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How thick is the layer of thermal volume surrounding the protein?

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Abstract

Investigation on the volume properties of protein hydration layers is reported. Presented results are based on combination of Monte Carlo modeling and available experimental data. Six globular proteins with known data are chosen for analysis. Analyzing the model and the experimental results we found that water molecules bound to proteins by hydrogen bond are preferentially located at the places with local depressions on the protein surface. Consequently, the hydration level is not strictly proportional to the area of charged and polar surfaces, but also depends on the shape of the molecular surface. The thickness of the thermal volume layer as calculated in the framework of the scaled particle theory is 0.6–0.65 Å for chosen proteins. The obtained value is significantly lower than that presented for proteins in earlier papers (where proportionality between the hydration level and the area of charged and polar surfaces was assumed), but is close to the value published for small solute molecules. Discussion including the influence of protein size and the thermal motion of the surface is presented.

Keywords: Globular proteins; Protein hydration; Interaction volume; Thermal volume; Monte Carlo simulation; Hydrodynamic parameters

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1. Introduction

A great variety of phenomena in molecular biology is essentially connected to the properties of water around great bio-macromolecules and their mutual interaction. As an example we can mention the competitive binding of water, ions, denaturants or osmolytes on the macromolecular surface (which is important for stability of proteins or DNA) [1–6], binding of signal molecules or drugs on the surface of proteins [7], aggregation of proteins [8], binding between the biological macromolecules [9], etc.

The partial specific volume is a well-defined thermodynamic quantity that carries information on the structure of the boundary layer of biological macromolecules in water environment. During the last decade high-precision U-tube density meters became available, which substantially improved the quality of experimental data on the partial specific volume [6,10–12]. The analysis of the specific volume, in combination with other experimental techniques, has had a basic impact on the theoretical description of protein hydration. Direct measurements of the partial specific volume are often combined with ultrasound measurements of adiabatic compressibility [10,13], which gives additive information on the geometry of the hydration layer and on the dynamics of protein's interior. Moreover, the hydration has a direct influence on the hydrodynamic properties of macromolecules, such as the friction coefficient, the diffusion coefficient and the intrinsic viscosity [14–16].

Precise theoretical analysis of dissolved macromolecules can be carried out using structural data of macromolecules based on NMR, X-ray and neutron diffraction measurements. Diffraction measurements were also applied directly to study the hydration of proteins [17,18]. These experiments have shown that there are some very stable places on the protein surface with long term bounded water molecules [17] and that the protein surface is preferentially hydrated in its concave part [17,19]. The dynamics of the hydration layer was investigated using thermodynamic [2–4] or molecular dynamic [19–21] calculations. Combination of molecular dynamic simulations with X-ray and neutron scattering data [21] has shown that the density of water in a 3 Å thick layer around a lysozyme is ~15% higher as compared to the free "bulk" water. This difference is in qualitative agreement with the results of Svergun et al. [22], who reported the density in the first hydration shell of lysozyme to be 6–11% higher than the bulk density.

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The early theoretical studies on partial specific volume of proteins were based on the simple approach of additive individual amino acid elements [23–25]. This approach does not take into account the essentially different interaction of the amino acid residue with its surroundings in an aqueous environment or in the interior of a protein. More recently, Kharakoz [26] has tested other possible approaches. As a result of his comparative studies he chose the scaling particle theory [27] to be the most adequate concept for the calculation of volumetric properties of macromolecules. In principle, this is an attempt to find some simple terms that give a satisfactory description of a complicated phenomena taking place in solutions. For the sake of simplicity, in the remaining part of the paper we refer to the volume terms, as it would correspond to a single particle (given in Å^3 units). Following the scaling particle theory, the partial volume V^0 of a macromolecule at an infinite dilution is equal to the sum of four components [11]:

$$V^0 = V_\mathrm{M} + V_\mathrm{I} + V_\mathrm{T} + \beta_{\mathrm{T}0} kT. \qquad (1)$$

The first term, V_M is the intrinsic volume of the solute inaccessible to water. Considering the physical model of both the protein atoms and water molecules, this term is usually described by the van der Waals (VdW) shell model. Different algorithms have been developed in the past for this purpose [11,28,29], approximating the solute atoms and the solvent molecules as VdW spheres. The interaction volume V_I represents the change in the solvent volume due to hydration. The thermal volume V_T corresponds to the apparently empty space around a solute molecule as a consequence of the mutual thermal motion of the solvent and the surface of the solute molecule. This term should always be taken into account when hard-core van der Waals surfaces are used to approximate the particles. Then the free motion of the particles in the thermal volume V_T is an approximation of their real motion in complicated potential wells. The last term is the ideal component of the partial volume resulting from the motion of the molecule along the translational degrees of freedom. It is the product of the isothermal compressibility of the solvent $\beta_{\mathrm{T}0}$, the Boltzman constant k, and the temperature T. This last term can be neglected in case of large macromolecules, such as proteins, in a water environment ($\beta_{\mathrm{T}0}kT = 1.83$ Å^3).

Note that the approximate picture of the thermal motion of particles presented above is a very simple one, and that an exact description of the real situation (e.g., in water environments) could be much more complicated. Nevertheless, this model allows one to carry out practical calculations of volume properties in solutions. The sizes of V_I and V_T of proteins in water environments are the major subjects of our present work.

Different algorithms have been used by other authors to calculate the intrinsic volume V_M of molecules in solvents. Kang et al. [30] described a method of V_M calculation where solutes represented by VdW spheres are located in a structureless continuum, while in other papers [11,28,29] both the solute and solvent molecules are represented by VdW spheres. In the later case the intrinsic volume is obtained as the volume inaccessible to the solvent "probe sphere" when it is rolled over the VdW surface of a macromolecule. One can also define the solvent-accessible surface of a macromolecule (to be used later) that is traced out by the center of the probe sphere in the same procedure [11,28].

