Clinical, biochemical and molecular genetic correlations in adenylosuccinate lyase deficiency

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INTRODUCTION

Adenylosuccinate lyase (ADSL) deficiency (MIM 103050) is an autosomal recessive inborn error of purine synthesis characterized by the accumulation in body fluids of succinylaminoimidazolecarboxamide (SAICA) riboside and succinyladenosine (S-Ado), the dephosphorylated derivatives of the two substrates of the enzyme. Because ADSL-deficient patients display widely variable degrees of psychomotor retardation, we have expressed eight mutated ADSL enzymes as thioredoxin fusions and compared their properties with the clinical and biochemical characteristics of 10 patients. Three expressed mutated ADSL enzymes (M26L, R426H and T450S) were thermostable and one (del206–218), was inactive. Thermostable mutations decreased activities with SAICA ribotide (SAICAR) and adenylosuccinate (S-AMP) in parallel, or more with SAICAR than with S-AMP. Patients homozygous for one of these mutations, R426H, displayed similarly decreased ADSL activities in their fibroblasts, S-Ado:SAICA riboside ratios of ∼1 in their cerebrospinal fluid and were profoundly retarded. With the exception of A2V, thermostable mutations decreased activity with S-AMP to a much more marked extent than with SAICAR. Two unrelated patients homozygous for one of the thermostable mutations, R303C, also displayed a much more marked decrease in the activity of fibroblast ADSL with S-AMP than with SAICAR, had S-Ado:SAICA riboside ratios between 3 and 4 in their cerebrospinal fluid and were mildly retarded. These results suggest that, in some cases, the genetic lesion of ADSL determines the ratio of its activities with S-AMP versus SAICAR, which in turn defines the S-Ado:SAICA riboside ratio and the patients’ mental status.


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and encodes a protein of 459 amino acids. As already mentioned (12) and recently described in detail (13), correction from C to A of the third nucleotide of this sequence has, however, revealed a first ATG, 75 nucleotides 5′ of the initially reported initiation codon. The open reading frame thus comprises 1452 nucleotides (E.A. Fon, EMBL accession no. X65867), and encodes a protein which is 25 amino acids longer at the N-terminus, thus containing 484 amino acids. Analysis of a first family with ADSL deficiency had revealed a T→C substitution, resulting in an S413P change (11), now labelled S438P (12,13). In accordance with the variability of the clinical symptoms, ∼20 missense mutations have been reported in apparently unrelated sibships (3,12,13). In about half of the families, the patients are compound heterozygotes. Most frequently encountered, accounting for about one-third of the alleles investigated, is an R426H mutation, previously identified as R401H.

Attempts presently are underway to establish detailed genotype–phenotype correlations in ADSL deficiency (13). In the present study, we have expressed eight mutated ADSL enzymes, compared their properties with those of the partially deficient enzyme in the cultured skin fibroblasts of 10 patients, and tried to correlate our findings with the patients’ S-Ado:SAICA riboside ratios and mental status.

RESULTS

Activities of ADSL in patients’ fibroblasts

Table 1 shows the activities of ADSL, measured with both S-AMP and SAICAR, in the fibroblasts of 10 patients belonging to 10 independent families. Also shown are the genotypes of the patients, the degree of their mental retardation and the ADSL activities in fibroblasts.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>Mental retardation</th>
<th>S-Ado:SAICA riboside</th>
<th>S-AMP</th>
<th>% of control</th>
<th>SAICAR</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5′-UTR T-49C/R426H</td>
<td>Severe</td>
<td>1.0</td>
<td>0.83 ± 0.06 (12)</td>
<td>33</td>
<td>0.65 ± 0.05 (4)</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>A2V/S395R</td>
<td>Severe</td>
<td>1.0</td>
<td>0.48 (2)</td>
<td>19</td>
<td>0.51 (2)</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>M26L/R426H</td>
<td>Severe</td>
<td>0.9</td>
<td>0.63 ± 0.12 (5)</td>
<td>25</td>
<td>0.43 ± 0.04 (4)</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>R141W/del206–218</td>
<td>Severe</td>
<td>1.5</td>
<td>0.43 (2)</td>
<td>17</td>
<td>0.29 ± 0.15 (3)</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>R303C/R303C</td>
<td>Mild</td>
<td>4.0</td>
<td>ND (2)</td>
<td>0</td>
<td>0.53 (2)</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>R426H/R426H</td>
<td>Severe</td>
<td>1.5</td>
<td>0.43 ± 0.17 (7)</td>
<td>17</td>
<td>0.26 ± 0.01 (4)</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>R426H/R426H</td>
<td>Severe</td>
<td>1.2</td>
<td>0.47 (2)</td>
<td>19</td>
<td>0.22 (2)</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>R426H/R426H</td>
<td>Severe</td>
<td>1.3</td>
<td>0.52 (2)</td>
<td>21</td>
<td>0.22 (1)</td>
<td>11</td>
</tr>
<tr>
<td>9</td>
<td>R426H/T450S</td>
<td>Moderate</td>
<td>2.5</td>
<td>0.43 (2)</td>
<td>17</td>
<td>0.29 (2)</td>
<td>15</td>
</tr>
<tr>
<td>Control</td>
<td>WT/WT</td>
<td></td>
<td>2.51 ± 0.17 (18)</td>
<td>100</td>
<td>1.95 ± 0.24 (7)</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

