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ORIGINAL PAPER

Brownian dynamics simulation of substrate motion near active site of enzyme entrapped inside reverse micelle

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Abstract Brownian dynamics simulation has been applied to analyze the influence of the electrostatic field of a reverse micelle on the enzyme-substrate complex formation inside a micelle. The probability that the enzyme-substrate complex will form from serine protease (trypsin) and the specific hydrophilic cationic substrate Nα-benzoyl-L-arginine ethyl ester has been studied within the framework of the encounter complex formation theory. It has been shown that surfactant charge, dipole moments created by charged surfactant molecules and counterions, and permittivity of the inner core of reverse micelles can all be used as regulatory parameters to alter the substrate orientation near the active site of the enzyme and to change the probability that the enzyme-substrate complex will form.

Keywords Computer modeling · Substrate orientation · Enzyme-substrate complex · Reverse micelle

Introduction

The first step in many biochemical processes is a diffusional encounter between ligand and receptor molecules. It has been widely shown (Gabdoulline and Wade 1997) that long-range electrostatic interaction is a driving force of encounter complex (EC) formation. For a successful binding or reaction to occur, encounters must be correctly oriented in space. Mutual orientation of two interacting molecules is determined not only by the electrostatic potential of the reagents themselves, but by the electrostatic potential of the microenvironment as well. In this paper, we analyze the effect of surface potential of reverse micelles on the substrate orientation near the enzyme active site.

Surfactant-based reverse micelles are widely used to study enzyme actions in an environment that, on the one hand, mimics some features of life systems (Chang et al. 2000; Thaler et al. 2006; Meersman et al. 2005), and on the other hand, brings new peculiar properties to enzymatic action (Wang 2009). A reverse micelle is a colloid particle that consists of a water droplet (core) surrounded by a monolayer of surfactant molecules. The surfactant polar head groups are in direct contact with the water core and their nonpolar tails protrude into a water-immiscible apolar solvent (i.e., oil), which forms the continuous phase of the system. Many enzymes display a substantial structural stability and a high chemical activity in reverse micelles and demonstrate numerous specific distinctive features in comparison with their behavior in water (Klyachko and Levashov 2003; Holmberg 1999).

Reverse micelles have a multiple-factor influence on solubilized substances and the mechanisms of interaction between them. The micellar environment induces alterations in protein structure (Valdez et al. 2001; Creagh et al. 1993), stimulates changes in concentration of reagents in the reaction zone (Garcia-Rio and Leis 2000; Aguilar et al. 2001), and evokes reaction catalysis or inhibition by counterions and surfactant and co-surfactant molecules (Oldfield et al. 2005; Das et al. 2005; Dasgupta et al. 2005; Stupishina et al. 2001). All these factors can amplify or compensate for each other, but it is impossible to separate the action of individual constituents within the overall micellar effect within the experimental study. However, it is possible to estimate them by means of computer simulations.

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In view of the intense use of reverse micelles in enzymatic studies, a number of attempts have been made at theoretical research of micellar media (Sheng and Tsao 2001), including the protein-filled reverse micelles (Chen et al. 2000) and small substrate molecules (Ermakova and Zuev 2005). However, the matter of the electrostatic effect of micelles on the interaction of substrates with enzymes remains practically open.

The process of enzyme-substrate complex formation has two stages: in the first stage, the enzyme and substrate molecules diffuse towards each other to form the intermediate encounter complex (EC), and in the second stage, the system is transformed from the EC into the reactive or bound complex. If the transition of the EC into the reactive complex is faster than the formation of the EC itself, the rate of the enzyme-substrate complex formation will be determined by the rate of the EC formation. The Brownian dynamics (BD) method (Northrup et al. 1987, 1997; Andrew et al. 1993) allows one to determine the rate and the probability of the EC formation and is widely used to study protein-protein interactions (Thomasson et al. 1997; Gabdoulline and Wade 1997; Ermakova 2005) but very rarely used for research into protein interactions with low-weight ligands.