The interaction volume V_I is related to the water molecules, which solvate the protein. These molecules can be divided into three groups: the water molecules firmly bound to the charged or polar protein surface by means of hydrogen bonds (HB), the water molecules contacting only the non-polar surface of the protein, and the molecules that are located in next hydration shells. Each of these groups is thermodynamically distinct from the bulk water; however, it is only the closely bond part that has a significant influence on the interaction volume. This feature of hydration — presented in the paper of Kharakoz [26] — is based on the comparison of partial molar volumes of small polar and non-polar solute molecules. Kharakoz found that the volume effect of each HB in solution is 3.66 Å^3. The effect of the electrostatic attraction is two orders smaller (see Ref. [26] and the literature cited in there). Negligible contribution of higher hydration shells to the interaction volume is shown in the analysis of X-ray and neutron scattering data of Merzel and Smith [21] in case of lysozyme. Based on these results one can draw a conclusion that the interaction volume V_I is related to the water molecules that are bound to the protein by means of HB. This assumption (also applied in the work of Chalikian et al. [11]) can be expressed via following equation

$$V_\mathrm{I} = V_\mathrm{h} - n_\mathrm{h} V_0^0, \qquad (2)$$

where V_h is the volume of the part of hydration shell containing n_h water molecules firmly bound by HB and V_0^0 is the mean partial volume of water in the bulk.

The knowledge of the partial volume V_h of the solvent in the hydration shell is principal when the partial volume V^0 of a protein or other macromolecule is to be calculated. In several cases, when the position of hydrating molecules is known, their volume can be evaluated using Voronoi polyhedrons [17,31] associated with the molecules in the hydration layer. This approach is only correct under certain conditions, namely when there is available data on the next layer of bound water covering the first hydration layer [17,31]. The general application of this method is limited; however, the obtained data are useful while comparing them with results of other theoretical models.

The thermal volume is a region of mutual thermal motion of molecules, so by nature it is a region shared by many molecules at the same time. Formally, in the bulk solvent environment the thermal volume can be equally divided between each solvent molecule. Then the average thickness of the thermal volume layer of bulk water molecules is $\Delta_\mathrm{w} \approx 0.5$ Å (we refer to the detailed discussion given by Kharakoz [26]). In the solution there is a thermal volume that is shared by the solute and the surrounding solvent molecules. For simplicity this volume can be divided between the interacting molecules in the following way. The thermal volume of solvent molecules is kept unchanged as compared to the bulk solvent regions and the remaining part is the V_T value assigned to a solute molecule. Note that Kharakoz [26] obtained the same value of thermal

Table 1

Hydration parameters of chosen proteins (δ_{MC} — hydration level i.e., ratio of bound water and protein mass determined by Monte Carlo simulation; $n_{h,MC}$ — number of bound water molecules; S_n, S_p and S_c — solvent accessible areas of nonpolar, polar and charged protein surfaces [11]; δ_{exp} — measured hydration level)

Protein	PDB entry	Mass [kDa]	S_n [Å^2]	S_p+S_c [Å^2]	$n_{h,MC}$	δ_{MC} [g/g]	δ_{exp} [g/g]
Cytochrome *c*	1 hrc	12.39	3446	2669	248±9	0.36	0.35 [47]
Ribonuclease A	5 rsa	13.69	3462	3328	298±7	0.39	>0.34 [48]
Lysozyme	4 lyz	14.31	3230	3455	296±10	0.37	0.33 [49–51], 0.34 [52], 0.38 [53]
Myoglobin	1 mbo	17.88	4772	2944	344±5	0.35	0.37 [54], 0.42 [47,52], 0.48 [55]
Chymotrypsinogen A	2 cga	25.66	5714	5101	505±16	0.35	0.34 [47,52]
Hemoglobin	1 had	64.60	15721	9784	1287±18	0.36	0.34 [56], 0.39 [55]

volume thickness $\Delta \approx 0.5$ Å for various low molecular mass solutes in water independently of their shape, charge and chemical nature.

Chalikian et al. [11] carried out a comprehensive study on the volume properties of proteins. The different contributions of the partial molar volume V^0 were calculated by supposing that the thermal volume V_T is proportional to the solvent accessible surface ($V_T \sim S_n+S_p+S_c$, where S_n, S_p and S_c are the nonpolar, polar and charged surface areas of the macromolecules) and that the interaction volume V_I is proportional to the sum of charged and polar surfaces ($V_I \sim S_p+S_c$). The parameters of the theory were fitted to experimental specific volume data of several proteins. In this way the value of the thermal volume thickness of $\Delta_p \sim 1$Å was obtained. This result was also mentioned in the review paper of Chalikian [32] and applied by Shimizu [33] in a calculation of excess hydration number in allosteric transition of hemoglobin and for the binding of camphor to cytochrome P450. The mentioned thickness is twice as large as 0.5 Å, which is the thermal volume thickness in case of water and which was also obtained by Kharakoz [26] for small molecules. Chalikian et al. [11] supposed that the disagreement is a consequence of additional modes of thermal motion in great molecules. If this is the case the question arises: How does the thermal volume thickness depend on the size or type of the molecule?

