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New development in the tritium labelling of peptides and proteins using solid catalytic isotopic exchange with spillover-tritium

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Summary. The mechanism of the reaction of high temperature solid state catalytic isotope exchange (*HSCIE*) of hydrogen in peptides with spillover-tritium at 140–180°C was analyzed. This reaction was used for preparing [^{3}H]enkephalins such as [^{3}H]DALG with specific activity of 138 Ci/mmol and [^{3}H]LENK with specific activity of 120 Ci/mmol at 180°C. The analogues of [^{3}H]ACTG$_{4-10}$ with specific activity of 80 Ci/mmol, [^{3}H]zervamicin IIB with specific activity of 70 Ci/mmol and [^{3}H]conotoxin G1 with specific activity 35 Ci/mmol were produced. The obtained preparations completely retained their biological activity. [^{3}H]Peptide analysis using ^{3}H NMR spectroscopy on a Varian UNITY-600 spectrometer at 640 MHz was carried out. The reaction ability of amino fragments in *HSCIE* was shown to depend both of their structures and on the availability and the mobility of the peptide chain. The reaction of *HSCIE* with the β-galactosidase from *Termoanaerobacter ethanolicus* was studied. The selected *HSCIE* conditions allow to prepare [^{3}H] β-galactosidase with specific activity of 1440 Ci/mmol and completely retained its the enzymatic activity.

Keywords: Tritium labelled – Amino acids – Peptides – Enkephalins – Spillover-hydrogen – Isotope exchange – Tritium NMR

Abbreviations: *HSCIE*, the reaction of high temperature solid state catalytic isotope exchange; *HS*, hydrogen spillover; ^{3}H NMR, tritium nuclear magnetic spectroscopy; CtxG1, conotoxin G1; AchR, acetylcholine receptor; HF, Hartree-Fock *ab initio* quantum-chemical calculation method

Introduction

Tritium labelled amino acids and peptides are mainly obtained by catalytic hydrogenation of unsaturated precursors or hydrogenolysis of precursors in solution with gaseous tritium. In the case of peptides, this method is primarily used for obtaining preparations with labelled Tyr residues through the dehalogenation of the corresponding diiodotyrosine derivatives (Zolotarev et al., 1982; Darula, 1998). Hydrogen atoms bound to the surface; metal atoms of applied catalysts can migrate on to the carrier. This effect is known as the hydrogen spillover (*HS*) (Sermon and Bond, 1973). We had suggested the reaction of high temperature solid state catalytic isotope exchange (*HSCIE*) (Zolotarev et al., 1992) between the hydrogen atoms of a solid organic compound and tritium spillover. This reaction proceeds with retaining the chirality and permits the preparation of peptides and proteins with a high tritium label (Zolotarev et al., 1995). *Ab initio* calculations were used to estimate the activation energies and structures of the transition states of this reaction (Zolotarev et al., 1999). The hydrogen exchange reaction at saturated carbon atoms occurs by a synchronous one-centre mechanism, with a transition state that is characterised by pentacoordinated carbon.

Material and methods

HSCIE of tritium for hydrogen in peptides

Aluminum oxide (100 mg) (Serva) was mixed with 5.0 mg of peptide in aqueous solution, and water was removed in a vacuum at 20°C. Aluminum oxide with the applied peptide was mixed with 10 mg of catalyst (5% Rh/Al_2O_3, Fluka). A part of the resulting solid mixture containing 0.5 mg of the peptide was placed in a 10-ml ampoule. The ampoule was evacuated and filled with gaseous tritium at 250 Torr. The reaction was carried out at 140–190°C for 40 min. The ampoule was cooled, evacuated, and blown through with hydrogen. The

peptide was desorbed with 20% aqueous ethanol and freed from the labile tritium by the two-fold dissolution in 20% aqueous ethanol and evaporation. The resulting the [^{3}H]peptide was successively purified on a Kromasil C_{18} (10 × 150-mm, Elsiko, Russia) and a Nucleosil C_{18} (4 × 250-mm, Macherey-Nagel, Germany) column. The specific radioactivity of the resulting peptide was determined using the liquid scintillation counting.

