the phenotypic heterogeneity are unclear. The main pathogenic effect is attributed to the toxic effects of accumulating succinylpurines (SAdo, SAICAr). Their concentrations in cerebrospinal fluid, particularly diverse SAdo/SAICAr ratio, do correspond with the phenotypic groups. It is hypothesized that it may result from a mutation specific and thus structural related non-parallel loss (or gain) of enzyme activity towards one of its substrates. The main goal of the thesis is to seek for biochemical and structural basis of the diverse SAdo/SAICAr ratios and thus explain a pathogenetic mechanism of ADSL deficiency.

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Dr. Burlina, Padova, Italy; Dr. Gil, Barcelona, Spain; Dr. Jakobs, Amsterodam, Netherlands and Dr. Spaagen, Maastricht, Netherlands: Providing commercially unavailable dephosphorylated ADSL substrates
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1. INTRODUCTION

Adenylosuccinate lyase (ADSL; EC 4.3.2.2.) is an enzyme acting in two pathways of purine nucleotide metabolism. It catalyses the conversion of succinylaminoimidazole carboxamide ribotide (SAICAR) into aminoimidazole carboxamide ribotide (AICAR) in the purine *de novo* synthesis pathway and the formation of adenosine monophosphate (AMP) from adenylosuccinate (SAMP) in the purine nucleotide cycle (Figure 1) [1, 2].

The native human ADSL has a homotetrameric structure with a subunit size of ~50 kDa. Its three dimensional structure has not been determined yet. Based on crystallographic studies on ADSL from *Thermotoga maritima* [3] and *Bacillus subtilis* [4] it is predicted that the protein homotetramer contains four active sites. Each of them is formed from regions of three different subunits.

The human *ADSL* gene consists of 13 exons [5], spans 23 kb on chromosome 22q13.1-13.2 [6, 7] and is ubiquitously expressed in two alternatively spliced mRNA isoforms [5].

Mutations in *ADSL* gene compromising the enzyme activity lead to an inherited metabolic disease with severe neurological involvement – ADSL deficiency (OMIM 103050).

Biochemically the enzyme deficiency results into intercellular accumulation of both enzyme substrates SAICAR and SAMP. Accumulated substrates are subsequently dephosphorylated to SAICAriboside (SAICAr) and succinyladenosine (SAdo) respectively (Figure 1), and they are excreted and detectable in plasma, urine and cerebrospinal fluid (CSF). For their detection, several simple screening procedures have been developed [8].

To date, 60 patients with ADSL deficiency have been reported world-wide [9]. Affected patients manifest mostly within the first year of life with psychomotor retardation, seizures, hypotonia, growth retardation, muscular wasting and, interestingly, also with a wide spectrum of behavioural changes such as autism, aggressiveness or self-mutilation. Two distinct clinical phenotypes can be distinguished based on onset and severity of symptoms:

i) Type I (severe form) presenting within the first months of life, encompassing the whole spectrum of disease symptoms and resulting in severe psychomotor retardation, developmental arrest, coma vigil and SAdo/SAICAr ratio in CSF ≈ 1.

ii) Type II (moderate or mild form) presenting within the first years of life either with psychomotor retardation or hypotonia and SAdo/SAICAr ratio in CSF ≥ 2.

The pathogenic mechanisms leading to the development of individual symptoms and underlying the phenotypic heterogeneity are unclear. No significant reduction in the rate of *de-novo* purine synthesis was found in fibroblasts of ADSL deficient patient [10]. No abnormality in purine nucleotide concentrations was found in several tissues of ADSL deficient patients [11]. No differences in residual enzyme activities were found

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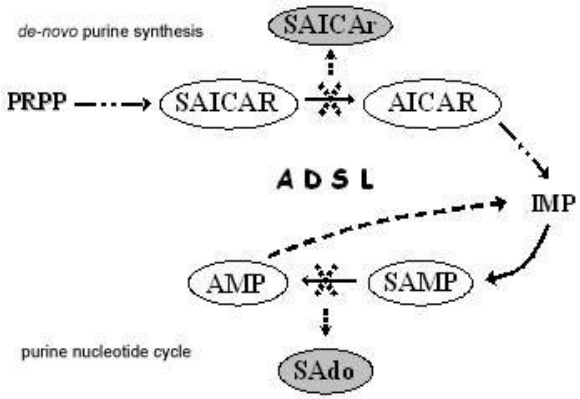
to be associated with severity of the phenotype. The main pathogenic effect is therefore attributed to the toxic effects of accumulating succinylpurines [12].

Figure 1: Metabolic pathways of ADSL



2. AIMS OF THE STUDY

The main pathogenic effect of ADSL deficiency is attributed to the toxic effects of accumulating succinylpurines [12]. Although, the absolute SAdo and SAICAr concentrations in body fluids do not correlate with severity of the phenotype, it has been found that diverse values of ratio between SAdo and SAICAr concentrations in cerebrospinal fluid (SAdo/SAICAr ratio) do correspond with the two main phenotypic groups [13, 14]. The mechanism(s) leading to the diverse ratios is (are) not clear. It is hypothesized that it may result from a mutation specific and thus structural related non-parallel loss (or gain) of enzyme activity towards one of its substrates.

