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Discussion Letter

Does biocatalysis involve inhomogeneous kinetics?

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Biocatalytic reactions can occur according to two very different mechanisms: homogeneous, which is described by standard transition state theory (TST) and its modifications, and inhomogeneous (polychromatic), which is characteristic for some of the charge-transfer reactions in liquids and amorphous solids. While most data published on enzyme reactions are interpreted on the basis of homogeneous kinetics, the important recent findings suggest the involvement of inhomogeneous kinetics mechanism.

Biocatalytic mechanism; Inhomogeneous kinetics; Transition state theory; Protein dynamics

1. INTRODUCTION

The mystery of the unique catalytic power of enzymes is a challenge to many researchers during several recent decades. Despite the great success in determining of protein structures at the atomic level of resolution, we are still unable to use these data for quantitative prediction of reaction rates. We need a better understanding of the origin of transition states, the role of protein structure, interactions and dynamics in substrate transformations. We have to re-examine our knowledge of enzyme catalysis and consider the possibility of correlation of substrate transformation event and protein dynamics on the basis of inhomogeneous kinetics mechanisms.

2. WHY OR WHY NOT THE TRANSITION STATE THEORY?

To explain the reaction mechanism the biochemical textbooks usually refer to TST even without critical discussion of its assumptions and limitations. One of them is the quasi-equilibrium assumption [1,2]. The transition-state species which originate from the reactant region are assumed to be present in equilibrium with reactants not only in total amount but also in the way they are distributed on their motion along the reaction coordinate. The other is the no-recrossing assumption which states that the substrate and the product parts of the phase space are separated and not recrossed. Solvent molecules and protein groups of atoms which do not participate in the primary catalytic event are considered as an 'adiabatic bath', which adjusts rapidly to changes in reaction coordinate of the primary system.

With these assumptions TST is most successfully applied to gas-phase reactions. To make it more appropriate to describe reactions in solutions a generalization by Kramers [3] and its modifications [4–6] are used. They treat the effects of reaction medium as an action of a random frictional force that retards the motion along the reaction coordinate and makes it diffusive. Being a substantial improvement the Kramers approach is still unable to describe properly many reactions in solutions, especially those which are fast, while the medium is slow, viscous and polar. The solid-state reactions are not adequately described either.

3. ARE PROTEINS MICROSCOPIC LIQUIDS OR SOLIDS?

Being accustomed to images of three-dimensional protein structures we usually believe that protein molecules in solutions are microscopic crystals. Meanwhile we apply TST, which is the theory best applicable to reactions in gases, less successfully to liquids and virtually non-applicable to solids. It is not easy, however, to find a simple macroscopic model for a protein [7] which in many respects behaves as amorphous solid and glass. Such a view is supported by the analysis of X-ray diffraction Debye-Waller factor, Mössbauer spectroscopy

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Abbreviations: TST, transition state theory; LE, locally excited; CT, charge transfer.

and scattering of Mössbauer radiation, low-temperature heat-capacity measurements and thermal expansion coefficient [8,9]. Convincing in this respect are the data on red-edge photoselection spectroscopy [10–12] which demonstrate an extended inhomogeneous broadening of protein electronic absorption spectra due to statistical distributions of protein aromatic groups on interaction energies with the environment. Such a distribution is typical for polar amorphous solids, and is of similar magnitude, about 100–500 cm^{-1}. The dielectric relaxations cause disappearance of the effect of inhomogeneous broadening with time. The relaxation rate, however, is slower by 3–5 orders of magnitude compared to aqueous solvent and to other low-viscous liquids at room temperature and usually is observed at nanosecond and longer times.

4. ORIGIN OF INHOMOGENEOUS KINETICS

Inhomogeneous kinetics is expected to be observed when the reaction rate depends strongly upon the interaction of the reactant with the surrounding and this interaction does not change fast enough with the motion along the reaction coordinate. Each reaction site (and each enzyme molecule if the biocatalysis is involved) is different from the others in terms of its intermolecular interactions. The population of these sub-states forms a distribution function on interaction energy in the reactant (or product) state and also in the transition state. The three characteristic cases can be singled out dependent upon the correlation between the rate of barrier crossing motion k_a and the relaxation rate of the reaction site environment, k_r.