We would like to discuss a problematic assumption made in Ref. [11], where it is supposed that the interaction volume is proportional to the sum of charged and polar surfaces ($V_I \sim S_p+S_c$). According to the same paper, V_I is also proportional to the n_h value (the number of water molecules firmly bound to the protein) — thus giving the ratio of $n_h/(S_p+S_c)$ to be a constant. We checked this assumption using the experimental hydration data presented in Table 1 and the S_p, S_c and S_n values taken from Ref. [11]. It is shown in Fig. 1 that $n_h/(S_p+S_c)$ significantly depends on the ratio of $S_n/(S_p+S_c)$. It seems likely that the hydration level and consequently the interaction volume cannot be estimated only using simple geometrical parameters such as the different surface areas.

In this paper a new method for the estimation of the partial specific volume of proteins is presented. A Monte Carlo method is used for modeling the position of water molecules bound to the protein by HB — the applied procedure represents a starting point for the estimation of interaction and thermal volumes. In order to see the possible mass dependence, proteins covering a wide range of masses are chosen for comparison with experiment. In the literature, we found six proteins with known distributions of surface areas and with the necessary experimental data available. Our aim is also to elucidate, at least partially, the influence of the intramolecular thermal motion of protein atoms on the studied value.

2. Methods

2.1. Model of hydration

In the present method of volume calculations, the hydration properties of the protein need to be determined in the first step. An advanced version of the MC procedure published in our previous paper [14] was applied to calculate the hydration level. Spheres with corresponding VdW radii are used to represent protein atoms and water molecules in the model. In accordance with most of other papers [11,17,28,31,29] a radius of 1.4 Å is applied in case of water. This value is half the smallest distance (2.8 Å) between oxygen atoms bound by HB in ice [11,29]. The radii of spheres representing protein atoms are chosen according to data presented in paper of Chalikian et al. [11]. This set of radii does not contain a value for hydrogen, the radius of which is taken from the paper of Zhou [16]. We use the Protein Data Bank as the source of atomic coordinates within the protein.

It is assumed in the model that water molecules near the surface can be in two states — bound or free. Bound molecules are fixed in position mainly due to HB but the weaker VdW interaction is also taken into account. A HB is supposed to occur between two neighboring bound water molecules or between bound water molecules and surrounding heavy

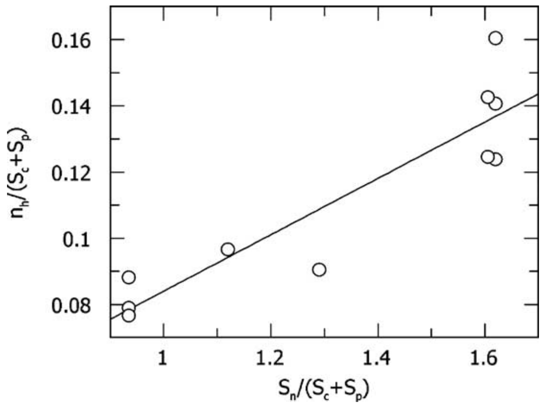

Fig. 1. Dependence of number of hydrating water molecules on nonpolar surface area. Both values are calibrated by sum of charged and polar surface areas. Experimental data are calculated from the hydration levels presented at Table 1, surface areas were calculated in Ref. [11].

charged or polar atoms (these are the atoms of the protein backbone and the atoms of charged or polar amino acids). The hydrogen bond potential is chosen according to papers [34–37]. It is set to be zero for distances longer than 3.5 Å, while the following effective values are assumed for smaller separation:

U_{hb}(O–H...O) = −10.0 kcal/mol (in case of water–protein interaction)
U_{hb}(O–H...O) = −4.5 kcal/mol (in case of water–water interaction)
U_{hb}(N–H...O) = −5.5 kcal/mol
U_{hb}(C–H...O) = −2.5 kcal/mol.

The significantly weaker VdW interaction calculated from Lenard–Jones potentials is also included in the model.

The basic idea of the iterative MC algorithm can be described as follows. The protein surface is covered with bound and free water molecules that contact the VdW surface of the macromolecule or the surface of other yet bound water molecules. Only the coordinates of bound water molecules are stored between consecutive iteration steps. The position of bound molecules can be shifted on the surface during the MC procedure to an energetically more favorable place (the maximal shift per iteration is less then the radius of the water molecule). From it follows that (in our model) the minimal distance between the bound water molecules and the surface atoms or other water molecules is equal to sum of their VdW radii. This distance is also equal to the minimal distance of the HB, while the maximal distance of the HB is limited by a cut-off at 3.5 Å. These conditions were chosen in agreement with the well-mapped situation in bulk water. They lead to shortening of water–(protein) surface and water–water distances and as a consequence to the higher density of the hydration layer compared to bulk water.

In each step of the MC algorithm first a single protein atom or one of the bound water molecules is chosen randomly (either of them is represented by the corresponding VdW sphere). The probability of selection is proportional to the VdW surface area of chosen atom or water molecule. Next, a new “water” sphere contacting the surface of the chosen particle is randomly generated with equal probability of all the possible orientations (allowing all kinds of overlapping with the neighbours). There are three different situations in the following procedure depending on the position of the generated new sphere:

a) If the new sphere does not overlap with any other atom of the macromolecule or any other bond water molecule: it is considered as a selected free water molecule and the corresponding probability of the (free → bound) state change is calculated (see below). Random numbers are used to determine the capture or unbending of this molecule.
b) If the generated sphere overlaps with any bound water molecules and the center of the new sphere is placed inside this bound molecule, and the new sphere does not overlap the macromolecule, then the generated sphere is considered to be a potential new position for the previously bound water molecule. The selected molecule is placed to the energetically more favorable position (old or new) and then the probability of the (bound → free) state change is tested and determined in a similar manner as case a).
c) Otherwise, the set of bound molecules remains unchanged.

