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

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1. Introduction

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 succinylaminomidazolecarboxamide 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.

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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 ($V_{\text{max}}$) 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 $V_{\text{max}}$ is that it gives a quantitative measure of the extent to which the enzyme acts on its designated substrates, SAI CAR and SAMP. Numerous studies attempt to relate $V_{\text{max}}$ 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 complete 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 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 $V_{\text{max}}$ 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 Adel 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-nitritolitriacetic acid-agarose, a QiAspin kit, and high throughput crystal condition screens were purchased from QiAGEN. QuickChange site directed mutagenesis kit was purchased from Stratagene. Centrifugal filter units were purchased from Millipore (Billerica, MA). SAICAR was prepared enzymatically from AICAR purchased fromSigma-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 Ndel restriction site and a 3′-end BglII 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 $E_{1%}^{1%} = 7.7$ [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 QuickChange (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
of SAICAR (1–100 μM). Specific activity was measured from the decrease in absorbance of SAICAR at 269 nm as it was converted to AICAR and fumarate. The assay was monitored over 30 s in a 1 mL volume. The difference extinction coefficient of 700 M$^{-1}$ cm$^{-1}$ between SAICAR and AICAR was used to calculate the specific activity. The initial velocities ($dP/dt$) and kinetic constants ($V_{\text{max}}, K_{\text{M},1}$, and $K_{\text{cat}}/K_{\text{M},2}$) were calculated by Mathematica templates. The initial velocity was put in terms of specific activity (μmol min$^{-1}$ mg$^{-1}$) by dividing $dP/dt$ by the total enzyme injected into the reaction volume. To determine the kinetic constants, the initial velocity data were fitted to the Hill equation, $dP/dt = (V_{\text{max},1} * [S]^n) / (K_{\text{M},1} + [S]^n)$.

### 2.4. Enzyme kinetics by HPLC–EC

Enzyme activity was measured by HPLC–EC analysis of AMP and AICAR formed from SAMP and SAICAR respectively. Assays were performed at 25 °C in 40 mM Tris/HCl (pH 7.4) with varying concentrations of SAMP and SAICAR (0–500 μM). Experiments were run in duplicate with approximately 250–500 μg ADSL injected into 1 mL reaction volume at 100% SAMP, 100% SAICAR, and different ratios of SAMP to SAICAR concentration: 3:1, 1:1, and 1:3. Every minute, 100 μL of the reaction mixture was extracted into 25 μL of 2.5 M perchloric acid on ice to stop the reaction. The solution was neutralized by adding 25 μL of 2.5 M potassium hydroxide. Samples were clarified by centrifugation in a Spectrafuge 16M at 14,000 rpm at –5 °C.

Separation of SAICAR, AICAR, SAMP, and AMP was achieved by HPLC–EC analysis similar to our previously described method [37]. Briefly, separation was obtained by a reverse phase HPLC–EC with a TSKgel ODS–80TM C-18 column (250 mm × 4.6 mm ID, 5 μM) protected by Tosoh Bioscience TSKgel guard cartridge. A column temperature of 35 °C was maintained throughout the analysis. A mobile phase consisting of 50 mM lithium acetate, 2% acetonitrile, and 5 mM tetrabutyl ammonium phosphate (TBAP) at pH 4.8 was delivered isocratically at a flow rate of 0.8 mL/min. Sample extracts and standards were kept at 10 °C until a 30 μL aliquot of each sample was injected using an ESA autosampler (model 542). After injection and separation, analytes were detected using a CouArray HPLC system (model 5600A, ESA) with three electrochemical detector modules. Each module contains four flow-through coulometric detectors in series. Nine EC channels were set to a range of potentials from 0 to 900 mV in 100 mV increments. Two channels were used for a high potential boron-doped diamond (BDD) amperometric detector set to 1700 mV and UV detector set at 265 nm.

This HPLC–EC software allows for quantitative measurement of the amounts of both substrates and products. Peak quantification was done using the CouArray software package with manual curation to assure appropriate peak calling by the software. Standard curves of concentration versus peak area in units of coulombs were generated for AICAR, and AMP. For these experiments the standard curves consisted of a concentration range of 2–150 μM for the products and fit with a linear trendline. Time points 0–5 min for each mixing ratio were analyzed. AMP and AICAR concentrations were calculated from the standard curve, plotted, and fit with a linear line. The activity was given by the slope of the line in units of molarity × min$^{-1}$ and changed into units of μmol × min$^{-1}$ × mg$^{-1}$.