(i) $k_a \ll k_r$. The dynamics is much faster than the barrier crossing. This is the case of application of TST and the Kramers theory. The reaction rate is sub-state independent and exponential with time.

(ii) $k_a \gg k_r$. The barrier crossing occurs in the rigid environment. The sites are distributed between different sub-states, and the activation energy E_a is site-dependent. There should be a distribution on E_a and thus, on k_a values. The reaction is non-exponential with time – the species with lower E_a react faster. Since the reaction rate is site-dependent, in case the selection can be produced at the starting point of the reaction, the site-selection-dependent kinetics should be observed.

(iii) $k_a \sim k_r$. The barrier crossing occurs simultaneously with the relaxation. This is the most complicated case since the site-dependent inhomogeneity in the reaction should exist initially and then it will vanish with time in the course of relaxation. For biocatalysis this is probably the most interesting case. If either unrelaxed or relaxed state produces extremely high barrier for the transition, and it changes with time, then the reaction rate could be easily modulated by the dynamics in protein matrix.

5. INDIRECT PROOF FOR INHOMOGENEOUS KINETICS IN ENZYME REACTIONS: NON-ARRHENIUS BEHAVIOUR

Linearity in the temperature dependence of the reaction in Arrhenius coordinates log k_a vs. $1/T$ is usually used as an argument for a mechanism described by TST. In enzyme reactions, however, the deviation from linearity is rather a rule than an exception [13–16]. Usually the slope changes so that the enzyme inactivation at low temperatures could be suspected. Instead of (or in addition to) temperature function of the exponential term $\exp(-E_a/RT)$, the change in temperature could influence the protein dynamics by changing the correlation between k_a and k_r and thus switching the reaction mechanism from inhomogeneous to homogeneous. The recent data [17] point to the fact of dramatic differences in temperature dependences of kinetic rates between the same enzymes isolated from normal and thermophilic strains of bacteria. These differences are probably not in activation energy E_a values since there could be no difference in positions and reactivities of catalytic groups, but in the rates of protein relaxations, which produce temperature-related time dependence of E_a by changing the correlation between k_a and k_r.

6. DIRECT EXPERIMENT FOR NON-ENZYME REACTIONS: REBINDING KINETICS OF MYOGLOBIN WITH LIGANDS

The kinetics of the rebinding of ligands (O and CO_2) with myoglobin can be easily studied by flash-photolytic technique. At temperatures below 200 K in glycerol–water mixed solvent the kinetics are highly non-exponential [8,18] and its temperature dependence is non-Arrhenius [19]. Similar non-exponential kinetics has been found for ligand rebinding to other heme proteins [8].

Following the flash photolysis a temporal shift in the near-infrared absorption peak of myoglobin was observed, and an universal, temperature-independent correlation between this shift and the rebinding kinetics was observed [20]. The probable explanation of this finding is that within inhomogeneous population of myoglobin molecules the species with lower electronic transition energies react faster giving rise to a shift and narrowing of the band. It was suggested that the protein structural relaxation alters the barriers for ligand rebinding [19–23]; this process is started from inhomogeneous distribution of protein environments which are unrelaxed and the relaxation occurs simultaneously with the rebinding.

