Hydrophobicity Scale of Amino Acids as Determined by Absorption Millimeter Spectroscopy: Correlation with Heat Capacities of Aqueous Solutions

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Hydration indexes of protein α-amino acids were measured by the new method of absorption millimeter spectroscopy (AMS) at 10 mm wavelength (1.05 cm^{-1}; 31.42 GHz). This region of electromagnetic waves, located between the region of high-frequency dielectric spectroscopy and that of far-infrared spectroscopy, allows to measure hydration on the basis of different rotational and reorientational mobilities of water in hydration shell. Contribution to hydration of the methylene group is shown to be significantly greater than that of polar -OH, $-CONH_2$ and -COOH groups, while the contribution of the charged $-CH(N^+H_3)COO^-$ group is even negative. The high sensitivity of AMS method to hydrophobic hydration allows to build up the hydrophobicity scale which gives an acceptable correlation (r=0.95) with the heat capacities of the aqueous solutions of amino acids. Thus, millimeter absorption spectroscopy, the method of hydration determination at a molecular level (determination of V-structure of water with lifetime ~50 ps), allows to quantitatively distinguish hydration of polar and non-polar groups as well as calorimetry does at a macroscopic level.

Introduction

The interaction of amphiphilic biomolecules with water results in two interrelated phenomena: hydrophobic and hydrophilic hydrations. The hydrophobic effect is usually associated with additional stabilization of H-bonds in the vicinity of a hydrophobic fragment. Additional ordering of water molecules near a non-polar group leads to decrease of entropy and to proportional increase in the hydration heat capacity, as was shown by Sturtevant (1977).

The balance between contributions to hydration of hydrophobic and hydrophilic groups is of great importance for estimation of various properties of chemical and biological water systems that can be quantitatively evaluated in different hydrophobicity scales. Different scales are necessary for description of different phenomena (Wilce *et al.*, 1995). For example a scale, which is based on the different hydration heat capacities of amino acid residues (Makhatadze and Privalov, 1990), allows to quantitatively study the thermodynamic stability of proteins and explains the significant increase in the heat capacity accompanying globule denaturation (Renner *et al.*, 1992). Manifestations of amino acid hydrophobic effect are also evident in peptide-receptor biorecognition processes, protein-protein interactions, enzymatic catalysis etc.

Because of the broad distribution of different rotational and vibrational motions of water, the studies of hydration phenomena were performed in a broad range of frequencies by different methods including infrared, millimeter and dielectric spectroscopies (Khurgin *et al.*, 1994a). In the millimeter range (1–10 mm), the high resolution of microwave gaseous spectra for simple molecules is lost when pressure is increased, and intermolecular collisions become more significant. For the condensed state of matter including water, the spectra are depicted by smoothed curves, and valuable information can be obtained by precise comparison of the absorption of pure water and that of solution at constant wavelength. Absorption millimeter spectroscopy (AMS) was designed as a spectroscopy technique to provide these measurements and to study how water molecules interact with surrounding molecules in different aqueous systems (Khurgin *et al.*, 1994 a, b; Vorob'ev *et al.*, 1996). The advantage of millimeter spectroscopy is a possible direct registration of hy-

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dration phenomena in the time-scale of reversible transfer of water molecules from bulk water to the hydration shell and rotation motions.

Previously, the absorption millimeter spectroscopy in the 3–10 cm^{-1} (300–100 GHz) range was proposed as a direct method for investigation of hydration effects in aqueous solutions of amphiphilic compounds (Khurgin *et al.*, 1994a). In this range of electromagnetic waves at frequencies comparable with rotational frequency of pure water (τ~10 ps), the absorption is almost completely determined by the freely rotated water molecules. Recently, a new modification of the AMS method in the 0.75–1.3 cm^{-1} (30–40 GHz) range based on the wave-guide dielectric resonance effect has been proposed (Khurgin *et al.*, 1994b). In this frequency range (τ~50 ps), the difference in absorption between water in hydration shell and bulk water is caused by their different rotational mobilities and differences in dielectric relaxation effects. Since the additional organization of water molecules is realized in the hydration shell of nonpolar group, one can anticipate that AMS method allows to detect a hydrophobic effect for amino acids at a molecular level.

In the present work this method was used to build up the hydrophobicity scale for protein α-amino acids on the basis of different rotational and reorientational mobilities of water molecules in the hydration shells of amino acid zwitterions. This investigation was designed to establish a relationship between the spectroscopically evaluated hydrophobicity and heat capacity as a thermodynamic measure of hydrophobicity.