### 2.5. Model

We carried out an enzyme mixing kinetics analysis to predict the rate of product formation when two substrates catalyzed by the same enzyme are present in solution at different concentrations. The analysis is based on the coupling of two independent enzymatic reactions via resource sharing. By independent reactions, we mean two separate reactions for each substrate in the absence of the other. However, resource sharing induces a coupling due to the fact that if one enzyme molecule is occupied by substrate one, it is not available for substrate two. Although the enzyme has multiple binding sites, we assume on a given enzyme that each site is occupied by substrates of the same type thus avoiding any other possible sources of coupling that may alter the equilibrium constant ($K_{\text{M}}$). Thus competitive binding has been enforced in the simplest form by conserving the total number of resources between the two substrates (see Eq. (8) in the appendix). This is the only source of interaction between the two reactions in our "resource sharing" model. Based on work by Ray, Deaton et al., cooperativity may play a small role in substrate binding and catalysis. Therefore, for simplicity, we disregard cooperativity in the present model [24]. From these assumptions, the rate of product formation for each substrate in mixing conditions can be predicted. The resulting Eqs. (1) and (2) are predictions for the rate of product formation when both substrates are present in solution.

$$\frac{dP_1}{dt} = \frac{V_{\text{max},1}}{K_{\text{M},1}} x + 1$$

$$\frac{dP_2}{dt} = \frac{V_{\text{max},2}}{K_{\text{M},2}} \frac{x}{3 + 1}$$

where $dP/dt$ is the rate of product formation of the substrate at a given ratio of substrates, $x = S_2/S_1$. The $V_{\text{max}}$ and $K_{\text{M},i}$ values refer to the pure reaction when there is only one substrate ($i = 1$ or 2).

### 3. Results

#### 3.1. Enzyme kinetics by UV

Enzyme kinetic parameters for WT and R303C have been previously reported [24]. $V_{\text{max}}$ values for the R426H mutant have been published for SAMP and SAICAR activities [13,19]. $K_{\text{M}}$ values have not been reported in the literature. In light of this, an initial investigation of enzyme activity using SAMP and SAICAR individually was performed by standard UV kinetic assays for the WT and for A291V, R303C, and R426H enzymes. All ADSL mutants with the exception of A291V have measurable activity that could be evaluated by monitoring the UV absorbance of SAMP or SAICAR. It has been shown previously that WT ADSL does not follow simple Michaelis–Menten kinetics [18]. Therefore, kinetic curves were analyzed with the Hill equation (Fig. 1). SAMP and SAICAR kinetic parameters can be found in Tables 1 and 2 respectively. The measured WT specific activities on SAMP and SAICAR are 13.9 and 23.3 μmol min$^{-1}$ mg$^{-1}$ respectively. The ratio of activity of SAICAR/SAMP is 1.7. This ratio is in agreement with the previously reported ratio of activities using the UV assay [17,23]. The R303C mutation reduces specific activity to 5% of SAMP and 38% of SAICAR relative to WT ADSL. This agrees with previous reports that the R303C mutation displays a nonparallel decrease in activity in vitro and in vivo [13,15,24]. The measured specific activities for R426H with SAMP and SAICAR respectively are 6.7 and 6.4 μmol min$^{-1}$ mg$^{-1}$. These correspond to a reduction to 45% for SAMP activity and 27% for SAICAR activity relative to WT and give a SAICAR/SAMP ratio of 0.96. This initially suggests the R426H mutation also causes a nonparallel decrease in activity, though not to the degree as R303C. Although the mutants reduce the activity on both substrates, R303C decreases the $K_{\text{M},1}$ while R426H decreases the $K_{\text{M},2}$. The decrease in $K_{\text{M},i}$ may indicate an increase in substrate affinity in order to maintain R426H enzyme efficiency at a level close to the WT ADSL. WT and R426H ADSL appear to have equivalent cooperative binding on both substrates, while R303C demonstrates...
no cooperativity. This may indicate that cooperative binding does not play a critical role in retaining enzyme activity and other factors such as substrate binding or alterations of the catalytic mechanism are more important to retain activity [24].