We previously applied this approach to studying Brownian diffusion of the substrate molecule to the active site of the enzyme from the oil phase into the reverse micelle when the substrate had to surmount the potential barrier of the charged micelle surface (Ermakova et al. 2008). It was shown that the surface potential of the reverse micelle could act as a specific electrostatic filter that facilitates or hampers the access of substrate into the reaction zone inside the micelle. In this work, we present results for the system where both the enzyme and the substrate are initially placed inside the water core of the reverse micelle to avoid the influence of the barrier properties of the micellar surface. The BD method was applied to study the influence of the micellar environment on the orientation of the substrate molecule relative to the active site of the enzyme under the influence of long-range electrostatic forces and thermal motion. We examined the influence of different factors such as the value and the sign of surface charge of the reverse micelle, the dielectric constant of the inner core of the reverse micelle, and the influence of counterions on the probability of the specific hydrophilic cationic substrate Nα-benzoyl-L-arginine ethyl ester (BAEE) and serine protease (trypsin) forming the EC.

Simulation model and methods

The BD method (Northrup et al. 1987, 1988) allows one to study thermal motion of two inflexible molecules of irregular form in solution under the influence of long-range electrostatic forces. We applied the MacroDox program (Northrup et al. 1997) (1) to calculate the electrostatic potential within the reverse micelle, taking into account the charge distributions all over the considered system, as well as the pH and ionic strength of the water core, (2) to run Brownian dynamics simulations, and (3) to calculate the interaction energy of the molecules in the EC and the probability of complex formation. The advantages and shortcomings of the BD method are discussed in detail elsewhere (Thomasson et al. 1997; Gabdoulline and Wade 1997; Ermakova 2005).

The structure of trypsin (code 1avw) was obtained from the Protein Data Bank (PDB; Bernstein et al. 1977), and the charge distribution for the protein was determined by the Tanford-Kirkwood method (Tanford and Kirkwood 1957). The dielectric constant of the protein molecule was taken as 4. The molecular structure of BAEE was optimized, and the charge distribution was determined by using the semi-empirical PM3 method (Stewart 1989a, b). The total charge of BAEE was equal to +e, being localized mainly on its guanidinium fragment. The ionic strength of the surrounding medium was chosen to be 0.1M, and pH value was 7.

The reverse micelle was modeled as a rigid sphere with point charges spread uniformly over its surface. The number of point charges per micelle was taken as equal to the surfactant aggregation number, i.e., $4\pi R^2/s$. The sphere radius (R) was defined in accordance with the known correlation (Lang et al. 1988; Bisal et al. 1990)

$$R = 3\left(\frac{v}{s}\right)W,$$

where v, s, and W are the volume of the water molecules, the cross-sectional area of the surfactant polar head (Bisal et al. 1990), and the water-to-surfactant molar ratio, respectively. R was taken as 30 Å, corresponding to a W of about 20. No barrier properties of the micelle, except its surface charge, were taken into consideration.

Dielectric properties of the water core in the reverse micelle differ from those of free water (Cohen et al. 2002). The dielectric constant of embedded water depends on the droplet size, type of surfactant molecule, solutes, or solubilizates, and it is spatially nonuniform, ranging from 9 to 78 (Mittleman et al. 1997; Lay et al. 1989). We particularly studied the influence of the dielectric constant on the probability of EC formation (see "Results and discussion"). In other calculations, we chose a dielectric constant of 30 to represent an averaged characteristic of the water phase. The electrostatic potential of molecules and micelle was determined by solving numerically the linearized Poisson-Boltzmann (PB) equation using the Warwicker-Watson (Warwicker and Watson 1982) method.

Brownian displacement of one particle relative to the other one under the effect of long-range electrostatic forces is defined according to the Ermak-McCammon algorithm (Ermak and McCammon 1978) by the equation:

$$r(t+\tau) = r(t) + \frac{D\tau}{KT}F + R(\tau)$$

where D is the spatially isotropic translational diffusion coefficient for relative motion of the particles, F is the direct interparticle (electrostatic) force, k is the Boltzmann constant, T is the temperature, and $R(\tau)$ is the random displacement vector used to simulate the solvent-mediated Brownian motion. A similar equation was used to describe the rotational motion of the particles. Translational (D_t) and rotational (D_r) diffusion coefficients for both molecules were defined by the MacroDox program using the Stokes-Einstein equations:

