Enhancing Human Spermine Synthase Activity by Engineered Mutations

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Abstract

Spermine synthase (SMS) is an enzyme which function is to convert spermidine into spermine. It was shown that gene defects resulting in amino acid changes of the wild type SMS cause Snyder-Robinson syndrome, which is a mild-to-moderate mental disability associated with osteoporosis, facial asymmetry, thin habitus, hypotonia, and a nonspecific movement disorder. These disease-causing missense mutations were demonstrated, both in silico and in vitro, to affect the wild type function of SMS by either destabilizing the SMS dimer/monomer or directly affecting the hydrogen bond network of the active site of SMS. In contrast to these studies, here we report an artificial engineering of a more efficient SMS variant by transferring sequence information from another organism. It is confirmed experimentally that the variant, bearing four amino acid substitutions, is catalytically more active than the wild type. The increased functionality is attributed to enhanced monomer stability, lowering the pKa of proton donor catalytic residue, optimized spatial distribution of the electrostatic potential around the SMS with respect to substrates, and increase of the frequency of mechanical vibration of the clefts presumed to be the gates toward the active sites. The study demonstrates that wild type SMS is not particularly evolutionarily optimized with respect to the reaction spermidine → spermine. Having in mind that currently there are no variations (non-synonymous single nucleotide polymorphism, nsSNP) detected in healthy individuals, it can be speculated that the human SMS function is precisely tuned toward its wild type and any deviation is unwanted and disease-causing.

Introduction

Polyamines are widely present in many organisms including mammals, plants as well as some bacteria, and play important roles in normal cell growth, differentiation, programmed death, and tissue repair [1,2]. A particular polyamine of interest in this manuscript, the spermine (SPM), was shown to be synthesized by the aminopropyltransferase enzyme - spermine synthase (SMS) through conversion of spermidine (SPD) to SPM [3]. Further structural and biochemical studies have revealed that SMS exists as a dimer and that the dimerization is crucial for its normal function [2,4]. Structurally each monomer of SMS contains three domains: an N-terminal domain to form the dimer contacts, a central domain serving as a lid for the C-terminal domain, and a C-terminal catalytic domain [2,4]. In wild type SMS, the amine substrate is coordinated via interactions between its terminal nitrogen atoms and acidic residues in the protein and by hydrophobic interactions of SMS with the alkane portions of the polyamine [2].

The importance of SMS for normal cell function is manifested by the fact that a malfunctioning of SMS results in the deficiency of SPM and affects the normal development in mice, humans and other organisms. In humans, this deficiency results in the Snyder-Robinson syndrome (SRS), which is a disease caused by the X-linked SMS gene defects. Patients with SMS show mild-to-moderate mental retardation, osteoporosis, facial asymmetry, thin habitus, hypotonia, and a nonspecific movement disorder [5]. The malfunction of SMS in patients with SRS was attributed to several missense mutations as c.267G>A (p.G56S) [6], c.496T>G (p.V163G) [7], c.550T>C (p.I184T) [8–10] and c.1084A>G (p.Y362C) (Zhang et al., in preparation). Computational modeling has revealed that these missense mutations affect SMS stability, flexibility, interactions, and formation of the dimer structures [9–12].

As mentioned above, the spermidine synthase (SRM) is widespread from E. coli, mammals, and plants to yeast. It is an aminopropyltransferase with a very high degree of specificity for putrescine as the amine acceptor and synthases SPD [13]. It was shown that the SRM of Thermotoga maritima, which is the only bacterium known to grow at a high temperature as well as 90°C [14], is remarkably stable to thermal denaturation particularly in the presence of the amine acceptor substrate putrescine with a half-life larger than 25 h at 90°C. In contrast, the human SRM (HsSRM) and SMS (HsSMS) are less stable under the same temperature [4,15]. The goal of this study is to probe the effect of transferring sequence information from Thermotoga maritima SRM (TmSRM) and SMS (TmSMS) to HsSMS and to reveal the effect of the amino acid substitutions on its stability and function. Previous in silico studies have indicated that mutations of the wild type amino acids may not be degrading the native properties
Author Summary

Proteins are constantly subjected to evolutionary pressure to assure the organism’s survival and reproduction. At the same time, the proteins’ amino acid sequence undergoes mutations, some of which may cause diseases while others may be reflecting natural differences within the population (non-synonymous single nucleotide polymorphism, nsSNP). In this study we examine the human spermine synthase (HsSMS), for which currently there are no nsSNPs, while rare disease mutations are known to cause Snyder-Robinson syndrome. What is so special with this protein? Maybe the HsSMS is so well optimized for its function that any change of the wild type sequence should be degrading its performance. To check such a possibility, we engineered a mutant of HsSMS with enhanced stability, electrostatic and mechanical properties. The mutant was confirmed experimentally to be a better enzyme than the wild type. Thus, the HsSMS is not evolutionally optimized with respect to its enzymatic reaction, its amino acid sequence differs only in sick individuals and so far its sequence was found to be identical in all healthy individuals. Therefore, it can be speculated that the HsSMS function is precisely tuned toward the wild type characteristics such so any deviation is unwanted and is disease-causing.

