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Inherent properties of adenylosuccinate lyase could explain S-Ado/SAICAr ratio due to homozygous R426H and R303C mutations

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ABSTRACT

Adenylosuccinate lyase (ADSL) is a homotetrameric enzyme involved in the de novo purine biosynthesis pathway and purine nucleotide cycle. Missense mutations in the protein lead to ADSL deficiency, an inborn error of purine metabolism characterized by neurological and physiological symptoms. ADSL deficiency is biochemically diagnosed by elevated levels of succinylaminoimidazolecarboxamide riboside (SAICAr) and succinyladenosine (S-Ado), the dephosphorylated derivatives of the substrates. S-Ado/SAICAr ratios have been associated with three phenotypic groups. Different hypotheses to explain these ratios have been proposed. Recent studies have focused on measuring activity on the substrates independently. However, it is important to examine mixtures of the substrates to determine if mutations affect enzyme activity on both substrates similarly in these conditions. The two substrates may experience an indirect communication due to being acted upon by the same enzyme, altering their activities from the non-competitive case. In this study, we investigate this hidden coupling between the two substrates. We chose two mutations that represent extremes of the phenotype, R426H and R303C. We describe a novel electrochemical-detection method of measuring the kinetic activity of ADSL in solution with its two substrates at varying concentration ratios. Furthermore, we develop an enzyme kinetic model to predict substrate activity from a given ratio of substrate concentrations. Our findings indicate a non-linear dependence of the activities on the substrate ratios due to competitive binding, distinct differences in the behaviors of the different mutations, and S-Ado/SAICAr ratios in patients could be explained by inherent properties of the mutant enzyme.

1. Introduction

Adenylosuccinate lyase (ADSL) is a homotetrameric enzyme involved in the de novo purine biosynthesis pathway and the purine nucleotide cycle. Its four active sites can accommodate the conversion of succinylaminoimidazolecarboxamide ribonucleotide (SAICAR) to aminoimidazolecarboxamide ribotide (AICAR) plus fumarate and succinyladenosine monophosphate (SAMP) to adenosine monophosphate (AMP) plus fumarate [1,2]. Missense mutations in the protein lead to ADSL deficiency, an inborn error of purine metabolism that manifests symptoms along a continuum of neurological and physiological symptoms such as psychomotor delay (PMD), expression of autistic features, structural brain abnormalities, seizures, and muscle wasting [3–9]. Approximately sixty cases of ADSL deficiency have been reported, but it is likely to be under diagnosed and may be more common than observed [10–13].

ADSL deficiency is biochemically diagnosed by elevated levels of the dephosphorylated derivatives of SAICAR and SAMP, succinylaminoimidazolecarboxamide riboside (SAICAr) and succinyladenosine (S-Ado), in urine, plasma, and cerebrospinal fluid [14]. The heterogeneity in disease phenotypes has made it difficult to understand the pathogenic mechanism behind the disease. In all known cases, the patients have significant residual enzyme activity. Three distinct phenotypic groups have been established, which correlate ratios of dephosphorylated derivatives with disease severity: 1) neonatal, 2) Type I, and 3) Type II. The neonatal form results in fatal neonatal encephalopathy and has a S-Ado/SAICAr ratio of <1. Type I patients experience early onset, severe PMD and

Abbreviations: ADSL, adenylosuccinate lyase; WT, wild type; SAICAR, succinylaminoimidazolecarboxamide ribonucleotide; AICAR, aminoimidazolecarboxamide ribotide; SAMP, succinyladenosine monophosphate; AMP, adenosine monophosphate; PMR, psychomotor retardation; SAICAr, succinylaminoimidazolecarboxamide riboside; S-Ado, succinyladenosine; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid tetrasodium salt dihydrate; Ap, ampicillin; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria-Bertani; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC–EC, high performance liquid chromatography with electrochemical-detection

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have a S-Ado/SAICAr ratio of ~1 [13]. Type II patients experience later onset with mild PMD and have a S-Ado/SAICAr ratio of 2–4 [15]. These ratios may be related to a leading hypothesis for a pathogenic mechanism: the toxic effects of accumulating succinylpurines, specifically SAICAR [16]. The accumulation of these succinylpurines may be caused by the residual enzymatic activity of the ADSL enzyme found in all patients with ADSL deficiency, regardless of mutation.

Enzymatic studies provide the specific activity and the maximum product formation rate (V_{max}) as a result of enzyme catalysis of the substrate. They also measure the equilibrium constant (K) between the free enzyme state and the enzyme substrate complex formation. The importance of the V_{max} is that it gives a quantitative measure of the extent to which the enzyme acts on its designated substrates, SAICAR and SAMP. Numerous studies attempt to relate V_{max} values with mutations associated with various types of ADSL deficiencies and the corresponding S-Ado/SAICAr ratio [13,17–23]. However, previously reported human ADSL kinetic parameters have been inconsistent. The wild-type (WT) SAICAR/SAMP activity ratio varies from 0.53 to 1.7 while SAMP and SAICAR K_m values vary from 1.9 to 4.9 and 1.8 to 3.6 μM, respectively [17,19,24]. Recent studies using each substrate independently have identified only one disease associated mutation, R303C, resulting in a nonparallel reduction of enzyme activity favoring SAICAR over SAMP [13,17–19,24]. An early study of fibroblasts taken from patients showing reduced ADSL activity showed that synthesis of completed adenine and guanine nucleotides remains possible in both cell types [15,25]. The affected patients used in these studies were originally reported in 1988 by Jaeken and Van den Berghe and the disease causing mutations reported later [23,26,27]. The fibroblasts used were from severely affected individuals including two patients homozygous for the R426H mutation and one mildly affected patient homozygous for the R303C mutation. R426H is the most common mutation occurring in 30% of all known patients; homozygous R426H mutations generally lead to severe disease type and result in a S-Ado/SAICAr value of 1.0–1.6 [3,28]. The homozygous R303C mutation leads to the mildest form of the deficiency and gives rise to a S-Ado/SAICAr ratio of 3.0–3.7 [3]. Interestingly, only fibroblasts from the individual with the R303C mutation accumulated SAICAR or SAMP as measured by radiolabeled formate incorporation despite having a similar reduction in SAICAR activity as fibroblasts from the R426H patient. This may help explain the elevated ratios of S-Ado/SAICAr seen in the extracellular fluid of the R303C patients, and led to the hypothesis that SAICAR accumulation may be toxic. A larger reduction in SAMP activity would result in more accumulation of intracellular SAMP, and subsequently more S-Ado. However, alternative hypotheses to explain this difference in S-Ado/SAICAr ratios have been proposed, including the age of the patients when tested, the dephosphorylation rate of each substrate (likely required for extracellular transport), or the rate of extracellular transport itself [13].