$$D_t = \frac{kT}{6\pi\eta R}; \quad D_r = \frac{kT}{8\pi\eta R^3}$$

where R is the radius of the protein or ligand and η is the viscosity of the medium. In our calculations, D was equal to 0.042 $Å^2\ ps^{-1}$, and D_r was $0.2 \times 10^{-4}\ ps^{-1}$ and $0.5 \times 10^{-3}\ ps^{-1}$ for the protein and substrate molecules, respectively. The electrostatic interactions (long-range forces) between the two reactants were included in this model together with the short-range repulsive forces, which were taken into account by means of excluded volume. No hydrophobic forces were taken into consideration, and the flexibility of both molecules was not considered in our simulations.

A large number of trajectories (the typical number was 50,000) gave statistically reliable results. The EC is accepted to be formed when two contact atoms of two molecules come as close as X Å, where X is the "reaction criterion" (RC). In this case, the trajectory was considered as successful. The fraction of successful trajectories was used to estimate the reaction probability for the EC formation.

Trypsin catalyzes the hydrolysis of peptide and ester bonds using the triad His-57/Asp-102/Ser-195 (Hedström 2002). The binding of the substrate to the active site of the enzyme is stabilized by the salt bridge between the positively charged amino acid residue of the substrate (guanidinium fragment in the case of BAEE) and the negatively charged binding pocket of trypsin (Asp-189). The charged atoms NE2 of His-57, OD2 of Asp-102, and OG of Ser-195 (according to the nomenclature of the PDB file for trypsin) were taken as contact atoms of the enzyme. For the BAEE molecule (Fig. 1), we selected the two oxygen atoms (carboxyl oxygen and oxygen of the hydrolyzed ester bond) (K1) and the two nitrogen atoms of the guanidinium fragment (K2) as the contact groups. We assume that

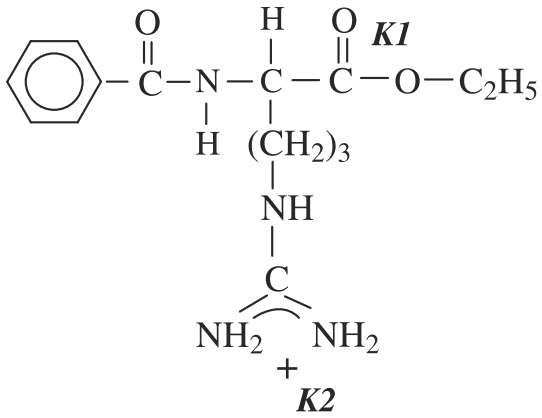

Fig. 1 Structure and contact groups of Nα-benzoyl-L-arginine ethyl ester

formation of the EC K2 is the necessary step for the enzymatic reaction since incorporation of the BAEE guanidinium fragment into the binding pocket of the enzyme has to be the initial stage in the multi-step reaction of the enzymatic catalysis. In the opposite case (EC K1), the substrate molecule is faced with the active site in a "nonworking" orientation.

The starting point for computation of the substrate trajectory was chosen inside the reverse micelle. The center of mass (COM) of the protein molecule was fixed in the center of the micelle. The COM of the substrate molecule was randomly placed at the spherical surface with a radius of 27 Å from the COM of the protein molecule. The substrate molecule was allowed to rotate and to translate. If the distance between the COMs of substrate and protein molecules expands to the escape radius (a standard parameter of 200 Å was used), the trajectory is terminated. Depending on the adjusted electrostatic potential of the micelle, a single calculation takes from 30 min to several hours of CPU time using a 3,300 MHz processor.

Results and discussion

The charge value of the micelle surface is determined by the degree of charge dissociation of the surfactant head groups. The overall charge of the micelle surface is defined by the surfactant ions and the screening action of counterions. In our model, we projected the charge of the micelle surface in two ways. In the first one, calculations were made for the uncompensated surface charge of the reverse micelle where each surfactant molecule is modeled as a point charge. This effective point charge reflects a different degree of screening effect from counterions. In the second case, the joint effect from the surfactant ions and counterions in the surface charge value is realized by a dipole moment at every surfactant molecule. Here, in addition to