ND, non detectable; WT, wild-type.

Activities of ADSL in patients’ fibroblasts Table 1. Patients’ genotype, mental retardation, S-Ado:SAICA riboside ratios in CSF, and ADSL activities in fibroblasts

and expressed S-Ado:SAICA riboside ratios between 0.9 and 1.5. Moreover, also in accordance with previous reports (8), the activities of ADSL measured in their cultured fibroblasts with both S-AMP and SAICAR were decreased in parallel, to 15–33% of normal. These four patients are compound heterozygotes, and two of them carry the most frequent mutation, R426H. The mutations on the other allele were a point mutation in the 5′-untranslated region (UTR) in patient 1 (14), a missense mutation in patients 2 and 3 and a splicing error in patient 4 (6,12).

The findings in patient 6, namely mild psychomotor delay, a markedly higher S-Ado:SAICA riboside ratio and a much more pronounced decrease of fibroblast ADSL activity with both S-AMP and SAICAR, were completely comparable with those observed in patient 5, already investigated by Jaeken et al. (7) and Van den Bergh et al. (8). Not unexpectedly, both patients, although unrelated, are homozygous for the same R303C mutation. Patients 7–9, also unrelated, are clinically, biochemically and enzymatically closely similar to patients 1–4, and carry the most frequent R426H mutation in homozygous form.

Patient 10 displays moderate mental retardation, intermediate between the severely and mildly affected patients, since she, and also her ADSL-deficient brother, are able to talk and to work in a specialized centre. Although the S-Ado:SAICA riboside ratio in her CSF was also intermediate between those of the severely and mildly affected patients, residual activities of her fibroblast ADSL, measured with S-AMP and SAICAR, were similar to those recorded in the severely retarded patients. She carries the R426H mutation, associated with T450S.

Expression and purification of recombinant ADSL

Major difficulties were encountered in expressing ADSL in a bacterial system. No expression could be obtained using the
pT7-7 plasmid (15) and *Escherichia coli* strain BL21(DE3):pLysS (16) with which Stone et al. (17) had succeeded in producing N-terminal truncated wild-type and S438P-mutated ADSL. With the pET-3a plasmid and the same *E. coli* strain, full-length ADSL could be expressed 5- to 10-fold above the basal bacterial activity, but, even in the insoluble fraction of the extract, no visible band could be visualized on SDS–PAGE, notwithstanding the use of a systematic array of culture times and inducer concentrations.

With the His-Patch ThioFusion expression system, involving insertion of the gene in vector pThioHis, ADSL could be expressed in *E. coli* TOP10 as a soluble fusion protein with thioredoxin (Trx) in the N-terminal position (Table 2). The activity of wild-type recombinant Trx–ADSL in soluble bacterial extracts, measured with S-AMP, reached 0.8–1.2 µmol/min/mg protein, compared with 0.005 µmol/min/mg protein for the basal bacterial activity. Purified protein was obtained to near homogeneity, as assessed by SDS–PAGE, after a single affinity chromatography step (Fig. 1). Seven of the eight mutations under investigation could be expressed similarly as soluble, active Trx–ADSL and purified. Del206–218 was the only mutation to which fusion with Trx conferred only limited solubility, since most of the synthesized protein remained in the insoluble part of the bacterial extract. Moreover, the 13 amino acid truncated enzyme was inactive.