Results

I) Computational results

a) Results of MSA. The sequences of HsSMS and TmSRM were aligned at the starting point of the investigation. The alignment is shown in Figure 1A, where conserved residues are marked by star ‘*’ and the bold italic letters indicate the four mutations among different species, and the residues corresponding to the mutation sites among 500 homologous proteins are given by Protein-BLAST [16]. As seen below, the sequences of HsSMS and TmSRM share many similarities but are also significantly different at many sites. The alignment (Figure 1A) was mapped onto HsSMS 3D structure and all sites being either invariant (with respect to amino acid substitution) or buried were given preference with respect to isolated non-conserved sites (Figure 1B). Thus, N160, M224 and C347 were deleted because N160 appear less than 50% in the MSA, the candidate was deleted. The site Q223 is gap (Table S3) and was deleted as well. Furthermore, N149 is just next to a disease-causing mutation site I150 [9,10] and quite close to the active site. Because of that, it is considered that its mutation could abolish the function of HsSMS and this candidate was removed from the list.

Additionally N149 is just next to a disease-causing mutation site I150 [9,10] and quite close to the active site. Because of that, it is considered that its mutation could abolish the function of HsSMS and this candidate was removed from the list.

Frequency and percentage of residue appearance at the mutation sites among 500 homologous proteins suggested the final selection: if the TmSRM→HsSMS substitution is found to appear less than 50% in the MSA, the candidate was deleted. Thus, N160, M224 and C347 were deleted because N160→V, M224→L and C347→F substitutions were found in less than 50% of cases. The site Q223 is gap (Table S3) and was deleted as well.

Table 1. Frequency and percentage of residue appearance at the mutation sites among 500 homologous proteins.

<table>
<thead>
<tr>
<th>Mutation Site</th>
<th>Residues and Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>S165D</td>
<td>Residues</td>
</tr>
<tr>
<td>Freq. (%)</td>
<td>428</td>
</tr>
<tr>
<td>L175E</td>
<td>Residues</td>
</tr>
<tr>
<td>Freq. (%)</td>
<td>380</td>
</tr>
<tr>
<td>T178H</td>
<td>Residues</td>
</tr>
<tr>
<td>Freq. (%)</td>
<td>203</td>
</tr>
<tr>
<td>C206R</td>
<td>Residues</td>
</tr>
<tr>
<td>Freq. (%)</td>
<td>386</td>
</tr>
</tbody>
</table>

Figure 1. Multiple sequence alignment (MSA). (A) MSA between HsSMS and TmSRM. Conserved residues are indicated by star ‘*’, and the four mutations are represented in bold italic letters; (B) MSA among different SMS species. The star ‘*’ indicates the conserved residue in Figure 1B. The whole comparison is quite long (Supplementary material Table S2), thus we only list the “four mutations” section in the figure. MSA of all homologous to HsSMS protein is shown in supplementary materials Table S3. The frequency of residue appearance for each of the four mutations sites is shown in Table 1.

Further refinement of the candidate list was based on the MSA (Figure 1B and Table S2). Sites located next to the conserved site were given preference with respect to isolated non-conserved sites (Table S2). This resulted in eight residues: N149, N160, S165, L175, C206, Q223, M224, and C347.

Table 1. Frequency and percentage of residue appearance at the mutation sites among 500 homologous proteins.
Table 2. pKa value of Glu175, His178 and Asp201 in C monomer of Pmut.

<table>
<thead>
<tr>
<th>Residue</th>
<th>WT</th>
<th>Pmut (L175E &amp; T178H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu175</td>
<td>N/A</td>
<td>2.926</td>
</tr>
<tr>
<td>His178</td>
<td>N/A</td>
<td>3.573</td>
</tr>
<tr>
<td>Asp201</td>
<td>0</td>
<td>14</td>
</tr>
</tbody>
</table>

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As result, only three sites were kept in the candidate list, namely S165→D, L175→E and C206→R to be mutated to the corresponding residues in TmSRM. In addition, the site T178 was included in the list because it is correlated with L175. The corresponding mutation is T178→H. Finally four mutations were selected (S165D, L175E, T178H and C206R) and for the purpose of this investigation, several tags are introduced, i.e. SDmut for S165D; CRmut for C206R; Pmut for the double mutant “L175E and T178H”; and Fmut for the 4.a.a. mutant “S165D, L175E, T178H and C206R”.

b) Effect on ionized states due to the four mutations. pKa calculations indicated only few titratable groups are affected by the mutations (Table 2). Particularly, the active site Asp201 is predicted to experience a large pKa shift due to the “pair” mutations (L175E and T178H). This shift was predicted for both Pmut (L175E and T178H) and Fmut (S165D, L175E, T178H and C206R) structures. No effect was calculated upon S165D and C206R substitutions and because of that only results obtained Pmut are shown.

The mutation at position 175 results in fully ionized Glu residue, simply because E175 side chain is fully solvent accessible. In contrast, the side chain of H178 is fully buried inside the protein, thus it is predicted to be deprotonated. Asp201 is fully ionized in the WT structure which is exactly the same as suggested in our previous work [9], but it is fully protonated in the mutant structure. Detailed analysis addressing structural reasons for these pKa shifts will be presented in the Discussion.

c) Effect on monomer stability due to the four mutations. In the following three sections we reported the computational results with respect to effect of mutations on monomer stability, dimer affinity, change of the electrostatic potential distribution.

Table 3 shows the calculated results for folding energy changes due to the mutations. The negative folding energy change indicates that the mutation results in a less stable monomer, while positive change indicates that the mutant is more stable than the WT.

The energy calculations predict that all mutants are much more stable than WT, especially the Fmut which is estimated to stabilize monomer structure by more than 30 kcal/mol. The last number is clearly an overestimation and should not be assumed to be confirmed experimentally, but rather should be considered as a tendency. In addition, all three force fields gave the same trend of change, i.e. increasing the monomer stability upon the mutations. It should be pointed out that the calculations with C monomer and D monomer always gave quite close results, demonstrating the robustness of the computational protocol.

