completely understood, but they seem to be expressed in pyramidal cells as well as in γ-aminobutyric acid (GABA)-containing interneurons (local-circuit neurons) and glutamate-containing nerve terminals that innervate the region[5].

Studies in non-human primates have begun to provide information about the functional consequences of dopamine-mediated neurotransmission in the prefrontal cortex. One view is that certain pyramidal neurons within the cortex form the cellular basis for working memory, and that dopamine can modulate this process largely through D1-like receptors[6]. There seems to be an optimal level of D1-like receptor activation, above or below which working memory is impaired, and similar mechanisms may operate in the cingulate cortex[7].

The reduced levels of D1-like receptors seen by Okubo *et al.*[1] in the prefrontal cortex of schizophrenic patients could, therefore, contribute to deficiencies in certain aspects of cognition. This is consistent with their observation that the lower the levels of D1-like receptors, the more severe the negative symptoms. On the other hand, antipsychotic drugs themselves decrease D1-receptor levels[2], so one must ask whether this drug-induced decrease mediates therapeutic or side effects of the drugs. And is the observed reduction in D1-receptor levels in schizophrenics part of the pathology of the disorder or a compensatory adaptation to that pathology?

Autopsy studies have also focused on cellular abnormalities in the prefrontal and cingulate cortices of schizophrenics. Variable results have been reported: a decrease in the number of GABA-containing interneurons[8]; a reduction in the amount of GABA made by these neurons, but no loss in their number[9]; a reduction in the dendritic processes of pyramidal neurons[10]; reductions in the levels of various presynaptic-nerve-terminal markers[11]; and increased levels of nerve-cell bodies in white matter, which suggests a defect in neuronal migration[12]. These discrepancies emphasize the need for further research. For example, it will be essential to determine whether schizophrenia involves the loss of cortical neurons and their inputs or, rather, a sustained impairment in the activity of existing neurons.

These autopsy studies are nonetheless encouraging because, for the first time, we have a lead as to the cellular pathophysiology of schizophrenia. The findings can also be reconciled with brain-imaging studies of the disorder. In general, autopsy studies point to a reduction in neuronal mass or activity, which could relate to the smaller volume of grey matter and reduced blood flow that are seen in the frontal cortex of schizophrenics[13,14]. The low levels of D1-like receptors reported by Okubo *et al.*[1] could reflect the reduction in dendritic processes of pyramidal neurons[10] (where the D1-like receptors are enriched[3]).

Nevertheless, it is critical to emphasize that many neurotransmitter and receptor types are probably involved in schizophrenia. For example, the serotonin 5-HT_{2A} receptors[15], which are localized to the distal dendrites of pyramidal neurons and some glutamate-containing nerve terminals (Fig. 1), modulate glutamate-mediated transmission. Hallucinogens — which mimic some of the positive symptoms of schizophrenia — are partial agonists at the 5-HT_{2A} receptors, whereas many antipsychotic drugs, particularly the 'atypical' drugs (defined by their improved efficacy and fewer side effects), are antagonists at these receptors.

One of the main questions in the field concerns the nature of the original insult that leads to cellular abnormalities in the prefrontal and cingulate cortices. Genetic and epidemiological studies indicate that there is a strong genetic component to the disorder, and that unidentified environmental factors are also required. Autopsy studies agree on the fact that there is no gliosis, inflammation or active neural degeneration, indicating that schizophrenia could involve an earlier insult during which time the damage is done. Alternatively, schizophrenia could involve a continuing pathophysiological process that impedes the functional activity of certain cortical neurons, but without obvious neurodegeneration. Reduced activity would be consistent with the clinical observation that some schizophrenics with severe chronic symptoms show dramatic improvement upon treatment with atypical drugs such as clozapine. If this model holds, it clearly offers much hope to schizophrenics as it should allow the development of more effective treatments.

Finally, it is important to emphasize that schizophrenia is probably a heterogeneous disorder, with several aetiologies resulting in a syndrome that has some shared clinical features. Brain-imaging and autopsy studies are based on the idea that, as well as some shared clinical features, there may be shared pathophysiological mechanisms. However, in medicine, the precedent is that similar-looking physiological and behavioural defects arise from distinct molecular and cellular abnormalities. In any event, sophisticated neurobiological hypotheses of schizophrenia are now emerging, which define the critical directions for future research. □

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Protein evolution

How far can sequences diverge?

Cyrus Chothia and Mark Gerstein

Protein interiors consist of closely packed amino-acid residues. Close packing places strong constraints on side-chain positions, and means that the large sequence changes that occur in some protein families over long periods of evolution lead to considerable structural variations — α-helices and β-sheets shift relative to each other, and loop regions change conformation[1]. These types of modifications are acceptable if they maintain the stability of the protein and do not adversely affect its function. Structural changes need not affect function if they are distant from the active site; and if they are close to the active site then they can be coupled in such a way that function is conserved[2]. But how do the conformational requirements of function limit the extent to which proteins can alter their sequences during evolution? Two experiments in protein engineering, reported by Gassner *et al.*[3] and by Axe *et al.*[4] in *Proceedings of the National Academy of Sciences*, provide results that are important for answering this question.