Relevant biochemical investigations answering this hypothesis are generally limited by the unavailability of one ADSL substrate SAICAR and a small number of patients and tissues. Another limiting factor is the unavailability of accumulating ADSL metabolites (SAICAr and SAdo) for diagnostics and relevant *in vitro* and *in vivo* studies.

To explain pathogenic mechanisms of ADSL deficiency we have therefore decided to:

1. Develop methods for preparation and purification of ADSL substrates and their metabolites :
    a) SAICAR
    b) SAICAr and SAdo
2. Identify more patients and collect relevant clinical, biochemical and molecular data by means of world-wide provided complex diagnostics of ADSL deficiency based on screening, biochemical investigation of body fluids and tissues, mutation analysis and assessment of mutation pathogenity identified.
3. Study genotype-phenotype correlation:
    a) Express recombinant mutated ADSL proteins (fmADSL) and to correlate their catalytic properties with clinical and biochemical data (e.g. different SAdo/SAICAr ratio).
    b) Correlate diverse SAdo/SAICAr ratios to biochemical and structural properties of corresponding mutant ADSL proteins:
        - Create a database of all published patients with ADSL deficiency
        - Express, purify and characterise recombinant mutant ADSL proteins selected according to their association with diverse SAdo/SAICAr ratios and different phenotypic groups
        - Prepare, purify and crystallize human ADSL protein
        - Use a human ADSL structure model in evaluation of structural alterations possessed by selected ADSL mutations.

b) We created a database of all published patients with ADSL deficiency. We collected clinical, biochemical and molecular data of 57 patients from 16 countries (47 different mutations).

c) Now we are studying various SAdo/SAICAr ratios by other biochemical and structural studies (This project is supported by the Czech Science Foundation). In terms of the project:
    - We focused to genotypes which correlate with various phenotype and SAdo/SAICAr ratios and prepared 21 appropriate mutated ADSL clones. The set of clones make us possible to characterized 17 patients with ADSL deficiency; 4 presented with neonatal, 6 with type I and 7 with type II phenotype.
    - For complementation studies we introduced and optimized a system for purification of human recombinant wtADSL. This method we will use to study intersubunit complementation in human mutant recombinant ADSL proteins.
    - We used the purification system for preparing human recombinant wtADSL in sufficient quantity and quality for crystallization, biochemical characterisation (analytical ultracentrifugation, circular dichroism) and antibody preparation.

6. ABBREVIATIONS

ADSL	adenylosuccinate lyase
AICAR	aminoimidazole carboxamide ribotide
AMP	adenosine monophosphate
cDNA	complementary DNA
CIAP	Calf Intestinal Alkaline Phosphatase
CSF	cerebrospinal fluid
ddNTP	dideoxynucleotides
DEAE	diethylaminoethyl
dNTP	nucleotides
DTT	dithiotreitol
fmADSL	recombinant fusion mutated ADSL protein
fwtADSL	recombinant fusion wt ADSL protein
HPLC	High Performance Liquid Chromatography
IIMD	the Institute of Inherited Metabolic Disorders
MBP	Maltose Binding Protein
PEI-TLC	polyethyleneimine-Thin Layer Chromatography
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAdo	succinyladenosine
SAICAr	succinylaminoimidazole carboxamide riboside
SAICAR	succinylaminoimidazole carboxamide ribotide
SAMP	adenylosuccinate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

5. CONCLUSION

The summary of achieved results:

1. Preparation and purification of ADSL substrates

a) We prepared commercially unavailable ADSL substrate, SAICAR, in the amount and purity sufficient for enzymatic studies in ADSL deficient patients and for kinetic characterisation of mutated ADSL proteins.

b) We developed methods for preparation and purification of SAdo and SAICAr, commercially unavailable dephosphorylated ADSL substrates, at a sufficient amount and purity for diagnostics and *in vivo* and *in vitro* toxic studies. These methods we publicised [17]. These substrates were provided to clinical and scientific institutions in Padova (Italy), Barcelona (Spain), Amsterdam and Maastricht (the Netherlands).

2. Complex diagnostics of ADSL deficiency:

a) We introduced a system for complex diagnostics of ADSL deficiency based on screening, enzymatic examination of tissues, mutation analysis and testing the pathogenity of mutation, and offered the diagnostics in the international system GeneTests (http://www.genetests.org).

b) We diagnosed 22 patients in 18 families (8 Czech Republic, 7 Poland, 5 Germany, 2 US) and obtained the worldwide unique collection of patients with ADSL-deficiency.

c) We characterised and published a prenatal and early neonatal form of ADSL deficiency [14]. This severe neonatal form of the disease seems to be associated with particular genotypes and, since these patients may escape the diagnosis, may form a significant proportion of ADSL-deficiency. However if the diagnosis is known, prenatal diagnosis is possible and can be offered to affected families.

d) We performed prenatal examination of 2 embryos.

e) We collected a unique set of 9 fibroblast lines and 2 tissues sets from ADSL deficient patients.

f) We determined catalytic activity of ADSL in fibroblasts of 9 patients.

g) We cloned full length *ADSL* cDNA and introduced a system for expression, purification and a catalytic properties study of fusion recombinant human ADSL in E.coli. The methods were published [5].

h) We cloned 16 mutated *ADSL* cDNA. Then we expressed, purified and characterised relevant fusion recombinant ADSL proteins.

