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ORIGINAL PAPER

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Homology modeling reveals the structural background of the striking difference in thermal stability between two related [NiFe]hydrogenases

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Abstract Hydrogenases are redox metalloenzymes in bacteria that catalyze the uptake or production of molecular hydrogen. Two homologous nickel–iron hydrogenases, HupSL and HydSL from the photosynthetic purple sulfur bacterium *Thiocapsa roseopersicina*, differ substantially in their thermal stabilities despite the high sequence similarity between them. The optimum temperature of HydSL activity is estimated to be at least 50 °C higher than that of HupSL. In this work, homology models of both proteins were constructed and analyzed for a number of structural properties. The comparison of the models reveals that the higher stability of HydSL can be attributed to increased inter-subunit electrostatic interactions: the homology models reliably predict that HydSL contains at least five more inter-subunit ion pairs than HupSL. The subunit interface of HydSL is more polar than that of HupSL, and it contains a few extra inter-subunit hydrogen bonds. A more optimized cavity system and amino acid replacements resulting in increased conformational rigidity may also contribute to the higher stability of HydSL. The results are in accord with the general observation that with increasing temperature, the role of electrostatic interactions in protein stability increases. Electronic supplementary material to this paper can be obtained by using the Springer Link server located at http://dx.doi.org/10.1007/s00894-001-0071-8.

Keywords *Thiocapsa roseopersicina* · Hydrogenase · Metalloenzymes · Homology modeling · Protein stability

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Introduction

Hydrogenases are redox metalloenzymes in bacteria that catalyze the uptake or production of molecular hydrogen. Many of them harbor a unique nickel–iron (NiFe) heterobinuclear active center and all of them contain two or three iron–sulfur (4Fe–4S) clusters. [1, 2] These enzymes have potential industrial applications including the production of molecular hydrogen, a clean and renewable energy source as well as the utilization of hydrogenases as redox catalysts in bioconversion reactions. [3] Two [NiFe]hydrogenases, HupSL and HydSL, were identified in the photosynthetic purple sulfur bacterium, *Thiocapsa roseopersicina* BBS that lives in cold marine environments. [3] One of them, HydSL, is characterized by an unusually high conformational stability: its temperature optimum is above 80 °C, it withstands the inactivating effects of O_2 and proteases, and is easy to purify in active form. [4] HupSL, on the other hand, shows marginal stability: it quickly loses activity even under mild conditions (e.g. at 4 °C) and it has withstood all purification attempts so far. [5] The optimum temperature of HydSL activity is estimated to be at least 50 °C higher than that of HupSL. [5] The two enzymes are homologous: both consist of one small and one large subunit (denoted by S and L, respectively) and the sequence identity between the two hydrogenases is considerable (46 and 58% for the S and L subunits, respectively). A detailed explanation of the large stability difference between these two highly similar enzymes clearly requires knowledge of their three-dimensional structures. Although such data are not available at present, the X-ray

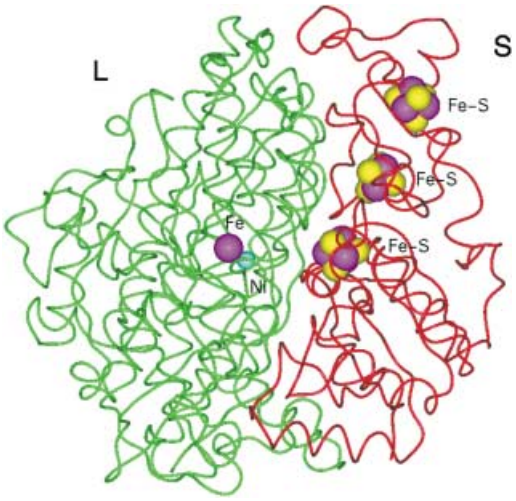

Fig. 1 The overall structure of [NiFe]hydrogenases. These enzymes consist of a small (S) and a large (L) subunit, shown here in different colors. The backbones of the polypeptide chains are represented by thin threads (*red* and *green*). The large subunit contains the NiFe heterobinuclear active center. The small subunit contains three iron-sulfur clusters. The figure was created using the *Desulfovibrio gigas* hydrogenase structure (PDB entry 2frv)

structures of homologous hydrogenases from some other microorganisms are now known (see Fig. 1). In this work, we used the available structures and homology modeling to construct models for HydSL and HupSL from *Thiocapsa roseopersicina* and performed a comparative analysis of the models to find the structural features underlying the stability difference between the two proteins. In addition, we also analyzed their amino acid compositions to identify some possible differences related to stability.

