The structural background of non arrhenius behavior of enzyme reactions – the role of conformational flexibility in the temperature adaptation of proteins

Statements of the Ph.D. thesis

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Budapest, Hungary

2009

Scientific background

The high functional specificity of enzymes is a result of their unique structure and surface, while their conformational flexibility and structural plasticity enable them to efficiently exert their catalytic power. These properties, which are characteristic of only protein molecules, are based on the forces and interactions found everywhere in nature.

One major peculiarity of enzymes is that their structural stability and activity can be upheld only under the appropriate environmental conditions, in a narrow temperature range. In our laboratory, we investigate the structural background of protein function. While investigating the influence of environmental factors on enzyme function, we observed several interesting phenomena. Because the conformation of individual proteins is only stable in a narrow physiological temperature range, I investigated the relationship between catalytic function, structural stability, and conformational flexibility on a series of orthologous enzymes of psychrotrophic, mesophilic and thermophilic origins, thereby broadening the experimental temperature range.

I aimed to address questions such as how the adaptation to environmental temperature is accomplished on the level of enzymes, and how the altered temperature optimum of catalytic activity is reflected in the conformational stability and structural flexibility of proteins. I also aimed to address the question what kind of physical interactions enable proteins to adapt to environmental conditions on the level of atomic interactions and motions.

The temperature dependence of chemical reactions follows the Arrhenius equation; the chemical rate constant grows exponentially as a function of temperature. The rate of enzyme-catalyzed reactions is modulated by the conformation and dynamic properties of the enzyme, hence the temperature dependence of the latter also influences that of the overall rate constant (k_{cat}), and, as a consequence, the temperature dependence of enzyme kinetic parameters often does not follow the usual linear relationships observed in the case of noncatalyzed chemical reactions. The possibility of coupling between protein dynamics and reaction kinetics emerged during investigation of the non-Arrhenius behavior, and the temperature dependence of conformational motions is suggested to influence the thermal behavior of the overall reaction rate of enzyme catalyzed reactions.

Aims

For proper enzyme function, the fine-tuned balance of two physical properties, structural stability and conformational flexibility, is required. Conformational motions are required for enzyme activity, while maintaining the native, three-dimensional enzyme structure is also a necessary condition. Enzymes are extremely vulnerable objects working only in a narrow temperature range. Comparative studies of enzymes with identical functions but adapted to environments with different temperatures are suitable for more in-depth investigations of potential correlations between flexibility, stability, and activity. I studied the temperature dependence of enzyme-catalyzed reactions, and the flexibilities of the catalyzing enzymes. My main objective was the detailed understanding of the correlations between enzyme activity and conformational flexibility. As experimental objects, I chose two dehydrogenases: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and isopropylmalate dehydrogenase (IPMDH).

- Through investigating a mesophilic and a thermophilic variant of GAPDH, I aimed to address the question how the adaptation to high temperature is accomplished in the conformational flexibility and stability of enzymes.
- Investigating the temperature adaptation of variants of IPMDH, I aimed to address the question: how general is the observation that the adjustment of conformational flexibility is the primary means of temperature adaptation? Do other strategies also exist?
- We observed that in the reaction catalyzed by *E. coli* IPMDH, the van't Hoff plot of the temperature dependence of the Michaelis-Menten constant of the substrate shows a distinctive sigmoid shape. I aimed to find the answer to the question what kind of physical interactions cause this previously unseen temperature dependence. We hypothesized that temperature-dependent changes in protein dynamics induce these changes. I aimed to find the exact reasons for the sigmoid temperature dependence experimentally.
- Previous studies showed that the enzymes with higher conformational flexibilities usually have higher enzymatic activities as well. I aimed to address the question how increasing the conformational flexibility of an enzyme by site-directed mutagenesis affects enzyme activity.

Methods

For the experiments, I used recombinant proteins produced in a bacterial expression system. The original plasmids were available, and I made several changes to them to achieve better protein expression efficacy. The site-directed mutagenesis was completed using a Quikchange mutagenesis kit. I applied the classical recombinant DNA techniques. Protein expression, purification, and analysis on SDS-PAGE gel electrophoresis are also routine methods. For the characterization of the enzymes, I designed kinetic measurements as a function of temperature, evaluated by assuming Michaelis-Menten kinetics. For the thermostability studies, I used differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy. The temperature-dependent changes were monitored by far-UV and near-UV CD spectroscopy. Fluorescence resonance energy transfer measurements were used for the determination of dissociation constants and for monitoring the large-scale domain-domain motions. H/D exchange monitored by FTIR was a powerful tool to monitor the local conformational fluctuations of proteins. The sequence alignments, graphical and comparative structural analyses were made done with assistance from my co-workers.