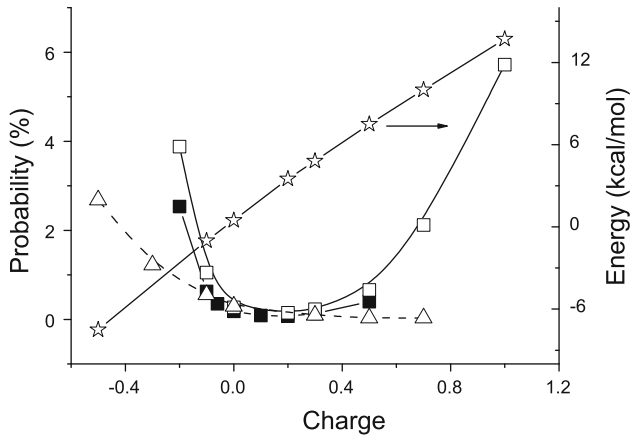

Fig. 2 Energy of enzyme-substrate interaction in the K2 complex (*open stars*), probability of EC formation for K1 (*filled squares*) and K2 (*open squares*) with a starting point for the substrate trajectory inside the micelle, and probability of EC formation for K2 (*open triangles*) with a starting point outside the micelle versus micelle surface charge (RC = 6 Å). The outside starting point models the system when the substrate molecule has to overcome the micelle barrier to reach the protein. The inside starting point reflects the system where the substrate is localized inside the micelle. Protein is assumed to be inside the micelle for both models

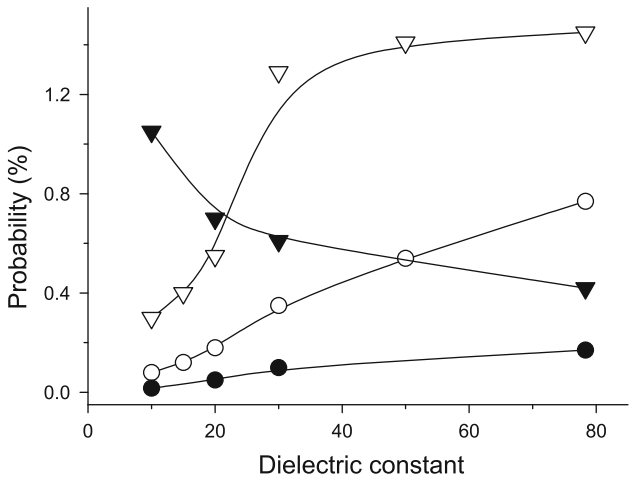

Fig. 3 Probability of the EC formation as a function of different dielectric constants of the micellar core for positively charged [K1 (*filled circles*), K2 (*open circles*)] and negatively charged [K1 (*filled triangles*) and K2 (*open triangles*)] micelles (RC = 6 Å)

the point charges uniformly placed over the spherical surface, the opposite charges are radially disposed at a fixed distance of 2 Å from the first charge family. In this case, the electrostatic field of the reverse micelle is determined by the dipole moments, which are spread over the spherical surface with the overall dipole moment of the micelle equal to zero. The positive dipole moment is related to anionic surfactant, whereas the negative one represents the case of cationic surfactant.

The influence of the uncompensated surface charge of the reverse micelle on the probability of the EC formation and on the energy of enzyme-substrate interaction is shown in Fig. 2. The probability of forming the complex K2 for an uncharged micelle ($q = 0$) is higher than forming the EC K1 complex because it is sterically less difficult. A negative potential of the micelle (anionic surfactant) increases the probability value of the EC formation for both contact groups. The probability of forming the EC K2 exceeds that of K1. The energy of both complexes is negative and, thus, they are stable. A positive potential (cationic surfactant) enhances the probability of formation for both complexes as well. However, the energy of enzyme-substrate interaction in the case of cationic surfactant is positive and increases as the positive potential of the micelle increases. Under these conditions, the EC is unstable and has to disintegrate rapidly without transition to the enzyme-substrate reaction complex. For comparison, the probability of K2 EC formation with a starting point for the substrate trajectory outside the reverse micelle is shown in Fig. 2 (Ermakova et al. 2008). In this case, the positive potential of the surfactant layer creates a barrier and keeps a positively charged substrate out of the reaction zone, which is localized inside the reverse micelle. The negative surface charge also hampers the penetration of substrate molecule inside the micelle, probably due to anchoring of the positively charged substrate at the micelle surface.

