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The effect of three-dimensional structure on the solid state isotope exchange of hydrogen in polypeptides with spillover hydrogen

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Abstract

The effect of the three-dimensional structure of polypeptides and proteins on their ability to undergo isotopic exchange under the action of spillover hydrogen (SH) in the high temperature solid state catalytic isotope exchange reaction (HSCIE) was theoretically and experimentally studied. The HSCIE reaction in the β-galactosidase protein from *Thermoanaerobacter ethanolicus* (83 kDa) was studied. The influence of the β-galactosidase structure on isotopic exchange as peptide fragments with spillover tritium was studied. The most accessible peptide fragment, which does not contribute to α-helix and β-strand formations (KEMQKE215–220), had the largest relative reactivity. The one located in the contact area between the subunits (YLRDSE417–422) showed the smallest relative reactivity. The relative reactivities of these peptides differ more than 150 times. Data collected during a study devoted to the HSCIE reaction of the β-galactosidase protein indicate that the HSCIE reaction might be employed for acquiring information about their three-dimensional structure and protein-protein interactions. The results of ab initio calculations have shown that α-helix formation in polypeptides decreases the reactivity in HSCIE. Hydrogen exchange in the α-helical fragment Trp1–Leu8 of zervamycin IIB was also analyzed using theoretical methods. It was shown by ab initio quantum-chemical calculations that the high degree of substitution of $C^{\alpha}H$ for tritium in Gln3

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might be associated with the participation of electron donor O and N atoms in transition state stabilization in the HSCIE reaction.

Keywords: Tritium labelled; Proteins and peptides; Galactosidase; Spillover-hydrogen; Isotopic exchange; Ab initio calculation

1. Introduction

Hydrogen atoms bound on the surface atoms of platinum metals can migrate on inorganic carriers such as alumina and barium sulfate. Such activated atoms were named spillover hydrogen (SH)[1] [1]. Intense isotopic exchange of hydrogen for gaseous tritium takes place at an elevated temperature between the organic compound on a non-organic carrier and the SH. One notable feature of HSCIE is the fact that it proceeds with no racemization, and some amino acids substitute hydrogen for tritium almost completely [2,3]. There are no other experimental approaches for the synthesis of such highly tritium labeled amino acids and peptides. This reaction has been experimentally shown to proceed with participation of spillover hydrogen [3,4]. It was shown that spillover hydrogen can migrate as proton particles on graphite surfaces [5]. The regioselectivity and stereoselectivity of hydrogen substitution at the solid state reaction have now been studied using ^{3}H NMR spectroscopy [6,7]. The HSCIE reaction proceeds according to the new mechanism, studied by ourselves, of hydrogen substitution at the saturated carbon atom. The substitution proceeds according to the synchronous one-center mechanism with a formation of a transition state with the pentacoordinated carbon atom. The incoming and the outgoing hydrogen atoms form a three-center bond between themselves and carbon. This mechanism accounts well for the lack racemization in HSCIE. Quantum mechanics calculations of the activation energy (E_{act}) and the geometry of transition states were used for analysis the mechanism of this reaction in amino acids [8]. Highly tritium labeled proteins were produced by the use of HSCIE and their enzymatic activities were completely retained [3,9,10].

2. Experimental procedures

2.1. Purification of β-galactosidase from Thermoanaerobacter ethanolicus

Escherichia coli cells TG1, transformed by a recombinant plasmid, were cultivated overnight at 37 °C Luria–Bertani broth containing of 100 μg/ml ampicillin. The plasmid were obtained from Institute of Molecular Genetics, Russian Academy of

[1] *Abbreviations used:* HSCIE, the reaction of high temperature solid state catalytic isotopic exchange; SH, spillover hydrogen; HF, Hartree–Fock ab initio quantum-chemical calculation method.

