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Towards Restoring Catalytic Activity of Glutamine Synthetase With a Clinically Relevant Mutation

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Glutamine synthetase (GS) is an essential enzyme in nitrogen metabolism and catalyses the ATP-dependent ligation of glutamate and toxic ammonia to glutamine. So far, three GS mutants with clinically relevant pathologies are known. These mutations lead to hyperammonemia and a global glutamine deficiency. Recently, we investigated the molecular mechanisms underlying these mutations. However, an adequate treatment for a patient carrying the R324S mutation, now eleven years old, remains elusive. Here, we investigate by means of unbiased molecular dynamics simulations if and how the compatible solute betaine, successfully used for the treatment of liver diseases, can restore GS catalytic activity in the case of R324S GS. Upon free ligand diffusion, betaine enters the R324S GS binding site and forms hydrogen bonds with S324 and weak electrostatic interactions with ATP. Thus, betaine could act as a non-covalently bound ATP binding-enhancer in the case of R324S GS.

1 Introduction

The human glutamine synthetase (GS, EC 6.3.1.2) is a homodecamer in which ten subunits form two pentameric rings that stack onto each other (Fig. 1A)¹. GS catalyses the conversion of glutamate and ammonia to glutamine under the use of adenosine triphosphate (ATP)². Thus, GS contributes to glutamate and ammonia detoxification^{3,4}, as well as to glutamine formation, which makes GS essential for the human nitrogen metabolism^{5,6}. Two GS mutations, R324C and R341C (sequence numbering of human GS¹), were linked to a reduced GS activity and led to severe brain malformations and multiorgan failure, which results in neonatal death^{9,10}. Another GS mutation, R324S, was identified in a boy, who is neurologically compromised due to the lack of ammonia detoxification and glutamine synthesis¹¹. The boy had been initially treated with glutamine supplementation¹². However, the treatment was stopped after the patient showed an increased irritability. Thus, no adequate medication is currently available to treat inborn GS deficiency caused by the R324S mutation. Recently, we showed that ATP binding is hampered in the R324S mutant because a salt-bridge interaction between R324 and ATP is lost in the case of the R324S mutant¹³. Additionally, the R324S mutant results in a gap between the S324 sidechain and ATP¹³.

In search of a treatment of this rare disease, we aim at identifying small molecules that can act as ATP binding-enhancing molecules, thereby restoring GS activity in the R324S mutant. Here, we focus on trimethyl glycine (betaine, Fig. 1B) as one such molecule. From a structural point of view, betaine could bridge the gap between S324 and ATP by