3. Study of genotype-phenotype correlation

a) We calculated a mean of homoallelic activities of fmADSL proteins by patients' genotypes and correlated it with severity of phenotype e.g. different SAdo/SAICAr ratio. However, all the mutant enzymes studied, if active, displayed proportional decrease in activity towards both substrates, and therefore, no ground for the varied SAdo/SAICAr ratio was found [5].

3. METHODS

3.1. PRODUCTION AND PURIFICATION OF SUBSTRATES

3.1.1. Production of substrates

SAICAR was generated in a reaction mixture (volume 1 ml, pH 7,5) containing 10 mM TRIS-HCl, 2 mM EDTA, 10 mM KCl, 20 mM fumaric acid, 2.6 mM AICAR and 10 U/ml of human recombinant ADSL (fwtADSL, see 3.2.3). Reactions were run at 37°C for 5 hours. To monitor reaction direction, aliquots taken every 30 minutes were analyzed by HPLC. Reaction was stopped after 5 hours of incubation by boiling in water bath for 5 minutes.

SAICAr was generated in a reaction mixture (volume 50 ml) of identical composition as the mixture used for SAICAR preparation. The reaction was run for 5 hours, stopped by boiling and the mixture was supplemented with 1 mM $MgCl_2$ and 10 U/ml of CIAP. Dephosphorylation was run at 37°C for 1 hour. Reaction was stopped by boiling and aliquot of the reaction mixture was analyzed by HPLC.

SAdo was generated according to Van den Bergh et al. [15]: in reaction mixture (volume 50 ml, pH 8.0) containing 10 mM TRIS-HCl, 1 mM $MgCl_2$, 2.6 mM SAMP and 10 U/ml CIAP. Reaction was run at 37°C for 1 hour and aliquot of the reaction mixture was analyzed by HPLC.

3.1.2. Purification of substrates

SAICAR: Reaction mixture (1 ml) containing SAICAR, AICAR and fumarate was concentrated by evaporation under stream of nitrogen at 40°C to final volume of 200 µl. Two 100 µl aliquots were applied on PEI-TLC plates (10 x 10 cm) and developed in 1 M ammonium acetate [16]. The UV-light spots corresponding to SAICAR (R_F 0.02) were scraped off and eluted with 1 ml of 1 M NH_4OH. The PEI-cellulose was removed by centrifugation at 10 000 rpm for 30 minutes. SAICAR was concentrated under stream of nitrogen at 40°C and its concentration was determined spectrophotometrically at 269 nm (UV-Vis Spectrometer Lambda 10, Perkin-Elmer, Beaconsfield, UK). The molar extinction coefficient 13.1 x 10^3 [15] was used for the calculation.

SAICAr: Reaction mixture (50 ml) containing SAICAr, AICAr and fumarate was applied on Dowex 50W column (150 x 10 mm). After washing the column with 200 ml of water, SAICAr and AICAr were eluted with 45 ml of 2 M ammonia. The pH of individual fractions was monitored. The alkalic fractions were collected and analyzed by HPLC. The fractions containing SAICAr and AICAr were pooled (30 ml) and ammonia was removed from the reaction mixture by stream of nitrogen at 40°C. Resulting mixture (pH 7) was applied on DEAE-Sephadex A50 column (160 x 10 mm). Column was washed with 100 ml of water and SAICAr was eluted with 100 ml of 0.01 M HCl. The pH of collected fractions was monitored. Acidic fractions containing SAICAr were pooled (20 ml) and neutralized with 1 M NaOH. To remove the pink coloured reaction by-product, 0.2 g of active carbon were added to the product solution. Active carbon was removed by centrifugation and clear, colour-less supernatant was lyophilized. The final product was dissolved in water and its concentration was

determined by UV spectrophotometry at 269 nm. The molar extinction coefficient 13.1 x 10^3 [15] was used for the calculation.

SAdo: Reaction mixture (50 ml) was applied on DEAE-Sephadex A50 column (160 x 10 mm). Column was washed with 50 ml of water and SAdo was eluted with 100 ml of 0.01 M HCl. Acidic fractions were pooled (20 ml) and neutralized with 1 M NaOH. The final solution was lyophilized. SAdo was dissolved in water and its concentration was determined spectrophotometrically at 269 nm. The molar extinction coefficient 19.2 x 10^3 [15] was used for the calculation.

HPLC analysis:
For HPLC analysis, Shimadzu 10A (HPLC) Liquid Chromatography System with PDA detector was used.
20 µl aliquots of reaction mixture were injected onto the reverse phase Prontosil 120-3-C18 AQ (200 x 4 mm id) 3-µm HPLC column (Bischoff) and eluted by linear gradient with acetonitril at a flow rate of 0.7 ml/min. Buffer contained 0.1 M KH_2PO_4 and 5 mM TBA.
UV absorbance of eluted compounds was monitored at 269 nm. The analyses were performed at ambient temperature (25 ± 1°C). All buffers were filtered and degassed by passage through a 0.22 µm filter under vacuum.