The size of the reverse micelle and the use of different additives give rise to the alterations in the water properties, including its dielectric constant. Figure 3 depicts the influence of the dielectric constant of the water medium on the probability of the EC formation. The decrease in dielectric constant inside the positively charged reverse micelle gives rise to the electrostatic repulsive interaction between the positively charged substrate and the potential created by both the enzyme and the positively charged surface of the micelle. As a consequence, the substrate molecule is pushed out from the reaction zone. Therefore, the probability of the EC formation decreases for both K1 and K2 complexes. The decrease in dielectric constant inside the negatively charged reverse micelle results in a more complicated dependence. For the EC K1, we detected the reverse picture in comparison with the positively charged micelle—as the dielectric constant decreases, the electrostatic attraction of substrate to the active site of the enzyme increases and the probability of the EC K1 formation increases. However, the probability of forming the EC K2 decreases with a decrease in dielectric constant.

We have assumed that such behavior might be caused by reorientation of the substrate molecule during its movement to the active site of the enzyme in the electrostatic field produced by the micellar surface and the enzyme. To prove this hypothesis, we analyzed the changes in the probability of the EC K2 complex forming when the

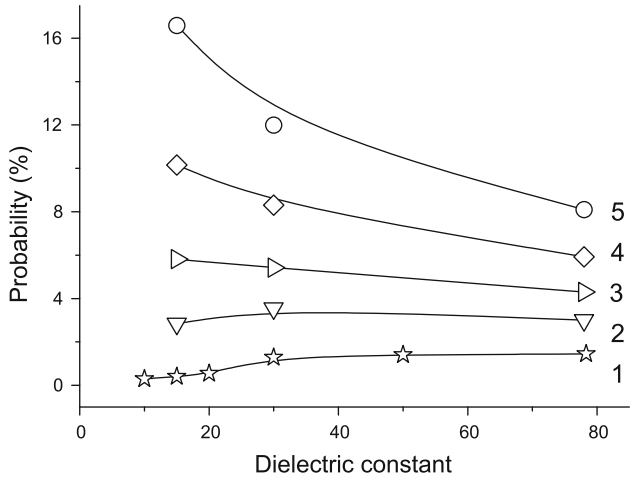

Fig. 4 Probability of EC formation for K2 as a function of different dielectric constants of the micellar core of negatively charged micelles for different RCs: 6 Å (*1*), 8 Å (*2*), 9 Å (*3*), 10 Å (*4*), and 11 Å (*5*). Different RCs reflect the different distances from the active site of the enzyme. *Lines* are shown for clarity

substrate approaches the active site of the enzyme (the alteration of the RC lets one characterize the radial distribution of the electrostatic potential inside the micelle core) in mediums with different dielectric constants (Fig. 4). At RC values equal to or exceeding 9 Å, the observed dependence looks the same as for the EC K1 in the negatively charged micelle (Fig. 3) when the increase in dielectric constant weakens the electrostatic interaction between the positively charged substrate and the electrostatic potential created by the negatively charged micelle. At distances smaller than 9 Å (RC 6 and 8 Å in Fig. 4), one can see that the dependence of EC K2 is similar to the substrate behavior in the positive electrostatic potential (Fig. 3). In our opinion, this is evidence of changes in the balance of electrostatic potentials of both the micelle and the irregularly charged enzyme in the vicinity of its active site. Consequently, our calculations show that irrespective of the potential sign, the strong electrostatic interaction in combination with the low dielectric constant of the medium decreases the probability of the EC formation for K2, which is a necessary step for the enzymatic reaction since the incorporation of the BAEE guanidinium fragment into the binding pocket of the enzyme is the initial stage in the multi-step enzymatic reaction (Hedström 2002).

Thus, the effect of the electrostatic field of the micelle surface on the probability of EC formation can vary in different ways: by alteration in the sign and the value of the micelle electrostatic potential and the dielectric constant of the micelle core. In real reverse micelles, the former can be achieved through the choice of surfactant and co-surfactant and the latter by changes in the size of the reverse micelle and by using different co-solvents.