The probabilities of (bound ↔ free) state changes are defined in a way that after a large number of computation steps the following probability condition is satisfied in any place on the surface:

$$p_b/p_f = A\cdot\exp\left(\frac{-\Delta U}{kT}\right), \tag{3}$$

where p_b and p_f are the probabilities of finding a bound and free water molecule on that particular place, respectively. ΔU is the difference of potentials between the bound and free states, k is the Boltzman constant, T is the temperature and A is a constant. Using this approach, the evolution of capture of water molecules on the protein surface is simulated until an equilibrium (characterized by a constant number of bound hydrogen molecules n_h) is achieved.

Once the extent of the hydration is obtained the volumes and hydrodynamic parameters of the “effective” particle (which contains both the macromolecule and bound water molecules) are determined.

2.2. Basic algorithm of volume calculation

All the volume calculations are based on a two-step process in which the macromolecule is represented by a complex of spheres with certain VdW radii and the surrounding water molecules are modeled by appropriate test spheres. In the first step an envelope is created around the particle. The envelope consists of many overlapping test spheres touching the particle; thus, it represents all the possible positions of free water molecules in the neighborhoods of the particle (see Fig. 2). Test spheres in the envelope are randomly generated with high density — the final distance between the centers of neighbors is significantly smaller then the applied radius. In the second step, the volume of the cavity enclosed by the envelope (representing the intrinsic volume) is evaluated by a conventional MC integration method. The principle of described calculation is

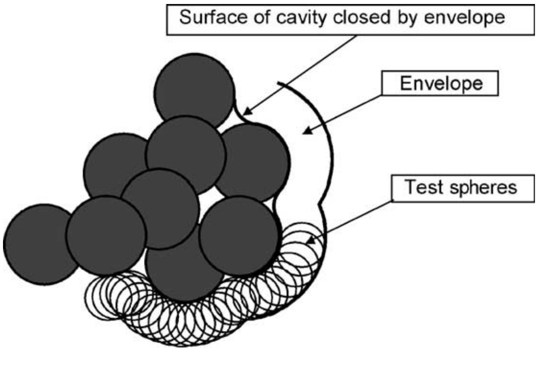

Fig. 2. Illustration of the intrinsic volume calculation in MC algorithm — idea is compatible with that presented originally by Lee and Richards [28].

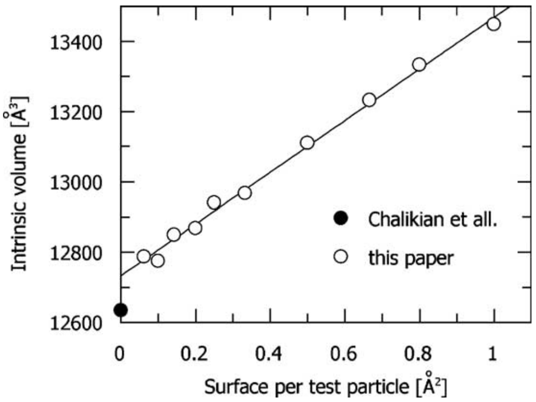

Fig. 3. Intrinsic volume of cytochrome *c* calculated with different number of randomly allocated test spheres in MC algorithm. The number of test spheres is represented by inverse value of the density of test spheres on the water accessible surface.

compatible with the procedure presented by Richards [38] and also used by Chalikian et al. [11]. In all the presented calculations the final volume of the enclosed cavity is obtained by extrapolation to infinite density of test particles (see Results section and Fig. 3).

2.3. Calculation of the partial volume

The partial volume of hydrated proteins is calculated from the expression:

$$V^0 = (V_\mathrm{M} + V_\mathrm{h} + V_\mathrm{T}) - n_\mathrm{h} V_0^0. \qquad (4)$$

Eq. (4) was derived from Eq. (1) by neglecting the ideal term and introducing Eq. (2). The last term in Eq. (4) can be calculated in a straightforward way based on the results of the hydration model, since both components are known. The term in the bracket represents the volume of the hydrated macromolecule (including the water molecules bound by HB) and the thermal volume of such a complex. Bound water molecules (generated by described MC procedure) touch the heavy atoms of the protein or other bound water molecules. No thermal vibration of HB is supposed in our calculations. The thermal volume is considered as being located around the whole effective particle composed of the macromolecule and the firmly bound part of the hydration shell. Of course, this does not mean that the thermal volume is really empty. This part of volume is the place where the mutual thermal motion is realized. In the applied approximation water molecules (including the firmly bound ones) are considered as being placed in their mean thermal positions related to the protein surface. The volume of the effective (hydrated) particle is determined using the same approach as it was described for the intrinsic volume at the beginning of this chapter. The radii of test spheres (creating the envelope) and the radii of spheres representing the effective particle are chosen in the following way. The radius R_t of test particles was discussed in many papers. It can be the VdW radius of water R_w, as it was proposed originally by Lee and Richards [28] and used later by Chalikian et al. [11] and other authors, or it can depend on the pressure as proposed by Paci and Velikson [29]. Taking into account that a volume assigned to a single molecule is a thermodynamic quantity that only has a sense after sufficient averaging over a longer time period, in our following calculations we prefer to use test spheres having the volume of free water molecules with radius $R_\mathrm{t}=R_\mathrm{w}+\delta_\mathrm{w}$. This value gives a correct result also in case when the solute molecule is replaced by a solvent molecule, thus giving a method, which is invariant to the exchange of these molecules. In this case, the centers of test particles represent mean positions of free water molecules near the protein surface averaged over a time interval, which is comparable (or moderately longer) than the characteristic time of their oscillation. The distance between test particles and the surface of the hydrated protein is chosen to be equal to the thickness of the thermal volume δ_p so that the gap width between the VdW surfaces of free water molecules and the hydrated protein is equal to the sum of their thermal volume thickness $\delta_\mathrm{w}+\delta_\mathrm{p}$. The corresponding schematic view is shown in Fig. 4. Ones the envelope of the effective particle was created according to the above procedure, the volume of the cavity closed by the infinitely dense envelope is equal to $(V_\mathrm{M}+V_\mathrm{h}+V_\mathrm{T})$.