Materials and methods

Model building

The amino acid sequences of the *Th. roseopersicina* hydrogenases were retrieved from the Swissprot database [6] (HupSL: entries Q56359 and Q56360; HydSL: entries O51820 and O51823). Using a BLASTP [7] search on Swissprot, all hydrogenase sequences homologous with the *Th. roseopersicina* hydrogenases were collected. A multiple alignment of the sequences was done using ClustalW. [8] The X-ray structures of [NiFe]hydrogenases from four organisms are known: *Desulfovibrio gigas* (PDB entry 2FRV [2]), *D. fructosovorans* (PDB entry 1FRF [9]), *D. vulgaris* (PDB entry 1H2A [10]) and *Desulfomicrobium baculatum* (PDB entry 1CC1; [11] this is actually a [NiFeSe]hydrogenase, which is somewhat different from the other members of this protein family). The structural alignment of these proteins was retrieved from the FSSP database [12] (FSSP entries 2frvA and 1h2rL for the small and the large subunit, respectively) and was used to adjust the ClustalW-based sequence alignment manually. The procedure was carried out separately for the small (S) and the large (L) subunits. Because of the high sequence similarities among the sequences, the alignment was fairly straightforward, and no need for making alternative alignments arose.

Models were constructed by comparative modeling using the method of satisfaction of spatial restraints as implemented in the program MODELLER. [13] Based on sequence similarity and structural considerations, the *D. gigas* hydrogenase structure (PDB entry 2FRV) was used as a template. Fe–S clusters and Ni and Fe ions were included in the models. The model structures are shorter than the full-length sequences because large portions of the C-terminus of the small subunit of both enzymes and a few residues at the N-terminus of the HupSL large subunit (HupL) could not be modeled for lack of a template structure. The C-terminal tail of the small subunit has been assigned to serve as a membrane anchor; [14] therefore it probably has a negligible contribution to the overall stability of the enzyme. Using a standard modeling procedure, ten slightly different models were generated with MODELLER for both HupSL and HydSL. This procedure is advantageous because one can select the best model from several candidates, and, more importantly, the variability among the models can be used to evaluate the reliability of the modeling. MODELLER produces models refined by molecular dynamics simulated annealing.

The quality of the models was assessed by the PROCHECK program [15] and by MODELLER itself (based on the value of the objective function calculated by MODELLER). Although the models proved to be of nearly equally good quality, the model with the lowest number of bad (i.e. close) contacts was selected from each group of ten models. These two models were considered the representative models for HupSL and HydSL, respectively (we do not call them the “best” models since the other ones are about the same quality). The coordinates of the representative models are available as electronic supplementary material.

Analysis of the models

A number of properties were calculated from each of the ten models constructed for HupSL and HydSL as well as from the four known hydrogenase structures.

Ion pairs

Ion pairs were defined using a simple distance criterion for oppositely charged residues. A limit distance of 6.0 Å was used to allow for both strong ion pairs (<4.0 Å apart) and weaker electrostatic interactions. Inter- and intra-subunit ion pairs were separately considered. Networks of ion pairs were categorized by the number of their constituent ion pairs.

Polarity of surfaces

Atomic accessible surface areas were calculated using the Molecular Surface Package (version 3.6) by Michael L. Connolly [16] with a probe radius of 1.4 Å. Buried surface areas were calculated as the difference between surface areas of the folded and unfolded chains; to calculate the latter, the subunit models were unfolded (i.e. an extended chain was produced) by setting all main-chain and side-chain torsion angles to the values given in [17]. Considering the N and O atoms polar and all other atoms apolar, the polar/apolar surface area ratios were calculated for both the exposed and the buried surface. Subunit contact areas were considered separately.

Hydrogen bonds

Hydrogen bonds were counted using the HB2 algorithm in the WHAT IF molecular modeling program. [18] Instead of using a simple yes/no criterion for hydrogen bonds, this sophisticated algorithm uses a special force field to find the optimum hydrogen-bonding network, and allows His, Asn and Gln side chains to flip. Intra- and inter-subunit hydrogen bonds were considered separately.

Cavities

Cavities inside the proteins were identified using the Molecular Surface Package (version 3.6) by Michael L. Connolly, [16] applying a probe radius of 1.4 Å. The number, total volume, total surface area and hydrophobic fraction of the total surface area of the cavities were calculated. Cavities inside each subunit and between the two subunits were considered separately.

