“Sequence-Structure Relationship in Proteins: a Computational Analysis of Proteins that Differ in Sequence but Share the Same Fold”

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Summary

Mapping between sequence and structure is currently an open problem in structural biology. Despite many experimental and computational efforts it is not clear yet how the structure is encoded in the sequence. Answering this question may pave the way for predicting a protein fold given its sequence.

My doctoral studies have focused on a particular phenomenon relevant to the protein sequence-structure relationship. It has been observed that many proteins having apparently dissimilar sequences share the same native fold. The phenomenon of mapping many divergent sequences into a single fold raises the question of which positions along the sequence are important for the conservation of fold and function in dissimilar sequences. Detecting those positions, and classifying them according to role can help understand which elements in a sequence are important for maintenance of structure and/or function. In the course of my doctoral research I have attempted to discover and characterize those positions.

The method I undertook was as follows: I constructed a database of structurally sim-
ilar, sequence dissimilar protein pairs, as a tool for detecting those positions. Aligned positions between pair-mates were examined for evolutionary conservation within their respective sequence families. Positions having a mutual conservation in the non-intersecting sequence families of each pair-mate are deemed to play a role in the conservation of fold and/or function between aligned pair-mates. The rationale being, that evolutionary conservation between aligned positions is due to the preservation of some critical aspect for fold or function.

In the initial phase of this work (Chapter 2), I have examined those positions which were structurally aligned, and possess identical residues. It was shown that out of Structurally Aligned, Identical ResidueS (STAIRS), 40% are only moderately conserved, suggesting that their maintenance as identical residues was coincidental. However, STAIRS with high mutual evolutionary conservation exhibit low solvent accessibility, and an over-representation of certain amino-acids. We also examined a subset of STAIRS which are spatially proximal (neighboring STAIRS or NSTAIRS). An itemized examination of the over-represented STAIRS which are spatially proximal has shown that an overwhelming majority can be assigned with a functional or a structural role: location in functional sites, and determination of secondary or super-secondary structure.

The evolutionary conservation of positions was determined automatically by multiple alignment of each sequence to homologues in the NCBI non-redundant sequence database using the PSI-BLAST program. PSI-BLAST was used to determine the conservation in each position over several iterations, in a way which enabled the expansion of
the “evolutionary horizon”, as each iteration provides alignments with sequences which are more evolutionary distant. I then proceeded to look at those positions which are persistently conserved in each pair-mate, and in both aligned structures. Persistently conserved positions have been defined as those positions which are conserved both at the first and last PSI-BLAST iterations for the following reason: conservation in only the first iteration might be due to evolutionary non-divergence, whereas conservation only in the last iteration might be due to a drift which sometimes occurs in PSI-BLAST, where the alignment generated by PSI-BLAST no longer holds information pertaining to the original query sequence. The intersection of the first and last iterations however, yields positions which are suspect of being conserved for a structural or functional reason. We have shown that few discreet positions are conserved in each pair. Those positions, dubbed MPCs (“Mutually, Persistently Conserved positions”), were shown to play a role in helix stabilization, hydrophobic core formation, and active sites. MPCs tend, in many cases, to form spatial clusters within their protein structures. We have derived a substitution matrix from the MPCs, which we believe might be conducive to protein engineering and design applications (Chapter 3).

The database of SSSD protein pairs has allowed us to assess the accuracy of alignments produced by PSI-BLAST. Alignment accuracy is important for the correct modeling of a sequence by the structure of a homologue. PSI-BLAST is often used for the detection of query sequence homologues, with a solved structure. In the SSSD database, several pair-mates are detected uni- or bi-directionally using PSI-BLAST, in the second
or following iterations. Having that data enabled the comparison of alignments produced by PSI-BLAST to the structural alignments, the latter being the “gold standard” for alignment evaluation. I have shown that from the 123 structurally similar, sequence dissimilar protein pairs, 52 pairs have detected their pair-mates, and for 16 of those, the detection was bi-directional. The alignment specificity was shown to be \( \approx 44\% \), and it does not improve significantly over consecutive iterations. The alignment sensitivity was shown to be \( \approx 51\% \) at best, and shows improvement over several iterations (Chapter 4). Based on these findings we concluded that the alignment accuracy produced by PSI-BLAST is as good as those produced by various threading algorithms, and that the alignment sensitivity may be improved over consecutive iterations.
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Chapter 1

Introduction

How does a protein sequence determine its structure? This question is known as the protein folding problem. Currently, this is an open problem in structural biology. The underlying assumption of the protein folding problem is that the native folded state is encoded in the amino-acid sequence. The goal is to decode this information, so that given a protein sequence, its structure may be made known.

The dogma stating that the amino-acid sequence determines the native folding state was formulated by Christian Anfinsen (Anfinsen et al., 1962; Anfinsen, 1973). It is based on studies in which Anfinsen et al. have shown that denatured RNaseA returns spontaneously to a fully functional form. Since then, this phenomenon was exhibited in many other proteins. It is known that proteins may be irreversibly denatured under severe conditions, or may not be able to achieve their native folding state without some kinetic assistance (e.g. chaperone-mediated folding). There are also numerous examples
of protein misfolding, in which a protein folds into a conformation other than its native state, or has “alternative native states”. The best-known cases are those in which protein misfolding is involved in diseases, e.g. Alzheimer’s disease and prion diseases. (Reviewed in Cohen, 1999). These cases, however, are not contradictory to the sequence determines structure dogma. In the case of chaperone-mediated folding, the information for achieving a global free energy minimum, exists in the sequence. Chaperones merely assist the folding process, which cannot take place spontaneously due to kinetic constraints. As such, they act as catalysts, and do not violate the central dogma of folding (Branden & Tooze, 1998). The “sequence determines structure dogma” still holds also in the other examples, although cases of discrete alternative conformations for a single sequence or subsequence do exist (Minor & Kim, 1996; Mezei, 1998).

An interesting observation associated with the protein folding problem is the existence of proteins having a similar structure, but with completely different amino-acid sequences. This phenomenon raises the following question: given two or more proteins with dissimilar sequences, but with similar folds, which positions along the sequence enable the preservation of the fold, and frequently, of function? In the course of my PhD studies, I have chosen to use bioinformatics methods to answer this question. The detection and characterization of those positions can contribute to the sum body of knowledge regarding the role of the sequence in determining structure.