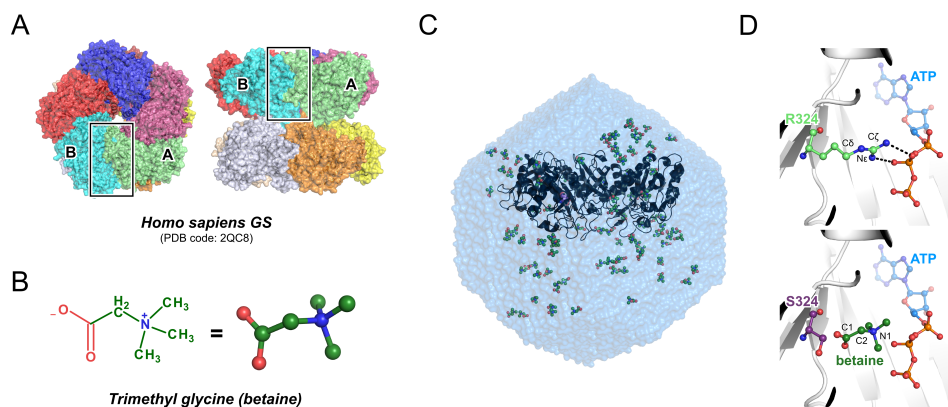


Figure 1. Human glutamine synthetase, betaine, and starting structures for unbiased MD simulations. A: Top view (left panel) and side view (right panel) of the human glutamine synthetase (GS) (PDB entry: 2QC8¹). Each subunit is coloured differently and shown in surface representation. Subunits A and B were used for a reduced dimeric model. The black box depicts the catalytic site. B: Schematic (left panel) and optimised 3D structure (right panel; ball-and-stick model without hydrogens) of the betaine molecule as used for MD simulations. C: Starting structure (“GS_{APO} model”) (black cartoon) of the R324S GS with ~ 150 mM of betaine (green spheres) in a truncated octahedron of TIP3P¹⁵ waters (blue surface representation). D: Close up view of the GS binding site. The upper panel shows wild type R324 (green) with labelled reference atoms and ATP (blue). Salt-bridge interactions are depicted as black dotted lines. The lower panel shows another starting structure (“GS_{ATP} model”) of the R324S GS mutant (magenta) with bound ATP (blue) and betaine (green) with labelled reference atoms.

forming hydrogen bonds and electrostatic interactions with S324 and ATP, respectively. From a clinical point of view, betaine is a safe, well tolerated, and inexpensive drug, and dietary betaine supplementation improved serum levels of liver enzymes in the context of fatty liver diseases¹⁴. We assessed if the GS binding site is accessible for betaine by means of unbiased molecular dynamics (MD) simulations of betaine diffusion in the presence of R324S GS. Moreover, we determined, if betaine simultaneously interacts with S324 and ATP and stabilises ATP binding.

2 Methods

To investigate if the R324S GS binding site is accessible for betaine, we performed unbiased MD simulations of R324S GS in the GS_{APO} state in the presence of ~ 150 mM of betaine (the preparation of the GS_{APO} state is described in Ref. 13). We randomly placed 92 betaine molecules around the R324S GS using *PACKMOL*¹⁶. Furthermore, to test whether a single betaine molecule can stably span the interaction between S324 and the phosphate groups of the GS substrate ATP, we generated an additional model with betaine and ATP bound to R324S GS (further referred to as GS_{ATP} state; for preparation of the GS_{ATP} state see Ref. 13). The coordinates of R324 atoms C δ , N ϵ , and C ζ were assigned to the betaine atoms C1, C2, and N1 such that the betaine’s carboxyl function is oriented towards S324 and the quaternary ammonium function towards ATP (Fig. 1D). After neutralising both the GS_{APO} and GS_{ATP} systems by adding sodium ions, the systems were placed in a truncated octahedron of TIP3P water¹⁵. Following thermalisation, both systems were subjected to

three independent MD simulations of 100 ns length using *Amber14*¹⁷ (simulation parameters are described in Ref. 13). Results are expressed as means. Mean standard error (MSE) is $< 0.02 \text{ \AA}$, unless specified differently. A one-sample Student's t-test was performed using the *R* software¹⁹.

3 Results and Discussion

To test if betaine can enhance ATP binding to the R324S mutant of GS and restore GS activity that way, we performed three unbiased MD simulations of 100 ns length of the GS_{APO} state of the R324S mutant in the presence of $\sim 150 \text{ mM}$ of betaine (Fig. 1C). Initially, we assessed whether the R324S GS_{APO} model is stable in the presence of betaine. The mean backbone root mean square deviation (RMSD) of R324S GS is $< 2.9 \text{ \AA}$, but some conformations showed RMSD values close to 5.0 \AA (Fig. 2A). Visual inspection of the trajectories revealed that those regions of the dimer are highly mobile where adjacent subunits would be located in the decamer¹³. Excluding these regions from the RMSD calculations (resulting in what is termed GS_{core} below) yielded backbone RMSD values that are largely constant and $< 3.0 \text{ \AA}$ with mean RMSD values for the $\text{GS}_{\text{core}} < 2.0 \text{ \AA}$ (Fig. 2B). As the catalytic site is located at the interface between the two subunits of the dimeric model (Fig. 1A) and, hence, in the centre of GS_{core} , we conclude that the dimeric GS_{APO} model is appropriate for investigating putative effects exerted by betaine at the catalytic site. Considering the first 20 ns as an extended equilibration phase, further analyses focused on the 20 - 100 ns interval.