Experimental

Materials

The amino acids were purchased from Sigma (St. Louis,USA). Solutions were prepared with twice distilled water that was degassed by boiling.

Absorption millimeter spectroscopy

Detailed description of principles of absorption measurements in millimeter range and equipment used was published (Khurgin *et al.*, 1994a,b).

New installation for measurements at 10 mm consists of the following consequently connected devices that were developed in the Institute of Radioengineering and Electronics (IRE), Russian Academy of Sciences, Moscow:

a) Stabilized generator G4–156 of extremely-high-frequency radiation in the 26–37.5 GHz region which is based on the Gunn-effect diode with output power of about 15 mW.

b) Waveguide with attenuator.

c) Waveguide measuring cell, where the polytetrafluorethylene tube with a sample solution (4 mm^3) was placed into the waveguide. The temperature of the cell was maintained with an accuracy of ±0.1 °C.

d) Semiconductor detector.

e) Amplifier UPI-2.

f) Voltmeter V7–40 and recorder LKB 6500 (LKB, Sweden).

G4–156, UPI-2 and V7–40 devices are of common use in laboratory work (Minpribor, Moscow, Russia).

Generator (a) with connected waveguide (b) was used for production of electromagnetic radiation at constant frequency (31.42 GHz) and its canalization into the measuring line. After passing through the measuring cell (c), the electromagnetic beam was passed to the detector (d). Amplifier (e) and recorder (f) were used for registration of the electric signal.

The absorption measurements for aqueous solutions of amino acids were performed at 30 °C. Absorptions α ($dB \cdot mm^{-1}$) were estimated using the formula $\alpha = -\log I/I_o$, where I_o is an intensity of the initial radiation, and I is the intensity of radiation after passing through the solution (Khurgin *et al.*, 1994b). The hydration effects of amino acids were calculated as the difference $\delta\alpha$ between the absorption of the solution α_{exp} and the theoretical contribution of an aqueous component $\varkappa_1 C_1$ using the equation:

$$\delta\alpha = \alpha_{exp} - \varkappa_1 C_1, \qquad (1)$$

where C_1 and $\varkappa_1$ are the concentration and the extinction coefficient of pure water, respectively.

Results and Discussion

The concentration dependencies of attenuation of the electromagnetic radiation at 1.05 cm^{-1} (31.42 GHz) by solutions of Gly and Leu are presented in Fig. 1. These two amino acids differ significantly in hydrophobicity of the side chains. As can be seen in Fig. 1, the linear dependence between the absorption and the concentration C_2 for Gly and Leu holds in the 0.01–0.1 M range, i.e. in the concentration region where the Lambert-Beer law can be applied (Khurgin *et al.*, 1994a,b). This indicates that the difference between the experi-

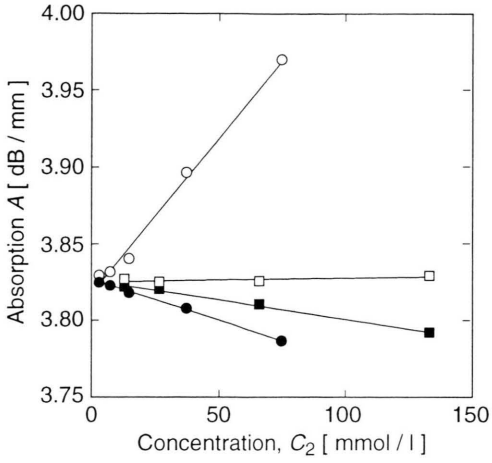

Fig. 1. Dependencies of absorption of millimeter radiation α_{exp} on the solute concentration C_2 for Leu (○) and Gly (□). Theoretically expected contributions to absorption of aqueous component $\varkappa_1 C_1$ for Leu (●) and Gly (■).