Science (IMG RAN) collection. After cultivation the cells were centrifuged, washed, and re-suspended in 1/10 of the initial 50 mM phosphate buffer solution amount, pH 6.0, containing phenylmethylsulphonyl fluoride (2 mM). The suspension was placed in an ice bath, and 100 ml portions were subjected to ultrasound using an UZDI-IM 3 US radiation source for five 1–2-min-long periods. Lysates were heated at 70 °C for 40 min and centrifuged (12,000*g* for 30 min). Subsequently, saturated ammonium sulfate was added to supernatant with stirring in an ice bath to reach 30% saturation. The mixture was kept in the ice bath for 1 h, and then the precipitate was removed by centrifugation (30 min, 6000*g*). A saturated solution of ammonium sulfate was once again added to the supernatant to reach 60% saturation and kept in a cold atmosphere for 12–24 h. After centrifugation the precipitate was dissolved in a minimal amount of 50 mM phosphate buffer, pH 6.2. The protein preparation (3 ml) was applied on a column (20 × 9000 mm) with a Toyopearl HW-60 (Toyo-Soda MFG, Japan), equilibrated with 50 mM phosphate buffer (pH 7.0), and eluted with the same buffer at a rate of 44 ml/h. Fractions (2 ml) were collected and tested for the presence of β-galactosidase activity. Fractions containing β-galactosidase activity were combined and concentrated and applied on to a 20 × 160 mm column with a Q Sepharose column (Pharmacia) pre-equilibrated with 50 mM phosphate buffer, pH 7.0. Elution was carried out using a NaCl solution (0–1 M NaCl) linear gradient in the same buffer. Fractions (2 ml) were collected and analyzed for β-galactosidase activity. Fractions with activity were combined and concentrated.

2.2. Determination of β-galactosidase activity using chromogenic substrates

Reaction mixtures (2 ml) containing 2 mmol of substrate *p*-nitrophenyl-β-D-galactoside (pNPGal from Sigma), 50 mM potassium phosphate buffer, pH 6.0, 0.1 ml enzyme solution were incubated for 10 min at 70 °C. The reaction was stopped by addition of 1 ml of 1 M Na_2CO_3 solution. The amount of *p*-nitrophenol (pNP) was assessed spectrometrically at 400 nm and compared with a solution without the enzyme. The quantity of enzyme forming 1 μmol of pNP during 1 min is diluted as 1 U of β-galactosidase activity. Protein concentration was measured by the Lowry-folin method [11]. HSCIE of tritium for hydrogen in proteins and peptides was done as described in [10].

2.3. Analysis of tritium distribution in peptide fragments of β-galactosidase

A solution of 1 mg protein or [^{3}H]protein in 200 μl of 0.1 Tris–HCl (Sigma), pH 8.5, and 6 M guanidine chloride (Sigma), was placed in a 1.5 ml Eppendorf tube and 10 μl 2-mercaptoethanol was added. Nitrogen was passed through the tube. After an overnight incubation at 37 °C, 15 μl 4-vinylpyridine was added. Upon 15 min incubation at room temperature, non-reacted 4-vinylpyridine was neutralized by the addition of 10 μl 2-mercaptoethanol. The pyridylethylated protein was dialyzed against 25 mM potassium phosphate buffer, pH 8.0. Subsequently, 10 μl of 0.2 mg/ml subunits of glutamyl endopeptidase in 20 mM Tris–HCl buffer, pH 8.4, containing

0.1 M NaCl, was added to 500 μl of 25 mM potassium phosphate buffer, pH 8.0, containing 0.5 mg pyridylethylated protein. After a 4-h incubation period at 37 °C, the solution was frozen and lyophilized.

The mixture of peptides, formed after protein hydrolysis, was separated on a Phenomenex Primesphere C18 (250 × 3.2 mm, 5 μm) using a gradient of acetonitrile (2–40% in 80 min) in the presence of 0.1% trifluoroacetic acid at a flow rate of 0.3 ml/min. The fractions containing product were subjected once more to chromatography on the same column using a gradient of methanol (2–80% in 80 min) in the presence of 0.1% trifluoroacetic acid at a flow rate of 0.3 ml/min.

2.4. The ab initio calculation procedure

The Hartree–Fock method and second order Møller–Plesset perturbation theory with the 6-31G* basis sets have been performed for the calculation of isolated molecular systems, fragments of the potential surfaces, and the transition states of HSCIE. The geometry of the systems under consideration was optimized using analytical gradients without considering point group symmetry. We used the quasi-Newton method of synchronous transition for the search of the transition states. The calculations were performed using GAUSSIAN 98 (Gaussian, USA) and GAMESS programs on a CRAY J-90 supercomputer.

2.5. The accessibility C–H bonds for the interaction with H_2O

The accessibility of the protein C–H bonds for the interaction was estimated from the van der Waals surface of the corresponding hydrogen atoms available for H_2O molecules. This was determined using the MOLMOL program [12] for the spatial structure of the peptide.