Secondary structure

Secondary structures were determined using the DSSP program. [19] The secondary structural composition (fractions of helices, beta strands and irregular regions) was also calculated separately for the two subunits.

Amino acid composition

Amino acid compositions were calculated from the parts of the sequences for which three-dimensional structures were available, i.e. not from the full-length sequences available in the Swissprot sequence database. The rationale for this is that the full-length sequences contain membrane anchors, signal sequences and other segments irrelevant to protein stability. The amino acid compositions of the two subunits were also considered separately.

Results

Comparison of HupSL and HydSL

Table 1 presents those structural parameters calculated for the HupSL and HydSL models that differ between the two proteins (the representative models of HupSL and HydSL were chosen for comparisons). The values calculated for the representative models and the averages over the ten HupSL and the ten HydSL models, respectively, are shown. The table includes the same parameters calculated for the four known hydrogenase structures, for comparison. Figure 2 shows the same data in the form of plots, and it also shows the individual values calculated for each HupSL and HydSL model. The parameters that are mentioned in the Materials and methods section but not included in Table 1 do not differ significantly between HupSL and HydSL. Most of the parameters related to interactions within the isolated subunits fall in this category.

The most conspicuous difference between HupSL and HydSL is found in the interactions linking the two subunits. In particular, ten ion pairs link the subunits in HydSL, as opposed to only two in HupSL (see Fig. 3). The total number of ion pairs, however, does not differ significantly between the two proteins (61 versus 64). There is a slight difference in the number of inter-subunit hydrogen bonds as well (50 in HydSL versus 45 in HupSL). The stronger interaction between the subunits of HydSL, in comparison with HupSL, is also reflected by the fact that the subunit interface area of HydSL is more polar (the ratio of polar/apolar surface areas is 0.638 in HydSL versus 0.572 in HupSL). This is also obvious when one looks at the distribution of the electrostatic potential on the surface (see Fig. 3). Since the interface area is somewhat smaller in HydSL than in HupSL, the increase in the polar fraction is partly a result of the elimination of some hydrophobic contact areas.

We also found a difference between HydSL and HupSL in the number and total volume of internal cavities within the large subunit. HupL contains about 1.6 times more cavities, with a proportionately larger total volume, than HydL.

We found some notable differences between the amino acid compositions of HupSL and HydSL. The most significant of these is the greater alanine content of the small subunit of HydSL. Besides, this subunit contains three more prolines and the large subunit has fewer glycines in HydSL than in HupSL. The cysteine content of HydSL is also smaller than that of HupSL.

Reliability of the results

Earlier experience shows that the reliability of models built by comparative modeling is largely determined by the sequence identity between the template used for the modeling and the modeled protein. [20] Since this is about 50% in our case, our homology models are likely to be fairly accurate. However, side chain and loop conformations may be inaccurate and this may influence the results of our analyses. Therefore, an analysis of the reliability of the results is required.

As mentioned earlier, we generated ten models for both HupSL and HydSL and calculated all the properties mentioned in the Materials and methods section for each model. When generating the models, MODELLER starts with random initial coordinates and uses an optimization procedure combining conjugate gradient minimization and simulated annealing with molecular dynamics to optimize an objective function combining CHARMM energy terms with distance and dihedral angle restraints derived from the template structure(s). [13] Running the program ten times (with different random seeds) results in ten models that slightly differ from each other, and regions with fewer template-derived restraints (mostly loops and exposed side chains) will be more variable, reflecting the uncertainty of the model obtained. Since our models proved to be of nearly equally good quality, the variance of the calculated properties among the ten models generated for the same protein is an indication of how reliably each property is predicted by the models. Table 1 shows (in parentheses) the averages of each property calculated for the ten models of each protein and its standard error. Besides, the data obtained for the models can be compared with the corresponding data calculated from the four known hydrogenase structures (see Table 1 and Fig. 2) to see whether all properties of the models are realistic. Although no stability data about these hydrogenases are available, obviously they are stable since their purification and crystallization was possible.