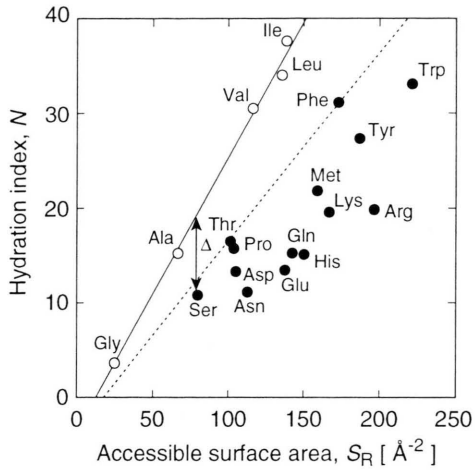

Fig. 2. Relationship between hydration indexes N and accessible surface areas of side chain residues S_R from Miller *et al.*, 1987. Solid line represents the linear correlation $N_{alph}=a+bS_R$ (Eq. (3)) for aliphatic amino acids (○). Dotted line corresponds to the hydration of aromatic amino acids without contributions of polar groups. Δ are the distances between lines (solid line for Ser, Thr, Asp, Glu, Asn, Gln, Met, Lys, Arg and dotted line for Tyr, Trp, His) and hydration indexes for polar amino acids.

mentally measured value α_{exp} and the expected contribution of the aqueous component to the absorption $\varkappa_1 C_1$ ($\delta\alpha=\alpha_{exp}-\varkappa_1 C_1$) is greater for hydrophobic Leu than for less hydrophobic Gly at any concentration (Fig. 1).

Previously, it has been shown that in the millimeter range the difference $\delta\alpha$ is not equal 0 in the result of the change in the state of water molecules in the hydration shell of the solute molecule (Khurgin *et al.*, 1994a,b). Significant difference in $\delta\alpha$ for hydrophobic Leu and less hydrophobic Gly (Fig. 1) shows that water disturbance measured by AMS method at 1cm^{-1} may be due to the hydrophobic effect. The validity of this assumption will be supported below by quantitative analysis of AMS data for other amino acids.

Functions α_{exp} and $\varkappa_1 C_1$ are the linear functions of C_2, and have the same intercept corresponding to the absorption of pure water (Fig. 1). Therefore, the difference $\delta\alpha$ is a proportional function of C_2. It allows to calculate the indexes of hydration:

$$N=\delta\alpha/\varkappa_1 C_2, \qquad (2)$$

as a concentration independent measures of hydrophobicity for protein amino acids (Table I).

The dependence of N on the accessible surface area (ASA) of the side residue S_R (Fig. 2) is linear function for aliphatic amino acids:

$$N_{alph} = a + b \cdot S_R,\ b=(0.284 \pm 0.012)\ \text{Å}^{-2},\ r=0.997. \qquad (3)$$

It follows from Eqn (3), that the contribution to hydration of the non-polar CH_2 group is $\Delta N_{np}=bS_{np}$ (9.1), where S_{np} is the ASA for CH_2 group, as accepted to be 32 Å^2. The a value from Eqn (3) corresponds to contribution of zwitterion fragment -CH (H_3N^+)COO^-, which is constant for all amino acids except Pro. The proportionality of hydrophobic hydration to ASA is, probably, a general property of hydrophobic hydration, which is recorded not only with AMS method but also by means of other methods (hydration heat capacity, compressibility etc.).

The presence of polar groups in polar amino acids leads to decrease of hydration indexes by the values $\Delta=N_{alph}-N$ (Fig. 2) comparatively to straight line N_{alph} corresponding to non-polar amino acids. It was shown (Vorob'ev *et al.*, 1996), that one can calculate the contribution to the hydration of a polar group (ΔN_p) according to the following simple equation:

$$\Delta N_p = S_p.b - \Delta, \qquad (4)$$

Table I. Hydration indexes for zwitterions of α-amino acids N and contributions of polar groups ΔN_p at 31.42 GHz (30 °C).

Amino acid type	C_2 [mol/l]	$N=\delta\alpha/\varkappa_1 C_2$	Δ	S_R [Å^2]	S_p [Å^2]	S_{np} [Å^2]	$\Delta N_p = S_p \cdot b - \Delta$
ALIPHATIC							
Gly	0.134	3.7±0.6	0	25	0	25	0
Ala	0.112	15±1	0	67	0	67	0
Val	0.0854	30±1	0	117	0	117	0
Leu	0.0762	34±1	0	137	0	137	0
Ile	0.0762	37±1	0	140	0	140	0
HYDROXY, ACID and AMIDE							
Ser	0.0954	11±1	9	80	36	44	2±2
Thr	0.0840	16±1	9	102	28	74	−1±1
Asp	0.0187	13±3	13	106	58	48	3±4
Glu	0.0340	13±1	22	138	77	61	−1±2
Gln	0.0685	15±1	22	144	91	53	3±2
Asn	0.0759	11±1	18	113	69	44	2±2
AROMATIC and HETEROCYCLIC							
Phe	0.0605	31±1	0	175	0	175	0
Tyr	–	27±1	6	187	43	144	2±2
Trp	0.0122	33±1	8	222	27	195	−2±2
His	0.0645	15±1	11	151	49	102	−2±2
BASIC							
Arg	0.0574	17±2	32	196	107	89	−2±4
Lys	0.0684	20±1	24	167	48	119	−11±2
SULFUR							
Met	0.0671	22±1	20	160	43	117	−8±2
Pro	0.0869	16±1	–	105	0	105	–