This chapter elaborates upon the question set in the previous paragraph, and places it within the context of the current body of knowledge. It begins with an overview of
current data which exists regarding protein sequences and structures. This is followed by a review of experimental and computational studies for the detection of critical amino-acid positions. Following that, the methods used to carry out this work are described. Finally, a synopsis of the following chapters, composed of published research papers is offered.

A technical note: Bioinformatics, being a discipline of its own, carries with it a unique lexicon. Being a relatively new discipline, some of these terms suffer from ambiguity. In order to overcome both problems of novelty and ambiguity, this work has those terms marked in **boldface** when first introduced.

### 1.1 Many sequences, few folds

In many cases, proteins of similar fold and function retain a significant sequence similarity. Common sequence alignment algorithms enable us to align and determine the evolutionary relationship between two or more proteins. A commonly used rule-of-thumb holds that for proteins over 80 residues in length, a 30% sequence identity (after alignment) is sufficient to infer a common fold. However, with the increase of sequence and structural data, it has become apparent that many proteins share the same fold, and possibly function, but do not display a detectable sequence similarity. When the structures of these proteins are examined, their structural similarity is obvious. Furthermore, in many cases their functional similarity is also apparent.
Awareness that structurally similar, sequence dissimilar proteins fold as a common, actually predominant, phenomenon has only been established recently (Rost, 1997). In that study, Burkhard Rost performed an alignment of all structural homologues from a database of structural alignments (FSSP, see section 1.4.2). He observed one peak at 8.5% ± 5% SD sequence identity. This was quite close to the random identity, established at 5.6% ± 3% SD. Furthermore, in four genomes, most close structural homologues have been determined to have less than 45% pairwise sequence identity. Two conclusions from Rost’s study relevant to this work are summarized here:

1. Many pairs of similar structures in the PDB have a sequence identity as low as expected from randomly related sequences. Most structural homologous have less than 45% pairwise sequence identity.

2. About 3-4% of the residues are crucial for protein structure and function. This was estimated by subtracting the mode of the random alignments from the mode of structural homologue alignments.

In another study it was shown that 196 domains released in 1998 to the PDB bore no sequence similarity to existing PDB sequences (Koppensteiner et al., 2000). However, 75% of the domains were shown to have structural similarity to previously known folds, and in two-thirds of the cases then similarity in structure coincided with related function. Brenner and Levitt have shown similar results in their analysis of new domains incorporated into the PDB over a period of 10 years (Brenner & Levitt, 2000).
1.1.1 Sequence family population in protein folds

A sequence family is a collection of sequences which share a sequence similarity. This is a collection of homologous proteins, sharing a related structure and function. Sequence families are discussed in Section 1.4.1. Different sequence families are assumed to be disjoint. How many sequence families populate a single fold? And how are they distributed among folds?

Most folds are populated by a single sequence family. However, there is a large number of folds which are populated by more than one sequence family. In one study (Wolf et al., 2000), the total number of folds populated by a single sequence family was estimated to be between 138 and 211. The total number of folds which are populated by more than one family was estimated to be between 176 and 226. (This was in 1999, when the total number of known folds was between 331 and 336, depending on the criteria used for fold classification). Thus, 52-56% of the protein folds are populated by more than a single sequence family. Some 12% of all folds were estimated to contain more than ten sequence families. Another study has placed the percentage of folds containing more than one family at 39%, with ~10% of all folds estimated to have more than eight sequence families (Zhang & DeLisi, 1998).

The distribution of sequence families in folds has been shown to be logarithmic, or near-logarithmic (Wolf et al., 2000; Zhang & DeLisi, 1998). Thus, while the one family-one fold phenomenon is predominant, still many are populated by multiple sequence
families.

In summary, many folds are populated by dissimilar sequences. It is not uncommon for proteins sharing only 10% sequence identity to assume a similar fold, and often related or identical functions. This suggests that many positions have no critical role in structure and function determination, and that folding determinants are restricted only to a certain small number of residues. Section 1.2 reviews the studies, experimental and computational, which were conducted in order to locate those positions.

1.2 Previous studies

The actual location of critical residues for fold and function can be found by experimental or computational means. In the experimental approach, a protein is selected, and mutated in various positions. Mutants are then assayed to determine how the various mutations affect function. The effect on structure is then derived from the effect on function. A more laborious approach, but feasible with certain proteins, would be to actually determine the structure of the mutants. The structural approach is the most direct and the results given may be considered “gold standard”. Experimental functional studies were the first which suggested that protein function is maintained by a small number of residues. Studies of the T4 phage lysozyme, the P22 Arc repressor and the *E. coli* lac repressor are reviewed in this section. However, experiments can only be performed with proteins for which functional assays exists. Even so, this is a laborious
process, in terms of work vs. amount of data yielded; and for most proteins, there exists no functional assay.

Experimental studies provide a family of related (if artificial) sequences, complete with bona-fide annotation regarding the role of mutated positions or subsequences. In a similar manner, evolutionary changes may be studied using computational methods. The issues which must be addressed when using a computational approach are: (1) criteria for the location of critical residues and (2) verification of results. In cases when the computational method is used in order to develop a predictive ability, then an assessment of prediction accuracy should also be provided.

Most computational approaches use evolutionary conservation (although not exclusively) as a criterion for the detection of key positions. The rationale is simple: if a position is conserved within a protein’s sequence family, then that is a result of selection, and this position is important for preservation of the protein’s viability. The methods vary in their definition of positional evolutionary conservation, and how the results should be interpreted. For example, Mirny & Shakhnovich’s conservatism-of-conservatism method differentiates between intra-family and inter-family evolutionary conservation, drawing conclusions regarding the function of the different types of conservation (Mirny & Shakhnovich, 1999). Typically, this information is obtained by automatically collecting and aligning sequence family members from one of the large protein sequence databases.

Structural information is also incorporated into computational analyses. For exam-
ple, positions which are spatially close and conserved may indicate a functional role, if located on the protein’s surface, or a structural role, if buried. All computational studies reviewed here use structural information, although in different manners. In the 3D cluster analysis, the predictive function incorporates spatial proximity data directly in order to assess positional importance. In the conservatism-of-conservatism and in the CKAAPs study, 3D information is used for structural alignments during family collection phase, where sequences are too distant to perform sequence-based alignment.