Next, we analysed if betaine can enter the catalytic site of R324S GS because this will be a prerequisite for any direct betaine effect on ATP binding. For this, we computed density maps from the MD trajectories showing the frequency of interactions of betaine on the surface of R324S GS. Regions of high betaine density across all three trajectories are labelled a to f in Fig. 2C. Additional regions are visible in one or two of the MD trajectories only, suggesting that sampling of the unbound state of betaine is not converged after 100 ns of MD simulations. One region was identified where betaine is frequently present within the ATP binding site (Fig. 2D). Monitoring the minimal distance between the two carboxylate oxygens of betaine and the sidechain oxygen of S324 reveals for one MD trajectory that the betaine molecule enters the binding site after $\sim 30 \text{ ns}$ and then remains there for the remainder of the trajectory, reaching a minimal distance to S324 of 6.2 \AA (Fig. 2D). As an explanation, betaine immediately binds to one of the Mg^{2+} ions within the binding site that would interact with ATP; the Mg^{2+} ions are important for GS activity²⁰. In agreement with the slow exchange kinetics of first shell ligands of Mg^{2+} ,²¹ no unbinding of betaine from Mg^{2+} is observed during $\sim 70 \text{ ns}$ of MD simulations. We hypothesise that the bound betaine will be displaced from the Mg^{2+} by subsequent binding of ATP; due to the low likelihood of observing two simultaneous binding events within the accessible time scale of MD simulations, we are at present unable to validate this hypothesis, however. Additional regions of high betaine density close to the glutamate binding site were identified in all three trajectories (Fig. 2E). However, no betaine molecule finally entered the glutamate binding site. This is likely because glutamate binding to GS is highly regulated by an ATP-dependent opening and closing mechanism of the glutamate binding site¹. As we disregarded ATP during MD simulations, the glutamate binding site is shielded by several loop regions (Fig. 2E), preventing betaine from entering it.