HSCIE of tritium for hydrogen in proteins

Solution of 1 mg of protein in 1 ml of 0.05 M K_2HPO_4 was added by 50 mg $CaCO_3$ and frozen in liquid nitrogen. Water was removed by lyophilising drying in vacuum. Solid reaction mixture was added to 20 mg of catalyst (5% palladium-on-barium sulphate) and was placed into a 10-ml glass vial. The vial was vacuumed, and then gaseous tritium was introduced to a pressure of 300 Torr. The vial was heated to a temperature of 60–160°C and was kept at this temperature during 10–60 min. Then the vial was cooled and vacuumed, the solid mixture was suspended in 2 ml of water, and the catalyst was removed by centrifugation. Chromatographic purification was conducted on Protein Column I-125 with eluent containing 0.05 M K_2HPO_4, pH 6.1; the eluent rate was 0.5 ml per minute, the wavelength of the flow spectrophotometer was 280 nm.

Membrane fraction from rat brain used in RRA

Cerebral cortex and midbrain preparations were isolated in the cold immediately after decapitation of animals and were immediately homogenized in 50 mM Tris-HCl buffer solution (pH 7.7) cooled to 4°C (the mass/volume ratio was 1/20) in a Potter-type (glass/Teflon) homogenizer. The homogenate was centrifuged (30,000 g, 30 min, 4°C). The precipitate was resuspended in the same volume of 50 mM Tris-HCl buffer solution (pH 7.7, 37°C) and incubated for 40 min at 37°C for proteolytic cleavage of endogenous opioids. The suspension was centrifuged under the same conditions and the supernatant was removed. The precipitate of membranes was frozen and kept at −20°C before the use in RRA.

Radioreceptor assay

We used flat-bottom plates (Linbro, UK) for the micro variant of RRA, as the control tests showed that they sorb no noticeable amounts of labelled ligands used in RRA. All the solutions were prepared using 50 mM Tris-HCl buffer (pH 7.7, 20°C). The incubation mixture (300 μl) contained the membrane preparations from rat brain (1.0–1.5 mg/ml of protein); 50 μg/ml bacitracin, a protease inhibitor (Sigma, United States); and OR ligands (0.5–3.0 nM): [^{3}H]peptide labelled with tritium by the procedure described above. The value of specific binding was determined from the difference in the binding of the labelled ligand with and without an excess unlabelled ligand. The samples were incubated for 40 min at 20°C under continuous stirring. The receptor-bound label was separated from the free label by applying the content of the plates with a Skatron (Norway) cell harvester on the fiberglass GF/B filters (Whatman, UK) presoaked in 0.25% polyethylenimine. The wells were washed with 4 ml of cooled 50 mM Tris-HCl buffer (pH 7.7, 4°C) for 10 s. The filters were air-dried, placed in 5 ml of ZhS-8 (Reakhim) scintillator, and kept overnight. The radioactivity level was determined using a MiniBeta (LKB-Wallac, Finland) liquid scintillation counter.

NMR spectroscopy

^{3}H NMR determined tritium distribution in peptides. [^{3}H]peptide (5–10 mCi) was dissolved in D_2O (600 μl, 100% deuterium, SIL, UK) at pH 7.5. ^{1}H and ^{3}H NMR spectra were recorded on a Varian UNITY-600 spectrometer (United States) at working frequencies of 600 and 640 MHz for protons and tritium, respectively. Before starting a pulse sequence, a 3-s delay was made for the relaxation of nuclei to the starting equilibrium state. The spectra were interactively treated and analyzed by the VNMR (Varian, United States) program. The resonances in ^{3}H NMR spectrum of [^{3}H]peptide were assigned using the corresponding ^{1}H NMR spectrum recorded under the same conditions.

The ab initio calculation procedure

The Hartree–Fock method in the 6-31G* basis was used 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 transit for the search of the transition states. The calculations were performed using GAUSSIAN-94 (Gaussian Inc., United States) and GAMESS programs on a CRAY J-90 supercomputer.

The accessibility C-H bonds for the interaction with H_2O

The accessibility of the CtxG1 C-H bonds for the interaction was estimated from the share of the van der Waals surface of the corresponding hydrogen atoms available for H_2O molecules; this was determined using MOLMOL program (Koradi et al., 1996) for the spatial structure of the peptide.

