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A Hydrophobic Site on the Surface of the Angiotensin-Converting Enzyme Molecule

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Abstract—Using the hydrophobic fluorescent dye 8-anilino-1-naphthalenesulfonic acid (8-ANS), a hydrophobic site on the surface of the protein globule of angiotensin-converting enzyme (ACE) from bovine lung was found. The dissociation constant of the ACE–8-ANS complex was estimated as 1.5 ± 0.2 μM. This hydrophobic site is far from the ACE catalytic sites because the binding of the hydrophobic dye does not influence ACE activity. Shielding of the ACE hydrophobic site due to the complex formation with 8-ANS or Triton X-100 resulted in pronounced stabilization of the enzyme against the action of water radiolysis products during γ-irradiation of dilute solutions of ACE.

Key words: angiotensin-converting enzyme, 8-anilino-1-naphthalenesulfonic acid, Triton X-100, γ-inactivation

Hydrophobicity is responsible for the fact that nonpolar substances tend to form nonspecific complexes in polar solvents. For proteins, hydrophobic interactions are driving forces for manifestation of their physiological functions. Such important "internal" features of protein as formation and maintenance of the spatial structure is mainly caused by a tendency to seal off the hydrophobic groups attached to a polypeptide chain from a thermodynamically unfavorable contact with water [1]. The surface hydrophobic sites are also responsible for "external" protein interactions—binding to cell membrane, protein–protein recognition, formation of complexes with biologically active compounds, etc. [2].

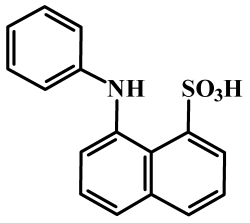
So, for proteins with unknown spatial structure mapping of surface hydrophobic sites supplemented with their quantitative characterization can be meaningful for structural and functional description of these proteins.

We present here an attempt to find and characterize hydrophobic sites on the surface of angiotensin-converting enzyme (ACE, peptidyl dipeptidase A, EC 3.4.15.1), which plays a key role in blood pressure regulation of mammals [3].

The newly synthesized ACE molecule consisting of 1306 amino acid residues has two hydrophobic sites—a signal peptide composed of 29 amino acids and a transmembrane domain composed of 17 amino acids [4, 5]. A soluble form of ACE does not have either of these sites: they are excised by proteinases. ACE is a glycoprotein with high (9-30%) content of carbohydrates [5], which should additionally increase hydrophilicity of the protein surface.

Both membrane and soluble forms of ACE are found as a monomer, various types of dimers, and tetramer in a system modeling biological membranes [6]. The carbohydrate chains of one ACE molecule interacts with the specific carbohydrate-binding site of another molecule [7]. However, for both ACE forms, this specific carbohydrate-binding site does not participate in formation of tetramers, and hydrophobic contacts between the interacting enzyme molecules are the most probable driving forces for this process.

We used Triton X-100 and fluorescent dye 8-anilino-1-naphthalenesulfonic acid (8-ANS) as the ligands able to bind to the possible hydrophobic sites on the ACE surface [8, 9]:



The main advantage of using 8-ANS as a hydrophobic probe is a significant increase in fluorescence quan-

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tum yield accompanied by a hypsochromic shift of the emission maximum upon sorption on hydrophobic sites of a protein surface [8]. This change in fluorescence characteristics of 8-ANS allows to determine free and bound in the equilibrium complex dye concentration [10]. Thus, using 8-ANS as a ligand, it becomes possible not only to state the formation of a hydrophobic complex, but also to give a thermodynamic description of the process of complex formation.

MATERIALS AND METHODS

The following reagents were used in this study: N^{α}-carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine (Cbz-Phe-His-Leu) from Bachem (USA); histidyl-L-leucine (His-Leu) from Serva (Germany); Triton X-100 from Ferak (Germany); *o*-phthalaldehyde from Koch-Light (Great Britain); 8-anilino-1-naphthalenesulfonic acid (8-ANS) from Sigma (USA); bovine serum albumin (BSA) from BelNIIEM (Belarus). Other reagents were of chemically pure and analytically pure grade.