Following is a review of experimental and computational studies of critical amino-acid positions. The experimental studies are discussed first. The importance of the experimental studies is not only in providing “gold standards” for the location of key positions, but foremostly in showing that many mutations may be introduced without any deleterious effect on structure or function. The computational studies are then provided, including recent ones. These are discussed also as a preamble to the research conducted during the course of my doctorate.

1.2.1 Experimental studies

T4 Phage Lysozyme

Rennel et al. (1991) have conducted a functional study of 2015 mutations along all 163 positions of the T4 phage lysozyme (T4L) (barring the initial methionine). They have shown that 55% of the positions along the T4L sequence tolerate a minimum of
13 different amino acid substitutions without any effect of the protein’s function. 328 mutations, affecting 45% of the residues were scored as deleterious. Comparing their findings to T4L’s structure, they have found that there is a high correlation between residue burial and substitution intolerance. Two striking exceptions of exposed positions were associated with catalytic function.

Critical positions, which were completely intolerant to mutations were found at the catalytic site, and in two buried salt bridges and their stabilizing residue network. Two others were found in exposed salt bridges. The total was 12 positions, ~7% of the protein’s length.

Subsequent experiments with the T4L system have been conducted by Brian Matthews, using functional assays and X-ray crystallography to examine the sequence-structure relationship in the T4L. In a 1995 review in Advances in Protein Chemistry, it was concluded that the protein can accommodate changes in many sites, while still maintaining structure (Matthews, 1995). This robustness is also featured while replacing core residues, sometimes leading to correct folding through repacking of the core region. Destabilizing core interactions included leucine to alanine replacements. The destabilizing effect was attributed to the creation of cavities leading to destabilization by loss of hydrophobic and van-der-Waals interactions. In one study up to 10 adjacent residues were substituted by methionines in the protein’s core region (Gassner et al., 1996). This study has shown that that multiple replacements with a single amino-acid in the core are possible. Although eventually leading to a loss of stability, T4L has retained its
structural and functional properties through most of the replacements. Another study, examining the structure-function relationship has shown that catalytic site positions may be substituted, leading to a reduction or loss of activity, but not of structural stability. Stability was actually increased, suggesting that catalytic site residues are not optimized for structural stability (Shoichet et al., 1995).

The following tenets were suggested: (1) A subset of the amino acids in a protein is of key importance for folding and stability. (2) This subset consists primarily of the interior residues. (3) The role and importance of a given residue depends on its context within the folded structure of the protein and can be evaluated by substitution of alternative amino acids at that site. (4) Catalytic site residues are not necessarily related to structural stability. In fact, the structure may be stabilized by mutating the catalytic residues, at the expense of function loss.

The bacteriophage P22 Arc Repressor

Milla et al. have studied single alanine-substitutions in the Arc repressor of bacteriophage P22 (Milla et al., 1994). The P22 Arc repressor provides an attractive system for sequence-structure relationships due to its small size of 52 residues. Fifty-one non-alanine positions were mutated to alanine in this study. Using melting temperatures as a measure, it was shown that twenty-five mutants had $t_m$ values near the wild-type, and 20 mutants were found to be less stable. Five mutations prevented protein folding altogether, and one mutant (P8A) was found to be more stable than the wild type. All
mutants were compared with the structure in order to elucidate the reason for structural disruption, or the lack of it. The reason for the increased stability of mutation P8A, was given as a relief of unfavorable packing interactions caused by a buried proline ring. The 25 neutral stability mutations affected side chains which are mostly solvent exposed, and have high B-factors (see also Markievicz’s et al. study below). Eight of the 25 mutations affected residues involved in hydrogen bonding, while two others affected residues in the hydrophobic core. The destabilizing mutations mostly affected glycines with positive $\phi, \psi$ values, hydrogen bonds and salt bridges. Those positions were found to be more buried and with lower B-factors than positions in the neutral mutations class. The five mutations which prevented folding altogether were at positions with low B-factors, four of the mutations affected hydrophobic core mutations and one a buried polar residue. The five wild-type side chains altered in the “unfolded” mutant class pack together in the native structure. The authors hypothesized that they may form a folding core, that may have been disrupted by the mutations.

Brown and Sauer studied mutants of the Arc repressor containing from 3 to 15 partially overlapping multiple-alanine substitutions (Brown & Sauer, 1999). (Five mutants with stretches of 3, 7, 11, 12 and 15 alanines, named 3A, 7A, etc.) Their choice of positions was purposefully targeted to those positions which in the previous study were shown individually to have little effect on protein stability. Twenty-two different residues in total were mutated in this study. Examinations of the spectral properties of the mutants (using CD and fluorescence) revealed that they were able to adopt native
structures with many similarities to the wild-type Arc repressor. Mutants 7A, 12A and 15A were unable to bind DNA, as revealed by footprinting probes. Mutants 3A and 11A functioned well in that respect. All of the five variants formed heterodimers with the wild-type Arc.

In summary, the Arc repressor mutant studies have shown that up to 55% of the residues can be mutated individually with no deleterious structural effect, and 26% of the positions can be mutated in concert, with the Arc repressor mutants still assuming the same fold as the wild-type.