3.2. METHODS FOR PATIENTS´ DIAGNOSIS

3.2.1. Biochemical methods

3.2.1.1. Screening

For screening of adenylosuccinate lyase deficiency we used a method based on thin-layer chromatographic (TLC) detection of urinary SAICAr with Pauli's reagent (sodium diazobenzenesulphate).
For final diagnosis, we used a HPLC analysis of urinary/CSF SAdo and SAICAr on Shimadzu 10A Liquid Chromatography System with PDA detector. 10 µl aliquots of diluted urine were injected onto the reverse phase Prontosil 120-3-C18 AQ (200 x 4 mm id) 3-µm HPLC column (Bischoff) and eluted by linear gradient acetate buffer at a flow rate of 0.7 ml/min. UV absorbance of eluted compounds was monitored at 269 nm. The analyses were performed at ambient temperature (25 ± 1°C). All buffers were filtered and degassed by passage through a 0.22 µm filter under vacuum.

3.2.1.2. ADSL activities in cultured fibroblasts

The ADSL activities were measured in lysate using the HPLC analysis of AMP and AICAR formed from both ADSL substrates, SAMP and SAICAR. The reactions were run for 20 minutes at 37°C in 180 µl containing 10 mM Tris pH 8.2, 2 mM EDTA, 10 mM KCl, 1 mM DTT, 4% glycerol and 32 µg protein. Substrate concentrations were 0.14 and 0.09 mM for SAMP and SAICAR, respectively. SDS–PAGE analyses were performed according to standard procedure; protein concentrations were determined using the Bradford assay.

mutation specific oligonucleotide primers. The constructs with the correct sequences were introduced into the *E.coli* strain DH5αF'IQ (Invitrogen).

In total, 21 mutant ADSL clones are available for intersubunit complementation experiments. These experiments enabled characterisation of 17 patients; 4 presented with neonatal, 6 with severe and 7 with mild phenotype.

Tab. I: ADSL mutations, SAdo/SAICAr ratios and associated phenotypes in patients with ADSL deficiency

Mutation 1	Mutation 2	SAdo/SAICAr (CSF)	Phenotype
R194C	R396C	nm	neonatal
R426H	Y114H	0,5	neonatal
Y114H	E376D	0,6	neonatal
Y114H	R396H	0,7	neonatal
E80D	D87E	2,4	mild
M1V	S448P	1,5	mild
R303C	R303C	3,0	mild
R426H	D430N	2,6	mild
Y114H	R190Q	2,1	mild
R426H	T450S	2,5	mild
R426H	D268H		mild
Y114H	T242I	1,1*	severe
R426H	S23R	0,8*	severe
P467R	D268N	nm	severe
R194C	D268N	1,0	severe
R426H	R426H	1,4	severe
D215H	I351T	1,4*	severe

4.3.2.3. Preparation and purification of wtADSL protein

To obtain a sufficient amount of purified protein, wtADSL was prepared as a fusion protein with a maltose binding protein (MBP) by the method previously described [5]. ADSL-MBP protein was expressed in a pMAL-C2 system. After sonication and centrifugation, lysate was applied on amylose affinity columns (NEB). The fusion protein was bound to the amylose sorbent. After washing, fusion protein was cleaved on the column using factor Xa protease. Then ADSL was eluted from the column and SDS–PAGE analyses were performed. Purity of isolated ADSL was 95%. ADSL was produced in sufficient amount, and purity for crystallography, analytical ultracentrifugation, circular dichroism and antibody were prepared.

compound heterozygotes and that probably two structurally different mutant enzymes are involved in tetrameric structure building *in vivo*.

In the case of ADSL protein, such limitation may be partly overcome by experiments in which mutant proteins corresponding to individual genotypes are mixed together before further characterisation. In this process, called intersubunit complementation [4, 18-20], it is assumed that both mutant proteins recombine randomly and an array of 5 possible tetrameric structures with 64 different active sites may be formed. Hypothetically then, in a situation where structural and catalytic mutant proteins meet together, unique catalytic sites having disproportional activity towards one of the substrates may evolve and form the structural basis for diverse SAdo/SAICAr ratios.

The main goal is to seek for biochemical and structural basis of the diverse SAdo/SAICAr ratios.

The work is planned in the following steps:

1. Creation of a database of all published patients with ADSL deficiency
2. Selection of ADSL mutants associated with various phenotypes (e.g. diverse SAdo/SAICAr ratios) and its cloning
3. Preparation and purification of wt ADSL protein
4. Crystallization of human wt ADSL
5. Elucidation of human ADSL structure
6. Implementation and optimization of methods usable in characterization of intersubunit complementation
7. Expression, purification and characterization of mutant proteins through complementation experiments
8. Modeling of selected ADSL mutations

This project is supported by the grant of the Czech Science Foundation number 301/07/0600. To date, the first three planned work steps were carried out.