As discussed above, most kinetic studies of ADSL mutant proteins have been done with each substrate individually. This does not reflect the in vivo situation, in which the presence of both substrates would be expected. Findings reported for the R303C fibroblasts by Van den Bergh et al. suggest that for the in vivo situation, at least for the R303C mutation, the elevated S-Ado/SAICAr is due to the intrinsic properties of the enzyme [25]. Under in vivo conditions both substrates bind with the ADSL enzyme. Competition due to resource sharing can mediate an indirect communication between the two substrates that do not interact otherwise. This hidden coupling can alter the activities of the substrates from the non-competitive scenario, i.e. in the absence of the other substrate. Emergence of complex patterns due to resource sharing has been reported in other biochemical networks [29,30].

In this study, we begin to explore the relationship between in vitro and in vivo environments and the possibility of hidden coupling and resource sharing. We describe a novel electrochemical-detection (EC) method to obtain the V_{max} for ADSL in solution with its two substrates at varying concentration ratios. Using this method we are able to more accurately determine the affects of hidden substrate coupling and resource sharing in dual substrate environments on the bi-functional ADSL enzyme and disease causing mutations. For this purpose we chose two mutations that represent extremes of the phenotype, R303C representing the mild form (Type II) and the R426H representing the severe form (Type I). Additionally, studies were done on the A291V ADSL mutation found in the CHO-K1 AdeI mutant. This mutant provides a useful control since its activity has previously been reported to be extremely low or undetectable with either substrate [31–33]. Finally an enzyme kinetic model was developed to predict SAMP and SAICAR activities from a given ratio of substrate concentration.

2. Material and methods

2.1. Materials

Chemicals, buffers, and solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), Fluka Chemical Corp. (Milwaukee, WI), or EM Science (Cincinnati, OH). Nickel-nitrilotriacetic acid-agarose, a QIAspin kit, and high throughput crystal condition screens were purchased from QIAGEN. QuikChange site directed mutagenesis kit was purchased from Stratagene. Centrifugal filter units were purchased from Millipore (Billerica, MA). SAICAR was prepared enzymatically from AICAR purchased from Sigma-Aldrich Chemical Co. as described by Zikanova et al. [34].

2.2. Site-directed mutagenesis, enzyme expression, and purification

The full description of the initial ADSL WT construct is described in Lee and Colman [35]. In short, the full length human ADSL gene (1–484 residues) was constructed in pET-14b vector containing a 5′ end *Nde*I restriction site and a 3′-end *Blp*I restriction site and a thrombin cleavable N-terminal histidine tag. In order to overexpress the human enzyme in *Escherichia coli*, the vector was transformed into *E. coli* Rosetta 2(DE3)pLysS. The WT and mutant ADSLs were purified to homogeneity using on a Qiagen Ni-NTA column. Purity was assessed by SDS-PAGE gel electrophoresis (data not shown). Protein concentration was calculated by absorbance at 280 nm using $E^{1\%}_{280} = 7.7$ [36]. After purification, protein was stored in enzyme storage buffer (50 mM potassium phosphate buffer, pH 7.0, containing 150 mM KCl, 1 mM DTT, 1 mM EDTA, and 10% glycerol) at −80 °C. Introduction of point mutations in the human ADSL plasmid was done by the QuikChange (Agilent) site-directed mutagenesis method. DNA sequencing performed at the University of Colorado Cancer Center DNA Sequencing & Analysis Core confirmed mutations.

2.3. Enzyme kinetics by UV

UV enzyme kinetics was performed on a UV–Vis Spectrophotometer Evolution 3000 from Thermo Scientific using 1 mL quartz cuvettes at 25 °C. ADSL with the His-tag intact was used for enzyme assays, as it has been shown that the His-tag does not affect enzyme activity [35]. Experiments were run with approximately 250 μg WT and R426H ADSL and 500 μg R303C and A291V ADSL. Frozen samples were incubated for ~2 h at 25 °C before measurements were taken to allow restoration of full activity [35]. Assays of ADSL with SAMP were measured in triplicate at 25 °C in 40 mM Tris–HCl (pH 7.4) with varying concentrations of SAMP (1–60 μM). Specific activity was measured from the decrease in absorbance of SAMP at 282 nm as it was converted to AMP and fumarate. The assay was monitored over 30 s in a 1 mL volume. The difference extinction coefficient of 10,000 M^{-1} cm^{-1} between SAMP and AMP was used to calculate the specific activity. SAICAR enzyme assays of ADSL were measured in triplicate at 25 °C in 40 mM Tris–HCl (pH 7.4) with varying concentrations