The two inter-subunit ion pairs found in the representative HupSL model are present in seven of the ten mod-

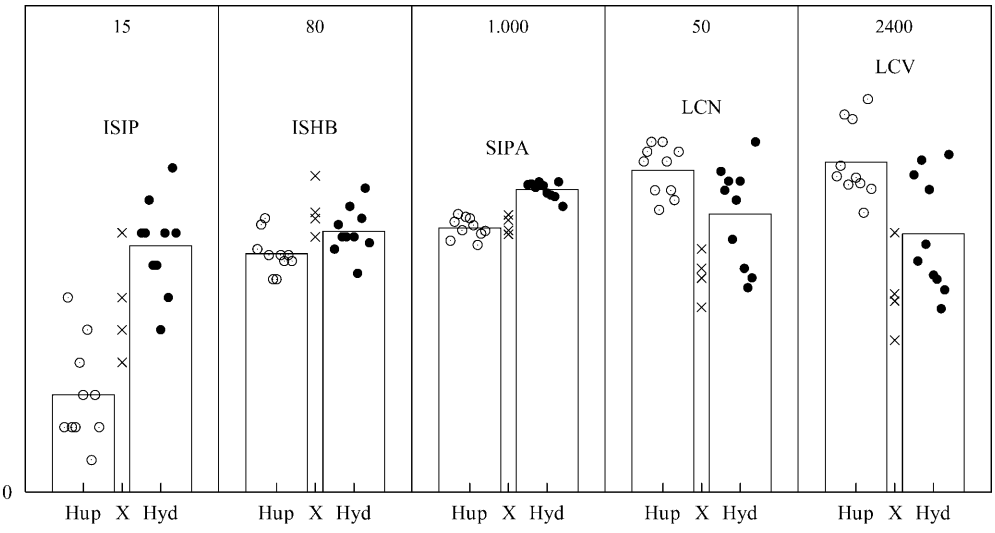

Fig. 2 The number of inter-subunit ion pairs (ISIP), inter-subunit hydrogen bonds (ISHB), ratio of polar to apolar surface areas calculated for the subunit interface area (SIPA), number (LCN) and total volume (LCV) of cavities in the large subunit, calculated for the ten HupSL models (*hollow circles*) and the ten HydSL models (*solid circles*) as well as the four known hydrogenase X-ray structures (*crosses*). The bars represent the averages over the ten HupSL and HydSL models, respectively. The parameter value corresponding to the top of the vertical axis is indicated at the top of each panel

Table 1 Properties differing between the homology models of HupSL and HydSL, possibly underlying the thermostability difference

	HupSL (unstable)	HydSL (stable)	2frv/1frf/1h2a/1cc1[a]
Number of ion pairs[b]			
Total	61 (60/1)	64 (57/2)	71/66/59/87
Inter-subunit	2 (3/0.5)	10 (8/0.5)	6/4/5/8
Number of H-bonds[b]			
Inter-subunit	45 (39/1)	50 (43/1)	46/42/52/45
Subunit interface[b]			
Total surface area (Å^2)	4219 (4403/54)	4149 (4341/46)	4547/4581/4459/4333
Polar/apolar ratio	0.572 (0.543/0.006)	0.638 (0.622/0.005)	0.538/0.530/0.559/0.570
Cavities[b, c]			
Number in L	36 (33/1)	22 (28/2)	22/19/25/23
Total volume in L (Å^3)	1863 (1627/59)	999 (1274/90)	943/978/1280/750
Amino acids[d]			
Alanine in S	18 (6.7%)	29 (10.7%)	23/24/31/25 (8.8/9.2/11.6/9.1%)
Proline in S	18 (6.7%)	21 (7.8%)	19/21/22/20 (7.3/8.0/8.2/7.3%)
Glycine in L	47 (8.2%)	42 (7.5%)	41/46/40/43 (7.7/8.5/7.5/8.8%)
Cysteine in S+L	20 (2.4%)	14 (1.7%)	21/22/19/19 (2.7/2.7/2.4/2.5%)

[a] The last column of the table shows the appropriate parameters calculated for the four hydrogenases with known structure (in the column heading, the appropriate Protein Data Bank identifiers are given). S and L denote the small and the large subunit, respectively
[b] For HupSL and HydSL, the number given in the table is the value of the property calculated for the representative model. The average value calculated for the ten models of each protein and its standard error are given in parentheses
[c] The number of cavities in the large subunit and their total volume are given
[d] For each named residue, the number of its occurrences is given, with the percentage in parentheses

els generated for HupSL. Therefore, these two ion pairs are quite reliably predicted. From the ten inter-subunit ion pairs found in the representative HydSL model, eight are present in at least half of the models generated for HydSL, i.e. they are reliably predicted. The average number of inter-subunit ion pairs is 3 and 8 for the HupSL and HydSL models, respectively, with only slight variation among the models. For the known hydrogenase structures, this number is 4 to 8, thus HupSL is close to the lower end of this range and HydSL is close to the upper end. We also examined whether the inter-subunit ion pairs present in HydSL can be generated in HupSL by adjusting the conformation of the corresponding side chains. We found that in seven cases, this is impossible because there is an uncharged residue in HupS in the place of a charged residue in HydS. In two other cases, the appropriate charged side chains are present in HupSL but they are too far apart to create an ion pair; of course, this observation is sensitive to possible inaccuracies of the model. In summary, we can state with high confidence that HydSL really contains at least five more inter-subunit ion pairs than HupSL, and this is