Results and discussion

The reaction of high temperature solid state catalytic isotope exchange (*HSCIE*) of hydrogen in peptides and proteins with spillover-tritium was studied. Dalargin (DALG, Tyr-*D*-Ala-Gly-Phe-Leu-Arg), a synthetic analogue of endogenous opioid peptide [Leu^5]enkephalin (LENK, Tyr-Gly-Gly-Phe-Leu), is a medicine used in gastroenterology. It was also shown to cause immunomodulating, anxiolytic, and a number of other biological effects (Zozulia et al., 1996, 1999). [^{3}H]DALG and [^{3}H]LENK with a specific radioactivity of 138 and 120 Ci/mmol, respectively, were obtained by the *HSCIE* reaction of tritium for hydrogen at 180°C. These tritium-labelled peptides were shown to completely retain their biological activity in the test of binding to opioid receptors from rat brain. The dissociation constants of the Dalargin-opioid receptor complex are 4.3 nM. Tritium distribution in [^{3}H]DALG and [^{3}H]LENK was analysed with ^{3}H NMR (Table 1).

The *HSCIE* of hydrogen for tritium in these peptides takes place virtually in all the amino acid residues, with tritium label distribution appearing to be similar in many ways. The share of tritium included in Tyr1 residues appears comparable in DALG and LENK and makes about 30%. It can be mentioned

Table 1. Tritium distribution in [^{3}H]enkephalins labelled with *HSCIE* with $^3H_2$ at 180°C. (DALG-Tyr-*D*-Ala-Gly-Phe-Leu-Arg, 138 Ci/mmol; LENK-Tyr-Gly-Gly-Phe-Leu, 120 Ci/mmol; ^{3}H NMR spectra in D_2O pH 7.0 at 30°C on a Varian UNITY-600 spectrometer at 640 MHz)

Amino acid	Part of tritium, %		Position	DALG		LENK	
	DALG	LENK		ppm	%	ppm	%
Tyr1	29.21	28.00	αY	4.15	4.63	4.20	0.53
			β,βY	3.09	1.35	3.08	1.81
			2,6Y	7.17	1.53	7.20	2.52
			3,5Y	6.87	19.70	6.91	23.14
Gly2	–	30.54	α,αG2			3.85	30.54
Gly3	30.57	31.78	α,αG3	3.80	30.57	3.78	31.78
Phe4	21.07	9.05	αF	4.60	15.33	4.60	1.52
			β,βF	3.08;2.95	2.00	3.12;2.96	2.92
			2,6F	7.29	0.99	7.29	1.28
			3,5F	7.40	1.99	7.40	2.38
			4F	7.35	0.76	7.35	0.95
Leu5	6.34	0.61	αL	4.30	6.34	4.19	0.61
Arg6	16.15	–	αR	4.29	14.80		
			β,βR	3.10	1.35		

that the *HSCIE* reaction calls forth a similar manner of isotope label distribution in Tyr1 and in free amino acid (Zolotarev et al., 1997). The isotope exchange in Tyr1 proceeds in all its C-H bonds, although it is much different in the molecule's aliphatic and aromatic portions. The substitution mostly takes place in the aromatic portion of the molecule, with the hydrogen exchange at the orto-position to the OH-group proceeding an order of magnitude rapider than at positions 2 and 6. For the peptides under study a similar reactivity of at C$^\beta$H of Tyr1 and considerable distinctions in the exchange ability of C$^\alpha$H of Tyr1 are observed. It follows from the data of Table 1 that C$^\alpha$H of Tyr1 of DALG exchanges for tritium in *HSCIE* twice as rapidly as in LENK. A similar considerable growth of C$^\alpha$H reactivity in DALG as compared with LENK takes place in Phe4 and Leu5. Since all these peptides' structures are close, the probable cause of such a sharp change of CαH reactivity might be an additional charge available on the Arg6-containing DALG molecule.

The share of tritium incorporated at C$^\beta$H and in the Phe4 aromatic part is close in the peptides studied, although a two-fold difference in the overall amount of tritium in this amino acid is observed. This difference can be associated with the varying reactivity of CαH. In the Phe4 residue of the DALG peptide, the larger portion of the label is incorporated at this position, and almost 70% substitution of ^{1}H for ^{3}H is observed. The substitution degree of this position in LENK is only about 6%.