C_2 is the maximum concentration of solute.
N is the hydration index.
Δ is the distance between N and the straight line (Fig. 2).
S_R is the accessible surface of side radical $S_R=S_p+S_{np}$ (Miller *et al.*, 1987).
S_{np} is the accessible surface of non-polar groups (Miller *et al.*, 1987).
S_p is the accessible surface of polar groups (Miller *et al.*, 1987).
N for Tyr was calculated from data for Gly-Tyr, because of low solubility of Tyr.

where S_p is the ASA for polar group in side residue, and b=0.284 Å^{-2} is the slope in Eqn (3). For amino acids with aromatic and heteroatomic cyclic R (Phe, Tyr, Trp and His), the Eqn (4) is also valid with b = 0.197 Å^{-2} which corresponds to the line passing through the point for Phe with the same intercept as the N_{alph} does.

The dependence of N on the accessible surface of non-polar groups of side radical $S_{np} = S_R - S_p$ is given in Fig. 3. If the contributions of polar groups into hydration may be neglected, the experimental points for all amino acids should be located on the corresponding straight lines (solid line for non-aromatic and dotted line for aromatic amino acids). The deviations of the points corresponding to the polar amino acids from the straight lines (Fig. 3), as it was shown Vorob'ev *et al.*, 1996), are equal to ΔN_p which are defined by Eqn (4). Thus, Fig. 3 represents in graphic form the contributions to hydration of different polar groups.

This quantitative approach was used for the calculation of contributions to hydration of different polar groups (Table I). ΔN_p values for -OH group (Ser, Thr and Tyr), for -$CONH_2$ group (Gln and Asn), and for -COOH (Glu and Asp) are close to 0 and are significantly less than the negative contributions of amino and -S- groups in Lys and Met respectively (Table I). These results indicate that AMS method records the difference in the

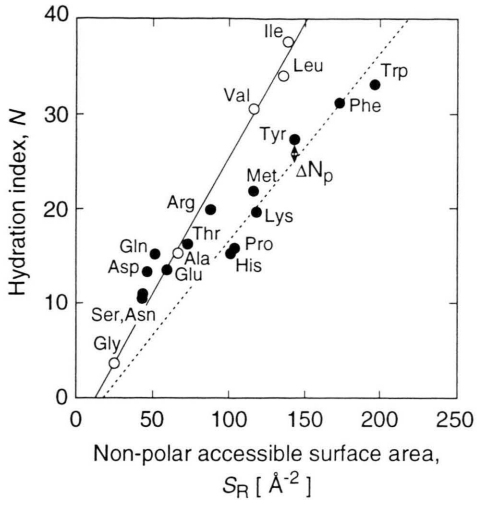

Fig. 3. Relationship between hydration indexes N and accessible surface areas of non-polar groups S_{np} of side radicals.

state of water molecules interacting with non-polar and polar groups, as well as of water molecules interacting with different polar groups. Thus, it is possible to build up the hydrophobicity scale on the basis of N indexes.

The method of differential scanning calorimetry (DSC) was used for the determination of partial molar heat capacity of solute $C_{p,2}$, and the hydration heat capacity $C_{p,h} = C_{p,2} - C_{p,g}$, where $C_{p,g}$ is the heat capacity of a molecule in gas phase (Makhatadze and Privalov, 1990; Vorob'ev and Danilenko, 1996). Heat capacity of solutions, as generally accepted, allows to quantitatively evaluate the hydration effects by DSC method. Relationships between hydrophobic hydration (CH_2 group), hydrophilic hydration of uncharged groups (-OH, -$CONH_2$ and -COOH) and hydrophylic hydration of charged -$CH(N^+H_3)COO^-$ group are presented in Table II, as were measured by AMS and DSC methods. According to AMS method, hydrophobic hydration gives highest N, showing significant decrease in rotational ability of water molecules in the hydration shells of non-polar groups. High hydration heat capacities of hydrophobic groups show that decrease of water rotational mobility may be compensated by the increase in low energy vibrations, which correspond to H-bonded clathrate-like hydrophobic structure. In contrast, the hydration contributions of -OH, -$CONH_2$ and -COOH groups give ΔN and $C_{p,h}$ values close to 0 (Table II), that correspond to the case when water in the hydration shells of polar groups looks undistinguishable from bulk water. Therefore, one can expect the correlation between the AMS and DSC scales because of the

Table II. Contribution to hydration of different groups.