Table 3 provides a row termed “Sum”, which shows the sum of predicted energy changes taken from individual mutants (note that that the pair 175–178 is taken together). The motivation of carrying calculations for “sum” is to test the additivity. Thus, one can see from Table 3 that the sum of individual energy change is almost the same as the combined effect of four mutations (last row in Table 3). Thus the effect of these mutations in current research is additive indicating that sites 165, 206 and 175 plus 178 do not interact with each other.

Furthermore the webserver Eris was also used for the prediction of folding energy change. Since Eris has a difficulty in modeling the disulfide bond, thus Cys related mutations are not supported. Therefore only SDmut and Pmut were submitted. The results are shown in Table 4. With the protocol of Eris, this result implied that SDmut and Pmut have more stable structure, which agreed with the prediction by sMMGB method.

d) Effect on dimer affinity due to the four mutations. Dimer formation was shown to be crucial for the function of HsSMS. Therefore we carried analysis of the effects of mutations on dimer affinity. Results are shown in Table 5. Positive values indicate that the mutation increases the dimer affinity while the negative values indicate that the mutant has a less stable dimer formation.

Table 3. The results of structure – based (3C6K) folding energy calculation on monomer stability changes under three force fields.

<table>
<thead>
<tr>
<th>Mutants ID</th>
<th>Monomer ID</th>
<th>AMBER98 (kcal/mol)</th>
<th>CHARMM27 (kcal/mol)</th>
<th>OPLSaa (kcal/mol)</th>
<th>Ave three force fields (kcal/mol)</th>
<th>Ave C &amp; D (kcal/mol)</th>
<th>Adjustment according to sMMGB [53]* (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDmut (S165D)</td>
<td>C</td>
<td>51.07</td>
<td>109.92</td>
<td>90.89</td>
<td>83.96</td>
<td>83.28</td>
<td>6.66</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>42.60</td>
<td>107.56</td>
<td>97.66</td>
<td>82.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRmut (C206R)</td>
<td>C</td>
<td>194.73</td>
<td>276.20</td>
<td>56.20</td>
<td>175.71</td>
<td>181.32</td>
<td>15.77</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>203.56</td>
<td>276.14</td>
<td>81.06</td>
<td>186.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pmut (L175E/T178H)</td>
<td>C</td>
<td>3.48</td>
<td>106.25</td>
<td>139.76</td>
<td>83.16</td>
<td>90.05</td>
<td>7.29</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>9.07</td>
<td>114.50</td>
<td>167.26</td>
<td>96.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum</td>
<td>C</td>
<td>249.28</td>
<td>492.37</td>
<td>286.85</td>
<td>342.83</td>
<td>354.65</td>
<td>31.89</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>255.23</td>
<td>498.20</td>
<td>345.98</td>
<td>366.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fmut (S165D/L175E/T178/C206R)</td>
<td>C</td>
<td>249.59</td>
<td>508.35</td>
<td>292.48</td>
<td>350.14</td>
<td>355.42</td>
<td>31.97</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>274.07</td>
<td>493.99</td>
<td>314.02</td>
<td>360.69</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Two parameters were used to adjust the prediction according to the experimental data. For details of sMMGB refer to [53].

doi:10.1371/journal.pcbi.1002924.t003
It can be seen that all of the predicted binding energy changes listed in the table are very small. According to our previous work [10], such small binding energy change is considered not to have effect on dimer affinity. Such a conclusion is expected from structural perspective, since all four mutation sites are located within the C-domain and quite far away from the dimer interface. Note that the original experimental work has shown that the N-terminal domain is the cause of dimerization [4].

e) Effect on potential distribution due to the four mutations. One plausibale reason why HsSMS functions as a dimer could be that the dimerization is necessary to provide guidance and steer the positively charged SPD/SPM in and out the active site. It can be speculated that there are two paths shown in Figure 2A as path “A” and “B”. The electrostatic field lines form a kind of funnel leading to the vicinity of the active sites. The path “A” is in the cleft between HsSMS domains and is a single path. In contrast, the path “B” is perpendicular to the dimer axis and is symmetrical (doubled) with an angle of symmetry 180° (Figure 2A). Neither of these paths, “A” or “B”, exist if one uses in the modeling the HsSMS monomer only, i.e. they are product of the dimerization. The mutations further increase the magnitude of the electrostatic potential along the both paths as it is illustrated in Figure 2B, where the change of the potential due to the mutations is shown. The potential difference is mapped onto molecular surface of the dimer. Red color patch indicates that the potential is more negative in the dimer as compared with the WT, and blue color the opposite. It can be seen that patches corresponding to both paths, “A” and “B”, are more negative in the mutant than in the WT, providing support of the hypothesis that the mutant enhanced activity is caused by better steering of the substrates to the active site.

f) Results of normal mode analysis. The ANM webserver calculates 36 smallest eigenvalues and each of them corresponded to a vibration mode. Out of them, the first 20 non-zero eigenvalues correspond to the 20 vibrational modes and are visualized in fmol at the ANM site. Considering the two plausible pathways of SPD/SPM and inspecting the corresponding motions, it was found that the first three modes correspond to relevant domain motions (See supplementary material Figure S1). The results for these normal modes calculated with different force field minimized structures are shown in Table 6. From the table, one can see that Fmut has a slightly higher frequency of domain motion than the WT. The change of the domain vibrational frequency is expected to affect the reaction by modulating the approach time of the substrates as they travel along either of the electrostatic funnels.