4.3.2.1. Database of all published patients with ADSL deficiency

We collected the unique set of 22 ADSL deficient patients. Because we needed a larger set for study of all ADSL deficiency forms and their relation with genotype, we created a database of all published patients with ADSL deficiency. The database is in a Microsoft Access programe.
We collected clinical, biochemical and molecular data of 57 patients from 16 countries (47 different mutations). The set of patients showed that ADSL deficiency is multiethnic.

4.3.2.2. Selection of ADSL mutants associated with various phenotypes (e.g. different SAdo/SAICAr ratios) and its cloning

Fifteen cDNA constructs containing mutations found in 22 ADSL deficient patients were prepared, cloned and characterised in our previous studies (Tab. I, white fields) [5, 14]. Additionally 6 mutations were selected according to their association with diverse SAdo/SAICAr ratio values (Tab. I, grey fields) and were cloned in pMAL-C2wtADSL clone using the GeneTailor™ Site – Directed Mutagenesis System (Invitrogen) with

3.2.2. Molecular biology methods

3.2.2.1. RT–PCR

Total RNA was isolated from peripheral blood lymphocytes, fibroblasts and skeletal muscle by the standard procedure. cDNA was reverse transcribed from total RNA using the SuperScript® First-Strand Synthesis System (Invitrogen) and oligo (dT) primer. *ADSL* cDNA was amplified in DNAengine Dyad termocycler (MJ Research) in a reaction volume of 25 µl containing 2.5U Klen*Taq*1 polymerase, 0.1 U DeepVent polymerase, 200 µM dNTPs, 0.15 µM primers and 1.5 mM $MgCl_2$ under the following conditions: the initial denaturation at 94°C for 1 minute followed by 40 cycles of denaturation at 94°C for 10 seconds, primer annealing at 61°C for 5 seconds and extension at 72°C for 1 minute. Final chain elongation was performed at 72°C for 5 minutes.

3.2.2.2. DNA sequencing

cDNAs prepared by RT–PCR were purified using the NucleoSpin Extract II kit (MACHEREY-NAGEL). Dideoxy cycle sequencing was performed in 7 µl containing 150 fmol of template, 1 pmol of the Cy5 labelled primer, 100 µM dNTP (dNTP:ddNTP ratio = 120), 5 mM $MgCl_2$ and Ampli*Taq* FS polymerase (PE Biosystems). Sequencing parameters were: initial denaturation (94°C for 2 minutes), 40 cycles of denaturation (94°C for 5 seconds), annealing of primers at specific temperature for 25 seconds and extension (68°C for 50 seconds), followed by 10 cycles of denaturation (94°C for 5 seconds) and extension at 68°C for 50 seconds. The samples were subsequently denatured in loading buffer (94°C for 3 minutes). Sequences were read on the ALFExpress sequencer (Pharmacia).

3.2.2.3. Mutation analysis

To verify the nature of mutations and to be able to screen for individual mutations in affected families, we designed PCR–RFLP-based assays on genomic DNA. These assays were all based on the PCR-based introduction of either one or both, diagnostic and control, restriction enzyme recognition sequence sites. The genomic DNA for PCR analysis was isolated from blood samples (Qiagen).

3.2.2.4. Genomic DNA sequencing

The PCRs were carried out according to standard procedure. Genomic DNA (100 ng) was amplified in 25 µl containing 2.5 U Klen*Taq*1 polymerase, 0.1 U DeepVent polymerase, 200 µM dNTPs and 0.15 µM primers. Amplification products were gel purified using the NucleoSpin Extract II kit (MACHEREY-NAGEL). Purified fragments were sequenced using the T7 and Rp primers. The dideoxy cycle sequencing was performed in a 7 µl reaction containing 150 fmol of template, 1 pmol of the Cy5 labelled primers, 100 µM dNTP (dNTP:ddNTP ratio = 120), 5 mM $MgCl_2$ and Ampli*Taq* FS polymerase. The sequences were read on the ALFExpress sequencer.

3.2.3. Expression and purification of ADSL recombinant proteins

3.2.3.1. Expression of ADSL protein in *E. coli*

The *ADSL* cDNA was prepared and cloned into the pCRII-TOPO vector (Invitrogen). The constructs of the correct sequence were used as templates for subsequent PCR amplification of *ADSL* cDNA using the 56S primer starting at the first ATG codon. PCR products were blunt end ligated into the pMAL-C2 vector (NEB). The constructs with the correct sequences were introduced into the *E.coli* strain DH5αF'IQ (Invitrogen) for fusion protein production. Briefly, a 600 ml culture of transformed bacteria was grown at 37°C in a rich medium, 100 μg/ml ampicillin and 0.2% glucose, to a density A_{600} of 0.5. IPTG was added to a final concentration of 0.3 mM and incubation continued for an additional 3 hours. Bacteria were harvested by centrifugation at 3000 *g* for 10 minutes. The pellets were resuspended in 10 mM Tris pH 8.2, 2 mM EDTA, 10 mM KCl, 1 mM DTT, 4% glycerol buffer and sonicated four times for 15 seconds at 40 W. Crude lysates were obtained by centrifugation at 8000 *g* for 30 minutes. Fusion proteins were isolated from crude lysate on amylose affinity columns (NEB). SDS–PAGE analyses were performed according to standard procedure; protein concentrations were determined using the Bradford assay (Sigma).