Type of hydration	Group	Contribution to hydration			
		AMS method		DSC method	
		ΔN	$\frac{\Delta N}{\Delta N(CH_2)}$	$\Delta C_{p,h}$ [$JK^{-1}mol^{-1}$]	$\frac{\Delta C_{p,h}}{\Delta C_{p,h}(CH_2)}$
Hydrophobic hydration	-CH_2(aliphatic amino acids)	9.1±0.5	1	75±4	1
Hydrophilic hydration (non-ionic)	-OH, -$CONH_2$ -COOH	1.4±1.9	0.15±0.20	−6.5±12	0.09±0.17
Hydrophilic hydration (ionic)	-$CH(N^+H_3)COO^-$	−3.5±1.3	−0.4±0.15	−119±11	−1.6±0.2
Bulk water	H_2O	0	0	0	0

ΔN is the contribution to hydration index of different groups as it was calculated by Eqs (3) and (4).
$\Delta C_{p,h}$ is the contribution to hydration heat capacity of different groups (Vorob'ev and Danilenko, 1996).
$\Delta N(CH_2)$ and $\Delta C_{p,h}(CH_2)$ are the contributions of methylene group.
AMS method is the absorption millimeter spectroscopy, and DSC method is the differential scanning calorimetry.

high sensitivities of both methods to the hydrophobic hydration, while the values for the hydrophilic hydration of uncharged groups are at least 7 times lower (Table II).

The difference in scales (Table II) is connected with different sensitivities of the two methods to the hydration of the zwitterion fragment $-CH(H_3N^+)COO^-$ (Table II). Investigations of hydration mechanisms for various ions have revealed two different mechanisms: structure making positive hydration (Na^+, Li^+, Ca^{2+}, CO_3^{2-}, etc.) and structure breaking negative hydration (K^+, Cs^+, NH_4+, Cl^- etc.), (Samoilov, 1965). According to both methods, contribution of the charged fragment $-CH(H_3N^+)COO^-$ has the sign which is opposite to that for the hydrophobic hydration indicating the possible mechanism of negative hydration for zwitterion fragment.

Since the negative value N was obtained by AMS method (1–5 cm^{-1}) also for urea, the well known agent of water structure breaking, one can propose that the interaction of zwitterion fragment with water can be accompanied by the increase in the content of water molecules capable of the free rotation (Khurgin *et al.*, 1994a; Khurgin and Maksareva, 1993). At 5 cm^{-1}, the study of hydration for different forms of ionization for Gly showed that the negative hydration is attributed to $-N^+H_3$ group (Khurgin *et al.*, 1994a). It was established that the negative hydration of $-N^+H_3$ group is accompanied by a decrease in dielectric relaxation (reorientation) time for solution in comparison to that for pure water (Pottel *et al.*, 1975). One of the possible mechanisms of negative hydration can be connected with closely neighbouring donor or acceptor atoms (with distance about 2.1–2.5 Å) forming H-bonds with two water molecules (diameter of H_2O is 2.8 Å) not simultaneously but alternately (Khurgin and Maksareva, 1993).

Heat capacity change $\Delta C_{p,2}$ corresponding to the process of zwitterion formation:

$$H_2NCHRCOOH \rightarrow H_3N^+CHRCOO^-$$

gives negative values -60÷170 J $K^{-1}mol^{-1}$ depending on amino acid and method of estimation (Cabani *et al.*, 1977).

Correlation coefficient between N and $C_{p,2}$ is 0.95 (Table III). By definition, hydration is the effect of interaction of water with the surface atomic groups of solute. Therefore, the comparison of hydration effects for molecules of different sizes is reasonable to perform with values that are normalized on ASA of solutes. Fig. 4 and Table III show also an acceptable linear correlation (r= 0.93) between AMS and DSC scales represented in the form of specific values per 1 $Å^2$ of total ASA of molecules.