Isolation of the soluble ACE form from bovine lung was performed by the method including Triton X-100 extraction of the enzyme and affinity chromatography on lisinopril-agarose as described earlier [11]. Electrophoretically homogeneous enzyme preparation was frozen and stored at −20°C.

The dissociation constants of ACE and BSA hydrophobic complexes with 8-ANS were determined using the titration curves of protein with 8-ANS. Protein concentration was determined by absorption of its solution at 260 and 280 nm and using a BSA protein assay kit from Pierce (USA). 8-ANS was dissolved in methanol and its concentration was determined via molar extinction $\varepsilon_{370} = 8 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ [12].

A solution of certain ACE or BSA concentration (about $5 \cdot 10^{-7}$ M) and various 8-ANS concentrations ($5 \cdot 10^{-7}$-10^{-4} M) in 50 mM phosphate buffer (pH 7.5) containing 150 mM NaCl and 1 μM $ZnCl_2$ was incubated for 3 h at 25°C to reach the equilibrium [10]; fluorescence intensity ($F\Sigma_i$ value) was measured then using a Hitachi MPF-4 spectrofluorimeter (Japan) at λ_{ex} = 350 nm and λ_{em} = 470 nm, slits 10 nm width. To calculate increase in fluorescence due to the complex formation, the fluorescence intensities of both 8-ANS solutions of corresponding concentrations in buffer (F_{ANSi}) and protein solutions in buffer (F_p) were also measured. For each of the 8-ANS concentrations used, increase in fluorescence due to the complex formation (ΔF_i) (Fig. 1) was determined by the equation:

$$\Delta F_i = F\Sigma_i - F_{ANSi} - F_p. \quad (1)$$

The specific maximal increase in fluorescence ΔF_{mi}(sp) on transfer of the 8-ANS molecule from aqueous to organic phase was determined from the plot of concentration dependence of fluorescence intensity of 8-ANS in benzene (F_{orgi}). For each 8-ANS concentration, the specific maximal increase in fluorescence (ΔF_{mi}) was determined as:

$$\Delta F_{mi} = F_{orgi} - F_{ANSi}. \quad (2)$$

The numerical value of ΔF_m(sp) was determined as the slope of the plot of ΔF_{mi} versus 8-ANS concentration.

The concentration of the 8-ANS–protein complex (C_b) was calculated using the equation:

$$C_b = \frac{\Delta F_i}{\Delta F m_i(sp)}, \quad (3)$$

and concentration of free 8-ANS (C_f) by the equation:

$$C_f = C_0 - C_b, \quad (4)$$

where C_0 is the total dye concentration.

The values of dissociation constants (K_d) and the number of binding sites (n) were obtained by processing the experimental data in Scatchard coordinates, C_b/C_f on C_b (Fig. 2).

The rate of hydrolysis of Cbz-Phe-His-Leu by ACE was monitored spectrofluorimetrically, determining the concentration of the reaction product His-Leu with *o*-phthalaldehyde [13]. The reaction was performed in 50 mM phosphate buffer, pH 7.5, containing 150 mM NaCl and 1 μM $ZnCl_2$, the substrate concentration 50 μM. The effect of 8-ANS on the rate of catalytic hydrolysis of Cbz-Phe-His-Leu was studied by measuring the rate of hydrolysis in the presence of $(0\text{-}5) \cdot 10^{-5}$ M dye.

The effect of 8-ANS and Triton X-100 on kinetics of γ-inactivation of ACE was studied by subjecting 10^{-8} M enzyme solution in 25 mM phosphate-borate buffer, pH 6.5, containing 150 mM NaCl, 1 μM $ZnCl_2$, and $(0\text{-}5) \cdot 10^{-5}$ M 8-ANS or 10^{-6} M Triton X-100, to γ-irradiation (^{137}Cs, 0.661 MeV, irradiation dose power 0.05 Gy/sec). According to kinetics of 8-ANS and Triton X-100 binding to ACE, the samples were incubated for at least 3 h prior to irradiation to establish the equilibrium. The absorbed dose was determined by irradiation time (kinetic integral dose at the constant irradiation dose power). The residual activity of preparations was estimated by the rate of catalytic hydrolysis of the substrate Cbz-Phe-His-Leu.