3.2.3.2. Expression of mutated ADSL in *E.coli*

All mutated cDNAs were first cloned into the pCRII-TOPO vector (Invitrogen). Individual mutated alleles were sequenced and subsequently subcloned into the pMAL-c2wt*ADSL* construct using the appropriate set of restriction enzymes. PCR product was blunt end cloned into the pMAL-C2 vector as described above. Resulting clones were sequenced and introduced into *E.coli* strain DH5αF'IQ. The protein expression experiments and enzyme activity measurements were performed in 20 ml cultures essentially as described above.

The ADSL activities were measured in isolated fusion protein using the HPLC analysis of AMP and AICAR formed from both ADSL substrates, SAMP and SAICAR. The reactions were run for 20 minutes at 37°C in 130 μl containing 10 mM Tris pH 7.5, 2 mM EDTA, 10 mM KCl, 1 mM DTT, 4% glycerol and 18 μg protein. Substrate concentrations were 0.14 and 0.09 mM for SAMP and SAICAR, respectively.

[19]. One of these histidines, His68, is spatially close to this conserved region. Based on the homology with the *B. subtilis* enzyme, it could be that the mutated histidine-114 residue in this region interferes with a proper active site formation. Both mechanisms, the hampered monomer association and improper active site formation, or even a combination of both, can thus lead to the observed enzyme instability and inactivity. The diminished activity of the D268N and D268N mutants is easily explainable as the wild-type aspartic acid residue resides in the active enzyme site and is directly involved in the catalytic mechanism where it serves as an electron donor of the acidobasic cleavage [16]. The exchange of this acidic residue for the uncharged asparagine leads to active centre disruption but does not seem to interfere with the enzyme protein stability. The effect of the severe and mild mutations is very hard to explain using the experimental data. There is no doubt that the mutations severely compromising the enzymatic activity are disease causing. To be able to make such a statement for the group of mild mutations, temperature activation profiles and kinetic parameters of the expressed proteins were studied. Three mutants, A3V, R190Q and D430N, compared with wild-type enzyme, showed modest changes in thermal stability and temperature dependence of ADSL activity suggesting the protein susceptibility to accelerated intracellular degradation.

4.3. GENOTYPE-PHENOTYPE CORRELATION

4.3.1. Determination of homoallelic activities and correlation with phenotype

It is hypothesized that the mechanism(s) leading to the diverse ratios of SAdo/SAICAr may result from mutation specific and thus structural related non-parallel loss (or gain) of enzyme activity towards one of its substrates. However, all the mutant enzymes studied, if active, displayed a proportional decrease in activity towards both substrates, and no ground for the varied SAdo/SAICAr ratio was therefore found [5].

During the studies, we identified and characterised a new neonatal form of ADSL deficiency [14] with a lack of spontaneous movements ("floppy infant") and intractable seizures. ADSL deficiency may also have a prenatal manifestation with a lack of fetal heart rate variability, hypokinesia (with all its consequences up to arthrogryposis and pulmonary hypoplasia) and prenatal growth restriction. ADSL-deficiency has thus been added to the differential diagnosis in this specific clinical context. This severe neonatal form of the disease seems to be associated with particular genotypes and, since these patients may escape the diagnosis, may form a significant proportion of ADSL deficiency. Once the diagnosis is known, however, prenatal diagnosis is possible and can be offered to affected families.

The results were published in journals *Human Molecular Genetics* in the year 2000 [5] and *Journal of Pediatrics* in the year 2007 [14].

4.3.2. Characterisation of genotype - phenotype correlations

In a previous study we calculated homoallelic activities of fmADSL proteins by patients' genotypes, but no ground for the varied SAdo/SAICAr ratio was found. The main limitation of the previous experiments was that they were performed on single isolated proteins. This situation has not reflected the fact that the patients are mostly

4.2.2. Molecular biology

4.2.2.1. Mutation analysis

In case of positive biochemistry we determine *ADSL* cDNA and gDNA mutations. We check the results by a PCR-RFLP method.
Most mutations found in ADSL-deficiency are missense mutations. The majority of them, except R426H, are private [9]. In our series of 22 patients, we identified 16 mutations [5, 14]. The mutation c.340C>T (Y114H) we detected in 10 patients. To date, this mutation was not published; however, it may be a prevalence mutation [5, 14].
Based on positive mutation analysis, prenatal diagnoses of two fetuses were performed in a subsequent pregnancy on genomic DNA isolated from native chorionic villi and subsequently from cultured chorionic villi. The mutation analysis of the first fetus showed heterozygosity for the 340T>C allele inherited from the father and carried the wild type allele from the mother. Pregnancy was continued and a healthy boy was born. Urinary excretion of both SAdo and SAICAr was normal. He is now 2.5 years old with normal growth and development. The mutation analysis of the second fetus showed heterozygosity for the 340T>C and 1128G>C alleles inherited from both parents. Pregnancy was terminated.