Different sensitivities of methods to negative hydration can not dramatically decrease the correlation, perhaps, because of the approximately constant contribution of negative hydration of $-CH(H_3N^+)COO^-$ fragment for different amino acids. Well known hydrophobicity scales give significantly less correlation coefficients between themselves. For example, correlation of the scale derived from Tanford's data (Tanford, 1962) with the scale based on the octanol/water partitioning data gives r=0.55 and the correlation of the Tanford's scale with the scale based on the accessibility for water of each amino acid residue in protein globules gives only 0.42 (Wilce *et al.*, 1995).

Table III. Correlation between hydration data obtained by absorption millimeter spectroscopy and differential scanning calorimetry.

Amino acid	N	$C_{p,2}$ [$JK^{-1}mol^{-1}$]	S [$Å^2$]	N/S [$Å^{-2}$]	$C_{p,2}/S$ [$JK^{-1}mol^{-1}Å^{-2}$]
Gly	3.7	39.2	173	0.021	0.23
Ala	15	141	203	0.074	0.70
Val	30	302	243	0.124	1.24
Leu	34	398	261	0.130	1.52
Ile	37	383	271	0.137	1.41
Ser	11	117	211	0.052	0.56
Thr	16	210	230	0.070	0.91
Asp	13	127	232	0.056	0.55
Glu	13	177	261	0.050	0.68
Gln	15	187	264	0.057	0.71
Asn	11	125	234	0.047	0.53
Phe	31	384	281	0.110	1.37
Tyr	27	299	296	0.091	1.01
Trp	33	420	308	0.107	1.36
His	15	241	265	0.057	0.91
Arg	17	279	310	0.055	0.90
Lys	20	267	295	0.068	0.91
Met	22	293	289	0.076	1.01
Pro	16	172	235	0.068	0.73
r		0.95[a]			0.93[b]

[a] Correlation coefficient between N and $C_{p,2}$.
[b] Correlation coefficient between N/S and $C_{p,2}/S$.
N is the hydration index (AMS method).
$C_{p,2}$ is the partial molar heat capacity of amino acid solute (DSC method).
S is the total accessible surface area of amino acid.
N/S and $C_{p,2}/S$ are the specific characteristics.

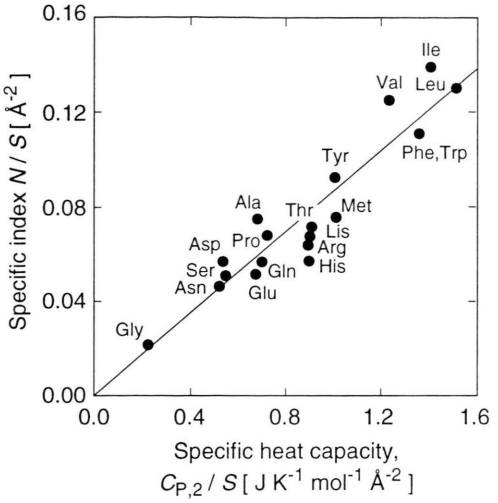

Fig. 4. Correlation between specific hydration indexes *N/S* and specific heat capacities $C_{p,2}/S$. Heat capacities of amino acid aqueous solutions were from Jolicoeur *et al.* (1986). Total accessible surface areas of amino acids *S* were calculated as sum of ASA for amino acid residues (main and side chains) and 99.4Å for terminal groups (Oobatake and Ooi, 1993).

Thus, the interaction of millimeter radiation with water structure (the V-structure) during some finite time interval (~ 50 ps) is significantly different for water molecules in the hydration shells of non-polar, polar and negatively hydrated groups. It means a heterogeneity of hydration shell in this scale of microscopic events. This, in turn, could be of interest for investigations of protein hydration with the high-frequency dielectric spectroscopy at frequencies higher than 1 GHz. It is evident, that the model of the protein hydration should be more complicate than the simple model (Yan-Zhen Wei *et al.*, 1994), which suggests the same state of all water molecules ("frozen" water) in the hydration shell of protein.

Hydration entropy, as accepted, is the most distinct quantity to distinguish polar and non-polar groups in water. The same is also valid for heat capacity, because of remarkably constant proportionality of this parameter to hydration entropy (Sturtevant, 1977). Our results show that the millimeter absorption spectroscopy, the method of hydration determination on a molecular level, allows to study hydrophobic effect as well as calorimetry does by measuring of the heat capacity.

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