3.2. Enzyme kinetics by HPLC–EC

To explore the effect on the specific activity when both substrates are present in solution we measured the levels of both substrates and products after separation by HPLC–EC and UV detection. The identities of the substrates and products were verified by co-chromatography with known compounds. To test the method, kinetic measurements on SAMP and SAICAR were run independently. The measured WT specific activities on SAMP and SAICAR are 13.4 and 7.4 μmol min⁻¹ mg⁻¹ respectively. This results in a SAICAR/SAMP activity ratio of 0.55 and is similar to results previously reported [11,19]. Interestingly, even though the Vₘₐₓ for SAICAR is different between the UV and HPLC–EC assays, and products after separation by HPLC–EC and UV detection. The identities of the substrates and products were verified by co-chromatography with known compounds. To test the method, kinetic measurements on SAMP and SAICAR were run independently. The measured WT specific activities on SAMP and SAICAR are 13.4 and 7.4 μmol min⁻¹ mg⁻¹ respectively. This results in a SAICAR/SAMP activity ratio of 0.55 and is similar to results previously reported using an HPLC based assay [11,19]. Interestingly, even though the Vₘₐₓ for SAICAR is different between the UV and HPLC–EC assays,

Table 1
SAMP UV enzyme kinetic parameters of ADSL at 25 °C. Kinetic parameters for SAMP activity, Vₘₐₓ, kₐₖ, K₀.₅, and the Hill coefficient, were determined by standard UV assays by varying substrate concentration and fitting the data to the Hill equation in Mathematica. Protein was reconstituted for 2 h at 25 °C prior to measurements. Activities are relative to WT SAMP activity. ND is “not detectable”. Values are shown along with their standard error.

<table>
<thead>
<tr>
<th>ADSL</th>
<th>Vₘₐₓ (%)</th>
<th>K₀.₅ (μM)</th>
<th>Hill coefficient</th>
<th>kₐₖ/K₀.₅ (s⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100 ± 4</td>
<td>2.05 ± 0.08</td>
<td>1.4 ± 0.1</td>
<td>2.52E + 07</td>
</tr>
<tr>
<td>A219V</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>R303C</td>
<td>5 ± 3</td>
<td>2.7 ± 0.2</td>
<td>0.97 ± 0.07</td>
<td>9.15E + 05</td>
</tr>
<tr>
<td>R426H</td>
<td>45 ± 3</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>1.78E + 07</td>
</tr>
</tbody>
</table>

Table 2
SAICAR UV enzyme kinetic parameters of ADSL at 25 °C. Kinetic parameters for SAICAR activity, Vₘₐₓ, kₐₖ, K₀.₅, and the Hill coefficient, were determined by standard UV assays by varying substrate concentration and fitting the data to the Hill equation in Mathematica. Protein was reconstituted for 2 h at 25 °C prior to measurements. Activities are relative to WT SAICAR activity. ND is “not detectable”. Values are shown along with their standard error.

<table>
<thead>
<tr>
<th>ADSL</th>
<th>Vₘₐₓ (%)</th>
<th>K₀.₅ (μM)</th>
<th>Hill coefficient</th>
<th>kₐₖ/K₀.₅ (s⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100 ± 2</td>
<td>1.74 ± 0.08</td>
<td>1.3 ± 0.1</td>
<td>5.09E + 07</td>
</tr>
<tr>
<td>A291V</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>R303C</td>
<td>38 ± 6</td>
<td>9.0 ± 1.0</td>
<td>0.9 ± 0.1</td>
<td>3.64E + 06</td>
</tr>
<tr>
<td>R426H</td>
<td>27 ± 3</td>
<td>0.65 ± 0.1</td>
<td>1.3 ± 0.3</td>
<td>3.75E + 07</td>
</tr>
</tbody>
</table>
R303C activities maintained the same percentage drop in activity, 5% SAMP and ~40% SAICAR. The HPLC–EC data for R426H show reduction in activities for SAMP and SAICAR to 47% and 51% respectively. As a further validation of the assay, we attempted to measure the levels of ADSL activity in the A291V mutant, previously found to be extremely low or not detectable with either substrate. This was found to be the case using the HPLC–EC assay as well.