Thermal inactivation of ACE was performed by incubation of 10^{-8} M enzyme solution in 25 mM phosphate-borate buffer, pH 6.5, containing 150 mM NaCl and 1 μM $ZnCl_2$, for time interval from 0 to 60 min at 55°C. Then the residual enzymatic activity was estimated by the rate of hydrolysis of the substrate Cbz-Phe-His-Leu under the standard conditions.

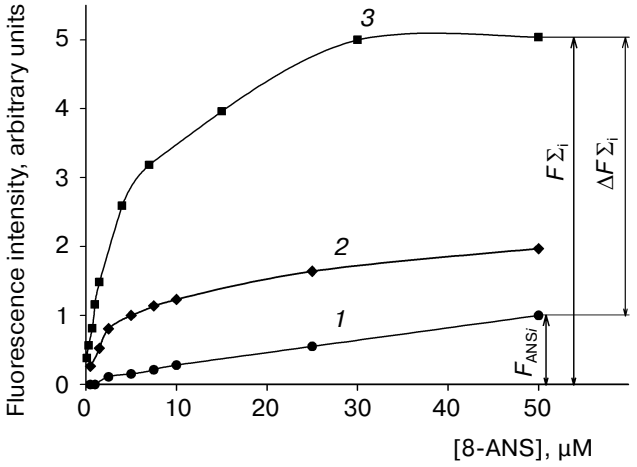

Fig. 1. Fluorescence intensity of 8-ANS and its complexes with ACE and BSA versus 8-ANS concentration: *1*) 8-ANS; *2*) 8-ANS in the presence of 0.5 μM ACE; *3*) 8-ANS in the presence of 0.5 μM BSA. Conditions: 50 mM phosphate buffer, pH 7.5, containing 150 mM NaCl and 1 μM $ZnCl_2$.

RESULTS AND DISCUSSION

Thermodynamics of formation of the hydrophobic complex. To determine the dissociation constant K_d and the number of binding sites n of the hydrophobic complex, it is necessary to estimate an intermediate value, $\Delta F_m(sp)$—the molar fluorescence increase provided that all dye molecules are bound in the complex. $\Delta F_m(sp)$ can be estimated by two methods: the first is to determine increase in 8-ANS fluorescence in organic solvent compared with that in water; the second is to determine a limiting value of increase in fluorescence on titration of a certain 8-ANS concentration with excess (up to 30-fold) protein [10].

Both techniques provide only apparent value of $\Delta F_m(sp)$. One of the common sources of errors is caused by the fact that microenvironment of the fluorescent dye molecule in various hydrophobic clusters of a protein depends on the nature and mutual position of a certain set of amino acids comprising this site. In organic solvent the microenvironment of 8-ANS will certainly differ from the microenvironment of the protein hydrophobic site, and while titrated with excess of protein $\Delta F_m(sp)$ will correspond to dye transfer from water to "the most hydrophobic" of all nonpolar sites on the surface of the protein. Besides this, uptake of 8-ANS molecules by the forming protein associates is possible on determination of $\Delta F_m(sp)$ by titration with high (up to 0.1 mM) protein concentrations. This results in overestimation of dye shielding from contact with water compared with experiments on determination of K_d and n in which significantly lower protein concentrations (about 0.5 μM) are used. It is also not completely correct to determine $\Delta F_m(sp)$ using low protein concentrations because dye molecules not bound in the complex are also present under these conditions.

Thus, in every case instead of the true $\Delta F_m(sp)$ value we can determine only overestimated effective value $\Delta F_m(sp)_{app}$; this results in underestimation of the experimentally determined n, but however, does not influence the K_d value. We consider the approach when $\Delta F_m(sp)_{app}$ is determined as increase in fluorescence on transfer of dye molecule from water to a nonpolar solvent to be more convenient because it does not require additional large amounts of protein.