Scientific results and conclusions

The Arrhenius plots of both thermophilic *Thermotoga maritima (Tm)* and mesophilic rabbit muscle (*Rm*) GAPDHs are nonlinear, and their break points are shifted relative to each other by 17 °C. By measuring the conformational flexibility of *Tm*GAPDH by hydrogen-deuterium exchange, and comparing the results with those obtained with *Rm*GAPDH, it is found that at room temperature, the conformational flexibility of *Tm*GAPDH is much less than that of *Rm*GAPDH, but the flexibilities are comparable at the respective physiological temperatures of the two organisms. This result suggests a correlation between non-Arrhenius behavior of enzyme activity and conformational dynamics. The analysis of B factors – which reflect the local mobility of protein atoms – demonstrates that the largest differences in flexibility are seen in the coenzyme and substrate binding regions.
The results suggest a general correlation between conformational flexibility and enzymatic activity: structural rigidity results in low activity; higher flexibility is associated with higher activity.

The temperature dependence of the activity parameters of the thermophilic, mesophilic and psychrotrophic IPMDH are highly similar. The Arrhenius and van't Hoff plots of the temperature

dependence of catalytic parameters have the same shape; the differences are found in the absolute values: the activities of the enzymes are inversely related to their thermostabilities. The conformational flexibilities, which are essential for the function of the enzymes, are similar at the respective physiological temperatures.

The cold-adaptation strategy used by the psychrotrophic *Vibrio* sp. I5 IPMDH differs from the common cold-adaptation strategy of psychrophiles achieved by high conformational flexibility and heat lability. The results demonstrate that cold-adaptation is in this case achieved by the creation of an extremely efficient enzyme, which has reduced but still sufficient activity at low temperature.

In the reaction catalyzed by *E. coli* IPMDH, the van't Hoff plot of the temperature dependence of Michaelis-Menten constant of the substrate shows a distinctive sigmoid shape.

My CD and DSC measurements indicate that the sigmoidal change is not a consequence of temperature-induced conformational changes. The temperature dependence of the ΔG_{mic} values determined by H/D exchange measurements indicates a substantial decrease of the free energies associated with local unfolding events at around 30^{o}C (the temperature corresponding to the midpoint of the sigmoid observed in the Van't Hoff plot of the dissociation constant), which reflects an overall increase in conformational flexibility, and suggests the appearance of new fluctuation modes.

Large-scale conformational motions were characterized by FRET measurements. The *f'* parameter (the FRET efficiency normalized with donor emission) provides information on the conformational dynamics of the protein, in our case on the amplitude of relative domain motions. The value of the *f'* parameter of the IPM-free NADH-IPMDH complex significantly increases at 30^{o}C, which can be explained by the larger amplitude of hinge-bending domain motions.

The catalytic efficiency (k_{cat}/K_M) of IPMDH varies little over a wide temperature range, due to the unusual temperature dependence of the enzyme-substrate interaction (sigmoid-shaped Van't Hoff plot). At higher temperatures, the substrate affinity is lower, and the sigmoidal change may be related to the temperature dependence of domain-domain motions and local conformational fluctuations. The experimental results suggest an induced fit mechanism for IPM binding. The more intense hinge-bending fluctuations at higher temperatures increasingly interfere with IPM binding, thereby abruptly increasing its dissociation constant in the 20-40°C range and leading to the observed unusual temperature dependence of the catalytic efficiency.

In the case of IPMDH, I found a direct link between a certain type of conformational flexibility – the flexibility related to relative domain motions – and a certain parameter related to enzyme

activity – the dissociation constant of the substrate. The more intense fluctuations interfere with substrate binding, thereby decreasing catalytic efficiency. The result shows that even the direction of the flexibility-activity correlation depends on which fluctuation mode and which activity parameter is studied.

My H/D exchange experiments demonstrated that conformational flexibility can be increased by mutating non-conserved proline residues to glycines. The thermostabilities of these *Thermus thermophilus* IPMDH mutants are lower than that of the wild type, in proportion to the number of glycines introduced. Correlation is found between the thermostability and conformational flexibility of the mutant enzymes, but the correlation is not perfect. There is no significant correlation between enzyme activity and conformational flexibility. These results indiciate that modification of the physical properties (thermostability, conformational flexibility) of enzymes is quite straightforward using site-directed mutagenesis, but this method is certainly insufficient for improving enzyme activity.

In summary, my findings show that there is no general, direct, and simple correlation between conformational flexibility and catalytic activity. Certain types of flexibility are essential for certain steps of the catalytic process; thus, correlation is seen. However, catalytic function is the result of an interplay of several factors, of which flexibility is only one; hence the observed diversity of strategies for environmental adaptation in nature.

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