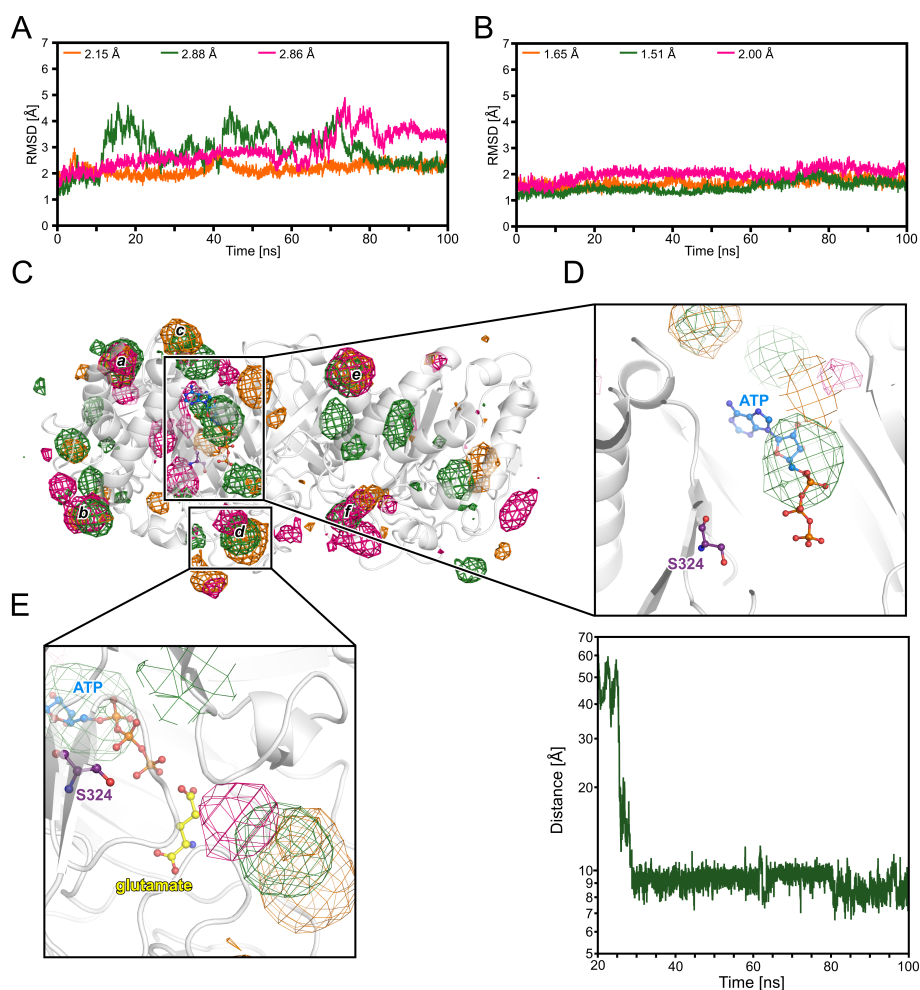


Figure 2. Unbiased MD simulations of betaine diffusion in the presence of R324S GS. A: Backbone RMSD of R324S GS in the GS_{APO} state for three trajectories. Mean values are provided in the legend. B: Backbone RMSD for GS_{core} residues of R324S GS in the GS_{APO} state for three trajectories. Mean values are provided in the legend. C: Overlay of density maps (depicted as isocontour mesh; contour level is 80 sigma) showing the frequency of betaine interactions on the surface of R324S GS_{APO} (white cartoon representation) of trajectory 1 (orange), 2 (green), and 3 (magenta). Labels a to f depict regions of high betaine density coherently found in all trajectories. D: The top panel shows a close up view of the ATP binding site with regions of high betaine density. The lower panel shows the minimal distance between the two carboxylate oxygens of the respective betaine molecule and the sidechain oxygen of S324 for the 20 - 100 ns interval of trajectory 2. E: Close up view of regions of high betaine density close to the glutamate binding site. For panels D and E, ATP (blue ball-stick model) and glutamate (yellow ball-stick model) were added only to visualise their location; both molecules were not present during the MD simulations. Residue S324 is depicted in magenta.

Next, to probe if betaine stably binds between S324 and ATP, thereby bridging the gap between the amino acid's side chain and ATP, we performed three additional MD simulations of 100 ns length in which a single betaine molecule was initially placed in the gap

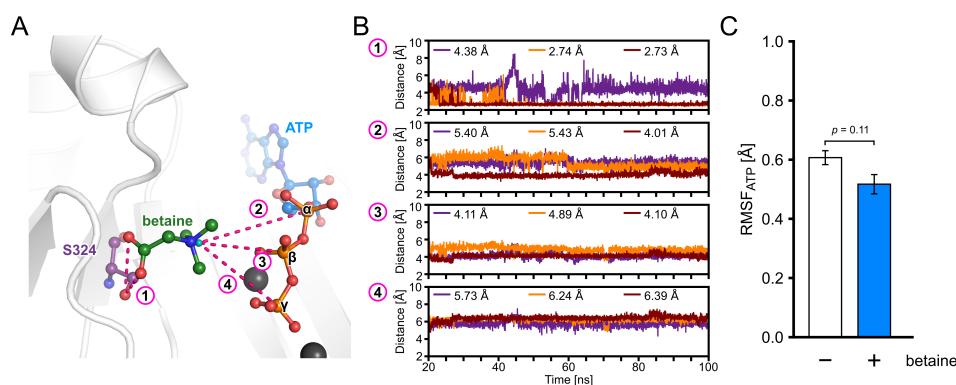


Figure 3. Structural analyses of betaine bound to the R324S glutamine synthetase binding site. A: Close up view of betaine (green) in the S324 GS binding site with residue S324 (purple) and ATP (blue) as blue ball-stick models. The geometric centre (GC) of the betaines trimethyl amine group is depicted as cyan sphere. Distances 1 - 4 (see text for definitions) are shown as dotted magenta lines. B: Distances 1 - 4 over trajectories 1, 2, and 3 (purple, orange, and red lines) with respective mean values in the legend. For distance 1, only the smallest distance to one of the oxygens of the carboxylate group of betaine, and for distance 2 - 4, only the smallest distance to one of the oxygens of the α , β , and γ phosphate groups in ATP are considered, respectively. C: Mean RMSF of ATP over three trajectories (mean standard error depicted as error bars). The white bar shows RMSF values in the absences (-) of betaine (MD simulations were previously described in Ref. 13), the blue bar in the presence (+) of betaine.