4.2.2.2. Expression studies

Since the majority of ADSL mutations are private, we must prove its pathogenity. Accordingly we introduced a system based on characterisation of recombinant ADSL proteins expressed in *E.coli.*

We cloned mutations into a pMAL-c2 vector and transformed *E.coli* DH5αF´IQ cells for protein production. Corresponding mutant proteins were affinity purified on amylose binding resin and analyzed by SDS-PAGE. Some protein had reduced expression. The possible explanation is a faster degradation by cells proteases. Then we measured activities of fmADSL using S-AMP and SAICAR as substrates. The proof of pathogenity is based on detection of reduced activities, altered catalytic properties (Km, Vmax) and/or altered enzyme thermal stability and temperature dependence properties.

We cloned mutations of all patients' diagnoses in IIMD except R337X (early stop codon). To see the functional effect of individual mutations we expressed 16 relevant amino acid sequence-altering alleles in *E.coli* and measured enzyme activities of the affinity-purified enzyme proteins with each ADSL substrate. According to the residual activities, the mutant enzymes were divided into 3 groups: null mutations free of detectable enzymatic activity (Y114H, D268N and D268H); 7 mutations with enzyme activities substantially compromised (E376D, S23R, R194C, R396C, R396H, R426H and P467R); and 6 mild mutations with activities comparable to that of the wild-type enzyme (A3V, R190Q, D215H, T242I, I351T and D430N). We propose the following mechanisms to explain the causative effect in the category of null mutations. In the case of the Y114H mutation it may be the pronounced protein instability. The wild-type tyrosine-114 resides in a highly conserved region common to all members of the fumarase enzyme superfamily. Based on molecular modelling, this region was predicted to be essential for the *Bacillus subtilis* ADSL subunit assembly [19] . Furthermore, the active site of the *B. subtilis* ADSL is formed by histidine–histidine residues pairing

4. RESULTS AND DISCUSSION

Screening for ADSL deficiency started in IIMD in 1993. Currently the diagnostic system is based on screening HPLC analysis of body fluids, enzymatic examination of available tissues, mutation analysis and characterisation of mutant recombinant proteins. This unique diagnostic system is provided world-wide to interested parties through GeneTests database (http://www.genetests.org). We collected a worldwide unique cohort of 22 ADSL deficiency patients from 18 families (8 CR, 7 Poland, 5 Germany and 2 USA). During their examinations, we identified a full-length coding sequence of *ADSL* cDNA, its isoform, 16 different mutations [5, 14] and characterised new a neonatal form of ADSL deficiency [14]. We also performed prenatal diagnoses in two pregnancies, collected a set of 9 ADSL deficient fibroblast lines and secured several post-mortem tissues from 2 ADSL deficient patients.

The pathogenic mechanisms leading to the development of individual symptoms and underlying the phenotypic heterogeneity are unclear. The main pathogenic effect is therefore attributed to the toxic effects of accumulating succinylpurines [12]. Although, the absolute SAdo and SAICAr concentrations in body fluids do not correlate with severity of the phenotype, it has been found that diverse values of ratio between SAdo and SAICAr concentrations in cerebrospinal fluid (SAdo/SAICAr ratio) do correspond with the two main phenotypic groups [13]. The mechanism(s) leading to the diverse ratios is (are) not clear. It is hypothesized that it may result from a mutation specific and thus structural related non-parallel loss (or gain) of enzyme activity towards one of its substrates. Relevant biochemical investigations answering this hypothesis are generally limited by the unavailability of one ADSL substrate SAICAR and by a small number of patients and tissues. Another limiting factor is the unavailability of cumulating ADSL metabolites (SAICAr and SAdo) for diagnostics and *in vitro / in vivo* toxic studies.

As an alternative approach, we therefore first cloned, expressed and purified fusion wt-human ADSL (fwtADSL) and used this enzyme for SAICAR preparation [5, 17]. Afterwards we cloned, expressed, purified and characterised catalytic properties of 17 fusion recombinant mutant ADSL proteins. We confirmed pathogenity of individual mutations, identified underlying structural and/or catalytic basis of the enzyme deficiency and found evidence that residual enzyme activity, calculated as a mean of homoallelic activities, correlates with the severity of the phenotype (e.g. different SAdo/SAICAr ratio). However, all the mutant enzymes studied, if active, displayed proportional decrease in activity towards both substrates, and no reason for the varied SAdo/SAICAr ratio was therefore found [5].

The main limitation of the experiments was that they were performed on single isolated proteins. This situation has not reflected the fact that the patients are mostly compound heterozygotes and that probably two structurally different mutant enzymes are involved in tetrameric structure building *in vivo*.

In case of ADSL protein, such limitation may be partly overcome by experiments in which mutant proteins corresponding to individual genotypes are mixed together before further characterisation. In this process, which is called intersubunit complementation [4, 18-20], it is assumed that both mutant proteins recombine randomly and an array of

5 possible tetrameric structures with 64 different active sites may be formed. Hypothetically, in a situation where structural and catalytic mutant proteins meet together, unique catalytic sites having disproportional activity towards one of the substrates may evolve and form the structural basis for diverse SAdo/SAICAr ratios.