3. Results and discussion

3.1. Experimental study of solid state isotopic exchange in proteins

In order to study the HSCIE reaction of hydrogen with spillover tritium in proteins, thermophilic β-galactosidase from *T. ethanolicus*, a homodimer with the 83 kDa subunit molecular mass, and mesophilic β-glucosidase from almond (Sigma), a heterodimer comprised of 117 and 66.5 kDa subunits, have been used. The dependence of both the radioactivity and the enzymatic activity of β-galactosidase and β-glucosidase under HSCIE conditions (Table 1) was determined. Table 1 shows that thermophilic β-galactosidase and mesophilic β-glucosidase are similar under HSCIE reaction conditions but under physiological conditions they differ in their thermal stability. The structural differences between these proteins affect the molar activity value of the labeled protein. Under the conditions more tritium is incorporated into β-galactosidase than β-glucosidase. For both, increasing the reaction time at

Table 1
The dependence of radioactivity and enzymatic activity on the HSCIE reaction temperature during production of tritium labeled proteins of thermophilic β-galactosidase from *T. ethanolicus* [11] and mesophilic β-glucosidase from almond (Sigma)

T	β-Glucosidase		β-Galactosidase	
	Radioactivity (Ci/mmol)	Enzymatic activity (%)	Radioactivity (Ci/mmol)	Enzymatic activity (%)
100	980	100	1090	100
120	1400	100	1440	100
140	1980	45	2050	59

temperatures of 100–120 °C leads to an increase in radioactivity with some decrease in enzymatic activity. It was also established that the rate of tritium incorporation into the protein does not depend on the $^3H_2$ pressure within the 5–45 kPa. The reaction was zero order with respect to $[H_2]$ under the studied conditions. This means that concentration of acidic catalytic centers on an inorganic support produced with SH did not depend on the tritium pressure.

In order to study the effect of the peptide fragment's spatial position in the β- galactosidase protein globule on its reactivity in HSCIE, a comparative study of tritium incorporation into the peptide hydrolysate of the protein and into native protein was conducted. Two series of peptides were obtained, which were separated by reverse-phase HPLC.

To purify the individual peptide fragments, the fractions collected using a methanol gradient were subjected once more to reverse-phase HPLC using an acetonitrile gradient and UV detection at two wavelengths. The fractions were analyzed by of MALDI mass spectrometry. The chromatography peaks were characterized by the retention time, peptide mass, and the extinction ratio coefficient for the two wavelengths. Then the relative activity of the corresponding peptide fragments was evaluated. To characterize the effect of the three-dimensional structure of the protein on its reactivity, the relative reactivity value obtained by normalization of the obtained values for the relative activity of the peptide was used.

The three-dimensional structure data studied β-galactosidase from *T. ethanolicus* were generated by multiple alignment of amino acid sequences of proteins with homologues. The structure of β-galactosidase cannot be solved by X-ray structural analysis. However, the three-dimensional structure of the *E. coli* β-galactosidase is available [13]. Therefore, a multiple sequence alignment from three galactosidases, the β-galactosidase from *T. ethanolicus*, β-galactosidase from *E. coli* with its known spatial structure, and the β-galactosidases from *Thermoanaerobacter thermosulfurigenes* EM1 [14], was performed in order to localize the studied β-galactosidase's portions found within the monomer and participating in dimer formation. A procedure to predict the protein's three-dimensional structure [15] was used. The sequence of the third β-galactosidase was used to enhance the validity of multiple alignment. The sequence identify between the studied β-galactosidase and the one from *T. thermosulfurigenes* EM1 is 42%. This indicates that the

three-dimensional structures of these two β-galactosidases have a similar architecture and belong to one and the same family of three-dimensional structures (the term "architecture" defines the number and the mutual location in space of second structure elements). Therefore, the presence of the β-galactosidase from *T. thermosulfurigenes* EM1 does not harm the multiple alignment results but helps to localize more reliably the inner regions and the contact-forming regions in the β-galactosidase's dimer on the basis of similar regions of the structurally known β-galactosidase from *E. coli*.