Several attempts were made to separate ADSL from its fusion partner Trx. The presence of an engineered enterokinase site allows liberation of free ADSL. However, even though enterokinase cleavage was 70% efficient, we did not succeed in separating ADSL from the remaining fusion protein, using various chromatographic techniques. All studies were therefore performed with the fusion protein Trx–ADSL. It was verified by gel electrophoresis that the molecular weight of one subunit of recombinant Trx–ADSL was 62 kDa, with ADSL accounting for 50 kDa and Trx for 12 kDa, and by gel filtration that the recombinant protein was in tetrameric form (Fig. 2).

Purified Trx–ADSL was stable for several hours when kept on ice. However, like with truncated ADSL (17), loss of activity was always recorded after storage at –20 or –80°C, even in the presence of glycerol or bovine serum albumin (BSA). Newly purified protein (wild-type or mutant) was therefore prepared for each experiment, which was repeated at least three times.

**Table 2. Expression of wild-type and mutant Trx–ADSL**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>A2V</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>M26L</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>R141W</td>
<td>12 ± 0.3</td>
</tr>
<tr>
<td>R303C</td>
<td>6 ± 0.5</td>
</tr>
<tr>
<td>S395R</td>
<td>6 ± 1.3</td>
</tr>
<tr>
<td>R426H</td>
<td>12 ± 0.2</td>
</tr>
<tr>
<td>T450S</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>del206–218</td>
<td>0.1</td>
</tr>
<tr>
<td>Empty bacteria</td>
<td>0.1</td>
</tr>
</tbody>
</table>

aMeans ± SEM of 3–4 expression experiments; given in U/100 ml culture.

**Figure 1.** SDS–PAGE analysis of expressed Trx–ADSL fusion proteins. Lane 1, molecular weight markers; lanes 2–4, crude lysates (lane 2, control cells; lane 3, wild-type ADSL; lane 4, del206–218); lanes 5–12, 4 µg of fusion proteins purified on proBond (lane 5, wild-type ADSL; lane 6, A2V; lane 7, M26L; lane 8, R141W; lane 9, R303C; lane 10, S395R; lane 11, R426H; lane 12, T450S).

**Figure 2.** Size exclusion chromatography of purified wild-type recombinant Trx–ADSL. Gel filtration was performed as described in Materials and Methods with 238 kDa (A), 158 kDa (B) and 67 kDa (C) mass standards.
contrast to the results obtained with fibroblasts (Table 1), in which ADSL activity was always higher with S-AMP, with the unique exception of the cells carrying the R303C mutation. As also shown in Table 3, all seven missense mutations decreased the $V_{\text{max}}$ of ADSL, measured with S-AMP, but never totally abolished activity. Mutation A2V had only a minor effect, with 80% residual activity. Mutations R141W, R426H and T450S had an intermediate effect, resulting in ~50% residual activity. The three other mutations, M26L, R303C and S395R, provoked a more marked decrease in activity, to 7–24% of the wild-type value.

Mutations A2V, R426H and T450S resulted in a grossly parallel decrease in the activity with SAICAR compared with that with S-AMP. Only mutation M26L displayed a distinctly lower activity with SAICAR than with S-AMP. Mutations R141W, R303C and S395R affected activity with SAICAR to a markedly lesser extent than that with S-AMP: mutation R141W displayed normal activity with SAICAR, mutation R303C resulted in 26%, and mutation S395R resulted in 60% residual activity. The parallel loss of activity with the two substrates observed in mutation R426H, and the non-parallel loss recorded in the R303C mutation, are in agreement with the measurements in fibroblasts of patients carrying these mutations in the homozygous form (Table 1, patients 7–9 and 5 and 6, respectively).

Only modest changes in affinity for the substrates were observed with the various mutant enzymes. Affinity for S-AMP was increased 2- to 3-fold for R141W and S395R, and affinity for SAICAR was decreased 2-fold for R303C.

### Thermal stability of recombinant ADSL

Pilot experiments revealed that at several temperatures below 53°C wild-type Trx–ADSL and several mutants were stable for at least 60 min (data not shown). This is in contrast to prior studies of recombinant N-terminal-truncated ADSL, which had shown thermolability of the wild-type enzyme above 35°C, and of S438P-mutated ADSL above 25°C (11). Figure 3 shows that the wild-type enzyme was stable at 53°C over a 60 min incubation. The A2V and R141W mutants were as stable as the wild-type protein. Two other mutants, R303C and S395R, displayed a partial, maximally 25% loss of activity, after only 5 min of incubation for R303C, and after 60 min for S395R. In sharp contrast, mutations M26L, R426H and T450S were markedly unstable, with 25% residual activity after 10 min and a nearly complete loss of activity after 30 min of incubation.