In the second model, we imagine each surfactant molecule as a dipole, and the overall charge of the reverse micelle equals zero. In this case, each surfactant molecule acquires a dipole moment (the positive dipole moment is related to anionic surfactant and the negative one represents cationic surfactant), and the radial distribution of micelle electrostatic potential becomes more complicated. Figure 5 depicts the changes in the probability of the EC formation as a function of the surfactant dipole moment. The dipole moment of the cationic surfactant creates an additional positive potential inside the micelle and hence in the region of the enzyme active site, thus obstructing EC formation, whereas the anionic surfactant produces an additional negative potential and contributes to EC formation. Our calculations show that micelles that are based on cationic surfactants increase the probability of EC formation for both K1 and K2, whereas for the anionic ones the tendency is the reverse. The probability of EC formation for the "correct" EC K2 exceeds that for the EC K1 in the examined range of dipole moment alterations.

Despite the wealth of experimental studies concerning the influence of micellar environment on the rates of enzymatic reactions, it is difficult to compare directly the obtained results with the known experimental data. The reason is that in the experiment the effects of all relevant factors are observed simultaneously. Therefore, we can only speak about the consistency of our calculations compared with the known experimental data. For example, we previously examined (Zakharchenko et al. 2008) the influence of reverse micelles in terms of the anionic surfactant bis(2-ethylhexyl) sodium sulfosuccinate on the elementary rate constants for BAEE hydrolysis catalyzed by trypsin and determined that the reverse micelles

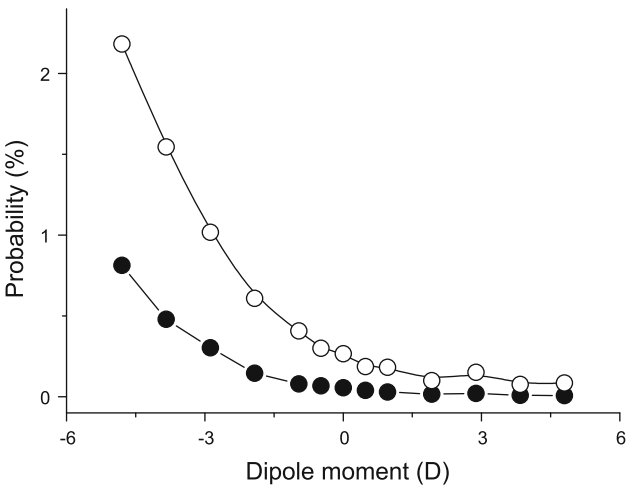

Fig. 5 Probability of EC formation for K1 (*filled circles*) and K2 (*open circles*) as a function of surfactant dipole moment (RC = 7 Å). The positive dipole moment is related to anionic surfactant, whereas the negative one represents the case of cationic surfactant

significantly modify the rate constant of the enzyme-substrate complex formation. Levashov et al. (1980) have shown a significant fall in trypsin activity in hydrolysis of the positively charged substrate in reverse micelles based on the anionic surfactant in comparison with the cationic one. However, the activity of the enzyme is usually characterized by the catalytic rate constant and the Michaelis-Menten constant, which are the complicated combination of the reaction elementary stage rates (Hedström 2002). Moreover, as mentioned in the "Introduction," the micellar environment results in alterations of protein structure, stimulates changes in concentration of reagents in the reaction zone, and evokes acceleration or inhibition of reactions by counterions and surfactant and co-surfactant molecules. At the same time, we have shown that the orientation of the substrate molecule depends on the distribution of electrostatic potential created not only by the enzyme itself but by the adjacent surface as well. The proposed model system and the applied simulation procedure can be useful not only in micellar enzymology and in the forecast of different biocatalytic applications but also for the modeling of protein-ligand pairing in living systems.

Conclusions

In this work, we applied the Brownian dynamics simulation to analyze the influence of the electrostatic field of the reverse micelle on the formation of the enzyme-substrate encounter complex. It was shown that surfactant charge, dipole moments formed by the charged surfactant molecules and counterions, and the dielectric constant of the inner core of the reverse micelle can be used as regulatory parameters to make alterations in the substrate orientation near the enzyme active site and to change the probability of the reactionary enzyme-substrate complex formation inside reverse micelles.

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