Localization of the inner and the outer regions of β-galactosidase from *E. coli* was executed using both the stereoscopic visualizer of protein molecules' spatial structures [14] and the MOLMOL program [12]. The results of the analysis are presented in Table 2 shows that the β-galactosidase fragment 215–220 located on the protein globule surface and not involved in secondary structure formation has the largest relative reactivity, whereas the inner region 338–347 in the protein globule has a reactivity three times lower (Fig. 1). It follows from the data presented for β-galactosidase fragments 392–398 and 501–509 that the participation of peptide fragments in the formation of α-helices and β-strands causes a reactivity decrease with respect to SH. Another marked reactivity difference is shown by fragments 417–422 located in the contact region of subunits. Its reactivity was over 150 times lower. The formation of intramolecular contact sharply reduces the possibility of isotopic exchange with SH. On the basis of our data, we infer that both the chemical nature of a peptide fragment and its position in the protein globule affect its ability to take part in interactions with tritium. The isotope label is preferentially incorporated in the peptide fragments situated on the protein globule's surface. To analyze the role of polypeptide α-helix formation on the reaction with SH, a theoretical study using ab initio quantum-mechanical calculations of hydrogen exchange was carried out.

Table 2
The effect of relative reactivity in the HSCIE of β-galactosidase peptide fragments on the attainable surface value in a subunit and their participation in the secondary structure of the protein (where α refers to α-helix, β refers to β-strand, A refers to the contact region of subunits)

Peptide	MW (Da)	Region in protein	Relative reactivity in the HSCIE	The attainable surface value in a subunit (%)
K E M Q K E	791.93	215–220	151.5	21.4
E I G M L V F E E I α α β β β β β	1179.40	338–347	43.3	13.7
D H D F Y K E α α α α α α α	952.97	392–398	50.5	18.2
Y L R D S E A	781.80	417–422	1	15.1
Y N T T S A F G S α α α α	946.96	501–509	60.6	13.8

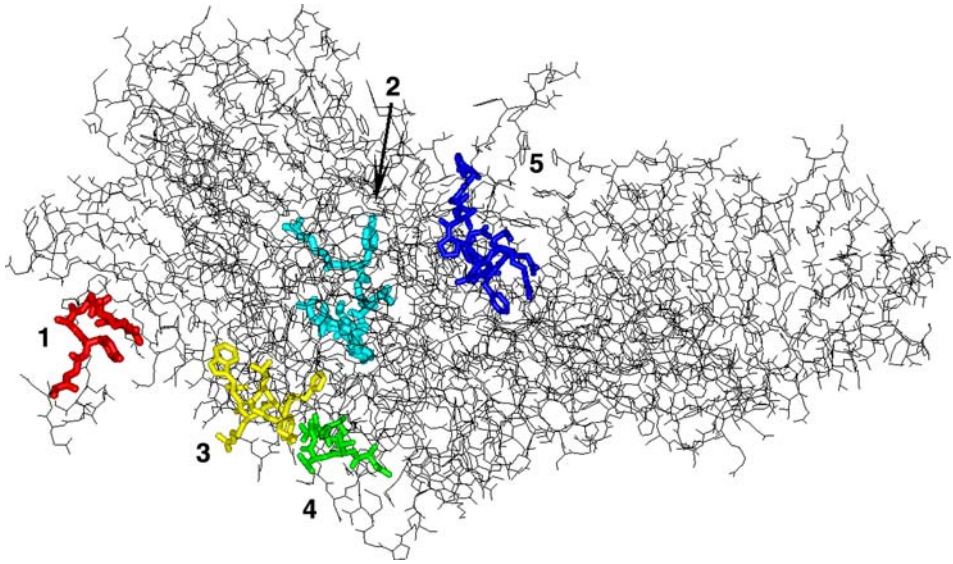

Fig. 1. The spatial structure of β-galactosidase fragments from Table 2 located on the protein globule surface of β-galactosidase from *E. coli* at high resolution by MOLMOL program. The numbers 1–5 refer to Table 2.