Finally, the thermal and hydrated volumes are also determined directly by the present algorithm. To obtain the thermal volume V_T, first the value of $(V_\mathrm{M}+V_\mathrm{h})$ is calculated repeating the above procedure with $\Delta_\mathrm{p}=0$. The difference between $(V_\mathrm{M}+V_\mathrm{T}+V_\mathrm{h})$ and $(V_\mathrm{M}+V_\mathrm{h})$ yields the thermal volume V_T. The hydrated volume V_h is obtained in a similar way calculating $(V_\mathrm{M}+V_\mathrm{T})$. In this case the envelope is created around the dry protein without the hydration layer.

2.4. The water density in a 3-Å layer

The water density in the hydration layer is directly connected to the interaction volume V_I. According to the work of Merzel and Smith [21] the density of the first hydration layer, represented by a 3 Å thick layer covering the protein surface, is higher as compared to free water density. The corresponding calculation can be done on a basis of known density ρ_f of free water and the density ρ_h of bound water, if the ratio of free and bound water volumes in this 3-Å layer is

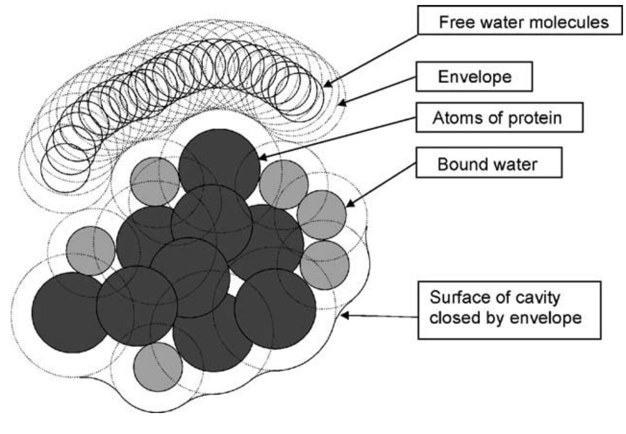

Fig. 4. Illustration of the volume calculation in presented Monte Carlo procedure. Volume of cavity represents the volume of hydrated macromolecule with its thermal volume.

known. The density of bound water ρ_h is calculated from the known number n_h of bound water molecules and the hydrated volume V_h. The (free/bound) volume distribution in a 3-Å thick layer is calculated using the envelopes of the dry and the hydrated protein. The total volume $V_{3Å}$ of the 3-Å layer is defined as that part of the dry protein envelope, in which the distance to the VdW protein surface is less than 3 Å (in standard calculations the envelope is 3.8 Å thick). In the same way, the volume of free water $V_{3Å,f}$ (in the 3-Å layer) is that part of the hydrated protein envelope, in which the distance to the VdW surface of dry protein is less then 3 Å. The difference $V_{3Å,h}=V_{3Å}-V_{3Å,f}$ is the volume of bound water in the mentioned layer. The water density in the 3 Å thick layer is calculated as

$$\rho_{3Å}=\left(\rho_h \cdot V_{3Å,h}+\rho_f \cdot V_{3Å,f}\right)/V_{3Å} \quad (5)$$

2.5. Calculation of hydrodynamic parameters

The friction coefficient, the diffusion coefficient and the intrinsic viscosity are calculated by the algorithm described in details in our earlier paper [14]. Here we only shortly remark that the applied method is based on the analogy between the basic equations of hydrodynamics and electrostatics. The pioneering works of Hubbard and Douglas [39] and Zhou [16,40] have been developed further.

The calculation procedure of hydrodynamic parameters utilizes the results of the hydration model. Again, the protein and the water molecules firmly bound by HB are considered as one effective particle moving in water environment.

3. Results

3.1. Test of hydration model

Six proteins are selected to test the results of the model. There are both experimental data and exact theoretical calculations available in the literature giving the area of molecular surfaces of these proteins. The selected globular proteins cover a wide mass range and there is also a significant difference between their ratio of non-polar to polar and charged surface areas — see the data taken from Chalikian et al. [11] in Table 1.

Hydration can influence the final result either by the number of water molecules bound by HB or by their localization. The number of firmly bound water molecules n_h is directly related to a well-measurable quantity — the hydration level δ, which is defined as the mass ratio of the bound water and the protein (expressed in grams of water per grams of protein). The results of hydration level calculations are presented in Table 1 together with the corresponding experimental values obtained by different methods. During the MC procedure, each protein was calculated 10 times with different starting values of the random generator. The measured hydration level data vary depending on the applied experimental method. However, within the limits of the experimental errors there is a reasonable agreement between the measured and the calculated values.

To characterize the position of a water molecule bound to the protein surface, one has to specify the shape of the surface around the binding place — if it is concave or convex. This feature can be characterized by different parameters. First, for each bound water molecule we define a spherical angle Ω, within which the fixed molecule can touch other water molecules (both free or bound). The spherical angle Ω defined this way can characterize the place in which the water molecule is localized — for molecules bound in a concave depression $\Omega<2\pi$. Mean values of Ω averaged for all the water molecules bound to a certain protein are found to be $\langle\Omega\rangle\in(5.09, 5.38)$ not showing any significant correlation with the protein mass.