The *E. coli* lac repressor

Markiewicz *et al.* studied a set of over 4000 single amino-acid replacements in the *E. coli* lac repressor protein [Markiewicz *et al.*, 1994]. Markiewicz *et al.* have located segments of tolerant regions, which they replaced with spans of 5 to 13 alanines, preserving repressor function. They concluded that 192 of 328 sites (59%) are generally tolerant to substitutions. Certain segments were found to be more restrictive in tolerance, i.e. tolerant of substitutions within a certain physico-chemical group. A multiple sequence alignment of the lacI protein with 13 known homologues has revealed a good correlation between positional conservation and substitution intolerance. Another phenotype analysis of the lac repressor was conducted [Suckow *et al.*, 1996] this time with reference to its published structure. The chief goal was to elucidate structural roles of substitution-intolerant mutations. In most cases, mutant effects could be correlated with structural
and functional features of the protein. Suckow et al. partitioned the amino-acid positions along the protein into 15 groups, based on their physico-chemical traits, location in the protein, DNA binding, ligand binding, participation in the dimerization interface, etc. They have found that the amino acids which are characterized solely by their solvent exposure, are generally tolerant to substitutions. However, solvent exposed residues participating in salt bridges were intolerant to substitutions. Positions which were composed of small amino-acids were tolerant to substitutions only by small amino-acids. Some parts of the protein, identified as spacer regions, were not only tolerant to single substitutions, but could completely be replaced with stretched of alanine residues, placing the alanine stretch replacements observed in the previous study in a structural context. Substitutions in positions participating in DNA contacts, or in ligand binding (isopropyl-β-d-thiogalactoside, IPTG, which lowers the repressor binding affinity to the operator DNA by three orders of magnitude), or in the dimerization interface were not tolerant to substitutions, resulting either in an inactive enzyme, or in the I\(^s\) phenotype (unresponsive to an inducer). Certain substitutions resulted in the I\(^s\) phenotype without being directly involved in inducer binding. Reasons that were given regarded a failure to transmit the inducer effect to the DNA binding domain of the protein, or in some cases, enhancement of DNA binding affinity so that the inducer can not force the protein to dissociate.

Some positions were identified as directly affecting the protein fold. Those are positions which are predominantly buried and small (Gly, Ala, Thr and Ser residues) for
which only small amino-acids are tolerated. Small exposed residues were also found to be intolerant, although the phenotypical effect was not as drastic as with the small buried ones.

Proline substitutions affecting secondary structure elements usually occurred at secondary structure elements involved in dimerization. Thus, even secondary structure element distortion was occasionally tolerated, or compensated for.

In conclusion, the lac repressor studies have shown a general robustness of the protein structure with regards to substitutions. Substitution-intolerant positions were shown to have a clear structural or functional role.

1.2.2 Computational studies

Conservatism-of-Conservatism

Mirny & Shakhnovich have studied five of the most populated protein folds (Mirny & Shakhnovich, 1999). They have attempted to separate between historic, structural and functional reasons for positional conservation. This was done by comparing intra- and inter-sequence family conservation, among sequences populating the same fold. In order to do that, representative structures of selected sequence families which populate a given fold were chosen, and the following steps were taken:

1. Construction of multiple sequence alignments of proteins homologous to each representative protein.
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2. Identification of positions which are conserved within each multiple alignment

3. Structural alignment of families to each other.

4. Identification of sites where conserved positions coincide between the families.

The phenomenon of consistent conserved positions amongst families was named conservatism-of-conservatism, or CoC. The analysis was performed using an alphabet of six letters, where the amino-acids were grouped by physico-chemical traits. This was done to factor out interchangeability which occurs between amino-acids of similar physico-chemical traits. Two measures of positional entropy were taken $S(l)$ and $S^{\text{across}}(l)$. $S(l)$ is the measure of CoC between families, and identifies conserved positions that may vary in residue identity among the families. $S^{\text{across}}(l)$ is the measure of CoC across families, which identified conserved residue types across all families.

The folds that were analyzed were the immunoglobulin (Ig) fold, the oligonucleotide-binding (OB) fold, the Rossman fold, and the TIM barrel. The parameters that were attempted to be correlated with high CoC were function, thermodynamic stability, and kinetics. For each of the folds studied, high scoring CoC positions were shown to form a dense cluster within the native structure. However, cluster location and participating residues were shown to vary between folds, or within families of the same fold. For example, in the Ig fold residues with high CoC form a cluster deeply buried in the fold. Some families of this fold stabilize this cluster by hydrophobic interactions, others by disulfide bonds. Generally, the study revealed a high correlation between CoC and
solvent accessibility: high CoC positions were usually buried. However, in those folds where data existed regarding folding kinetics, many clusters were shown to form nucleation centers. In two cases, the analysis of $S^{across}$ revealed the existence of super-sites, that is a common denominator which can be attributed to function, regardless of the actual function which the protein assumes.

**Conserved Key Amino-Acid Positions**

Reddy et al. have studied conserved key amino acid positions (CKAAPs), which were derived from common substructures in proteins from the entire PDB (Reddy et al., 2001). The subsequences derived from the substructures are aligned, and the positions in the multiple alignments score according to conservation.

In this study, several folds were also particularly examined, in order to find the function of conserved residues according to the CKAAPs method. Interestingly, one of the folds examined was the Ig fold. Reddy et al. have discovered that the CKAAPs analysis found all the residues found by the CoC analysis, plus some others. In contrast with the CoC analysis, the CKAAPs analysis was performed on many families of the PDB. This gave Reddy et al the opportunity to select those proteins in which mutations were well-documented, and compare them with the CKAAPs findings. The well-documented Arc repressor was examined. It was found that Arc mutations in positions designated as CKAAPs exhibit more severe perturbations in protein stability.

In a whole-database analysis, CKAAPs were shown to be no more buried than the
normal pattern of solvent accessibility. However, CKAAPs were found to be predominant in the terminal regions of rigid secondary structural elements. Examination of Ooi numbers shows that CKAAPs are mostly surrounded by other amino acids and that charged groups on the amino acids are better neutralized by hydrogen bonding interactions.

3D cluster analysis

Landgraf et al. used representative structures and multiple sequence alignments in a method called three dimensional cluster analysis (Landgraf et al., 2001). In this study, the regional conservation score $C_R(x)$ defines the conservation of each residue and its spatial neighbors relative to the rest of the protein. A high $C_R(x)$ value means that position $x$ is located within a conserved spatial cluster within the sequence family. The similarity deviation score $S(x)$ detects clusters with sequence similarities deviating from the similarities of the full-length sequences. A high $S(x)$ score indicates a strong deviation between the similarity relationships within the regional alignment of the structural neighbors of residue $x$ and the similarity relationships obtained for the full-length sequences.