Gassner *et al.*[3] studied T4 lysozyme, which has an active site in a groove between two domains (Fig. 1, overleaf). They replaced ten residues that are buried in the core of the second all-helical domain (six leucines, two isoleucines, one valine and one phenylalanine) with methionine residues. These mutations make little or no change to the volumes of the side chains that they replace, but they do change their shape. They found that the individual mutations had small effects on function — the mutant proteins had between 70% and 105% of the native activity, and a loss in stability of 0.4–0.9 kcal energy per mol of protein. But a protein with seven of the mutations showed 43% of the native activity and a 5.0-kcal loss in stability.

By comparing the atomic structure of the

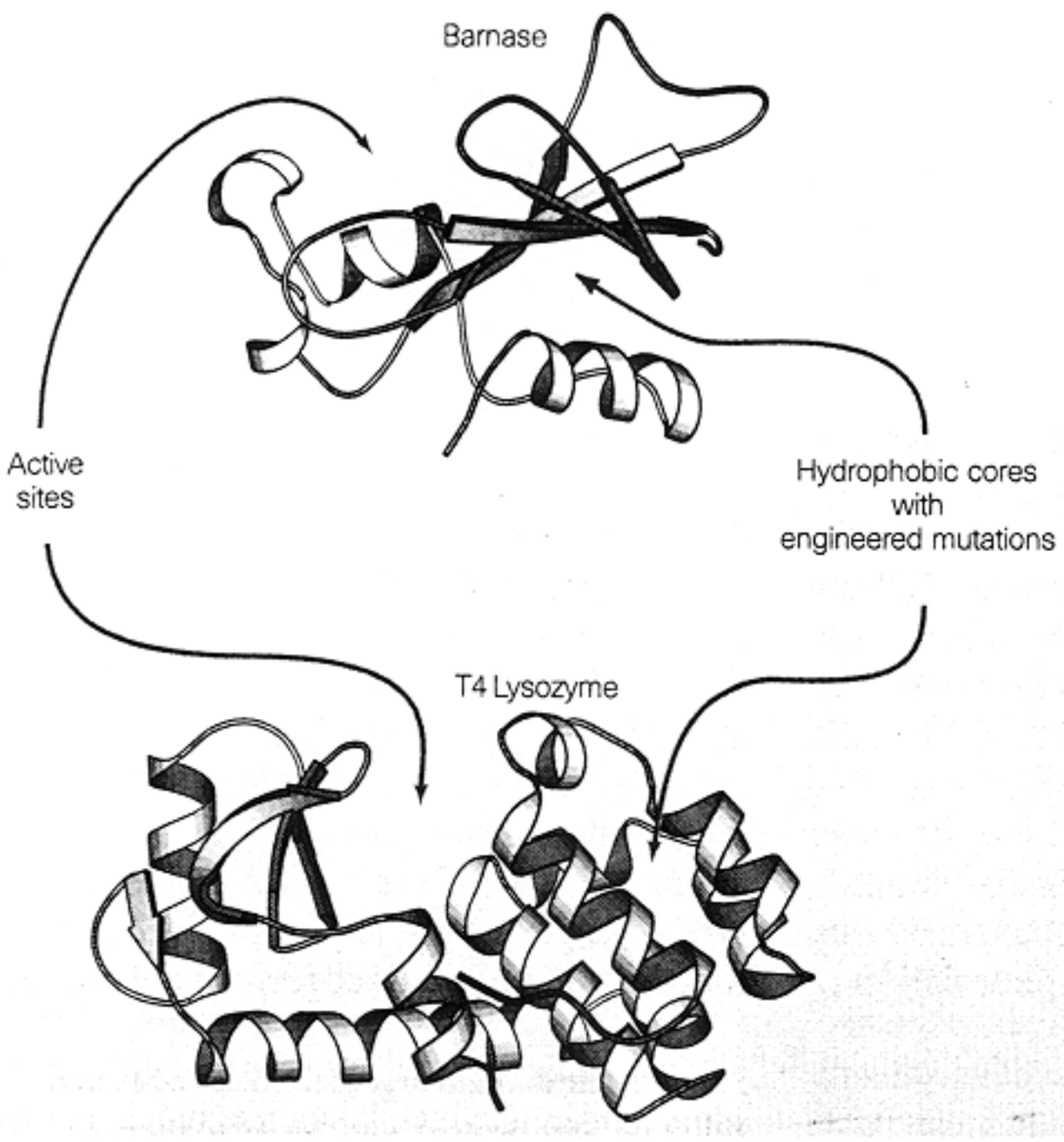

Figure 1 **The molecular structure of barnase[9] (top) and T4 lysozyme[10] (bottom). Coiled ribbons represent α-helices and extended ribbons represent strands of β-sheet. Gassner *et al.*[3] engineered mutations in T4 lysozyme at the sites of ten residues that pack between the α-helices that form the second domain, and Axe *et al.*[4] engineered mutations in barnase at the sites of 13 residues that pack between the amino-terminal α-helix and the β-sheet. The picture was drawn using MOLSCRIPT[11].**

