STRUCTURAL BIOLOGY APPROACH TO STUDY THE SPORULATION PROCESS

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Bacillus subtilis is a model organism for the study of one of the simplest cell differentiation process, called sporulation. A rich amount of genetic, biochemical and molecular biology data have been obtained during the study of this process. Recently, this study has advanced to the solving the tertiary structure of key protein regulators. This contribution focuses on the progress in protein crystallography oriented toward the understanding of sporulation mechanisms, that have been achieved in the last couple of years.

The phosphorelay is the main regulatory network in the initation of sporulation. Recently, the tertiary structures some of the components of this network were solved. The structures of response regulators Spo0F [5], Spo0A [2,3,4] and phosphotransferase Spo0B [7], together with biochemical and mutational data provide an important framework for further understanding of their biological function at the molecular level.

Structural data were also obtained from some proteins involved in activation of the first cell-type specific factor - ^{F} (structures of anti-anti- factor SpoIIAA and anti-factor SpoIIAB in the complex with ^{F}) [6,1].

The structures of only a small number from more than 100 sporulation specific proteins are known due to problems associated with the crystallization of these proteins. Structures for many of the interesting candidates remain a challenge. Among them are the membrane bound proteins and proteins with highly flexible domains.

Especially interesting for understanding of the mechanism of the transient gene expression asymmetry during sporulation would be the detailed study of sporulation septa formation by solving the crystal structure of key proteins involved in this cell division and chromosome translocation processes such as phosphatase SpoIIE, DNA translocase SpoIIIE or division protein DivIVA.

Work in authors laboratories is supported by grant 2/1004/22 from the Slovak Academy of Sciences and Wellcome Trust Project and Collaborative Research Initiative Grants (056247/Z/98/Z and 066732/Z/01/Z, respectively).

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STRUCTURAL BASIS OF PROTEIN METASTABILITY

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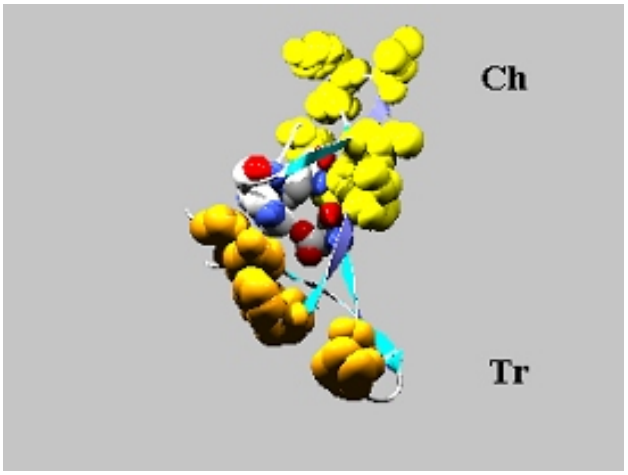
All information for the three dimensional structure of proteins and their functionality is encrypted within their amino acid sequences. The natural amino acid sequences of proteins have been perfected by evolution not only for their functional structure but also for a rapid and highly directional acquisition of their folded, fully functional state. An unambiguous distinction between these two possibilities is important for a clear-cut interpretation of consequences of amino acid replacements in protein engineering experiments.

The double-headed Bowman Birk serine protease inhibitor (BBI) built up from two triple stranded b-hairpin domains directed against trypsin and chymotrypsin was selected as a model protein. The double-headed arrangement of two independent subdomains facilitates the detection of long-range irregularities transmitted from the trypsin- into the chymotrypsin-inhibitory region used as a reference.

The exposed hydrophobic patches on the protein surface and a polar protein interior appear as structural peculiarities more reminiscent of the kind of structural features that have been postulated to occur in partially folded proteins rather than their folded state.