The difference between this study and the CKAAPS or CoC studies is that positions in this study were initially scored based both on their conservation and their clustering with other conserved positions. The incorporation of three-dimensional information in that manner enabled Landgraf et al. to detect clusters which cannot be detected sim-
ply by multiple sequence alignment, and which have a potential for controlling protein function. In this study, 35 different folds were analyzed. The main goal of the analysis was to evaluate the ability of 3D cluster analysis to predict functional interfaces, as defined by cocrystal structures. It was found that $C_R(x)$ identifies the majority of the residues in interfaces. The identification of interface residues increases as the sequence diversity within the family increases. The reason being that the signal-to-noise ratio increases with increased overall sequence diversity: the conserved clusters remain conserved, whereas the overall sequence identity decreases.

The $S(x)$ score was used for a different purpose. Here, the question was whether proteins could possess residue clusters for which the global sequence similarity relationships might not adequately reflect evolutionary and functional relationships. Landgraf et al. suggest that increased $S(x)$ scores may indicate regions controlling the specificity of protein functions.

In addition to the 35 families analysis, 3D cluster analysis was performed on the MAP-kinase ERK2, and on aldolase. In the ERK2 analysis, $C_R(x)$ identified the P1 site, the ATP-binding pocket, and the dual-phosphorylation site. There was a considerable overlap between $C_R(x)$ and $S(x)$, however the highest $S(x)$ scores were exhibited in the specificity-conferring P1 region and the ATP-binding pocket. In the aldolase analysis, the area showing the highest $C_R(x)$ scores was found to be located in the core of the $\alpha/\beta$-barrel and includes all key residues known to be involved in catalysis.
1.2.3 This study’s approach

This study approaches the problem of critical residue location by studying a whole set of proteins, without restriction to any given fold or sequence family. The studied data set is compiled of protein pairs where the pair-mates are widely different in their sequence, but have the same fold. The hypothesis being that there is a cryptic common denominator, present at the sequence level, which causes such proteins to assume the same fold. This sequence-encoded common denominator is not overtly detectable by sequence alignment methods (see section 1.4.1), as by definition the collated protein pairs are not sequence-alignable.

As a first step, a database of structurally similar, sequence dissimilar protein pairs (SSSD, see 1.4.3) was constructed. We then proceeded to locate and study aligned positions which are suspect of being critical to the fold and function of the proteins in the database. Initially (Chapter 2) we have looked at positions which are structurally aligned and identical. Following that, we studied positions which are aligned and conserved, using a novel method of detecting evolutionary conservation in close and distant sequence family members of the studied proteins (Chapter 3). Computational studies published previous to and concurrently with this study which aimed to locate critical positions, have used either a single structure and its sequence family members (3D clustering), or structural alignment of proteins from the same structure and sequence family (CoC, CKAAPs). In contrast to those studies earmarked by being family spe-
cific, this study makes a point of examining pairs of proteins which have no detectable sequence similarity, sifting in only those sequence determinants which would play a role in assuming the same fold/function for the studied protein pair.

In another part of this study, we have examined the accuracy of alignments produced by PSI-BLAST, when compared with the structural alignments we already had from our database. PSI-BLAST is commonly used to detect remote homologues. Having a database of structurally similar, sequence dissimilar protein pairs enabled us to assess the ability of PSI-BLAST to detect remote homologues, and to evaluate its alignment accuracy.

1.3 The Importance of Determining Protein Structure

A protein’s structure is much more biologically informative than sequence only. The reasons for that are:

1. **Functional**: Structure solution provides knowledge regarding the biochemical mechanism by which the protein carries out its function. The roles and mechanisms of elements of a protein become apparent only when viewed in a structural context. Understanding the mechanism of catalytic sites, binding sites, protein-protein interfaces, kinetics and thermodynamics of folding all require a structural
solution of the protein.

2. **EVOLUTIONARY:** Structure is well conserved over evolutionary time, and thus it enables the recognition of evolutionary relatedness with other proteins, undetectable by sequence comparison.

Automated sequencing techniques have inundated us with protein sequences. Even before the coordinated genomic sequencing efforts, large repositories of sequence data have been created simply by the contributions stemming from ongoing research. There are currently ~600,000 protein sequences in the protein sequence databases SwissProt and TREMBL (Bairoch & Apweiler, 2000). However, with regard to protein structure, the amount of data we have is much smaller. The reasons for that are: (1) the lower throughput of structure determination methods: X-ray crystallography and NMR; (2) Structure determination methods are currently limited almost exclusively to globular proteins; (3) Even among those proteins there are certain proteins which cannot be crystallized, or are too large for NMR spectroscopy.

As a result of those limitations, targets for structure determination were chosen much more selectively than targets for sequence determination. When compared with sequencing, structure determination is a costly and lengthy undertaking. The Protein Data Bank (PDB) (Berman *et al.*, 2000) currently holds ~16,000 structures.

The importance of producing structural solutions is exemplified by the recently launched effort of **structural genomics** (Brenner, 2001). Structural genomics aims to
provide tractable solutions to all proteins, by creating a library of solved representative structures, and using computational means to assign a fold to all known sequences.

1.4 Methods and Materials

This section explains, and when necessary elaborates upon, the methods used in this study.

1.4.1 Homology by sequence, and sequence families

The most common way of inferring and studying an evolutionary relationship between proteins is by sequence alignment. There are currently several algorithms which enable us to align two or more protein sequences, in order to determine their similarity. Pairwise alignment, the alignment of two sequences, is commonly performed using the Smith-Waterman ([Smith & Waterman, 1981]) or Needleman-Wunsch ([Needleman & Wunsch, 1970]) algorithms. Pairwise alignment of protein sequences is initially performed in order to determine whether they are homologous, that is, originating from a common ancestor. Homology which is a qualitative trait, is inferred from the degree of similarity, a quantitative trait, between the sequences. Inferral is performed by setting a threshold on the degree of similarity between the sequences, beyond which homology is considered to be established. Once homology has been established, additional questions may be asked and answered based on the examination of the alignment. For example, are the
sequences homologous throughout their length, or in some conserved local region? Are functional areas preserved?

Dynamic programming is used for pairwise sequence alignment. The rationale is that the optimal solution for the alignment of the two sequences stems from the optimal solution of the previous alignment step. Using recursion, it is possible to establish the optimal shortest distance between any two sequences. The score of the alignment achieved can be assessed by placing it within a distribution of mean random alignments, establishing a statistical significance for the alignment score.