The next parameter we have expressed is the number of hydrogen bonds n_{HB} connecting a certain bound water molecule to the protein surface. The average value of n_{HB} obtained for different proteins is $<n_{HB}>\in(3.04, 3.57)$, which is in agreement with results of Makarov et al. [20]. Finally, we define a contact between a bound molecule and a non-polar group of the protein when the distance between the centers of the VdW spheres is less than 3.5 Å (H atoms are excluded). The mean number n_n of such contacts (not bonds) to non-polar groups is $<n_n>\in(0.56, 0.66)$. All these results show that firmly bound water molecules as calculated by the MC procedure are mostly localized in depressions of the surface having contacts not only to charged and polar atoms but also to non-polar groups. All these facts are in agreement with the results of many other papers [17,19–21,41].

3.2. Tests of the basic algorithm of the volume calculation

Comparison of our MC algorithm with the method published by Chalikian et al. [11] was done for the case of the intrinsic volume V_M applying same conditions. The intrinsic volume of cytochrome *c* (albacore tuna — PDB entry 5cyt) was calculated using same VdW atomic radii. Test particles ($R=1.4$ Å) touched the surface of the dry protein neglecting the hydrogen atoms. Fig. 3 shows the calculated volume closed by the envelope as a function of the reciprocal surface density of test particles (water accessible surface/number of test particles). It is evident that the obtained intrinsic volume rises linearly with the average area of surface elements belonging to a single test particle. Due to this fact, it is possible to extrapolate our data into the zero value on the *x*-axis-related to an infinite density of test particles on the molecular surface. The zero limit value (12,733 ± 10 Å^3) is slightly higher than the one determined by Chalikian (12,634 Å^3). This negligible but evident deviation represents a mean shift of the molecular surface (of approx. 0.016 Å), which can arise from the finite dimension of triangles representing atomic surfaces (with average area of 0.062 Å^2) in the method used in paper [11].

3.3. Test of water density near the protein

The calculated water density $\rho_{3Å}$ in a 3Å thick layer around a protein exceeds the free water density by some $(15 \div 25)\%$

Table 2
Hydrodynamic parameters of chosen proteins (diffusion coefficient D_{MC} and intrinsic viscosity $[\eta]_{MC}$ determined by Monte Carlo simulation and measured experimentally D_{exp}, $[\eta]_{exp}$)

Protein	$D_{MC} \cdot 10^{11}$ [m²/s]	$D_{exp} \cdot 10^{11}$ [m²/s]	$[\eta]_{MC}$ [ml/g]	$[\eta]_{exp}$ [ml/g]
Cytochrome *c*	12.18 ± 0.04	12.0 [57]	2.85 ± 0.02	2.74 [63], 2.91 ± 0.09 [64]
Ribonuclease A	11.41 ± 0.02	11.2 ± 0.2 [58]	3.15 ± 0.02	3.30 ± 0.04 [65]
Lysozyme	11.48 ± 0.02	11.2 ± 0.2 [59]	2.94 ± 0.02	2.98 [66], 3.00 [59]
Myoglobin	10.65 ± 0.02	10.3 [57], 10.6 [60]	2.96 ± 0.02	3.15 [67]
Chymotrypsinogen A	9.45 ± 0.02	9.01 – 9.48 [61]	2.92 ± 0.02	2.5 [68], 3.13 [69]
Hemoglobin	6.88 ± 0.01	6.43 [62], 7.04 [60]	3.00 ± 0.01	3.6 [70]

(see Table 3). The value $\rho_{3Å}$=1.15 g/ml obtained for the lysozyme is in an excellent agreement with that reported by Merzel and Smith [21] for this protein. This result is also in qualitative agreement with results obtained by Svergun et al. [22]. It is interesting to remark that there is a positive correlation between the $\rho_{3Å}$ density and the protein mass $M - \rho_{3Å}$ increases towards higher mass values. This effect is directly related to another phenomenon: the hydration level δ is not correlated with the protein mass. Taking into account that hydration is a surface effect, δ should be proportional to $M^{-1/3}$ in a first approximation, however neither experimental nor MC data show such correlation. It seems to be likely that a denser hydration layer builds up on the surface of heavier proteins.

3.4. Test of hydrodynamic parameters

The hydrodynamic parameters are affected by the shape of the outer surface of particle, the results being mostly sensitive to "radial" shifts of the surface. An equidistant 0.1 Å radial shift of the whole surface changes the diffusion coefficient and the intrinsic viscosity by ~0.6% and ~1.8%, respectively [16]. Taking into account that bound water molecules are located mainly in concave parts of the surface [19,21] the calculated hydrodynamic parameters are partially sensitive also to the tangential allocation of water molecules on the surface.

Calculated and measured diffusion coefficients and intrinsic viscosities are presented in Table 2 — showing a reasonable agreement.

3.5. Width of the thermal volume layer

The presented method has only one free parameter — the width Δ_p of the thermal volume layer, which can be used as a fitting parameter to reproduce the experimental partial specific volume data. As there is a reliable set of consistent experimental data on the partial specific volume of cytochrome *c* available in the literature (measured with modern techniques — see Table 3) this molecule is chosen to determine Δ_p in the first step. A strong dependence of the calculated partial specific volume on the assumed value of Δ_p is found. The modeling results are compared with the experimental data as it is shown in Fig. 5. Each MC calculation is repeated ten times with different starting value of the random number generator. The obtained results clearly show that the experimental data are reproduced when using thermal volume thickness of Δ_p=0.6 Å in the model. In the next steps the value Δ_p=0.6 Å (obtained

Table 3
Volumetric properties of chosen proteins determined by Monte Carlo simulation at Δ_p=0.6Å (n_h — number of bound water molecules; $V_M + V_T + V_h$ — sum of the protein intrinsic volume V_M, thermal volume V_T and volume of hydration layer V_h; v^0_{MC} — apparent specific volume; $\rho_{3Å}$ — water density in 3Å layer surrounding protein surface) and experimental specific volume v^0_{exp}