7. FLUORESCENCE EMISSION KINETICS

In the case of no inhomogeneity in the ground-state fluorophore interactions and the absence of excited-

state reactions the fluorescence decay should be a mono-exponential function of time. However, the decay kinetics of fluorescence emission in proteins even with single tryptophan residue is usually non-exponential [24]. This peculiarity may arise from different probabilities of quenching between protein sub-states which vary in interaction of tryptophan chromophore with surrounding groups of atoms. If this is the case, then the transitions between sub-states should be slower than the rate of fluorescence emission, which is of the order of 10^{-9} s. The description of non-exponential decay by a continuous distribution of decay rates [25–27] allows one to observe the sharpening of this distribution on the increase of temperature which probably reflects thermoactivated recrossing between sub-states. The rare earth terbium ion bound to calmodulin molecule displays a transition of fluorescence decay from mono-exponential to nonexponential at temperatures below 200 K [28], indicating the change of fluorescence quenching mechanism.

8. A MODEL ELECTRON-TRANSFER REACTION IN THE LIGAND-BINDING SITES OF PROTEINS

An advantageous approach to study inhomogeneous kinetics is the investigation of reactions in the electronically excited states. This allows to start the reaction by a short light pulse and observe the fast kinetics in time domain [29] simultaneously with the kinetics of dielectric relaxations in the protein or solvent matrix [30]. Moreover, since the excited-state lifetime, τ_f, serves an intrinsic time marker in the nanosecond time range, a valuable piece information can be obtained in conventional steady-state experiments [30,31]. Site-selectivity can be achieved very easily by modulating the excitation wavelength at the red (long-wavelength) edge.

We applied this approach to study the excited-state electron transfer reaction in bianthryl, liganded to protein molecules [32]. Bianthryl is an anthracene dimer which undergoes an electron transfer from the hydrophobic locally-excited (LE) to highly polar charge-transfer (CT) state, and these states have substantial differences in fluorescence emission spectra. In rigid environments (e.g. in low-temperature propylene glycol glass) the inhomogeneous kinetics is spectacular [30,31]. The fluorescence spectrum changes from that of LE state to structureless and long-wavelength shifted spectrum of the CT state by changing the excitation wavelength. If the same solvent is liquid, the spectrum of the CT state shifts further to longer wavelengths, and the kinetics becomes homogeneous (excitation-wavelength independent). With bianthryl liganded to different proteins we clearly observe the inhomogeneous kinetics of electron transfer: similarly to rigid model environments the relative contribution of the CT form in fluorescence spectrum is a function of excitation wavelength. For bianthryl complex with serum albumin the inhomogeneous kinetics is temperature-independent in the range 10–40°C [32]. The studies of bianthryl complexes with specific anti-bianthryl antibodies and with apomyoglobin are now in progress in our laboratory.

9. ELECTRON-TRANSFER KINETICS IN PHOTOSYNTHETIC REACTION CENTER

The kinetics of electron transfer in bacterial photosynthetic reaction center is exponential at room temperature, but it becomes strongly non-exponential when the centers are cooled under illumination (in the charge-separated state) [33,34]. This suggests the distribution of reaction centers with different rate constants. It could be the result of configurational inhomogeneity of the electron-transfer chain [35], which at higher temperatures is averaged due to rapid cycling between the sub-states. In favour of such an explanation are the results on temperature dependence of fluorescence and phosphorescence spectra. The relaxational changes in these spectra correlate with the changes in the pattern of reaction kinetics [36].

An interesting result has been obtained recently: the low-temperature kinetics can be modulated by the pulses of the far infrared light [37], which suggests the reaction to be coupled with protein dynamics (the transitions between sub-states), and these dynamics can be activated by light pulses.

10. DEPROTONATION STEP IN THE PHOTOCYCLE OF BACTERIORHODOPSIN

Bacteriorhodopsin produces pumping of protons across the membrane upon the absorption of light. The deprotonation step (transition from L_{550} to M_{412} intermediate) occurs at the time of $=60$ μs. Its kinetics is inhomogeneous and can be decomposed into two rate constants differing by one order of magnitude [38]. The retinal pocket contains a cluster of charges which should produce highly inhomogeneous and anisotropic electric field [39]. Variations in reaction rates can be achieved by even small displacements of charges and dipoles and their slow motions compared to characteristic time of the reaction step.