II) Experimental results
The HsSMS is highly specific for SPD as an amine acceptor. Kinetic analysis of the HsSMS reaction indicated that the Km values for SPD and dcAdoMet are 0.8 mM and 0.45 μM, respectively, and that the activity is 3776±494 nmol/h/mg (Table 7). On the other hand, the activity of four mutants HsSMS (Fmut) is over ten times higher than that of WT, whereas Km for both substrate of dcAdoMet and SPD are much less affected by the mutations. Still the decrease of the Km for SPD indicates that the mutant affinity toward SPD increases in HsSMS (Fmut), while the affinity toward dcAdoMet decreases. The dramatic increase of the activity, therefore, cannot be attributed to the change of the affinity of mutant HsSMS to the reactants. Rather it should be associated with a change of the maximum rate (Vmax) which in turn may indicate better ability of reactants to find their way to the active site and to bind there.

Discussion
The experimental data shows that the designed HsSMS mutant is more active than the WT HsSMS and computational investigations indicate that biophysical characteristics of the mutant are altered as compared with the WT characteristics. What are the structural origins for the predicted changes? The MSA among homologous proteins implied that the sites 175 and 178 are correlated. In the WT HsSMS, L175 and T178 are quite close, and the polar hydrogen of T178 makes a hydrogen bond (hydrogen bond length 1.81 Å) with OD2 of Asp 201 (Figure 3A), and thus providing support for the ionized form of Asp 201. The substitution with a charged residue Glu at site 175 introduces extra negative charge, but the side chain of E175 is almost fully exposed to the water phase and thus is solvated. However, the substitution causes slight backbone reengagement and at the same time, the negative potential of E175 suppresses the ionized form of D201 and makes D201 protonated. There is no hydrogen acceptor in the vicinity of D201 and Thr at position 178 is replaced with hydrogen donor/acceptor with a longer side chain, His, to make a hydrogen bond with the polar hydrogen of D201 (Figure 3B). The negative charge of E175 also supports the tautomeric orientation of the side chain of His at position 178 by orienting the polar proton of H178 toward the negatively charged OE atoms of E175 (Figure 3B). It can be speculated that by favoring the protonated form of D201, the mutations weaken the interactions between the product, the spermine, and the protein moiety and thus facilitates the release of the product (note that the product, the spermine, has one extra positive charge as compared with the reactant, the spermidine). In addition, the protonation of D201 lowers a bit the pKa value of D276 (from pKa(WT) = 9.7 to pKa(Fmut) = 9.4) and thus reduces the work need to be done to protonate D276 upon substrate binding. It can be speculated that it will enhance the turnover of the reaction and will increase the reaction rate.

The energy calculations indicated that all mutations stabilize the monomeric structure of HsSMS. The couple, E175 and H178, increases stability by lowering the desolvation penalty for D201, which is protonated in the mutant, and by providing stabilizing hydrogen bonds. The other two mutations, S165D and C206R, also stabilize the monomer, especially C206R is predicted to increase the monomer stability by 15.77 kcal/mol. In the WT structure, C206 forms a hydrogen bond with D239 (Figure 4A); while in the CRMut structure, R206 forms hydrogen bonds with the backbone oxygens of both D239 and G238 (Figure 4B). The extra hydrogen bond is the main reason for increased stability of CRMut. The S165D stabilizes the monomer as well, because the WT residue, Ser 165 is not involved in any specific interactions, while in the mutant, the Asp 165 interacts with Lys 163 and Lys 167 while being exposed, does not pay any desolvation penalty.

At the same time, the analysis showed that neither of the mutations nor the Fmut affects the HsSMS dimer affinity.
showed that if N-domain is removed the HsSMS does not form terminal domain (path ''B'') (Figure 2A, Figure 2B). There is no dimer cleft (path ''A'') and within a patch at the bottom of the C-terminal domain. Because of that, from structural perspective they are not expected to alter the dimer affinity. The fact that computation protocol predicted no effect is reassuring and indicates that numerical protocol is robust.

How the increased stability may be related to enhanced activity is not an obvious question. Typically a decrease of the stability (or folding free energy) caused by mutation is considered to be bad for the function of proteins and frequently associated with diseases [17–22]. However, the opposite effect of increasing stability was also shown to cause disease [23,24]. Perhaps the key factor of estimating the relations between stability and function is the understanding the mechanism of the corresponding reaction. The HsSMS needs to bind two reactants and to release two products, while being a dimer. The less conformational changes are involved in the reaction, the faster the reaction will be. It can be speculated that such predicted overall rigidification will enhance the reaction rate, while allowing the necessary local and small conformational changes still to be allowed to carry the conversion SPD to SPM. Similar effect, an increase of the reaction rate upon mutations to non-synonymous amino acids was also shown in various enzymes [25,26].

One of the most important finding is that the four mutations, Fmut, result in a structure which carries more negative charge than the WT. The distribution of the extra negative charge is not uniform and results in stronger (negative) potential within the dimer cleft (path “A”) and within a patch at the bottom of the C-terminal domain (path “B”) (Figure 2A, Figure 2B). There is no experimental data to provide insights why the dimerization is necessary for the HsSMS function, except the original work which showed that if N-domain is removed the HsSMS does not form dimer and does not function [4]. However, each of the HsSMS monomers carries an active site and in principle should be capable to function as a monomer. Perhaps the dimerization is needed not for the reaction itself, but to promote the delivery of the reactants and the release of the products of the reaction. Electrostatic calculations support such a notion. Indeed in both the WT and the mutant, we identified two plausible electrostatic funnels, marked as path “A” and “B” in Figure 2A, however the potential is more negative in the mutant. It can be speculated that this extra negative potential provides better steering of the SPD to enter the dimer cleft and to increase the concentration of SPD in the proximity of the active sites. Indeed, the experimental data suggests that the affinity of HsSMS to SPD is higher in the mutant (Table 7). In addition, the dramatic increase of the reaction rate could be attributed to the increase of the maximal rate (Vmax) in the mutant, which in turn can be effect of the increased effective concentration of SPD at the entrance of the active site.