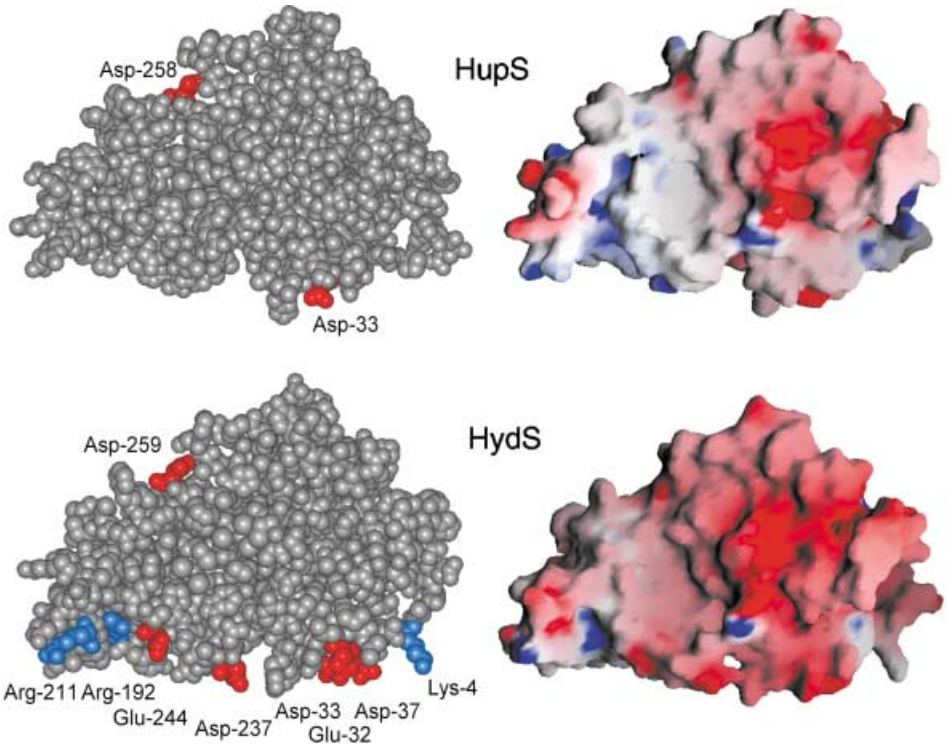

Fig. 3 The subunit interface areas of the small subunit of HupSL (top) and HydSL (bottom). On the left, space-filling models are shown, with the residues making an ion pair with the large subunit colored *red* (negative charge) and *blue* (positive charge). On the right, the molecular surfaces are shown, colored according to the calculated electrostatic potential (images were created susing GRASP [29])

mainly due to the presence of some extra charged residues in the small subunit of HydSL. Two of these extra ion pairs are strong in the sense that they are even detected with a stricter distance criterion (4.0 Å) to define ion pairs.

The averages and standard error of the number of inter-subunit hydrogen bonds (Table 1 and Fig. 2) suggest that the difference found in this property between HupSL and HydSL is only slightly significant.

On the other hand, the difference in the average polarity (polar/apolar surface area ratio) of the subunit contact surface is highly significant statistically. In addition, HydSL has a more polar subunit contact surface than any of the hydrogenases with known structure.

The number and total volume of cavities in the large subunit is quite variable among the models and even among the hydrogenases with known structures (see Table 1 and Fig. 2). The difference in this parameter between HupSL and HydSL is only slightly significant.

Discussion

The nickel–iron hydrogenases HupSL and HydSL from *Thiocapsa roseopersicina* are an intriguing example of two homologous enzymes with the same function and in the same organism but having substantially different conformational and thermal stabilities. The purpose of this work is to find a structural explanation of this observed stability difference between the two enzymes. To this end, we constructed homology models for both enzymes and used the models to calculate a number of protein properties that are known to influence stability as indicated by numerous experimental and theoretical studies (see e.g. [21] and references therein).