Gly3 residues in DALG and LENK show high reactivity. In the selected *HSCIE* conditions, the degree of hydrogen-tritium substitution was 65 to 70% in these residues. Isotope exchange intensity in LENK's Gly2 was found to be approximately the same as that in Gly3. In this connection, it seems of interest to disclose the causes of the absence of the isotope exchange reaction in D-Ala2 of DALG. The isotope exchange in the free amino acid Ala takes place mostly in the methyl group (Zolotarev et al., 1997). Possibly, during the sorption process on a non-organic carrier, the D-Ala2 methyl group appears inaccessible to the carrier's catalytic acid centres in the *HSCIE* reaction.

Isotope exchange in Leu5 of DALG and LENK proceeds selectively at C$^\alpha$H, with the substitution in the charge-carrying DALG being of a higher degree than in LENK. In the Arg6 residue of the DALG peptide, over 90% of the label incorporates at CαH. The degree of hydrogen substitution for tritium at this position of Leu5 makes about 70%.

To optimize the therapeutic effect, to study pharmacokinetics and interaction with the target cells was synthesized [^{3}H]Met-Glu-His-Phe-Pro-Gly-Pro with specific activity of 80 Ci/mmol. This newly produced medicinal preparation exerts selective effects on the central nervous system and has no analogues in the world parmacoipea. Some properties of this

Table 2. Chemical shifts of resonance in ^{3}H NMR spectrum of [^{3}H]Met-Glu-His-Phe-Pro-Gly-Pro, their relative integral intensity, and assignment. ^{1}H and ^{3}H NMR spectra were recorded in D_2O pH 7.5 at 30°C on a Varian UNITY-600 spectrometer (United States) at working frequencies of 600 and 640 MHz for protons and tritium, respectively

Amino acid residue	Part of tritium label in residue, %	Label position in the residue	δ, ppm	Part of tritium label in the position, %
Met1	2.37	α	3.6	2.37
Glu2	5.62	α	4.1	1.42
		β	2.2	2.50
		γ	2.3	0.5
His3	54.99	α	2.5	2.50
		β	2.9	0.2
		4	6.9	18.39
		2	7.65	33.90
Phe4	1.38	α	4.9	0.18
		3,5	7.65	1.2
Gly6	27.80	α	4.05	27.80
Pro7	7.02	α	4.4	0.43
		β	2.1	2.00
		δ	3.65	4.59

synthetic peptide named SEMAX allow this preparation to be referred to nootropic medicine. This synthetic peptide is the analog of $ACTG_{4–10}$ completely devoid of any hormonal activity. It was assumed that the mechanism of biological activity of SEMAX is related to inhibitory activity of enkephalin-degrading enzymes (Kost et al., 2001). It was shown that Met-Glu-His-Phe-Pro-Gly-Pro specifically bind with rat brain membranes. Using this labelled peptide the kinetics of its hydrolysis in cells and the kinetics of the hydrolysis of all its fragments were analysed.

The *HSCIE* reaction gives possibility to synthesise [^{3}H]peptide containing tritium in most amino acid fragments. Peptide analysis using ^{3}H NMR spectroscopy was carried out (Table 2). The resonances in 3H NMR spectrum of [^{3}H]peptides were assigned using the corresponding ^{1}H NMR spectrum recorded under the same conditions.

Over 50% of the label in this peptide are incorporated in His3, and the hydrogen is substituted by a larger part of the tritium in the molecule's imidasol fraction. The substitution takes place both at position 2 and at position 4. The Gly6 amino acid shows an equally high reactivity as the one observed in DALG and LENK. Tritium incorporation in other amino acid residues is much smaller. Substitution at $C^{\alpha}H$ is observed in Met1, predominately at $C^{\alpha}H$ and $C^{\beta}H$ in Glu2, and at $C^{\delta}H$ in Pro7. The observed incorporation selectivity in these amino acid peptide residues corresponds to the isotope label distribution during *HSCIE* in free amino acids (Zolotarev et al., 1997). The share of ^{3}H incorporated in Phe4 is considerably less than the one observed for Phe4 in DALG and LENK. This considerable decrease of reactivity may reflect the effect of Phe accessibility to the *HSCIE* after peptide sorption on a non-organic carrier.