3.2. Theoretical studies of solid state isotopic exchange in polypeptides

Within the frame of these experiments, a theoretical study of the effect of α-helical fragment formation in peptides on the reactivity of their component amino acids was accomplished. Table 3 shows the results of an estimation of the activation energy for hydrogen exchange in free amino acids and for the amino acids which are the part of the β-galactosidase protein's α-helix regions. Fig. 2 presents the spatial structure of

Table 3
The calculation of the transition states of HSCIE

Amino acid	The activation energy (kcal/mol)	
	HF/6-31G*	MP2/6-31G*
α-Helical Ser, α	49.2	–
α-Helical Ser, β	37.0	–
Free Ser, α	31.8	16.9
Free Ser, β	37.7	24.9
α-Helical Ala, α	52.6	–
α-Helical Ala, β	35.6	21.4
Free Ala, α	27.0	20.0
Free Ala, β	22.8	16.6

The activation energy of hydrogen isotopic exchange between amino acids and model acidic centre H_3O^+, calculated by the Hartree–Fock method in the 6-31G* basis (HF/6-31G*) and second-order Møller–Plesset perturbation theory in conjunction with 6-31G* (MP/6-31G*).

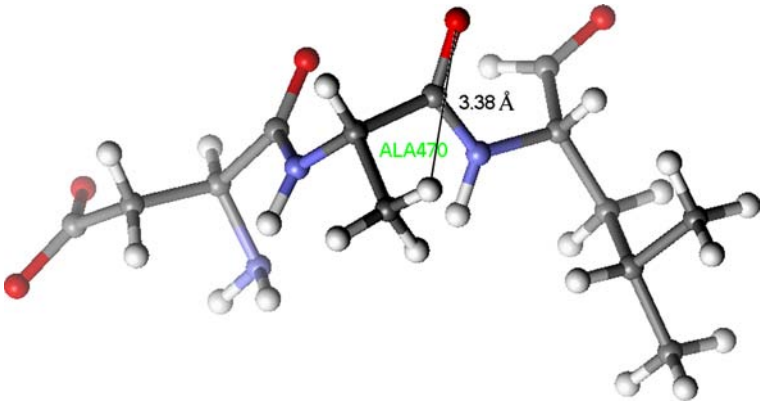

Fig. 2. The spatial structure of Ala470 from α-helix fragment of β-galactosidase at high resolution by MOLMOL program.

the α-helical fragment of β-galactosidase containing Ala470. The distance between carbonyl O and $C^{\beta}H$ was 3.38 Å. For comparison, in free alanine the distance between the carbonyl O atom and the $C^{\beta}H$ exchanging atoms in the transition state is only 2.16 Å (MP2/6-31G*). The carbonyl group of Ala470 was unable to contribute to isotopic exchange. It was shown using Ala and Ser as examples, that the participation of amino acids in the formation of α-helix portions results in a considerable decrease of their reactivity in solid state hydrogen exchange with spillover hydrogen (Table 3). These data help one to understand the reason of the tritium distribution in [^{3}H]zervamycin IIB previously obtained with HSCIE [10].

An interesting feature of this peptide is that it contains the Trp1–Leu8 and Aib9–Phe16 fragments having the α-helical and helical β-ribbon conformations, respectively (Fig. 3). Fig. 3 and Table 4 show the stereochemistry and degree of hydrogen substitution for tritium in [^{3}H]zervamycin IIB. Most of the amino acids forming the Trp1–Leu8 fragments with α-helical conformations show a low reactivity. The α-helical fragment Trp1–Leu8 has a relatively rigid conformation [16]. So that the geometry of the heavy atoms in the Ile2–Gln3–Iva4 fragment was fixed according to its conformation in order to calculate the transition state of HSCIE. The activation energy calculated for hydrogen exchange at the Gln3 $C^{\beta}H$ was 26.2 kcal/mol (MP2/6-31G*), whereas the E_{act} for $C^{\alpha}H$ and $C^{\gamma}H$ was 30.9 and 35.3 kcal/mol (MP2/6-31G*), respectively. The spatial structure of more stable transition states of the hydrogen exchange reaction between Gln3 and SH is shown in Fig. 4. It follows from the quantum-chemical calculation results for the hydrogen exchange transition state that the involvement of the electron donor O and N atoms in interaction with Gln3 $C^{\beta}H$ leads to a significant reactivity increase of this position. It was found previously that the unfavorable conformation of Ala6 and the absence of interaction between exchanged hydrogen atoms and electron donor atoms in a transition state of HSCIE reaction in conotoxin G1 tend to decrease the probability of this reaction to occur in Ala6 $C^{\beta}H$ [17].