From the normal mode analysis, we can see the Fmut has a slightly higher frequency of domain motion than the WT, the difference being 170–260 MHz. Such difference most probably will affect the rate and efficiency of the reaction by affecting the substrate approach to and release from the active site. Following the original work of Zhou and coworkers [27], the higher the frequency of the gate is, the better is the chance that the substrate will successfully pass the gate, since the gate will be more frequently open when the SPD happens to be at the entrance. The same speculation can be made for the release of the products, i.e. the increased frequency of gate switching should increase the efficiency of product releasing [27].

Taking all these effects together, seems to us that increased activity in the mutant is due to a combinations of factors, ranging from enhanced access of the substrates to the active site via better electrostatic guiding and gate switching, structural stabilization and better protonation environment for the reaction. This indicates the complexity of the reaction process taking place in SMS and shows the complex interplay between structural and thermodynamic factors.

At the same time, it should be noticed that the amino acid sequence of HsSMS currently does not have known variants. A search in various databases (as for example dbSNP at NCBI, NIH [28–30]) resulted in no hits. The only mutations found in human population are the missense mutations causing Snyder-Robinson syndrome [6–9]. Our recent computations analysis [10] indicated that HsSMS should be able to tolerate mutations even at the disease-causing sites; however, such variants are not seen in the human population yet. Combining these observations with the result of this manuscript, it can be speculated that the function of

### Table 5. The results of structure – based (3C6K) binding energy calculation on dimer affinity changes under three force fields.

<table>
<thead>
<tr>
<th>Mutants ID</th>
<th>AMBER98 (kcal/mol)</th>
<th>CHARMM27 (kcal/mol)</th>
<th>OPLSaa (kcal/mol)</th>
<th>Ave three force fields (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDmut (S165D)</td>
<td>−1.71</td>
<td>−1.61</td>
<td>−0.22</td>
<td>−1.18</td>
</tr>
<tr>
<td>CRmut (C206R)</td>
<td>3.79</td>
<td>0.78</td>
<td>1.41</td>
<td>1.99</td>
</tr>
<tr>
<td>Pmut (L175E/T178H)</td>
<td>0.50</td>
<td>−1.47</td>
<td>−1.20</td>
<td>−0.73</td>
</tr>
<tr>
<td>Sum</td>
<td>2.58</td>
<td>−2.3</td>
<td>−0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>Fmut (S165D/L175E/T178H/C206R)</td>
<td>0.36</td>
<td>−2.11</td>
<td>−0.07</td>
<td>−0.61</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pcbi.1002924.t005

Structurally they are far away from the dimer interface and are situated in the C-terminal domain. Because of that, from structural perspective they are not expected to alter the dimer affinity. The fact that computation protocol predicted no effect is reassuring and indicates that numerical protocol is robust.

Enhancing Human Spermine Synthase Activity

Figure 2. Potential distribution. (A) Electrostatic field lines for the WT HsSMS; Drawing method: FieldLines; GradientMag: 1.45; Min Length: 35.31; Max Length: 50.90; Coloring Scale Data Range: −1; 1; (B) Potential difference (mutant − WT) mapped onto HsSMS surface; Coloring method: Volume; 1. Drawing method: Surf; Coloring Scale Data Range: −1; 1 (blue – positive, red – negative).

doi:10.1371/journal.pcbi.1002924.g002

Enhancing Human Spermine Synthase Activity

Figure 2. Potential distribution. (A) Electrostatic field lines for the WT HsSMS; Drawing method: FieldLines; GradientMag: 1.45; Min Length: 35.31; Max Length: 50.90; Coloring Scale Data Range: −1; 1; (B) Potential difference (mutant − WT) mapped onto HsSMS surface; Coloring method: Volume; 1. Drawing method: Surf; Coloring Scale Data Range: −1; 1 (blue – positive, red – negative).
HsSMS is precisely tuned toward its wild type value to satisfy some currently unknown constraints in vivo. Perhaps, any deviation from the wild type sequence is unwanted and disease-causing. However, caution should be used in the interpretation of the in silico results, since the threshold indicating a deviation is perhaps specific for each protein and reaction involved.

Methods

1) In silico modeling

a) Protein structure. The wild-type (WT) structure of human spermine synthase protein crystalized with SPD (PDB ID 3C6K) [4] were downloaded from the Protein Data Bank (PDB) website. There are four molecules (A, B, C, and D) forming two dimers (A–B dimer and C–D dimer) in each asymmetrical unit. In the simulations only C–D dimer was used as a biological unit, whereas the A–B dimer is removed because of significant van der Waals (vdW) clashes presented in the X-ray structure. The residues not resolved in the X-ray structures were rebuilt with the program Profilix in Jackal package (http://wiki.c2b2.columbia.edu/honiglab_public/index.php/Software:Jackal).