Protein	$V_M + V_T + V_h$ [Å³]	V_h [Å³]	V_T [Å³]	$\rho_{3Å}$ [g/ml]	v^0_{MC} [ml/g]	v^0_{exp} [ml/g]
Cytochrome *c*	22 539	4872	3474	1.144	0.733 ± 0.006	0.725 [43] 0.730 [6] 0.733 [44] 0.735 [11]
Ribonuclease A	25 093	5593	3847	1.165	0.711 ± 0.003	0.702 [11] 0.704 [43]
Lysozyme	25 938	5832	3853	1.150	0.718 ± 0.006	0.699 [11] 0.712 [43] 0.725 [45] 0.726 [6]
Myoglobin	32 467	6635	4539	1.176	0.746 ± 0.003	0.730 [45] 0.742 [11] 0.747 [43]
Chymotrypsinogen A	46 353	9203	5930	1.204	0.733 ± 0.005	0.717 [43] 0.727 [11] 0.731 [12]
Hemoglobin	117 197	23607	13 168	1.251	0.733 ± 0.003	0.743 [11] 0.745 [43] 0.746 [45] 0.751 [46]

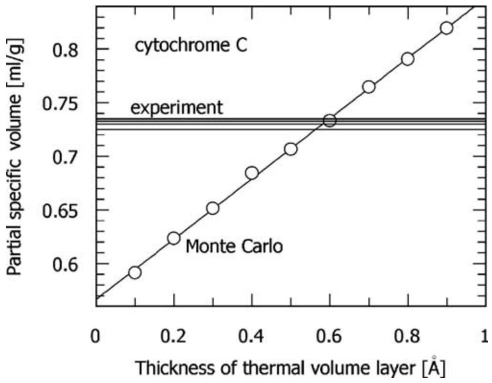

Fig. 5. Partial specific volume vs. thickness of thermal volume layer calculated for cytochrome c. Monte Carlo results are compared to experimental data presented at Table 3.

previously for the cytochrome c) is incorporated into the model. The calculation method is tested for the remaining five proteins with this fixed value of Δ_p. The partial specific volume v^0_{MC}, the hydration layer volume V_h, the thermal volume V_T and the water density $\rho_{3\text{Å}}$ in a 3 Å thick layer around a macromolecule are calculated for each protein. The calculations are repeated 10 times (in case of hemoglobin 5 times) using different seed points of the random number generator. Obtained results are presented in Table 3 together with the available experimental data of the partial specific volume v^0_{exp}. Within the limits of the experimental errors the calculated and measured data are in good agreement. The only exception is the greatest molecule — hemoglobin, for which the experimental partial specific volume is significantly higher than the corresponding MC result. Scanning the value of Δ_p in the model nearby 0.6 Å we found that the discrepancy in the case of hemoglobin can be eliminated while using a higher value of $\Delta_p=0.65$ Å.

4. Discussion

The results of our MC calculations indicate that in case of six chosen proteins the thickness of the thermal volume layer is $\Delta_p=(0.6–0.65)$ Å. This value is significantly lower then the thermal layer thickness of proteins reported by Chalikian et al. [11] but is close to the value found by Kharakoz [26] for water and low molecular mass compounds in water.

A principal question arising from this result is connected to the possibility that the absolute value of the (negative) interaction volume is underestimated in our model and consequently in real situation the thermal volume is greater and its layer is thicker. One possible verification of our results can be based on the data of Merzel and Smith [21] for the case of lysozyme. Using their approximation, that a $\delta=3$Å thick layer around a protein contains the first hydration layer with a modified density, Eq. (2) can be transformed to a form

$$V_I \cong V_{3\text{Å}}\left(1-\rho_{3\text{Å}}/\rho_f\right) \cong \delta\cdot\left(S_n+S_c+S_p\right)\left(1-\rho_{3\text{Å}}/\rho_f\right). \qquad (6)$$

Using the available surface area data [11] ($(S_n+S_c+S_p)=6685$ Å^2) and the mean relative density of the hydration layer [21] ($\rho_{3\text{Å}}/\rho_f=1.15$), one obtains an experimental approximation of the interaction volume to be $V_I=-3008$ Å^3. This approximation can be compared with the result of our calculation using Eq. (2) and the data presented in Table 1 ($n_h=296$) and Table 3 ($V_h=5832$ Å^3). The obtained interaction volume is $V_I=-3048$ Å^3, which is in very good agreement with the mentioned approximation based on experimental data.

In order to find an explanation for the above differences one has also to check other physical parameters that are related to the thermal volume layer. In the next part we give a discussion on some consequences of the different Δ_p values. Recall that there are two basic assumptions made by Chalikian et al. [11] concerning the behavior of the interaction and the thermal volume, namely that $V_I \sim S_p+S_c$ and $V_T \sim S_n+S_p+S_c$. We tested the relation between V_I, V_T and Δ_p as follows:

a) The interaction volume V_I is related to the volume of a single bound water molecule $V^0_h=V_h/n_h$. This value was determined by crystallographic studies (using the Voronoi polyhedra method) to be 22.9 and 24.5Å^3 for buried and near surface molecules, respectively [17]. The same quantity calculated on the basis of our MC model from V_h is $V^0_h=(18.2–22.2)$ Å^3. Taking into account that the results of Gerstein and Chothia [17] apply only for water molecules covered by a next layer of bound water and that there are also problems with the application of Voronoi polyhedra for atoms (molecules) on the surface [31], the agreement is satisfactory. Bound water molecules in our model touch the VdW surface of the protein and are mostly localized in concave parts of the surface. It follows that the calculated values of V^0_h are close to the minimal possible volume in the frame of the presented model.