The autonomous refolding competence of the parent protein was abolished as s result of amino acid replacements, resulting in heterogeneous populations of conformers greatly differing in their activity. In contrast to the autonomous refolding competence of the parent protein, the variants require the presence of trypsin-Sepharose as a template with complementary structure in order to reach their fully active state. The fully active state of the variants attained by means of this method returns to the initial mixture of conformers upon subsequent incubation in the refolding buffer in a slow first order reaction. Therefore, the fully active state of the variants may be regarded as local energetic minima surrounded by high barriers of activa-



The exposed hydrophobic patch belonging to the trypsin-inhibitory region (Tr) is shown in ochre and that projecting out from the chymotrypsin-inhibitory region (Ch) is shown in yellow. Residues belonging to the buried polar interior are highlighted in CPK.

tion. The appearance of apparently metastable state supports a kinetic reaction control for the variants on the template although it cannot rule out a thermodynamic reaction control. In fact, the template facilitates folding not only kinetically, by reducing the high barrier of activation in solution but also thermodynamically by stabilising the fully active state by means of protein-protein-interactions. Protein metastability has also been documented for certain proteolytic enzymes after removal of their prosequences and for the native conformation of viral hemagglutinins. Presently, it is unknown whether the native conformation of soybean BBI corresponds to a global energy minimum or a metastable state on its conformational landscape. However, the inside-out situation in BBI and the conformational changes that are induced with reducing agents even in the absence of denaturants seem to be more in favour of the second possibility.

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SATURATION MUTAGENESIS OF L177 IN HALOALKANE DEHALOGENASE LINB

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Enzyme LinB is the haloalkane dehalogenase from bacterium *Sphingomonas paucimobilis* UT26. It is involved in a biochemical pathway for degradation -hexachlorocyclohexane. LinB catalyses hydrolytic dehalogenation of broad range of halogenated aliphatic compounds [1]. The amino acid in position 177 was identified as a very important determinant of catalytic properties of LinB by structural analysis [2] and by comparison of its protein sequence with other family members: (i) L177 is positioned in the mouth of the entrance tunnel leading the enzyme active site and is pointing directly to the tunnel and (ii) L177 is the most variable residue of the active site pocket among different haloalkane dehalogenases. L177 of the wild type enzyme was therefore replaced by every other amino acid and then the effect of mutations on enzyme activity was studied. Construction of the protein variants was conducted in two successive rounds. In the first round, L177 was replaced by A, C, G, F, K, T and W, respectively. The specific activities of the first set of mutants were statistically analysed but results from this analysis were not statistically significant. For that reason the first set of mutants was complemented with the second set of mutants comprising replacement of L177 by D, E, H, I, M, N, P, Q, R, S, V and Y, respectively.

All seven protein variants of the first set could be overexpressed in *Escherichia coli* and showed activity with at least some of the substrates used for characterization. In the second set, two out of twelve protein variants (L177E and L177N) could not be overexpressed in *E. coli*, while other two variants (L177P and L177I) did not show activity with any of the substrates. Circular dichroism spectra were recorded for all proteins purified in the second set and two inactive mutants showed spectra different from wild-type LinB and other mutants (Fig. 1), suggesting a decrease in the number of amino acids in -helical conformation and protein unfolding.

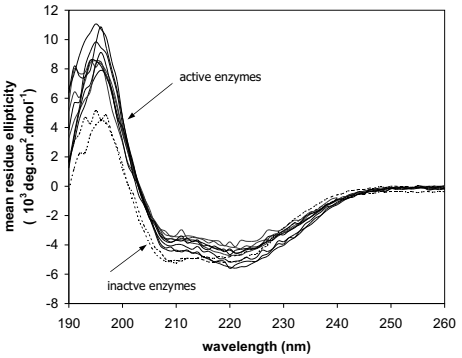

Fig. 1. Far-UV circular dichroism spectra of the wild-type haloalkane dehalogenase LinB, L177D, L177H, L177M, L177Q, L177R, L177S, L177V, L177Y mutants (solid lines) and L177P, L177I mutants (dashes lines). The spectra were measured at room temperature with the protein concentration 0.3 mg/ml in 50 mM phosphate buffer (pH 7.5) using the JASCO J-810 spectropolarimeter.

Successfully purified enzymes from both sets were kinetically characterized using a gas chromatography. Compounds 1-chlorobutane and 1,2-dibromoethane were selected as the substrates for steady-state kinetic measurements because they often serve as the reference compounds for characterization of the haloalkane dehalogenases. Dehalogenation of 1-chlorobutane showed typical Michaelis-Menten dependence, while dehalogenation of 1,2-dibromoethane showed substrate inhibition. Furthermore,