Comparing the protein sequence of HsSMS and TmSRM, four mutations were made in HsSMS: p.S165D, p.L175E, p.T178H, and p.C206R (they are p.S180D, p.L190E, p.T193H and p.C221R in the amino acid sequence, respectively). The selection was based on several considerations: a) mutations sites should be on the surface of the HsSMS so that their substitution would not cause any significant structural perturbations. Thus, S165D, L175E and C206R were selected since they are located at the surface of HsSMS; b) any residue predicted should be correlated with the above three to address the cooperativity. Thus, the T178H was selected since it is predicted to be correlated with L175E; c) Since the goal is to alter the properties of HsSMS, only substitutions resulting in a change of the biophysical properties such as the polarity or the hydrophobicity between the WT residue and the mutant residue were considered; d) In order to avoid abolishing the function of HsSMS, only sites that were not conserved in multiple sequence alignments were analyzed. However, preference was given to non-conserved sites neighboring other well-conserved sites since it was expected that such a substitution would have a larger effect on HsSMS than a substitution at an isolated non-conserved site; (e) Preference was given to amino acid substitutions that occurred with the highest frequency in the multiple sequence alignment. Taking in all these considerations, the four amino acid substitutions mentioned above are subject of the investigations described in this work, with the goal of enhancing the WT properties of HsSMS.

The mutant structures were built in silico by side chain replacement through the program SCAP in Jackal package [31]. Several mutant structures were generated in both C chain and D chain: a) the mutant with a single mutation S165D, which is named “SDmut”; b) the mutant with a single mutation C206R, which is named “CRmut”; c) the double mutant with two mutations L175E and T178H together. Making such “PAIR” mutations was prompted by the analysis of multiple sequence alignment (MSA), which will be introduced in a latter section. This mutant is named “PAIRmut”; d) the mutant with all of the above four mutations S165D, C206R, L175E and T178H. This mutant was named “Fmut”. The mutation sites are shown in Figure 5.

b) Multiple sequence alignment. To investigate the evolutionary relationship of the above four mutations among different species and homologous proteins, the multiple sequence alignment (MSA) was performed by Constraint-based Multiple Alignment Tool (COBALT) [32], which is currently provided by National Center for Biotechnology Information (NCBI) [33]. Three datasets were selected to do such an investigation: a) MSA only between HsSMS and TmSRM; b) MSA among SMS from different species; c) MSA among all the homologous to HsSMS proteins.

Protein sequences between HsSMS and TmSRM were aligned by COBALT since these four mutations were suggested by TmSRM. Additionally this comparison is intended to give a global view of sequence differences between these two species.

The pioneering work on HsSMS [4] analyzed MSA of representative members of SMS family among different species: human (Homo sapiens; NP_004586), chicken (Gallus gallus; NP_001025974), zebrafish (Danio rerio; NP_571831), fruit fly (Drosophila melanogaster; NP_729798), mosquito (Anopheles gambiae; XP_315341), bee (Apis mellifera; XP_393567), sea urchin (S. purpuratus; XP_789923), sea anemone (N. vectensis; XP_001636780), M. brevicollis (Joint Genome Institute protein ID 30201), A. thaliana (NP_568785), and Saccharomyces cerevisiae (AAC19368). In the present work, we use the same species mentioned above. The FASTA sequences were downloaded from NCBI and uploaded to COBALT for the MSA analysis.

MSA among the homologous proteins were also investigated with the same tools. Protein-BLAST (Basic Local Alignment Tool) and MAFFT (Multiple Alignment with MAFFT) were used to perform the MSA for the above four species. The results of normal mode analysis. Table 6.

Table 6: The results of normal mode analysis.

<table>
<thead>
<tr>
<th>Structures</th>
<th>AMBER98 (GHz)</th>
<th>CHARMM27 (GHz)</th>
<th>OPLSaa (GHz)</th>
<th>Ave three force fields (GHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modes</td>
<td>Model/2/3</td>
<td>Model/2/3</td>
<td>Model/2/3</td>
<td>Model/2/3</td>
</tr>
<tr>
<td>WT</td>
<td>29.21/34.77/39.55</td>
<td>24.93/27.26/32.77</td>
<td>28.98/34.05/37.96</td>
<td>27.11/32.02/36.76</td>
</tr>
<tr>
<td>Fmut (S165D/L175E/T178H/C206R)</td>
<td>30.00/35.56/40.76</td>
<td>24.38/27.34/32.71</td>
<td>29.26/33.66/37.59</td>
<td>27.88/32.19/37.02</td>
</tr>
</tbody>
</table>

Table 7: Comparison of SMS activity between WT and Fmut.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity (nmol/h/mg)</th>
<th>Km for dcAdoMet (µM)</th>
<th>Km for SPD (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3776.4 ± 494</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Fmut (S165D/L175E/T178H/C206R)</td>
<td>40418.4 ± 3247</td>
<td>0.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Fmut (S165D/L175E/T178H/C206R)

Table 7: Comparison of SMS activity between WT and Fmut.
Search Tool [16] was used to search the homologous proteins. The max target sequences were set as 500 to include the sequences of all of the above SMS from difference species along with the sequence of other homologues proteins.

c) pKa calculations. Since all of the above four mutations involve titratable group, pKa calculations were carried out to investigate the ionized states of each titratable group in both WT and mutants. All titratable groups were analyzed, because it is known that mutations may affect the ionized states of neighboring residues by either perturbing the original dielectric boundary of the protein or altering the hydrogen bond network [34,35]. The differences of pKa value of each titratable group between the WT and mutants were calculated by Eq. (1):

$$\Delta pK_{ai}(\text{mutation}) = pK_{ai}(WT) - pK_{ai}(\text{mutant})$$  

where $\Delta pK_{ai}(\text{mutation})$ is the pKa change of amino acid $i$ due to the mutations; while $pK_{ai}(WT)$ and $pK_{ai}(\text{mutant})$ are the pKa values of amino acid $i$ in the WT and mutants respectively.