Having validated the HPLC–EC assay for each individual substrate, we then assayed the ADSL activity for WT, R303C, and R426H mutant enzymes in the presence of varying ratios of SAICAR and SAMP. Chromatograms showed a preserved separation of products and substrates as well as the growth of product and conservation of substrate from 0 to 5 min (Fig. 2). Accumulation of products was linear for the five time points as shown in Fig. 3. A summary of the results is shown in Fig. 4.
in Table 3 and represented graphically in Fig. 4. For WT and R426H, as SAICAR concentration increases, SAICAR activity increases as SAMP activity decreases as expected. For the R303C mutation, SAMP activity remains fairly constant as SAICAR concentration increases. To quantitatively analyze the data, we calculated the ratio of activity rates (SAMP activity/SAICAR activity) for ADSL as a function of substrate mixture (Table 4). For SAMP/SAICAR mixtures of 3:1, 1:1, and 1:3, the ratio of activity rates for WT decrease from 3.4, to 1.3, to 0.39 respectively. R426H follows a similar decrease in ratio of activity rates: 3.2, to 0.85, to 0.36. R303C ratios also decrease, but have values smaller than WT or R426H: 1.7, to 0.44, to 0.29. This implies that mutants can behave similarly or differently to WT in solution with both substrates and still result in succinylpurine accumulation. Interestingly, the relative rates of activity (mutant activity/WT activity) of R426H remain fairly constant while R303C relative rates change for any given substrate ratio (Table 4). Both SAMP and SAICAR relative rates for the R426H mutation remain about 50% of WT activity. The R303C mutation on the other hand has an increase in SAMP relative rate, 5–21%, as well as in SAICAR relative rate, 18–43%.

3.3. Prediction of the specific activity when both substrates are present

To test the prediction of the rate of product formation for a given substrate ratio for WT and mutant ADSL, $V_{max}$ values from the HPLC–EC assay and $K_m$ values from the UV assay were used in Eqs. (1) and (2). Specific activities from the HPLC–EC assay were used since it was the method employed to measure rate formation under mixed substrate conditions. The overlay of the prediction to the experimental data can be seen in Fig. 4. The black line is the calculated rate of product formation for AMP while the red line represents the rate of AICAR formation. The black and red data points and error bars are the experimental data for AMP and AICAR respectively. The blue line is the $V_{max}$ value for SAMP activity. Even though Eqs. (1) and (2) were generated assuming no change in equilibrium constants due to the presence of the other substrate, the predictions are in good agreement with the experimental data. These results suggest that the rate of product formation is mainly dependent on the ratio of $K_m$ values for pure SAMP and SAICAR and the stoichiometry of the two substrates (Eqs. (1) and (2)).

4. Discussion

4.1. Quantitative analysis of mixing kinetics

Upon mixing substrates in solution at different ratios, the specific activity depends on the amount of substrate present in a non-linear fashion. Eqs. (1) and (2) explain such non-linear dependence based on a competitive binding model including the presence of both substrates. Although the enzyme has multiple binding sites, we assumed that for a given enzyme molecule only one type of substrate can bind...
to these sites, allowing us to ignore the effect of modified equilibrium constants ($K_m$) due to the presence of the second substrate on the same enzyme molecule. Thus, enzymes bound to the first substrate are not allowed to complex with the second, leading to a reduced pool of enzyme molecules available for the second reaction. This is the only allowed source of coupling between the two reactions accounted by Eq. (8) in the appendix and the basis of our simple “resource sharing” model. The reasonable agreement between prediction and data supports the validity of our assumption as well as our assumption that cooperativity can be disregarded. Although modified reaction constants and Hill coefficients due to mixed binding of two different substrates on a given enzyme cannot be ruled out, they may be smaller effects compared to the primary effect of sharing limited resource modeled by Eq. (8). It is also worth noticing, as a result of competitive binding, the R303C relative activity (defined as a ratio of mutant $V_{max}$ to WT $V_{max}$) is no longer a constant but a function of SAMP/SAICAR ratio whereas R426H is constant for any given ratio of SAMP/SAICAR.

It has been observed that there is inconsistency in enzyme activities reported for ADSL, even from the same lab using the same assay. The only discrepancy reported here is the specific activity on SAICAR from two different assays. Since in the case of the HPLC based assay we quantify the growth of the product AICAR, which is identified by both retention time and EC-profile, we believe that this is a direct method to measure enzyme activity. The HPLC–EC data also indicates that R426H does not cause a nonparallel decrease in activity in agreement with the results from Race et al., Kmoch et al., and Zikanova et al. [13,19,23].