11. TIME-CORRELATION OF EVENTS

Thus inhomogeneous kinetics can be observed in different protein reactions. Usually such kinetics is the slow kinetics in which the transition state is separated from the reactant state by high solvent–reorganizational barrier. The protein matrix should be considered as a specific 'solvent', many properties of which may derive from unique three-dimensional construction of the molecule. For instance, the performed protein construction may allow creating local non-zero electric

fields which may modulate the energy profile in order to facilitate catalysis [40–42]. If the dynamics of the neighbouring protein dipoles are fast, this effect should be less efficient due to dielectric screening. The benefit of the fast relaxations, however, is that many configurations of the reactive site surrounding can be realized during single reaction step and the reaction event could occur at the most effective moment. The very high catalytic efficiency of enzymes drives the reaction to faster time scale, while the slowing down of dynamics (which is characteristic of proteins [10–12]) should produce the effect in opposite direction. This could enforce the enzyme evolution in the direction of convergence of the rates of protein dynamics and the rates of the catalyzed reactions. The idea on time correlation of these events was suggested earlier on the basis of general stochastic considerations [43] and it should be animated in view of the new findings.

Remarkable in this regard is the present state of understanding of ion conductivity and selectivity by membrane channel proteins. There are attempts to describe this reaction by both ion diffusional models and the models based on the concept of ion binding sites and application of TST [44]. Meantime we have to consider that the energy profile in the channel should substantially depend upon the relation between the rate of ion movement and the rate of relaxation of protein dipoles [45], and this is probably the way by which protein dynamics can modulate the ion transfer rate and produce ion-selectivity.

12. CONCLUSIONS AND PROSPECTS

The arguments presented above point to the necessity of revision of basic concepts in the description of enzyme kinetics. It seems probable that we are unable to find explanation to the rates and mechanisms of biocatalytic reactions, especially those which involve charge transfer, without introducing the new concepts that are being developed in the field of molecular photochemistry in liquids and amorphous solids [29,31]. The reaction rates can be controlled by dipole-relaxational dynamics of the protein matrix, and the basic mechanisms of the reactions can be very different from that described by TST. One of the important features of this mechanism is, probably, the inhomogeneous kinetics.

The theory is needed which could describe inhomogeneous kinetics in terms of sub-state distribution in the enzyme–substrate system, the rates of dielectric relaxations, the intrinsic barrier heights, the contribution of quantum-mechanical tunneling. This is the field of active research by theoreticians [46–48]. Most of them, however, consider the simple case of the reaction in homogeneous medium, for which the so called dielectric continuum approximation could be applied. How relevant is this approach if applied to protein reactions? This question should be resolved by comparative experimental studies of charge-transfer reactions, biocatalytic, and uncatalyzed in model media.

For low-activation-barrier electron-transfer reactions the solvent coordinate looks to be unidimensional, i.e. the isoenergetic solvent configurations have the same reaction probabilities [31]. Does this regularity hold true in organized and anisotropic protein environment? We have the powerful tool now relevant for this purpose – the site-directed mutagenesis, which can allow one to produce the desired changes in the protein structure and observe their effect on catalysis.

We really understand very little of the mechanisms of those protein reactions in which allosteric regulation, effects of memory and feedback control are involved. We have to learn how to discriminate the general features of protein behaviour (e.g. dielectric relaxations) from the specific ones which fit to a specified structure and reaction mechanism. This could simplify the analysis and decrease the number of important variables. If this is done, the description of protein function in conformational variables and the analysis of coupling of conformational motions with vibrations and transfer of charge [35,49,50] can be made at a more advanced level.

Computer-simulation studies of protein molecular dynamics are very important [51,52], and there is a belief that they should continue to play a substantial conceptual role in this field, especially if the simulations are started at inequilibrium initial conditions and conducted at different temperatures and longer times. This should require a new jump in the computational power.

Thus the new step of understanding biocatalysis is the step towards complexity. This is the necessary step, however, for better understanding nature.

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