pKa was calculated by Multi Confirmation Continuum Electrostatics (MCCE) [36–38], version 2.4 [http://134.74.90.158]. The default parameters were used and the dielectric constant of protein was set as 8.0. For both of the WT and mutants, the calculation was performed on monomer C, monomer D and dimer CD respectively. It should be mentioned that standard rigid body pKa’s calculations are very sensitive to the initial structure used, especially in terms of side chain orientations. The importance of accurate predictions of side chain rotamers for molecular biology is illustrated by numerous studies [39–44] including developing tools such as SCWRL [45,46] and SIDEpro [47]. MCCE takes advantage of these developments and uses Dunbrack conformer libraries [40,42] to sample the side chain rotamers within the calculations of the pKa’s of titratable residues and thus the predictions are independent of the initial side chain positions.

d) Energy calculations. The effects of the mutations were modeled on two energies, folding energy and binding energy. In order to save the calculating time and reduce unwanted artifacts, we removed N-domain (Residue number: 2–109) in the calculation since all of these four mutations were located in the C-domain and far away from the N-domain (Figure 5) [9,10]. For each of the energy calculations in the present work, the TINKER package [48] was applied and three force fields (Amber 98 [49], Charmm27 [50] and OPLSaa [51]) were used and results averaged. The solvent was modeled with the Still Generalized Born model [52], and the internal dielectric constant was set up to 1.0. The structures of the dimer and the corresponding monomers were energy minimized with the “minimize” module of TINKER package utilizing Clemson’s high performance computing cluster, Palmetto (HPC). The minimization was done via Limited Memory BFGS Quasi-Newton Optimization algorithm within the TINKER package [48]. The convergence criteria applied was RMS gradient per atom equal to 0.01. After successful energy minimization, the potential energies for the minimized structures were obtained with “analyze” module of TINKER package.

Change of folding energy due to mutations corresponds to the change of stability of monomers. Negative folding energy change indicates that the mutant monomers are less stable compared with

![Figure 3. Interaction networks among product SPM, active sites: D201 & D276, and pair mutation sites. (A) WT; (B) Pmut. Pair mutation sites were shown with sticks: magenta represented L175 in WT and E175 in mutant; blue represents T178 in WT and H178 in mutant. The active sites (D201 and D276) and SPM are shown in ball and sticks: yellow represents SPM; orange represents D276 and cyan represents D201. The short red lines indicate hydrogen bonds. doi:10.1371/journal.pcbi.1002924.g003](#)

![Figure 4. Effects on hydrogen networks surrounding the mutation site C206. (A) WT; (B) Mutant C206R. The residues are presented in sticks: green sticks represent C206 in WT and R206 in the mutant; yellow sticks represent G238; and cyan sticks represent D239. The red dash indicates the hydrogen bonds. doi:10.1371/journal.pcbi.1002924.g004](#)

![Figure 5. 3D structure of HsSMS dimer in ribbon presentation. Four mutation sites are shown with ball representation: Yellow: S165D; Magenta: C206R; Cyan: L175E; and Orange: T178H. doi:10.1371/journal.pcbi.1002924.g005](#)
WT monomers, while positive energy change means increasing stability of mutant monomers. In order to calculate the folding energy change due to the missense mutations, we applied the scaled Molecular Mechanics Generalized Born (sMMGB) method described in our previous works [9,10,53]. It should be mentioned that this approach uses three different force fields (AMBER98, CHARMM27, and OPLSaa) to deliver the predictions and was shown to perform better that any of these force field alone [53]:

$$
\Delta G(\text{folding}) = G(\text{folded}) - G(\text{unfolded})
$$

$$
= G(\text{folded}) - G_0(\text{unfolded}) - G_3(\text{unfolded})
$$

where $G(\text{folded})$ is the potential energy calculated with the energy minimized structure through simulating with TINKER, and $G(\text{unfolded})$ is the potential energy of unfolded state. The unfolded state energy is separated into $G_0(\text{unfolded})$ and $G_3(\text{unfolded})$. $G_3(\text{unfolded})$ is calculated on a three-residue segment taken out from the corresponding WT and mutant structures, whereas $G_0(\text{unfolded})$ is considered to be mutation independent and cancels out in Eq. (3).

$$
\Delta AAG(\text{folding,mut}) = \Delta G(\text{folding : WT}) - \Delta G(\text{folding : mut})
$$

$$
= \left[ G(\text{folded : WT}) - G(\text{folded : mut}) \right] - \left[ G_3(\text{unfolded : WT}) - G_3(\text{unfolded : mut}) \right]
$$

where $\Delta AAG(\text{binding : WT})$ is the binding energy of the WT monomers and $\Delta AAG(\text{binding : mut})$ is the binding energy of the corresponding mutant monomer chains.

**e) Potential map generation and analysis.** Since HsSMS reaction involves highly charged substrates, it can be expected that electrostatics and potential distribution play significant roles in the HsSMS functions. To assess the effects of the mutations on the electrostatic properties of SMS, the wild type and mutant HsSMS proteins were subjected to continuum electrostatic potential calculations using DelPhi [57]. The following parameters were used: scale – 1 grid/Å; percent of protein filling of the cube – 70%; a dielectric constant of 2 for the protein and 80 for the solvent; the ionic strength 0.15 M; the water probe radius 1.4 Å; and the Stern ion exchange layer 2.0 Å. The results were outputted into a file in CUBE format (http://compbio.clemson.edu/delphi.php) that represents the electrostatic potential at each grid point within the grid. To determine the changes in electrostatic potential due to the mutation, the electrostatic potential map for wild type protein was subtracted from one of mutant type. The results were then visualized with VMD software [58]. The red color represents area with negative potential difference, and blue color represents that with positive one. Since both substrates are positively charged, the enhancement of the negative potential is expected to facilitate their approach to active site of SMS.