4.2. Potential relevance to S-Ado/SAICAr ratios observed in ADSL deficiency

The current clinical diagnosis of ADSL deficiency is based on the detection of the succinylpurinines in body fluids. It has been questioned whether the ratio of these dephosphorylated derivatives of the substrates is predictive of phenotypes or not, but rather depends on the life stage of the cell or patient that determines the S-Ado/SAICAr ratio [13]. Since the in vivo condition involves presence of both the substrates it is imperative to consider the relative drops in activity of the enzymes under mixed conditions as opposed to independent conditions. This has been experimentally assessed in whole cells for the R303C mutation, but not for WT or R426H. Our present study provides quantitative analysis of this scenario in vitro, complementing earlier in vitro or cell extract studies reporting the percentage drop in activities when only one substrate is present. Thus, with the whole kinetic picture available, it is possible to determine if the S-Ado/SAICAr ratio could be due to intrinsic properties of mutant ADSL. If the amount of S-Ado to SAICAr in body fluid was due to mutations of ADSL, there would be an inverse relationship between the ratio of activities (SAMP activity/SAICAr activity) and the accumulated substrates (SAMP/SAICAr) within the cell. Using the mixing kinetics model, it is possible to predict the intracellular concentrations of SAMP and SAICAr. R426H causes a S-Ado/SAICAr ratio of ~1. From the prediction curves, we would expect the SAMP/SAICAr ratio to be 13:10 where the SAMP and SAICAr activities are equal. Whereas R303C causes a S-Ado/SAICAr ratio of ~4. This corresponds to a SAMP/SAICAr ratio of 2:5 where the SAMP activity is four times less than SAICAr. These are quite different predicted intracellular ratios. Quantitative accumulation of these substrates has not been studied in mammalian cells [38,39]. Normal levels of purine nucleotides and variable levels of residual enzyme activity from various tissues have been reported in tissues of ADSL deficiency patients [40,41]. However, human WT and mutant ADSL were notoriously unstable at the time of the study.

One of the leading hypotheses for the pathogenic mechanism of ADSL deficiency is the toxic effect of accumulating succinylpurinines, specifically SAICAR. Therefore, it is important to understand how SAICAR might accumulate and to understand the consequences of this accumulation. Similarly, it has been hypothesized that the accumulation of SAMP might ameliorate the toxic effects of SAICAR accumulation. The observation that both SAICAR and SAMP accumulate in fibroblasts derived from an ADSL patient homozygous for the R303C mutation is consistent with this hypothesis.

Recently, SAICAR has been shown to activate pyruvate kinase M2 (PKM2) under conditions of limiting glucose, thus enhancing energy (ATP) production [42]. SAMP also activates PKM2 but at a much higher concentration. PKM2 is expressed in human brain and glucose uptake in the brain of ADSL deficient patients may be reduced [39]. Interestingly, ADSL deficient cells were shown to produce more ATP and higher pyruvate kinase activity than cells with normal levels of ADSL [42]. PKM2 has been considered to be present primarily in cancer cells, embryonic tissues, and in cells with a high proliferation rate [43]. However, there is evidence that PKM2 is the prominent isoform in most tissues [44]. If PKM2 is present in most tissues, then the relative amounts of accumulation of excess SAICAR and SAMP may cause aberrant regulation of PKM2 leading to toxic effects. Different relative amounts may regulate PMK2 differently, which could play a role in the heterogeneity of ADSL deficiency. Alterations in the activity of PKM2 in embryonic tissues may also contribute to defects in brain maturation and overall development in early stages of life when purines are mainly supplied by purine biosynthesis [45]. If aberrant glucose metabolism occurs because of SAICAR accumulation and leads to toxicity, it may be possible to ameliorate this effect by modulating glucose metabolism or by lowering accumulation of SAICAR. Clearly the ability to control the amount of SAICAR activity would be useful to maintain a low intracellular SAICAR concentration. If enzyme activity is measured in healthy individuals, then Eqs. (1) and (2) could be used to introduce appropriate inhibitors to alter the concentration of the substrates in the cell to gain desired activities for SAMP and SAICAR.

5. Conclusions

In this study, we provide a model that predicts the dependency of product rate formation ($dp/dt$) as a function of substrate mixing that was verified by experimental HPLC–EC measurement for WT ADSL and two clinically associated mutations, R303C and R426H. This strongly suggests competition due to resource sharing mediates indirect communication between the two substrates. This hidden coupling causes a non-linear dependency of the activities on the substrate ratios. Electrochemical detection is a robust method for this purpose because of its ability to detect low concentrations of chemical compounds and two degrees of separation: retention time and electrochemical profile. From in vitro results, it is clear that mutations behave differently when mixtures of substrates are present. R426H retains a constant relative activity ratio while R303C does not. We propose that making inferences about intrinsic properties of ADSL should be based on how it functions in the presence of both substrates as opposed to two isolated activities may more accurately reflect the in vivo situation. Currently, the only mutation that has been experimentally verified to accumulate succinylpurinines in whole cells is R303C while accumulation in R426H was undetectable. HPLC–EC could provide a more sensitive assay to quantify intracellular concentrations of succinylpurinines. The prediction model could then be used to calculate ADSL relative activity to determine if the S-Ado/SAICAr ratio is caused by inherent properties of mutant ADSL.