Our main finding is that the interactions linking the two subunits of the enzyme are stronger in HydSL than in HupSL. This strengthening is mainly brought about by electrostatic interactions as reflected by the presence of at least five extra inter-subunit ion pairs in HydSL. The increased polarity of the subunit interface and a few extra inter-subunit hydrogen bonds in HydSL also support this conclusion. This finding is in accord with the general observation that, with increasing temperature, the role of electrostatic interactions in protein stability increases; this is supported by analyses of proteins from thermophilic organisms [21] as well as theoretical considerations. [22] In an earlier work, we successfully increased the thermal stability of isopropylmalate dehydrogenase by introducing extra ion pairs linking the two subunits of the enzyme. [23]

It should be noted that ion pairs in proteins could be stabilizing or destabilizing, depending on the local conformation and environment of the affected side chains. To make sure that the extra inter-subunit ion pairs found in HydSL are stabilizing, their contribution to the free energy of association should be measured. An estimation of this contribution could also be given using free energy calculations. Most treatments of electrostatic contributions to binding energies have been based on continuum electrostatics, often combined with molecular dynamics simulations. [24] Although this approach is sometimes highly successful, its accuracy is limited.

The calculations are extremely sensitive to geometry and it is essential that highly accurate structures be used. [24] In addition, subunit–subunit association often involves conformational change, which should be accounted for. Since these requirements obviously cannot be fulfilled in our case (we have two homology models, whose accuracy is inherently limited), a calculation of binding free energies would provide highly uncertain results. Therefore, we did not perform such calculations. However, in an earlier study, Xu et al. [25] analyzed a number of protein–protein complexes (with accurate structures) and found a strong positive correlation between the number of electrostatic interactions across the binding interface and the binding free energy. They also argue that salt bridges linking subunits are much more often stabilizing than intra-subunit ones because the contributing side chains are less solvated before forming the bridge; thus, the desolvation penalty is lower. [25] In addition, another study comparing mesophilic and thermophilic proteins indicated that ion pairs on the protein surface are often stabilizing, especially when they form networks. [26] Since the inter-subunit ion pairs in HydSL are at the surface (see Fig. 3) and are also part of ion pair networks, it is highly probable that they do stabilize the complex.

In addition to the increased subunit–subunit interactions, we found a marked decrease in the number and total volume of cavities in the large subunit of HydSL, in comparison with HupSL. Since internal cavities in proteins are known to be destabilizing, this result might suggest that the higher stability of HydSL is partly due to the elimination of some of the cavities present in HupSL. However, we found that cavity parameters are predicted with low reliability by our models and are obviously very sensitive to small differences in atomic coordinates. In fact, the large subunit of [NiFe]hydrogenases contains a large number of cavities, some of which are water-filled. These cavities are believed to be involved in enzyme function: internal hydrophobic channels are likely to serve as pathways for gas access to the active site. [9] Because of the complex cavity and channel system within the large subunit and the low reliability of predicted cavity parameters, the suggested relationship between stability and cavities should be treated with caution.

Finally, there are differences between the amino acid compositions of HupSL and HydSL that might be related to the stability difference between them. The small subunit of HydSL contains more alanines, and since alanine is known to have a strong helical propensity, they may stabilize helices. A slight increase in the number of prolines (which make the polypeptide backbone rigid) and a decrease in the number of glycines (which make the backbone flexible) point to a more rigid and thereby more stable structure; an inverse relationship between stability and flexibility is suggested by a number of theoretical and experimental studies. [27, 28] The elimination of some cysteine residues, which are chemically unstable at higher temperatures, may also have a stabilizing effect. Obviously, these differences between the amino acid compositions are relatively small, and are likely to have only a small contribution to the stability difference.

The present modeling study demonstrates that the stability difference between HupSL and HydSL can be attributed to intrinsic factors, i.e. structural differences between the two enzymes. Although extrinsic factors cannot be excluded, they are unlikely to play a role since HupSL and HydSL are at the same subcellular location in the same organism.

Since HupSL is hard to isolate and crystallize, an X-ray structure of it is not expected to become available in the near future. Therefore, for the current time, homology modeling remains the only way to get useful structural information about it. The models also allow us to design mutants to test our findings and possibly to design HupSL mutants with increased stability. The best candidates for mutagenesis are the residues that contribute to inter-subunit ionic interactions (see Fig. 3).

Supplementary material The coordinates of the representative models are available as electronic supplementary material.

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