**Amino acid substitution matrices**

Amino acid substitution matrices such as the PAM (Dayhoff, 1978) or BLOSUM (Henikoff & Henikoff, 1992) series are typically used in the process of protein sequence alignment. When there is call for an amino-acid replacement (as opposed to an insertion/deletion event), the cost of this replacement is assessed using the substitution matrix. Each entry in a log-odds substitution matrix contains the following value:

\[
M_{i,j} = \frac{\lambda}{\log_2(\frac{P_{i,j}P_i P_j})}
\] (1.1)

Where \(i\) and \(j\) are any two given amino acid types, or the same one. \(P_{i,j}\) is the observed frequency of replacements between \(i\) and \(j\) in the database, \(P_i\) and \(P_j\) are the respective probability of occurrence for amino acids \(i\) and \(j\) in the database. Thus \(P_i P_j\) is the probability of a random replacement between \(i\) and \(j\). This is placed on a
logarithmic scale, typically base 2, and the resulting score $M_{ij}$ is said to be expressed in \textbf{bits}. $\lambda$ is a scaling factor.

Consider the BLOSUM series of matrices: each BLOSUM$X$ matrix is composed of a subset of aligned sequences with $\leq X\%$ identity from the BLOCKS database of multiply aligned sequences. Thus BLOSUM85 contains the log-odds replacement frequencies for all aligned sequences in BLOCKS with a 85\% identity and less. These replacements may be compared to the ones exhibited in BLOSUM35 (constructed from BLOCKS of 35\% identity or less), for example, and conclusions may be drawn regarding the differential frequency of replacements given different evolutionary distances.

Substitution matrices may also be used for the analysis of databases of aligned proteins. An important measure which may be drawn from a substitution matrix regarding the set of alignments it represents is its \textbf{relative entropy}. A matrix’s $M$ relative entropy is calculated as:

$$H(M) = \sum_{i \leq j} P_{ij} \log_2 \left( \frac{P_{ij}}{P_i P_j} \right)$$

Relative entropy provides a measure of how stringent or promiscuous are the substitutions in the alignments represented by the matrix. A larger relative entropy represents a more stringent distribution of substitutions. BLOSUM85 has a relative entropy of 1.085 bits, whereas BLOSUM35 has a relative entropy of 0.34 bits. The reason is that the smaller the evolutionary distance between the proteins, the less frequent are the non-
synonymous replacements. Thus, studying a matrix’s relative entropy, and comparing it with those of other matrices, is a good measure by which to evaluate substitution strictness within a data-set. Of course, the matrix should also be studied in a particular manner, in order to get detailed results.

**Multiple sequence alignment, and sequence families**

Several protein sequences may be aligned together, to form a multiple sequence alignment. The considerations for multiple sequence alignment are the same as that for pairwise sequence alignment, except for the major caveat that dynamic programming cannot be used due to its time and memory inefficiency. Therefore, heuristics such as the Clustal (Thompson *et al.*, 1994) algorithm are used, based on a progressive alignment of pairwise alignments.

Using combinations of pairwise or multiple sequence alignment techniques, it is possible to map the known protein sequence space into families. Indeed, several databases containing sequence families, of whole proteins or of protein domains exist. In the recent Nucleic Acids Research database issue (January, 2002) 18 such databases were listed. ([http://www3.oup.co.uk/nar/database/cat/12](http://www3.oup.co.uk/nar/database/cat/12)). Although some of the databases listed there deal solely with sequence motifs, most of them are a compilation of sequence families. A sequence family is loosely defined as a collection of sequences which can be aligned in a significant manner, and which have a functional and evolutionary relationship. The definition is considered loose, as varying alignment definitions and
thresholdings can serve to create different families from the same pool of sequences.

1.4.2 Homology by structure, and structure families

Another way of inferring a relationship among proteins is by structural considerations. This is considered to be a much stronger approach than sequence-based approaches, as structure is better conserved than sequence (Lesk & Chothia, 1980). It is used less often, however, due to the paucity of structures available, and until recently, the paucity of structural alignment programs, and the lack of accessibility to those which do exist. There are several automated structural alignment algorithms e.g., [Orengo & Taylor, 1996] [Holm & Sander, 1993] [Leibowitz et al., 2001], for comprehensive reviews and assessments see [Godzik, 1996] [Gerstein & Levitt, 1998]. Automated structural alignment tools are an important part of bioinformatics research, and more so once it has been realized that many proteins which share a common fold have no detectable sequence similarity. As stated in section 1.3, most of the information we have regarding proteins is sequence information, whereas only for a minority do we have solved structures. However, we do have enough structure solutions so that we can examine relationships among proteins based on structural considerations. That is, superimpose whole proteins, or structural domains using structural alignment techniques, in order to discover the relationship among them. Structural domains are defined as autonomous folding units within a single protein chain, which are usually
CHAPTER 1. INTRODUCTION

associated with a given function.

The representation of a structure —3D information— is much more complex than sequence 1D information. Consequently, The alignment of two structures is quite a complex problem. The problem is compounded by the lack of a representative model for structure, and consequently of a single model of similarity for structure alignment. Regardless of the method employed, all sequence alignment methods assume that the sequences to be aligned have a common ancestor, and that the two sequences are related to each other by a series of quantifiable steps, which are indels (insertion and deletions of sequence elements) and substitutions. The methods vary in algorithmic application (various heuristics vs. dynamic programming), and indel/substitution penalties applied. Furthermore, the representation of sequences is always performed using a string of characters. In contrast, there is no requirement for an underlying evolutionary hypothesis for structural alignments. Indeed, some alignment algorithms, such as the geometric hashing, rely on rigid body superimpositions, and are sequence independent (Nussinov & Wolfson, 1991).

Different algorithms can be used for structural alignment. Among those used are graph theory, geometric hashing (Bachar et al., 1993), distance plot comparison (Holm & Sander, 1996) and double-dynamic programming (Orengo & Taylor, 1996). Graph theoretical approaches are based on the representation of the protein as a graph, e.g. using the distance and angles between secondary structure elements, the latter being the nodes of the graph. Partial matches between proteins are performed using sub-graph isomorphism algorithms.
CHAPTER 1. INTRODUCTION

Geometric hashing, introduced as a method for structural alignment by Nussinov & Wolfson (1991) decomposes the proteins to be aligned, representing them as partial rigid objects. The combinatorial extension (CE) algorithm uses aligned fragment pairs (AFPs) (Shindyalov & Bourne, 1998). AFPs are pairs of fragments from the proteins to be aligned. Combinations of AFPs which represent possible continuous paths are selectively extended or discarded, eventually producing a single optimal alignment.