b) The above results can also be compared with the consequences that follows from the work of Chalikian et al. [11], where $\Delta_p=\sim 1$ Å. From the experimental specific partial volume of the cytochrome c ($v^0=0.733$ ml/g, Table 3) the partial volume of $V^0 \cong 15080$ Å^3 is obtained. The total surface area $S_n+S_c+S_p$ of 6115 Å^2 as calculated by Chalikian et al. for the cytochrome c yields $V_T=6115$ Å^3 (assuming $\Delta_p=\sim 1$ Å). Neglecting the last term in Eq. (1) and using $V_M=(12{,}634 \div 12{,}733)$ Å^3 (see the discussion connected to Fig. 3 in the Results section and also the work of Chalikian et al. [11]) the interaction volume can be calculated as follows: $V_I=V^0-V_M-V_T=-3720$ Å^3. The number of bound water molecules is $n_h \cong 248$, a value consistent with the experimental hydration level δ_{exp} data (Table 1). Assuming water density of 0.997 mg/ml, the volume of free water molecules is $V^0_0=30$ Å^3. Taking all these data into account the volume of a bound water molecule $V^0_h \cong 15$ Å^3 is obtained from Eq. (2), which is significantly smaller than that reported by Gerstein and Chothia [17]. The discrepancy (indicating a questionable V_I value) is a direct consequence of the supposed value of $\Delta_p=1$ Å.

c) We also calculated the quantity (V_T/Δ_p) using our MC results and compared it with theoretical total water accessible surfaces of non-hydrated proteins as given in paper of Chalikian et al. [11]. It was found that these values are almost identical, our model yields (V_T/Δ_p) only a few percent lower than $S_n+S_c+S_p$. This comparison supports the assumption that the thermal volume is proportional to the size of solvent accessible protein surface as a first approximation. Based on the presented comparisons we conclude that there is no principal difference between the method of our calculations of the thermal volume and that presented by Chalikian et al. [11]. In both cases the thermal volume is approximately equal to the product of the water accessible surface area and the thickness of the thermal volume layer.

In summary, according to our calculation the thermal volume V_T is proportional to the total surface of the molecule, but the proportionality between the interaction volume V_I and S_p+S_c is in contradiction with both the experimental hydration data presented in Fig. 1 and with data on the volume of bound water molecules. It seems likely that the high value of $\Delta_p=1$ Å obtained for proteins in Ref. [11] (as compared to 0.5 Å for small solute molecules and 0.6–0.65 Å according to our calculations) is a consequence of this invalid proportionality.

More detailed analysis shows that the observed violation of proportionality is a consequence of preferred binding of water molecules in concave depressions on the macromolecular surface [17]. At these depressions the water molecule touches not only charged and polar surfaces, but also overlaps with some non-polar groups of the protein. Effectively, this means that even if water molecules are not bound to the non-polar atoms via HB, they can partially cover the non-polar surface. On the other hand, binding of water molecules over convex surface areas is less probable. This implication is also true over charged and polar surface places, since a single HB cannot ensure a long-term binding action. For such binding it is necessary to make more hydrogen bonds simultaneously with atoms on a distance smaller then the threshold of the HB. Here it should be stressed that water binding on convex surface areas is not excluded; only its probability is lower. Moreover, also a long-term HB between a water molecule and a charged or polar atom with great amount of convex surface can be very probable on the side of the atom where it contacts other atoms of the protein surface (since there is a high probability to realize next hydrogen bonds here). It follows that the hydration level depends in a complicated way on the combination of the surface shape and the distribution of charges on it.

Note that the radius of test spheres has only minor effect on the final value of the calculated thickness of the thermal volume. The influence of the radius size of test particles was checked using test spheres with VdW radius of water $R_t=R_w$. This change yields a surprisingly small deviation from presented results ($\Delta_p=0.635$ Å for cytochrome *c* — instead of 0.6 Å).

A substantial result can be obtained dividing the interaction volume V_I by the number of total hydrogen bonds between the protein and water molecules. The obtained ratio represents the volume effect of the formation of one hydrogen bond. For different proteins we obtained values in the range of ($-2.9 \div -3.8$) Å^3 with the corresponding mean value of -3.5 Å^3 (averaged over all the hydrogen bonds). Very similar value (-3.66 Å^3) was reported for small solute molecules by Kharakoz [26]. This agreement also supports our calculation method of the interaction volume.

Within the frameworks of the presented model only the static structure of hydrated molecules is taken into account (after allocation of water molecules on their mean position). Molecular dynamic studies can help one to estimate the influence of the intramolecular motion of component atoms on the surface of the macromolecule. It was shown in several papers [7,42] that the root mean square deviation (RMSD) of position vectors of native protein atoms (averaged over time) is in the range of ~1 Å and in rare cases exceeds 2 Å. This motion covers a wide range of time scales and amplitudes. It is evident that different modes of surface motion may have different contribution to the thermal volume.

In the first approximation, the contribution of the thermal motion as a whole can be neglected since the displacement of the surface is symmetric around a mean position (which is used in the present calculation), thus on average the volume differences are compensated. However, the volume of the free and/or bound water follows the quick movement of the protein surface in the direction towards the protein interior only partially. This dynamic effect depends on the frequency of the motion and also on the shape of the interaction potential between the surface and water molecules. This potential has a strong repulsive region (hard core) and a region with weak attractive forces. Finally, the local curvature of the surface may also influence the effect of the surface fluctuations on the thermal volume. Precise evaluation of the outlined ideas would be complicated but we suppose that this effect may be the reason for the slightly higher Δ_p in the case of proteins as compared to the free water or small solute molecules.

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