**f) Normal mode analysis.** The synthesis of SPM requires the substrate SPD to come inside the enzyme and after the catalytic reaction, the product SPM will be released. Bearing in mind that domain formation is shown to be critical for HsSMS function, it is natural to assume that clef formed between HsSMS units in the dimer is the pathway of substrates. Because of that, the frequency of the cleft “opening” and “closing” is expected to be related to the efficiency of the catalytic reaction. This mechanism, described as “conformational gating”, was investigated by Zhou et al. in the case of acetylcholinesterase [27]. It was shown that the frequency of the gate opening and closing affects the substrate entering to the active site.

The theory and methodology of normal mode (NM) is well developed and described elsewhere [39,60]. In the NM model, the protein is presented as an elastic network [61] and each residue of protein is modeled as a node in the network and the connections between nodes are described as elastic potentials. In particular, the anisotropic network model (ANM) [61,62] considers the molecule as a collection of N sites (one site for each residue, resulting in ensemble of 3N-6 independent modes). An important parameter in the ANM analysis is the force constant $c$, describing the strength of intramolecular potentials. The optimal value of $c$ has been obtained by comparing the theoretically predicted mean-square fluctuations of alpha-carbons with those indicated by the X-ray crystallographic B-factor and it was found that the best value is $1.0 \pm 0.5$ kcal/(mol\(^{-1}\)Å\(^2\)) [61]. In our analysis we used 1.0 kcal/ (mol\(^{-1}\)Å\(^2\)) as the optimal value.

The cutoff distance $r_c$ is another parameter in the ANM model which requires optimization. It specifies the threshold distance within which the residues/atoms are considered to interact with each other. In this work, $r_c$ and distance weight for interaction between $C_b$ atoms are set as the default parameters shown on the website ($r_c = 15$ Å and the distance weight is 0). All of the TINKER minimized structures were submitted to the ANM webserver (http://igmtest.ccb.pitt.edu/cgi-bin/annm/annm.cgi) [62]. Analysis of the predicted vibrational modes with respect to the plausible substrate pathways (pathways A and B in Figure 2) made us select mode 1, mode 2, and mode 3 for further investigations. The predicted motions (the directions of the vibrational vectors) and their plausible implications for the SPD/
SPM pathways can be seen in the supplementary material (Figure S1). The corresponding frequencies were calculated as:

\[ \omega_i = (\gamma_2 z_i)^{1/2} \quad \text{and} \quad f_i = (\omega_i / 2\pi) \]  

where \( \gamma \) is the force constant; \( z_i \) is the ith eigenvalue, for \( 1 \leq i \leq 3N-6 \) (for 3D or N-1 for 1D) and \( f_i \) is the frequency.

**II) Experiments**

a) **Expression and purification of HsSMS.** A DNA fragment encoding HsSMS was amplified by PCR and subcloned into the pQE-30 vector downstream of the polyhistidine coding region [63]. The resulting plasmids were used to transform XL1-Blue cells. Recombinant human SS was purified by immobilized metal affinity chromatography using TALON affinity resin (Clontech Laboratories, Palo Alto, CA, U.S.A.), in accordance with manufacturer’s instruction.

b) **Assay of HsSMS activity.** Activity was measured by following the production of [35S]MTA from [35S]dcAdoMet in 100 nM sodium phosphate buffer (pH 7.5), in the presence of SPD [64]. The reaction was stopped by acidification and [35S]MTA separated from [35S]dcAdoMet using phosphocellulose columns. All assays were conducted with an amount of protein and for a period of time in which product formation was linear with time. Reactions were run with an amount of enzyme that gave a linear rate of MTA production over the assay time period. Assays were conducted for 1 h at 37°C. All assays were carried out in triplicate, and results agreed within ±5%.

c) **Production of 1HsSMS mutants.** The four amino acid mutated HsSMS was generated by PCR and subcloned into the pQE30 vector. The following oligodeoxynucleotides (with mismatches underlined) were used to generate the mutant indicated:

- 5'-ATCGCATTGCTGCAATAATGACATCCCCATCAAGGAAGTGATG-3' (antisense) for S165D
- 5'-TTGGAGGAGAAGTGATGACATATACCGGCGCCAATGATGATAC-3' (sense) for L175E and T178H
- 5'-GGAGGATTTCCGCTGAAAATGATGACATATCACCGGCGCCAATGATGATAC-3' (sense) for C206R
- 5'-TTTGAATTTTTCAGCGATATGCTCC-3' (antisense) for C206R

**Supporting Information**

Figure S1 The first three vibrational modes calculated with the ANM server. The plausible pathways of substrates SPD/SPM are indicated with the black arrows. The letter code of pathways “A” (the cleft between C-terminal domains) and “B” (the cleft between adjacent C- and N-terminal domains) corresponds to Figure 2 in the main body of the manuscript. The directions of vibrational vectors of the three modal vibrations are shown with orange arrows.

(DOCX)

Table S1 Candidate sites for engineered mutations selected based on 3D structure of HsSMS.

(DOCX)

Table S2 The MSA among different species. The highly conserved residues are marked with * in the alignment.

(DOCX)

Table S3 MSA among 500 homologous proteins.

(DOCX)

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**Author Contributions**

Conceived and designed the experiments: ZZ AP YI EA. Performed the experiments: ZZ YZ YI EA. Contributed reagents/materials/analysis tools: ZZ YI EA. Wrote the paper: ZZ YZ YI EA.

References

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