### DISCUSSION

In this study, we have attempted to correlate the pronounced variability of the clinical and biochemical characteristics of 10 ADSL-deficient patients with the residual activity of the enzyme in their cultured fibroblasts, and the properties of seven recombinant mutated enzymes, expressed as fusion proteins with Trx.

In accordance with previously published findings (7,8,13), severe psychomotor delay was associated with CSF S-Ado:SAICA riboside ratios of ~1 and parallel loss of the activity of fibroblast ADSL with S-AMP and SAICAR (Table 1). Also in accordance with previous reports (7,8), and now confirmed in a second case (patient 6), mild mental retardation was associated with significantly higher S-Ado:SAICA riboside ratios, reaching 3–4, and a non-parallel loss of both activities, with very low to undetectable residual activity for S-AMP. Although patient 10 displayed moderate mental retardation and an intermediate S-Ado:SAICA riboside ratio, her residual fibroblast ADSL activities with S-AMP and SAICAR were comparable to those recorded in the severely retarded patients. Similar observations were made by Kmoch et al. (13). Except in extreme cases, such as patients 5 and 6, fibroblast ADSL activities thus do not reflect adequately S-Ado:SAICA riboside ratios in body fluids. Measurements of the ADSL

### Table 3. Kinetic properties of wild-type and mutant Trx–ADSL

<table>
<thead>
<tr>
<th></th>
<th>S-AMP</th>
<th></th>
<th></th>
<th>SAICAR</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>% of control</td>
<td>$K_m$ (µM)</td>
<td>$V_{\text{max}}$</td>
<td>% of control</td>
<td>$K_m$ (µM)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>5.4</td>
<td>100</td>
<td>12</td>
<td>8.7</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>Mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2V</td>
<td>4.3</td>
<td>80</td>
<td>10</td>
<td>6.1</td>
<td>70</td>
<td>12</td>
</tr>
<tr>
<td>M26L</td>
<td>1.3</td>
<td>24</td>
<td>12</td>
<td>0.8</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>R141W</td>
<td>2.2</td>
<td>41</td>
<td>6</td>
<td>9.0</td>
<td>103</td>
<td>18</td>
</tr>
<tr>
<td>R303C</td>
<td>0.37</td>
<td>7</td>
<td>13</td>
<td>2.2</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td>S395R</td>
<td>1.1</td>
<td>20</td>
<td>4</td>
<td>5.2</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td>R426H</td>
<td>2.9</td>
<td>53</td>
<td>13</td>
<td>4.5</td>
<td>52</td>
<td>ND</td>
</tr>
<tr>
<td>T450S</td>
<td>2.7</td>
<td>50</td>
<td>9</td>
<td>3.1</td>
<td>36</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.

Activities are expressed as µmol/min/mg protein. Values shown are means of 3–5 values. SEM were <5% of the value to which they refer.
homozygous patients 7–9 and tion, with <20% of control activity in fibroblasts of the fibroblasts. This is particularly striking for the R426H mutation, as shown in Table 3, all seven mutations resulting in extreme instability of the enzyme and/or residual activities tended to be higher than in the patients’ protein. Nevertheless, and possibly due to fusion with Trx, complete loss of its activity. As shown in Table 3, all seven mutations displayed less activity than the wild-type protein. Nevertheless, and possibly due to fusion with Trx, residual activities tended to be higher than in the patients' fibroblasts. This is particularly striking for the R426H mutation, with <20% of control activity in fibroblasts of the homozygous patients 7–9 and ~50% of control activity in the recombinant form. It is noteworthy that wild-type and mutant MBP–ADSL recombinant enzymes displayed higher activities under the form of fusion proteins than after cleavage (13). Also in accordance with Knoch et al. (13), none of the missense mutations had a major effect on substrate affinity.

The least severe mutation is A2V, which causes only a 20–30% loss of activity, and is thermostable. An A3V mutation was also found to affect neither activity nor thermal stability of recombinant ADSL (13). This is in agreement with the results of Stone et al. (17), who expressed a truncated enzyme, with 25 amino acids missing in its N-terminal part, which was active and stable, as if this part of the protein was not necessary for full activity. It is therefore surprising that, when associated with S395R (patient 2), the A2V mutation led to a very severe clinical presentation with early death. The apparently most severe missense mutation is M26L, which has a marked extent with SAICAR. In contrast, with the exception of A2V, the mutations R303C, S395R and S438P cause severe mental retardation in the homozygous patients 7–9 and ~50% of control activity in the recombinant form. It is noteworthy that wild-type and mutant MBP–ADSL recombinant enzymes displayed higher activities under the form of fusion proteins than after cleavage (13). Also in accordance with Knoch et al. (13), none of the missense mutations had a major effect on substrate affinity.