[^{3}H]zervamicin IIB (Ac-Trp-Ile-Gln-Iva-Ile-Thr-Aib-Leu-Aib-Leu-Hyp-Gln-Aib-Hyp-Aib-Pro-Phl, where Aib = 2-amino-isobutyric acid) with specific activity of 70 Ci/mmol was produced. The obtained preparations completely retained their biological activity. Tritium distribution in [^{3}H]zervamicin IIB is shown in Table 3.

The Gln3, Hyp10, Hyp13 and Pro 15 residues seemed to be the most reactive in this peptide. Over 95% of the total isotope label are incorporated in these amino acids. Hydrogen atoms at the β-position of Gln3 appear to be almost in equal degree involved in the isotope exchange reaction. Stereo chemistry of chemical shifts of resonance of β-γ-δ-methylen protons were assigned in article (Balashova, 2000) and were shown in order 2, 3. For $C^{\beta 2}H$ and $C^{\beta 3}H$ of Gln3, the degree of hydrogen substitution for tritium makes 40 to 45%. Stereo selective substitution at the β- and δ-positions is observed for the heterocyclic molecules of Hyp10, Hyp13 and Pro15. For Hyp10, the $C^{\beta 3}H$-position is the most reactive one, incorporating five times as much tritium as at $C^{\beta 2}H$. In Hyp13 and Pro15, hydrogen substitution mostly occurs at $C^{\beta 3}H$, with

Table 3. Chemical shifts of resonance in ^{3}H NMR spectrum of [^{3}H]zervamicin IIB, their relative integral intensity, and assignment. ^{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

Amino acid residue	Part of tritium label in residue, %	Label position in the residue	δ, ppm	Part of tritium label in the position, %
Trp1	1.96	z2	7.55	0.45
		δ	7.42	1.51
Ile2	1.42	α	3.85	1.42
Gln3	38.78	$\beta 2$	2.40	18.94
		$\beta 3$	2.15	16.76
		$\gamma 2$	3.50	3.18
Leu8	0.36	α	4.42	0.36
Hyp10	10.73	$\beta 2$	2.12	1.91
		$\beta 3$	2.55	8.82
Hyp13	11.74	$\beta 3$	2.54	9.23
		γ	4.18	2.51
Pro15	33.81	$\beta 3$	2.22	25.28
		$\delta 2$	4.02	8.53

$C^{\beta 2}H$ not at all involved. It is worth mentioning that the maximal substitution of the C-H bond, 60%, in this peptide is observed at Pro15 $C^{\beta 3}H$. The manner of isotope label distribution in the zervamicin residue Pro15 is essentially different from the distribution in the free amino acid and in the peptide SEMAX. This change in the hydrogen exchange ability may be due to an additional interaction taking place during peptide sorption on the solid phase surface. The spatial distribution of tritium label in [^{3}H]zervamicin was analysed with high resolution by MOLMOL program (Koradi, 1996). The most of tritium-carbon bonds were situated on the same side of peptide molecule. This data may be connected with interaction between adsorbed peptide and active catalytic centre of inorganic support.

To analyse the dependence the reaction ability of amino fragments on their structure, the availability and the mobility of the polypeptide chain the *HSCIE* reaction of [^{3}H]peptide conotoxin G1 (CtxG1) was studied. Conotoxins are effective acetylcholine receptor (*AChR*) blockers. The spatial structures at high resolution are reported for CtxG1 (Maslennikov, 1998; Guttat, 1996).

We obtained [^{3}H]CtxG1 with specific activity 35 Ci/mmol from the synthetic CtxG1 by the *HSCIE* reaction with gaseous tritium at 140°C. This level of tritium incorporation is quite sufficient for the use of the resulting preparation in the study of its biological target, the nicotinic *AChR* of muscle type. [^{3}H]CtxG1 retained its biological activity, which was confirmed by

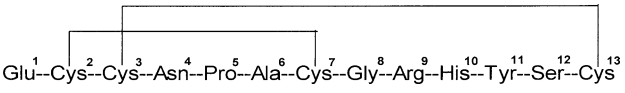

Fig. 1. α-Conotoxin GI

the data on its binding to the receptor. The curves of the specific binding of [^{3}H]CtxG1 and [^{125}I]CtxG1 to *AChR* membrane preparations from the electric organ of *Torpedo californica* were analyzed. The labelled preparations are similarly bound to the receptor and exhibit close K_d values (about 66 nM for [^{3}H]CtxG1 and 59 nM for [^{125}I]CtxG1). The substitution of tritium for hydrogen in the biologically active compound is more advantageous than the introduction of iodine, as the physicochemical properties of the [^{3}H]compound remain practically unchanged.