Thermodynamics of complex formation of ACE with 8-ANS (Fig. 1) was compared with that of BSA—well studied, available and earlier characterized model protein [10]. The K_d value for 8-ANS–BSA complex was determined as 2.2 ± 0.2 μM (Fig. 2). It should be noted that this value is close to earlier published 1.2 μM, obtained with $\Delta F_m(sp)_{app}$ determined by titration of 8-ANS by protein [10]. The K_d value for the 8-ANS–ACE complex is 1.5 ± 0.2 μM which practically coincides with K_d for the 8-ANS–BSA complex. Comparing this with K_d values (1.1-80 μM) for other proteins given in [10], the 8-ANS–ACE complex can be considered as one of the most high-affinity ones.

For BSA, the effective number of hydrophobic binding sites per one protein molecule appeared to be 2.4 ± 0.2. If the planar 8-ANS molecule is supposed not to be positioned in the hydrophobic pocket exactly corresponding to it in size but to be "glued" to the protein surface, screening only one of its sides from the contact with water, then the $\Delta F_m(sp)_{app}$ value determined for transfer of

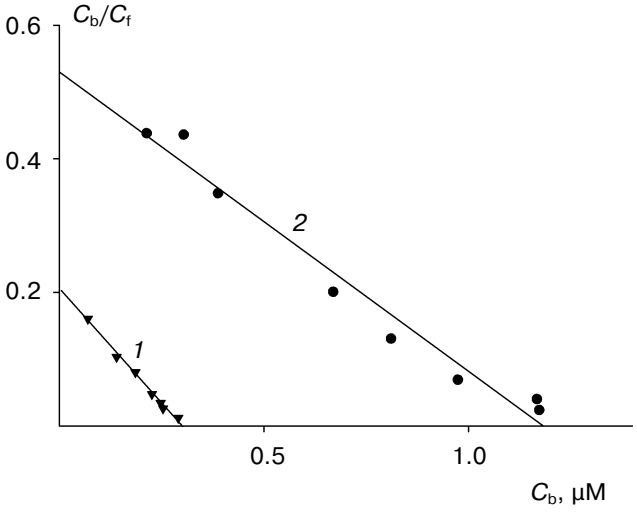

Fig. 2. Analysis of complex formation of ACE (*1*) and BSA (*2*) with 8-ANS in Scatchard coordinates. Conditions: 50 mM phosphate buffer, pH 7.5, containing 150 mM NaCl and 1 μM $ZnCl_2$.

the 8-ANS molecule from water to benzene can be at least two times overestimated. Thus, it becomes possible to state that there are not less than five hydrophobic sites on the BSA molecule. Cardamone and Puri evaluated *n* to be 10 [10]. The difference can be addressed to the use of different methods for determination of $\Delta F_m(sp)_{app}$ and the effect of buffer components (we used phosphate, while Cardamone and Puri [10] used Tris-HCl buffer). For ACE, the *n* value is 0.6 ± 0.1; this suggests that one enzyme molecule has only one hydrophobic site.

Sorption of a hydrophobic molecule of 8-ANS has no effect on the catalytic activity of the enzyme; this suggests that the discovered hydrophobic site is not related with the substrate-binding region of the ACE active site.

γ-Inactivation of ACE in the presence of 8-ANS or Triton X-100. In diluted solutions of enzymes, the irradiation effect is caused by reactions of the active products of water radiolysis, mainly $OH^{\cdot}$ and $H^{\cdot}$ radicals and also hydrated electrons e^{-}_{aq} [14]. The aromatic residues of tryptophan and tyrosine and also the histidine residue are considered to be the most sensitive to radiation [15]. We showed earlier that ~30% tyrosine and tryptophan residues (mainly at the cost of the solvent-exposed residues) can be lost due to γ-irradiation without any loss of ACE catalytic activity [16]. We supposed that if these residues are not only chaotically distributed on the protein surface but are incorporated into the hydrophobic site, their shielding with the hydrophobic molecule 8-ANS could be a significant stabilizing factor.