The main goal is to seek for biochemical and structural basis of the diverse SAdo/SAICAr ratios and thus extend our previous studies on genotype – phenotype correlation in ADSL deficiency.

4.1. PRODUCTION AND PURIFICATION OF ADSL SUBSTRATES

The strategies used for production of the commercially unavailable ADSL substrate SAICAR and of the dephosphorylated SAICAr and SAdo were based on the availability of fwtADSL [5] and CIAP.

SAICAR

SAICAR was produced from fumarate and AICAR in a reaction catalyzed by recombinant human ADSL. The maximal SAICAR concentration in a reaction mixture was reached after 5 hours of incubation. After that period of time, concentration of SAICAR started to decrease due to a reverse reaction direction. Using purification on PEI-TLC plates we isolated 2 μmoles of SAICAR, which represents a production yield of about 76%. HPLC analysis of the final product showed no presence of AICAR or fumarate.

SAICAr

SAICAr was produced by adding the CIAP to the SAICAR containing reaction mixture (50 ml) as described under the Experimental Section. Dephosphorylation of AICAR and SAICAR was 100%, so the final reaction mixture contained only AICAr, fumarate and SAICAr. SAICAr and AICAr were separated from fumarate by treatment with strong cation-exchange resin, and SAICAr was subsequently isolated from AICAr on a column filled with weak anion-exchange resin. During the latter purification step the preparative column became pink coloured. The pink coloured fraction co-eluted with SAICAr. To reveal the origin of pink colour fraction we analyzed eluent on HPLC with UV diode-array detection, but only a single peak corresponding to SAICAr was detected. Similar observations were reported by Van den Berghe [15]. The pink colour probably arises from the reaction of the SAICAr amino group at weak acidic pH. Based on this assumption we neutralized the pink coloured SAICAr containing fraction and used active coal, which efficiently removed contaminating pink product. The amount of isolated SAICAr was 60 μmoles, which represents a production yield of about 46%.

SAdo

SAdo was produced from the SAMP containing reaction mixture by adding the CIAP. Dephosphorylation of SAMP was almost 100%. The final reaction mixture also contained negligible amount of aglycon. After purification steps on the DEAE – Sephadex column, the concentration of SAdo was estimated spectrophotometrically at 269 nm. The amount of isolated SAdo was 60 μmoles, which represents a production yield of about 46%.

In summary, we modified reaction conditions previously described [15, 16] for SAICAR preparation and employed the full-length recombinant human ADSL enzyme [5]. This approach resulted in shorter incubation time, more efficient substrate conversion and higher yield. Using described methods we prepared SAICAR in amount and purity sufficient for enzymatic studies in ADSL deficient patients and for kinetic characterisation of mutated ADSL proteins as described in our previous study [5].
SAICAr and SAdo, dephosphorylated forms of both ADSL substrates accumulate in body fluids of patients with ADSL deficiency. Various *in vitro* and *in vivo* experiments are therefore indicated to study eventual toxic effects of these compounds and their role in pathogenesis of severe neurological symptoms observed in patients. To prepare both compounds at sufficient amount and purity, we developed reaction schemes and purification procedures based on separations on cation- and anion-exchange resin columns, which are opposite to the preparative TLC method previously used, and are more efficient and easily scaleable. Described procedures are relatively simple and resulting products may be of use in a number of ADSL and purine de novo synthesis related studies.

4.2. DIAGNOSIS OF ADSL DEFICIENCY

4.2.1. Biochemistry

Patients with ADSL deficiency are diagnosed at IIMD during a screening of unexplained developmental delay using TLC chromatography with Pauli reagent detection (SAICAR assay) [8]. We are examining urine of about 1000 patients every year. However, since TLC chromatography can cause false negative results, we use the HPLC analysis for general diagnosis showing accumulation of both SAICAr and SAdo. According to SAdo/SAICAr ratios we determine type I or type II ADSL deficiency.

Next, we determine catalytic activities of ADSL with both substrates in ADSL deficient fibroblasts. The activities are reduced.

Some patients examined had a low SAdo/SAICAr ratio, consistent with severe type I presentation. Interestingly, in available CSF samples, the SAdo/SAICAr ratio reached the lowest values we have had observed so far. Activities of ADSL in fibroblasts were also low (especially for SAICAR). They all shared severe muscular hypotonia necessitating mechanical ventilation, intractable seizures and early death. The majority of affected patients had impaired intrauterine growth, microcephaly and a lack in fetal heart rate variability. We thus characterized a new lethal neonatal variant of adenylosuccinate lyase (ADSL) deficiency.

In one patient, we did not detect SAdo and/or SAICAr, but we found its hydrolyzed products SAdenin and SAICA (ratio 1.6). Consecutively, we added SAdo and SAICAr to urine. After several hours it was also hydrolyzed. We think that there is probably a new unpublished specific ribosidase present in patient's L1 urine. Maybe this ribosidase degrades toxic SAdo and SAICAr.