The solid state isotope exchange with *HS* is known to occur with the comparable rates for all free amino acids (Zolotarev et al., 1997). Therefore, we expected that tritium would incorporate in all CtxG1 residues. However, one can see from the Table 4 and Fig. 2 that tritium substituted for hydrogen only in 10 of 13 amino acid residues of this peptide. No isotope exchange was observed in Asn4-Pro5-Ala6 site. A decreased reactivity of these residues is likely to be due to special features of the CtxG1 spatial structure. The distribution of isotope label in a peptide chain is probably determined by the reactivity of C-H bonds in particular amino acid residues and by the accessibility of these bonds to *HS*. The accessibility of the CtxG1

Table 4. Chemical shifts of resonance in ^{3}H NMR spectrum of [^{3}H]CtxG1, their relative integral intensity, and assignment. ^{1}H and ^{3}H NMR spectra were recorded in D_2O pH 7.5 at 30°C on a Varian UNITY-600 spectrometer (United States) at working frequencies of 600 and 640 MHz for protons and tritium, respectively

Amino acid residue	Part of tritium label in residue, %	Label position in the residue	δ, ppm	Part of tritium label in the position, %
Glu1	3.33	$C^{\gamma}H_2$	2.18	3.33
Cys2	6.80	$C^{\alpha}H$	4.60	6.80
Cys3	8.93	$C^{\alpha}H$	4.65	8.93
Asn4	0			
Pro5	0			
Ala6	0			
Cys7	7.80	$C^{\alpha}H$	4.48	7.80
Gly8	2.61	$C^{\alpha}H$	3.78, 4.03	2.61
Arg9	1.64	$C^{\gamma}H_2$	1.25	1.64
His10	50.56	C^2H	7.70	46.57
		$C^{\alpha}H$	4.73	3.99
Tyr11	4.08	C^3H, C^5H	6.77	1.36
		$C^{\beta}H_2$	2.88	2.72
Ser12	3.55	$C^{\alpha}H$	4.34	3.55
Cys13	8.92	$C^{\alpha}H$	4.63	8.92