Under the experimental conditions used, ACE γ-inactivation in diluted solutions is described by the first-order kinetics with the rate constant k_{in} $2.5 \cdot 10^{-2}$ sec^{-1} (Fig. 3, curve *1*).

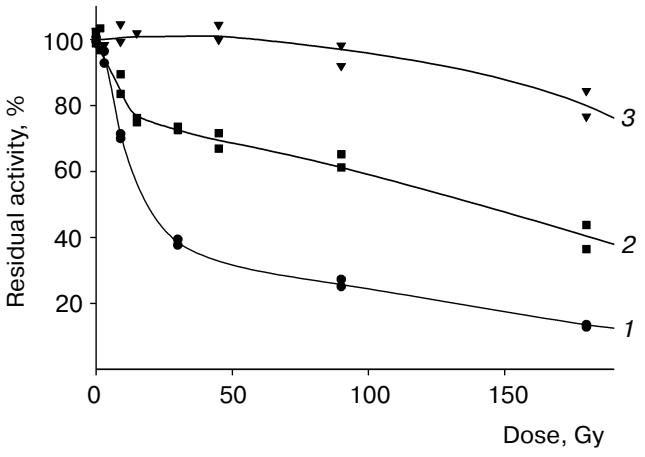

Fig. 3. γ-Inactivation of ACE: *1*) ACE; *2*) ACE + 50 μM 8-ANS, without preincubation; *3*) ACE + 50 μM 8-ANS, after 3 h incubation. Conditions: 50 mM phosphate buffer, pH 6.5, containing 150 mM NaCl and 1 μM $ZnCl_2$.

To determine the effect of 8-ANS on kinetics of ACE γ-inactivation, we chose conditions under which the hydrophobic site in more than 95% of the enzyme molecules should be shielded by the adsorbed dye molecule (50 μM 8-ANS, 3 h incubation, according to the K_d value and kinetic data from [10]). As a consequence, the kinetics of ACE inactivation could not be described by the first-order equation (Fig. 3, curve *3*). In this case the D_{80} value (an irradiation dose when enzyme retains 80% catalytic activity) increases from 6 to 180 Gy, what indicates a significant stabilizing effect.

In order to show that the observed stabilization is not only caused by the "effect of radicals uptake" provided by 8-ANS, but also caused by the shielding of hydrophobic site, we studied nonequilibrium γ-inactivation of ACE solution containing 8-ANS. In this case we began to irradiate immediately after addition of the dye, not waiting for achievement of the equilibrium which takes about 2 h [10]. As shown (Fig. 3, curve *2*), at short-time irradiation in the presence of 8-ANS enzyme stabilization is practically not observed and only after the longer irradiation time the stabilizing effect is increased probably due to the accumulated binding of 8-ANS. The result indicates that ACE stabilization against irradiation effect observed in the presence of 8-ANS is at least partly due to the shielding of hydrophobic site of the enzyme.

To exclude specific radiolytic effects, we obtained similar γ-inactivation data when a nonionogenic surfactant Triton X-100 at the concentration of 1 μM (lower than the critical constant of micelle formation) was used instead of 8-ANS. For this protector, a stabilizing effect also developed with time: the D_{80} value estimated under nonequilibrium condition increased 3 times and under equilibrium conditions 7 times compared with the intact enzyme.

It should be noted that the presence of the hydrophobic compounds in the medium had no effect on ACE thermostability; this points to the absence of conformational changes in the protein molecule as a result of hydrophobic interactions.

So, the observed stabilization of the enzyme against the action of the products of water radiolysis can be rationalized by the protector effect of 8-ANS shielding of the ACE hydrophobic site. Incorporation of the tryptophan residues into this site seems to be even more probable, because this is binding of aromatic compounds with the electron-acceptor properties to the tryptophan residues thus forming the charge-transfer complexes, which results in significant shift in the protein spectra [17].

It cannot be also excluded that the observed stabilization to γ-inactivation is not only due to the shielding of hydrophobic site, but also due to the changes in the accessible enzyme surface because of the minor local changes in position of the amino acid residues and oligosaccharide chains.

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