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Mutation R303C is the only mutation for which a striking correlation is observed between ADSL activities in fibroblasts, activities of the recombinant enzyme and the S-Ado:SAICA riboside ratio in body fluids. Indeed, the highly depressed activity with S-AMP compared with SAICAR provides an explanation for the much more important accumulation of S-Ado compared with SAICA riboside. Interestingly, R303C is the mutation which is located most closely to the fumarate lyase signature, a sequence of 15 well conserved residues in the lyase superfamily of enzymes, which catalyse β-elimination of fumarate from different substrates and include argininosuccinate lyase (20), class II fumarase (21), aspartase (22), δ-crystallin (23) and 3-carboxy-cis,cis-muconate lactonizing enzyme (24). Seven residues in the signature are absolutely conserved among the different lyases, and are underlined in the following human sequence: 288YKSAMPYKRPN298. A neighbouring residue, E302, located next to the R303C mutation, is also conserved in all ADSL species. It is therefore possible that the R303C mutation causes a structural change, leading to a different handling of the two substrates. (i) The contact amino acids for SAICAR and S-AMP are not exactly the same, notwithstanding the structural similarity of both compounds, and the mutation affects the binding of S-AMP more than that of SAICAR, resulting in a non-parallel loss of activity with the two substrates. (ii) The mutation causes a structural change which modifies the apertures of the catalytic cleft and thereby its accessibility for the substrates. Owing to its closed purine ring, the S-AMP molecule might be less flexible than that of SAICAR, rendering its accessibility to the catalytic site more sensitive to structural changes.

Mutations M26L, R426H and T450S render Trx–ADSL markedly thermostabile (Fig. 1). A similar thermostability of R426H, and to a lesser extent of another C-terminal mutation, D430N, has been reported for MBP–ADSL (13), and for the closely located S438P mutation, expressed as a truncated protein and termed S413P by Stone et al. (11). Both the R426H and S438P mutations cause severe mental retardation in the homozygous state, associated with S-Ado:SAICA riboside ratios of ~1, and parallel loss of fibroblast and recombinant ADSL activity with S-AMP and SAICAR. It is therefore surprising that R426H causes only moderate retardation when associated with T450S (Table 1), as well as with D430N (13). Since ADSL is tetrameric, and its catalytic site is formed by the association of three subunits (19,25), it is possible that in heterozygote patients subunit complementation can recreate a more stable protein than the homotetramer, or a more active catalytic site, as demonstrated with mutated ADSL of Bacillus subtilis (19).

Taken together, our studies show that some of the gene lesions identified in ADSL deficiency cause thermostability of the enzyme, whereas others barely affect its stability. As a rule, the unstable mutations decrease the activity of the enzyme with S-AMP and with SAICAR in parallel, or to a more marked extent with SAICAR. In contrast, with the exception of A2V, the stable mutations decrease activity with S-AMP to a more marked extent than with SAICAR. This is particularly striking for mutation R303C. In accordance with these data, patients homozygous for the unstable R426H and S438P mutations display S-Ado:SAICA riboside ratios of ~1, whereas patients...
homozgyous for the stable R303C mutation present with significantly higher S-Ado:SAICA riboside ratios, reaching 3–4.

Both the present study and the recent report by Kmoch et al. (13) show that, although patients with moderate mental retardation display S-Ado:SAICA riboside ratios between 2 and 3, no correlation could be found between the latter ratios and S-AMP:SAICAR activity ratios in fibroblasts and expressed enzymes. Evaluation of genotype–phenotype correlations in these patients is hampered by the fact that they are compound heterozygotes, and that ADSL is a tetramer, potentially allowing subunit complementation. More precise evaluation of the consequences of the individual mutations will require their identification in the homozgyous form. Nevertheless, the present results suggest that, in some rare patients, the genetic lesion of ADSL determines the ratio of its activities with S-AMP compared with SAICAR. In turn, this ratio defines the S-Ado:SAICA riboside ratio in these patients' body fluids, which seems a determining factor in the degree of mental retardation.