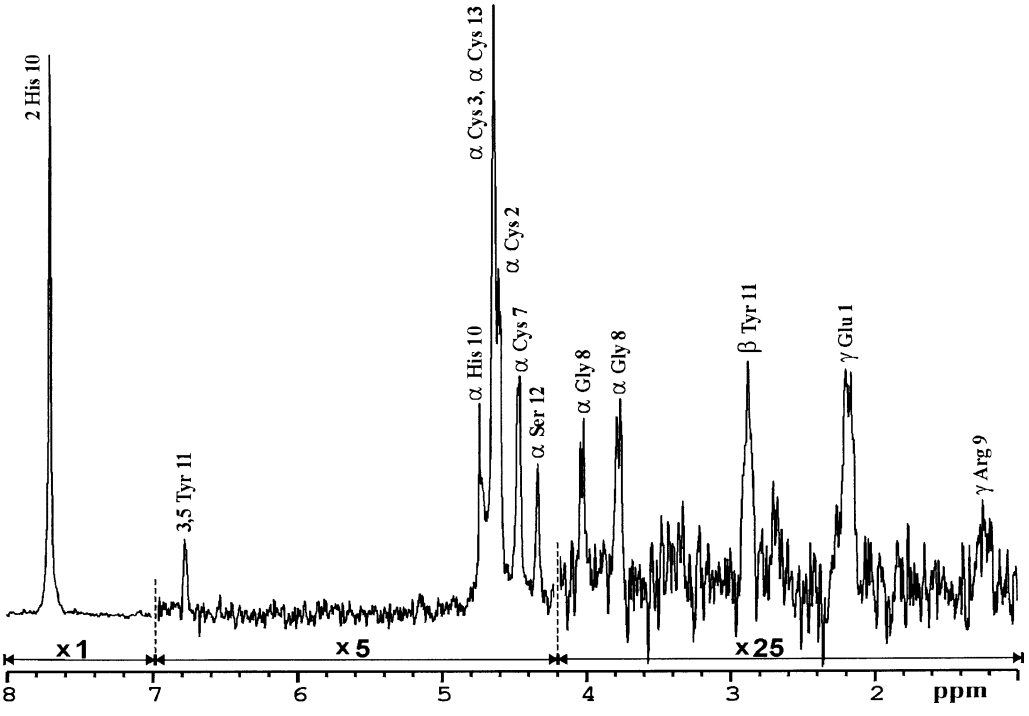

Fig. 2. Parts of ^{3}H NMR spectrum (640 MHz) of [^{3}H]CtxG1 (D_2O, pH 7.5, 30°C). The spectrum intensity increased 5 times and 25 times at 7.0–4.2 and 4.2–1.0 ppm, respectively. The resonance assignments are shown (see also the Table 4)

C-H bonds for the interaction with the H_3O^+ acidic centers of catalyst was estimated from the share of the van der Waals surface of the corresponding hydrogen atoms available for H_2O molecules. This was determined using MOLMOL program (Koradi et al., 1996). To analyze the reactivity of C-H bonds in CtxG1 the *ab initio* quantum-chemical calculation of transition state of *HSCIE* reaction in fragments of CtxG1 was done by the Hartree-Fock (HF) method.

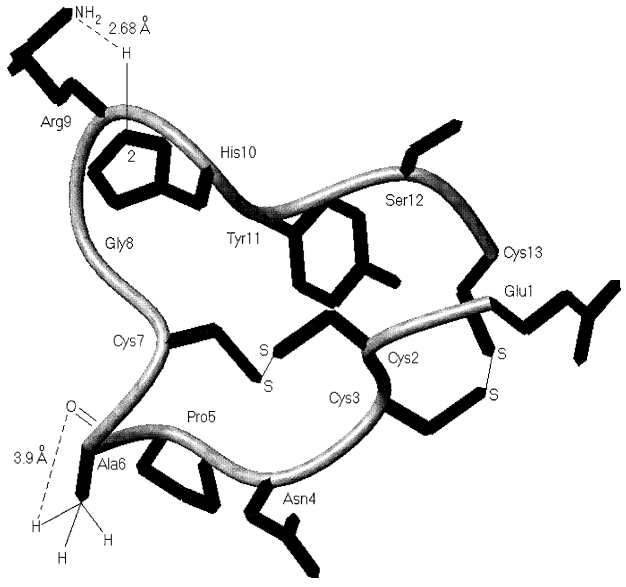

Fig. 3. The spatial structure of CtxG1 at high resolution by MOLMOL program

The $C^{\delta}H$ atoms of Pro5 in CtxG1 are shielded and this appears to explain the absence of tritium in this residue. The low reactivity is also observed in the case of Asn4 whose $C^{\alpha}H$ and $C^{\beta}H$ are also poorly exposed. The methyl group of Ala6 is completely accessible. Nevertheless, tritium is not incorporated into this moiety (see Table 4). This means that the accessibility of one or another peptide moiety is the necessary but not sufficient condition for *HSCIE*.

According to ^{1}H NMR, the Asn4-Cys7 fragment has a rigid conformation, which differs from that of the remainder of the CtxG1 molecule (Maslennikov et al., 1998). This permitted us to fix the geometry of heavy atoms of the main chain of the model fragment CH_3-C(O)-NH-$C^{\alpha}H(CH_3)$-C(O)-NH_2 according to the conformation of the Pro5-Ala6-Cys7 fragment of CtxG1 Fig. 3. When computing the geometric parameters of the transition state of the model fragment, the geometry of the remainder of the reaction complex was completely optimized. The activation energy (E_{act}) calculated for the reaction of hydrogen exchange at the Ala C^{β} atom was 33.7 kcal/mol (HF/6-31G*). For comparison, the calculated E_{act} of the isotope exchange of $C^{\beta}H_3$ in free alanine are 16.6 kcal/mol (HF/6-31G*) (Borisov and Zolotarev, 1999). The decreased E_{act} for $C^{\beta}H$ exchange in free alanine is due to the involvement of carbonyl oxygen in the transition state; in this case, the distance between the carbonyl O atom and the H^{β} exchanging atoms is only 2.16 Å. In the case of CtxG1, the high E_{act} of the hydrogen exchange in the Ala6 methyl group is connected with the rigid conformation of this site of the peptide that hinders the interaction of carbonyl O with H^{β} in the transition state (the distance between O and H^{β} is 3.9 Å). Thus, using CtxG1 as an example, we were the first to show that limited mobility of a peptide fragment can cause a significant decrease in the rate of isotope exchange.