In the course of my work I have used two different structural alignment methods. Those were the DALI (Holm & Sander, 1996) and SSAP (Orengo & Taylor, 1996) algorithms. Those methods were chosen as they have been producing credible results, for their flexibility and ease of use. Results produced in this work as a result of both alignments do not differ significantly from each other (for details on this see Chapter 2). Both structural alignment algorithms shall be briefly described here.

The DALI (Distance-matrix ALIgnment) algorithm uses a distance matrix representation of a protein structure, which is obtained as follows: a matrix is drawn, with the protein sequence running along the top and side of the matrix. For each pair of residues that are determined to be in contact (below a certain distance threshold), a mark is made in the cell representing those two residues. It follows that for two structurally similar proteins, two similar distance matrices will be drawn. In order to align them, the distance matrices are first decomposed into elementary contact patterns, e.g. hexapeptide-hexapeptide submatrices. Then, similar contact patterns in the two matrices are paired and combined into larger consistent sets of pairs. This methods allows
for the accumulation of indels.

SSAP (Sequential Structure Alignment Program) uses the Needleman-Wunsch algorithm originally developed for sequence alignment. However, in this case, three-dimensional geometry is compared to identify equivalent positions. This is done as follows:

1. Construct a view for each residue. The view is a set of vectors from each Cβ atom to Cβ atoms of all other residues in the protein. A common frame of reference is defined for each residue based on the tetrahedral geometry of the Cα atom.

2. The optimal pathway aligning the views is obtained, similarly to sequence alignment, by dynamic programming.

Alignment of views is suggested to be more informative than the alignment of distance plots (e.g., DALI), vectors give more information on relative positions than distances. Similarly, Cβ atoms give more information than Cα atoms.

As in protein sequence space, families of protein structures based on their structural similarities may be obtained. It follows that a map of protein structure space can be generated using structural alignment techniques, or a combination of sequence and structure alignment techniques. The three best known and often used maps are SCOP \cite{murzin1995structure}, CATH \cite{orengo1997classification} and FSSP \cite{holm1996structure}. SCOP and CATH contain a hierarchical representation of the structural information.
in the PDB. FSSP is constructed differently, and contains a clustered representation of protein structures.

**Structural databases**

Although seemingly few when compared with the number of sequences, there is a need to order and classify the ~16,000 structures resident in the PDB. Structural classification is the initial step necessary for recognition of evolutionary and functional relationships among proteins.

The three main structural classification databases are reviewed here. The hierarchical databases, CATH and SCOP partition the protein structure space in a hierarchical fashion. CATH clusters proteins at four major levels, Class(C), Architecture(A), Topology(T) and Homologous superfamily (H) (*Orengo et al., 1997*). Class, derived from secondary structure content, is assigned for more than 90% of protein structures automatically. The partitioning is into all-alpha helix structures, all-beta strand structures, and alpha+beta. Architecture, describes the gross orientation of secondary structures, independent of connectivities. The topology level clusters structures according to their topological connections and numbers of secondary structures. The homologous superfamilies cluster proteins with highly similar structures and functions. The assignments of structures to topology families and homologous superfamilies are made by sequence and structure comparisons.

SCOP (Structural Classification of Proteins) is partitioned in a similar manner
as CATH. However, the structure curation and classification is performed manually [Murzin et al., 1995].

FSSP (from which SSSD, the database in this work is derived, see section 1.4.3) is described here. FSSP stands for Fold classification based on Structure-Structure alignment of Proteins [Holm & Sander, 1996]. FSSP is constructed as follows:

1. Representative sequences from the PDB are chosen. The sequences are chosen so that there is no more than a 25% similarity between any two sequences.

2. The representative sequences are structurally aligned, using DALI, to all other PDB structures.

3. For each representative structure, an entry in the FSSP database is created. Each entry contains the alignment of that structure to all PDB structures for which a statistically significant alignment exists, according to DALI.

DAPS is a subset of FSSP [Mallick et al., 2001], which contains alignments from those entries which have a low sequence identity percentage (25% or less).

1.4.3 The SSSD database

A central implement used for my work was a rigorously compiled database of 118 structurally similar, sequence dissimilar protein pairs. (The SSSD database). The database is composed of pairs of proteins whose structures have been determined by X-ray crystallography. The structural alignment of these proteins has been determined by the
DALI algorithm, and they have been extracted from the FSSP and DAPS databases. The SSSD database has the following traits:

1. A minimal protein length of 30 residues. The reliability of the structural solution of shorter peptides is often dubious, and they are more prone to radical structural changes based on single-residue substitution.

2. Each protein has been determined by X-ray crystallography with a resolution of \( \leq 3.5 \, \text{Å} \). This ensures that a reasonably accurate structural solution was obtained.

3. Difference in pair member lengths does not exceed 50% of the shorter member’s length, and is at least 60% of the longer pair-member’s length. These criteria ensure a large mutual overlap length of the alignments, so that the structural alignment would be of at least one structural domain.

4. The Smith-Waterman algorithm was used to check the statistical significance of pairwise sequence alignment of the pair-mates. Pairs whose local alignment was found to be statistically significant, were excluded from the database. Therefore, the pair-mates in the SSSD database do not have any detectable sequence similarity.

Taken together, these traits ensure that SSSD contains pairs of proteins which are well-aligned structurally, but have no sequence similarity between the pair-mates. The pairwise structural alignments in SSSD were used as the starting point for the location
and characterization of positional determinants which are suspected of being important for protein fold / function. Initial analysis of SSSD shows that the distribution of folds within it parallels that of the entire PDB.