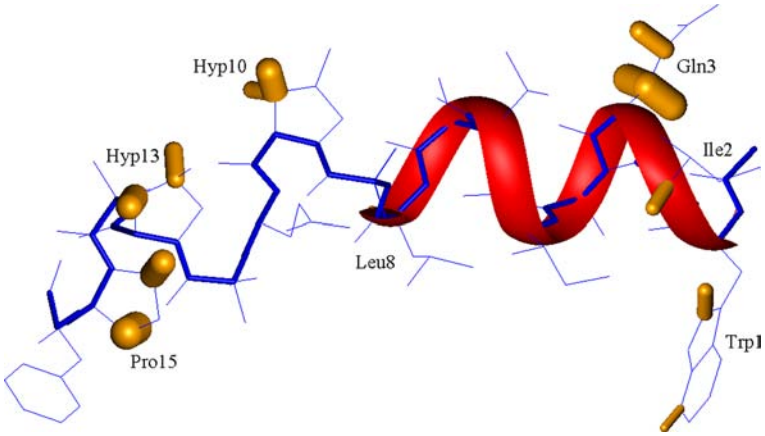

Fig. 3. The spatial structure of [^{3}H]zervamicin IIB and tritium distribution at high resolution by MOLMOL program. The value of width of C–H bond is proportional log of tritium contents which is reported in Table 4.

Table 4
Tritium distribution in [^{3}H]zervamicin IIB produced by HSCIE according to ^{3}H NMR spectral data

Amino acid residue	Label position in the residue	δ (ppm)	Degree of substitution C–H in the position (%)
Trp1	C7H	7.55	1.1
	C2H	7.42	3.6
Ile2	C$^{\alpha}$H	3.85	3.4
Gln3	C$^{\beta}$H2	2.40	45.8
	C$^{\beta}$H3	2.15	40.4
	C$^{\gamma}$H2	3.50	7.7
Leu8	C$^{\alpha}$H	4.42	0.9
Hyp10	C$^{\beta}$H2	2.12	4.6
	C$^{\beta}$H3	2.55	21.3
Hyp13	C$^{\beta}$H3	2.54	22.3
	C$^{\gamma}$H	4.18	6.1
Pro15	C$^{\beta}$H3	2.22	61.0
	C$^{\delta}$H2	4.02	20.6

^{1}H and ^{3}H NMR spectra were recorded in CD_3OD at 30 °C on a Varian UNITY-600 spectrometer (United States) at working frequencies of 600 and 640 MHz for protons and tritium, respectively.

4. Conclusions

1. The effect of the peptide fragment's spatial position in the β-gal protein globule on its reactivity in HSCIE reaction with spillover hydrogen was analyzed. The isotopic label is preferentially incorporated in the peptide fragments situated on the protein globule's surface.

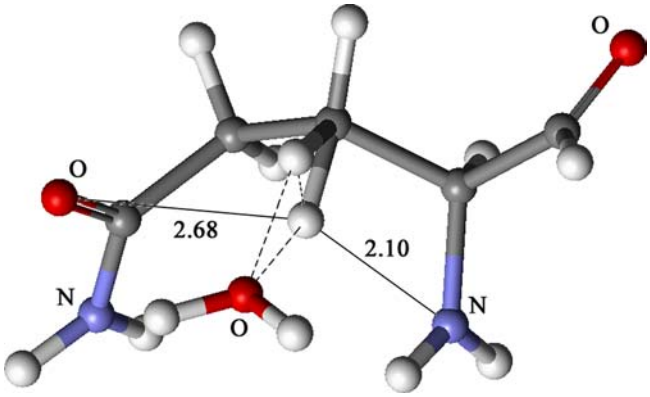

Fig. 4. The spatial structure of transition states of the hydrogen exchange reaction between Gln3 $C^{\beta}H$ in Zrv IIB and H_3O^+ at high resolution by MOLMOL program. Distance between amid N and $C^{\beta}H$ in the transition state was equal 2.1 Å.

2. It was shown that the formation of intramolecular contact of protein globules sharply reduces the possibility of isotopic exchange with SH, which can be used for the definition of sites of protein globule participating in the interaction with others macromolecules.
3. The influence of the formation of α-helical peptide structures on the hydrogen isotopic exchange reaction with spillover hydrogen was studied using ab initio calculations. The previously studied mechanism in polypeptides correlates with experimental data.
4. It was shown that thermophilic and mesophilic proteins in the HSCIE reaction have comparable high stability at elevated temperatures.

Acknowledgments

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