(Fig. 1D). To analyse hydrogen bond formation with S324 and formation of an electrostatic interaction between the positively charged trimethylamine function in betaine and the negatively charged phosphate groups of ATP, we measured four distances (Fig. 3A). Distance 1, measured as minimal distance between the two carboxylate oxygens of the betaine molecule and the sidechain oxygen of S324, is rather constant in trajectories 2 and 3 with mean distances of 2.73 Å and 2.74 Å (Fig. 3B). In these trajectories, a hydrogen bond (using a distance of 3.2 Å between the two donor and acceptor atoms and an angle (donor atom, H, acceptor atom) of 120° as cutoff criteria¹⁸) is present between both functional groups in at least 90% of all conformations. The smallest distances between the geometric centre of the quaternary ammonium group in betaine to one of the oxygens of the α , β , and γ phosphate groups in ATP, respectively, show the overall smallest mean values across all three trajectories in the case of the β phosphate group (values between 4.10 Å and 4.89 Å). In the case of trajectory 3, a small mean value of 4.01 Å is found with respect to the α phosphate group, too. Considering that the radial distribution function between nitrogen atoms of the choline cation and oxygen atoms of alanine anions revealed favourable interactions between both groups in a distance range from 4.00 Å to 6.0 Å²², we suggest that such interactions also exist between betaine and at least the β phosphate group of ATP. Together with the hydrogen bond analysis above, our results suggest that betaine can bridge the gap between S324 and ATP and, thus, enhance ATP binding.

To investigate whether betaine has a stabilising effect on ATP binding to R324S GS, we calculated the root mean square fluctuation (RMSF) of ATP in the presence of betaine after superimposition of backbone atoms of residues within 5 Å of ATP in the starting structure (Fig. 3C, blue bar). We compared values to those extracted from MD simula-

tions of R324S GS in the GS_{ATP} state without betaine, which were described in Ref. 13 (Fig. 3C, white bar). The RMSF value decreases from 0.61 Å (± 0.03 Å) without betaine to 0.52 Å (± 0.04 Å) in the presence of betaine (Fig. 3C); the difference is weakly significant ($p = 0.11$). As the RMSF is a measure of atomic mobility, this indicates that betaine weakly stabilises ATP binding in the R324S GS binding site, in agreement with the above geometric analysis of intermolecular interactions.

4 Conclusion and Outlook

The clinically relevant R324S mutation is suggested to reduce catalytic activity of GS¹², which leads to hyperammonemia and a global glutamine deficiency¹¹. Previously, we found that ATP binding is hampered in the R324S mutant¹³. Here, we investigated by MD simulations of in total 600 ns length if betaine can act as an ATP binding-enhancing molecule. Our results show that betaine can enter the ATP binding site of R324S GS_{APO} state and come close to S324. In the GS_{ATP} state, betaine simultaneously forms hydrogen bonds with S324 and electrostatic interactions with at least the β phosphate group of ATP. In agreement, betaine binding reduces the mobility of ATP in R324S GS. In all, this indicates that betaine weakly stabilises ATP binding in the R324S GS binding site.

From our results, several suggestions arise for how to strengthen an ATP binding-enhancing effect. First, the interactions with the phosphate group may be strengthened if, instead of the bulky trimethylammonium group of betaine, a positively charged group such as an amidino or guanidine moiety is chosen that can form salt bridge interactions with the phosphate oxygens in addition. Glycocyanine (2-guanidino acetate), a metabolite of glycine, or creatine (2-[amidino(methyl)amino]acetate), a methyl derivative of glycocyanine, which are both used as dietary supplements to treat creatine deficiency^{23,24}, contain such guanidine groups. Second, the negatively charged carboxylate group of betaine, which results in disfavoured electrostatic interactions with the phosphate groups, may be replaced by a neutral hydroxymethyl group, resulting in the positively charged molecule choline. Choline is used as a dietary supplement²⁵ and in the treatment of liver diseases²⁶. We intend to investigate these alternatives further by MD simulations and experimental validation in order to aid in the identification or development of ATP binding-enhancing molecules by which the R324S GS mutant can be repaired extrinsically²⁷.

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