The 57% degree of substitution of tritium for hydrogen at C2 of His10 in [^{3}H]CtxG1 was determined from ^{3}H NMR. It is evident from Table 4 that the His10 imidazole ring turns out to be the most reactive in the *HSCIE* reaction of CtxG1. The reason for this is probably the participation of some electron donor atoms in the stabilization of the transition state in *HSCIE*. An analysis of the spatial structure of CtxG1 shows that only guanidine group of Arg9 can be such an electron donor moiety for the His10 imidazole ring. To test this proposal, we carried out a quantum-chemical calculation of the transition state at hydrogen exchange in the CtxG1 fragment including Arg9 and His10. The geometrical parameters of the heavy atoms were taken fixed (Fig. 3) (Guddat et al., 1996). The distance between the exchanging H atom at His C2 and N atom of the Arg9 guanidine group in the transient state was 2.68 Å, and the E_{act} of this reaction was very low (7 kcal/mol, HF/6-31G*) according to this calculation. Therefore, it is the proximity of the His10 imidazole residue and the Arg9 guanidine group that is the reason for about a half of tritium incorporated at C2 of the His10 imidazole ring.

The reaction of *HSCIE* of hydrogen in protein with spillover-tritium was studied with the β-galactosidase from *Termoanaerobacter ethanolicus*, protein with molecular mass of 83 kDa. The dependence of specific radioactivity and the retention of the β-galactosidase enzymatic activity on the condition of *HSCIE* were determined (Table 5).

The selected *HSCIE* conditions allow the enzymatic activity of β-galactidase to be completely retained at isotope exchange temperatures of 40–120°C during 10 min. A larger increase of the reaction time up to 60 min at 100–120°C leads to tritium incorporation growth with a certain decrease of the molar activity. It may be stated that even after 10 min at 160°C a small part of protein enzymatic activity can be retained. This data demonstrated "softness" of the labelling process in *HSCIE* and the high retention of the enzymatic activity. The selected *HSCIE* conditions allow the

Table 5. Tritium labelled β-galactosidase obtained by *HSCIE* with tritium in presence 5% Palladium on $BaSO_4$

Temperature, °C	Time of reaction, min	Specific radioactivity, Ci/mmol	Retention of the molar activity, %	Chemical yield, %
40	10	54	100	56
80	10	960	100	54
120	10	1,440	100	49
140	10	2,050	59	47
160	10	3,870	6	32
100	60	3,190	88	81
120	60	2,330	55	64
160	40	–	0	10

enzymatic activity of β-galactosidase to be completely retained and to prepare [^{3}H] β-galactosidase with specific activity of 1440 Ci/mmol. Specific radioactivity of tritium labelled β-galactosidase may be close to the same value as for ^{125}J, but this [^{3}H]protein may be also used for analysis its fragmentation.

Conclusion

It was shown that the reaction high temperature solid-state catalytic isotope exchange (*HSCIE*) of tritium for hydrogen could be used for preparation of highly tritium labeled peptides with specific radioactivity of 100–150 Ci/mmol with completely retaining its biological activity. The mechanism of the *HSCIE* reaction in peptides was analyzed. The participation of electron donor O and N atoms in the stabilization of the transition state is the necessary condition for the *HSCIE* reaction in peptides. The electron donor atoms can belong both to the residue in which the exchange occurs and to other spatially close amino acid residues. Thus, the *HSCIE* reaction in peptides differs from that in free amino acids. The reaction ability of amino fragments in *HSCIE* was shown to depend both of their structure and on the availability and the mobility of the polypeptide chain. The spatial accessibility of exchanging protons for the interaction with the acidic centers formed by *HS* and the conformational flexibility of the peptide fragment that permits to form the transition states with the participation of the electron donor atoms plays here an important role. The selected *HSCIE* conditions allow to prepare [^{3}H]proteins with specific activity about 1000 Ci/mmol and completely to retain their enzymatic activity.

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