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Appendix A

The analysis starts with two independent kinetics reactions

\[ [E] + [S_1] \xrightarrow{k_1} [E \cdot S_1] \xrightarrow{k_{cat,1}} [E] + [P_1] \]  
\[ [E] + [S_2] \xrightarrow{k_2} [E \cdot S_2] \xrightarrow{k_{cat,2}} [E] + [P_2] \]

where \([E] = \text{ADSL}, [S_1] = \text{SAICAR}, [P_1] = \text{ALCAR}, [S_2] = \text{SAMP}, \) and \([P_2] = \) AMP. The two independent enzyme reactions indicate that a single enzyme molecule binds \([S^1] \) or \([S^2] \). Using our assumption no cooperativity, the rate of product formation, \(\frac{d[P]}{dt} \), in mixing conditions can be predicted in the following manner: Applying the law of mass action to \([P_1] \) and the \([E \cdot S_1] \) complex in Eq. (1)

\[ \frac{d[P_1]}{dt} = k_{cat,1} \cdot [E \cdot S_1] \]  
\[ \frac{d[E \cdot S_1]}{dt} = k_1 \cdot [E][S_1] - k_{cat,1} \cdot [E \cdot S_1] \]

Assuming a steady-state approximation, \(\frac{d[E \cdot S_1]}{dt} = 0\), leads to

\[ \frac{[E][S_1]}{[E \cdot S_1]} = k_{cat,1} \cdot k_1 \]

Applying the same to Eq. (2)

\[ \frac{d[P_2]}{dt} = k_{cat,2} \cdot [E \cdot S_2] \]  
\[ \frac{[E][S_2]}{[E \cdot S_2]} = k_{cat,2} \cdot \frac{k_2}{k_2} \]

If both substrates are present, the total enzyme concentration, \([E_{total}]\), can be written as

\[ [E_{total}] = [E] + [E \cdot S_1] + [E \cdot S_2] \]

Solving for \([E] \) from Eqs. (5) and (7)

\[ [E] = \frac{[E \cdot S_1][K_{m,1}]}{[S_1]} + \frac{[E \cdot S_2][K_{m,2}]}{[S_2]} \]

Utilizing Eq. (9) to write Eq. (8) in terms of \([E \cdot S_1] \)

\[ [E_{total}] = \frac{[E \cdot S_1][K_{m,1}]}{[S_1]} + \frac{[E \cdot S_2][K_{m,1}][S_2]}{[S_2]} \]

Solving for \([E \cdot S_1] \) from Eq. (10)

\[ [E \cdot S_1] = \frac{[E_{total}]}{k_{cat,1} + \frac{k_1}{k_{cat,1} + 1}} \]

Substituting Eq. (11) into Eq. (3)

\[ \frac{d[P_1]}{dt} = \frac{k_{cat,1}[E_{total}]}{k_{cat,1} + \frac{k_1}{k_{cat,1} + 1}} + \frac{V_{max,1}}{k_{cat,1} + \frac{k_1}{k_{cat,1} + 1}} \]

Since our experiment was always in saturating conditions, \(K_{m,1} \ll S_1 \)

\[ \frac{d[P_1]}{dt} = \frac{V_{max,1}}{k_{cat,1} + \frac{k_1}{k_{cat,1} + 1}} \]  
\[ Setting \quad x = \frac{k_1}{k_{cat,1} + 1} \]

Utilizing Eq. (9) to write Eq. (8) in terms of \([E \cdot S_1] \), solving for \([E \cdot S_1] \), and substituting into Eq. (6) results in the rate for formation for \([P_2] \)

\[ \frac{d[P_2]}{dt} = \frac{V_{max,2}}{k_{cat,2} \cdot \frac{k_2}{k_{cat,2} + 1} \quad (14)} \]

Eqs. (14) and (15) are predictions for the rate of product formation, \(d[P]/dt\), as a function of substrate ratio, \(x\). \(V_{max}\) and \(K_{m}\) values are obtained experimentally by enzyme kinetic assays on each substrate independently.

References