T4 lysozyme containing seven mutations with that of the native protein, Gassner *et al.* were able to show how the protein had responded to the mutations. Six of the mutations involved the replacement of leucine by methionine, the side chains of which have the same volume but different shapes: whereas the leucine side chain is branched ($-CH_2-CH(CH_3)_2$), that of methionine is linear ($-CH_2-CH_2-S-CH_3$). The authors showed that the first three side-chain groups in the methionine residues ($-CH_2-CH_2-S-$) occupied the same positions as three of the side-chain groups ($-CH_2-CH-CH_3$) in the leucines that they replaced. However, the different stereochemistries of methionine and leucine meant that the terminal methyl groups in the six methionine residues occupied quite different positions (2.6–3.9 Å) from the branched methyl groups of the six leucines.

Gassner *et al.*[3] next superposed the helical domains of the mutant and native proteins, and they found that the overall root-mean-square difference in the position of the main-chain atoms between the two structures is 0.2 Å. They suggest that this close similarity in the structures of the two proteins is not compatible with the precise spatial complementarity that is implied by the close-packed description of the protein interior.

Stimulated by these findings, we analysed the mutant and native structures. Although the overall difference in the position of the main-chain atoms is small, we find that there are significant local changes. In particular, the main-chain atoms of two regions that contain a mutation (residues 82–84 and 153–155) shift by 0.4–0.7 Å. Also, in a region that is adjacent to one mutation and in contact with two others, one helix and an adjacent turn (residues 107–116) are slightly distorted, and they shift by 0.4–1.0 Å. Buried side chains that are adjacent to the mutations rotate by 10–23°, and an adjacent surface side chain rotates by 58°.

We also determined the volumes that are occupied by the buried atoms of the mutant methionine residues. Two-thirds of the atoms in the six methionines are totally buried, and they occupy a total of 612 Å^3. The volume that would be expected for these atoms — if they are close-packed in the usual manner[5] — is 614 Å^3. (This volume implies a greater packing density than that in amino-acid crystals[5].) So the effect of the structural changes is to go from a set of close-packed leucines in the native structure to a set of close-packed methionines in the mutant. In other words, by using a variety of subtle changes in its conformation, T4 lysozyme can accommodate simultaneous changes in the shape of buried residues, and maintain close packing.

Axe *et al.*[4] carried out a much more extensive series of mutations to the main hydrophobic core of barnase, a bacterial ribonuclease. The major secondary structure in this protein is a six-stranded β-sheet, one face of which, together with certain loop regions, forms the active site. A helix packs against the other face of the β-sheet, and the region between these two secondary structures forms the main hydrophobic core of the protein (Fig. 1).

The authors made random hydrophobic substitutions at the 13 residues that form the hydrophobic core. In the native structure, these residues are: two alanines, one valine, four isoleucines, two leucines, one phenylalanine, two tyrosines and one tryptophan. They were randomly replaced by valine, leucine, isoleucine, methionine or phenylalanine, and Axe *et al.* found that a surprisingly high proportion of the mutants — close to a quarter — had enzymatic activity. Even in some cases where all 13 sites had non-native residues, mutants were highly active.

The experiments by Axe *et al.*[4] and Gassner *et al.*[3], together with previous studies by Lim *et al.*[6,7], imply that protein structures can often adapt to changes in the shape of buried residues, and that many different combinations of hydrophobic residues in the core are able to maintain structural integrity.

Gassner *et al.*[3] have shown that the presence of seven mutations within the active site of T4 lysozyme reduces the activity by half, even though the structural changes are small. When single mutations are introduced, we would expect the structural changes to be even smaller, yet function is again affected, with activities from 70–105% of that of the native protein. The mutations introduced by Axe *et al.*[4] affect the activity of barnase to a lesser degree and the mutant proteins are highly active, even though the mutations must produce changes that are much larger than those in T4 lysozyme. This must be because the active site is on the opposite side of the β-sheet to the mutations, and it will only be affected if conformational changes are transmitted across the β-sheet.

Conformational changes allow individual structures to accept a range of mutations, especially those that retain the chemical character of the residue and do not involve large changes in volume in one step. Limitations on the extent of this process in actual proteins arise from their functional requirements. For proteins such as histones and actin, whose functions involve extensive interactions with several different proteins or DNA, there are severe limitations — the rate of change in their sequences is about 10% per billion years[8]. Conversely, for the different domains in the immunoglobulin superfamily, where duplicated genes have different functions, less than a third of the domain structure is conserved, and the only sequence conservation is the hydrophobic character and approximate size of some 12 residues. Essentially, the extent of sequence divergence in proteins is inversely related to the extent of their functional requirements. □

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