MATERIALS AND METHODS

Patients

Ten ADSL-deficient patients with varied ethnic backgrounds were studied (Table 1). Diagnosis was based on the presence of S-Ado and SAICA riboside in CSF and urine. Both compounds were quantified by high performance liquid chromatography (HPLC) with ultraviolet detection (1). Genetic analysis of seven patients (nos 2–5 and 7–9) was reported previously (12). Three new patients were included in this study (nos 1, 6 and 10). Their genetic analysis had revealed two novel mutations: one missense mutation, T450S, and a mutation in the 5′-UTR (14). Mental retardation was assessed as severe, moderate or mild, according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edn (DSM-IV). Clinical information and samples were kindly provided as follows. Patient 1, Dr M.C. Nassogne (Brussels, Belgium); patients 2, 5–7, 9 and 10, Dr J.G.M. Huijmans (Rotterdam, The Netherlands) and Dr B.T. Poll-The (Utrecht, The Netherlands); patients 3 and 4, Dr G.F. Hoffmann (Heidelberg, Germany); and patient 8, Dr P. Edery (Bicêtre, France).

Fibroblast culture

Skin fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 1 mM ultraglutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum. After 7 days of culture, cells from three 75 cm² flasks were harvested by trypsinization and centrifugation. Homogenization was performed with a Kontes conical glass homogenizer in 300 µl of 20 mM Tris–HCl pH 7.4, 1 mM dithiothreitol (DTT). Cell debris was removed by centrifugation. Protein concentration was determined with the Bio Rad (Hercules, CA) protein assay, using BSA as the standard.

The assay of fibroblast ADSL was performed as described previously, using both SAICAR and S-AMP as substrates, and measuring the products of the reaction by HPLC (26).

Expression and purification of recombinant ADSL

The E.coli expression vector pThioHis (Invitrogen, Carlsbad, CA) was used to produce recombinant ADSL as a fusion protein with Trx. Full-length inserts starting at the ATG were obtained by PCR and cloned in the blunted KpnI site of pThioHis. TOP10 E.coli cells transformed with the recombinant vectors were cultivated for 5 h at 37°C in 100 ml of LB medium in the presence of 100 µM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation at 10 000 g for 15 min, and resuspended in 5 ml of lysis buffer (20 mM NaH₂PO₄/Na₂HPO₄, pH 7.8, 10 mM NaCl, 5 µg/ml antipain and leupeptin, 1 mg/ml lysozyme). After 20 min on ice, cells were lysed by three cycles of freezing–thawing. DNase I (0.1 mg/ml) and 10 mM MgSO₄ were then added. After 30 min on ice, a soluble extract was obtained by centrifugation at 20 000 g for 30 min at 4°C. The extract was either used immediately for purification or stored at −20°C after addition of 20% glycerol.

Purification was performed in one step by affinity chromatography on ProBond resin (Invitrogen), a nickel-chelating Sepharose resin. Soluble extract (2 ml) diluted with 8 ml of buffer A (20 mM NaH₂PO₄/Na₂HPO₄, pH 7.8, 0.5 M NaCl) was passed over a 2 ml ProBond column pre-equilibrated with buffer A. The column was washed with 10 ml of buffer A, then 20 ml of buffer B (20 mM NaH₂PO₄/Na₂HPO₄, pH 6, 0.5 M NaCl), then 10 ml of buffer B with 0.1 M imidazole, before elution with 10 ml of buffer B with 0.3 M imidazole. One milliliter fractions were collected, and assayed for ADSL activity. The active fractions were pooled and used the same day for kinetic and stability studies.

Assay of recombinant ADSL

The activity of the enzyme was measured with its two substrates, S-AMP and SAICAR, as described by Schultz and Lowenstein (27), using an Aminco DW2 dual-wavelength spectrophotometer (American Instrument Company, Silver Spring, MD).

Size exclusion chromatography

Gel filtration of recombinant Trx–ADSL was performed on a Sephacryl S-200 Superfine column (1.5 × 50 cm) in 20 mM HEPES buffer pH 7.0, 50 mM NaCl, 1 mM DTT. Approximately 0.8 U of Trx–ADSL was loaded on the column which was eluted at a flow rate of 20 ml/h, and fractions of 1.5 ml were collected. The column was calibrated with three mass standards: catalase (238 kDa), aldolase (158 kDa) and BSA (67 kDa).

Assessment of thermal stability

The mutant and the wild-type ADSL were incubated at 53°C for times ranging from 5 to 60 min. After chilling on ice, residual activity was assayed rapidly. Protein concentration was 150 µg/ml, and was adjusted with BSA when necessary.

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