1.4.4 PSI-BLAST

Position Specific Iterated Basic Local Alignment Search Tool (Altschul et al., 1997) (PSI-BLAST) is a program used for searching sequence similarities in protein databases. Briefly, PSI-BLAST works in a series of repeated iterations. First, a protein sequence (the query sequence) is given to the program. PSI-BLAST searches for sequence similarities in whatever database the user specifies. Second, the results of the search are aligned, and a position specific scoring matrix (PSSM) or profile is generated. The PSSM is a matrix which indicates the frequency of each amino acid in each aligned position. Thus, for each position in the alignment, the PSSM contains information both about the amino-acid types in that position, and the overall conservation of the position. In the third step, the database is searched again with the PSSM. Searching with a PSSM instead of searching with a query sequence sensitizes the search to include more sequences, as the PSSM contains information from the alignment of several sequences, thus representing a sequence family rather than a single sequence. The second and third steps are reiterated, for a predetermined number of iterations, or until the search converges: no new sequences are found in the queried database. In my work I
have used PSI-BLAST as a tool for determining positional conservation along protein sequences (chapters 2 & 3). The positional frequency of amino-acids in PSI-BLAST is not readily available, and I was required to modify PSI-BLAST’s source code in order to extract that information. One important parameter in PSI-BLAST, which is referred to in Chapters 3 is the e-value. The e-value (“Expect-value”) is a parameter that describes the number of hits one can “expect” to see just by chance when searching a database of a particular size. Essentially, the e-value describes the random background noise that exists for matches between sequences. The e-value is used as a convenient way to create a significance threshold for reporting results.

1.4.5 Materials

Most of the code independently written for this study was developed in Python (python.org), using the the Biopython toolkit (biopython.org). A minority was developed in C. Computations were performed on RH Linux 6.2 and 7.1 (Red Hat Inc.) Intel 686, and on Silicon Graphics (Silicon Graphics, Inc.) Indy IRIX 6.5. Databases used (CATH, FSSP, DAPS, NCBI non-redundant) for this study were downloaded from their respective sites, with updates and citations noted in the papers presented. The standalone version of PSI-BLAST, blastpgp, was used for PSI-BLAST runs. I have modified the source code of blastpgp in order to obtain observed and expected residue frequencies making up the PSI-BLAST PSSMs.
Database parsers and program output parsers were developed independently, or taken from the Biopython toolkit. Program suites such as GCG 10.1 (Accelrys, Inc.) and EMBOSS (Rice et al., 2000) were occasionally used for the processing of sequence data.

1.5 The Challenge of Fold Prediction

Fold prediction is the technique of predicting a fold for a given sequence which shares no similarity with other sequences in the database. Methods of fold prediction vary. Threading uses pseudo-energy functions to determine whether a sequence can be aligned using energy considerations, with any of the known folds. PSI-BLAST, and more sophisticated methods derived from PSI-BLAST, may be described as extremely sensitive sequence detectors, and use PSSMs to detect remote sequence similarities, which may be used for modeling. In any case, these fold-prediction techniques can rarely predict new folds. Ab-initio methods try to predict the fold given energy considerations alone, so hypothetically they may be able to predict novel folds. However, they are currently quite impractical, both in terms of computational time, and in success in target prediction.

Much effort in fold prediction is given to the training and refinement of the techniques. This work is not directly concerned with prediction, but rather with understanding the mechanisms leading to a given fold. Specifically, with the detection and
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characterization of specific positions which can serve as anchors for folding and function. The ability to locate and understand the role of these positions can improve the construction of fold predictors.

1.6 Synopses of following chapters

Chapter 2 is a published paper describing the study of identical, aligned conserved residues between the SSSD pair-mates. It shows that among structurally aligned protein pairs identical residues which are conserved in evolution tend to be located in buried positions, and many are found in positions critical for maintenance of structure or function.

Chapter 3 is a published paper describing the study of all aligned, conserved residues between SSSD pair-mates. This paper introduces the novel concept of persistent conservation, that is, an assessment of conservation based on close and distant sequence family members. A significant fraction of these mutually, persistently conserved positions (MPCs) are shown to be located in positions which are conducive to secondary structure determination, are mostly buried, and many of them form spatial clusters within their protein structures. A substitution matrix based on MPCs shows distinct characteristics which may prove valuable in protein design experiments.

Chapter 4 is a published paper which examines the accuracy of PSI-BLAST alignments vs. structural alignments. Notably it shows improvement in alignment sensitivity
over consecutive iterations with no discernible specificity loss. It also shows that in terms of alignment accuracy, PSI-BLAST performs as well as threading methods.
Chapter 2

Paper: Glimmers in the Midnight
Zone: Characterization of Aligned
Identical Residues in
Sequence-Dissimilar Proteins
Sharing a Common Fold

Friedberg I, Kaplan T and Margalit H Proc Int Conf Intell Syst Mol Biol. (2000) 8:162-70.
Chapter 3

Paper: Persistently Conserved Positions in Structurally-Similar, Sequence Dissimilar Proteins: Roles in Preserving Protein Fold and Function

Friedberg I and Margalit H Protein Science (2002) 11(2):350-60
Chapter 4

Paper: Evaluation of PSI-BLAST alignment accuracy in comparison to structural alignments

Friedberg I, Kaplan T and Margalit H Protein Science 2000 9(11):2278-84.
Chapter 5

Additional Results

This chapter elaborates upon another case study, in the same manner as in Chapter 3. The protein pair chosen from the database was Methionine synthase (MetS, PDB:1BMTA) (Drennan et al., 1994) and CheY (PDB:3CHY) (Volz & Matsumura, 1991) from E. coli.

5.1 Description of the Enzymes

5.1.1 Methionine Synthase

MetS is composed of two domains, a Rossman fold, the C-terminal domain, and an orthogonal α-helix bundle, the N-terminal domain. Sandwiched between the two domains is the cobalamin prosthetic group. Free methylcobalamin, a derivative of vitamin B12, is composed of a heme-like corrin ring, and a dimethylbenzimidazole nucleotide
moiety. In the center of the corrin ring lies a cobalt atom, that is hexacoordinated by four nitrogen ligands provided by the corrin macrocycle, a methyl group in the upper (β) axial position, and a nitrogen (N3) from dimethylbenzimidazole in the lower (α) axial position. However, when bound to Methionine synthase, the dimethylbenzimidazole nucleotide is displaced from the cobalt to form a “nucleotide tail”. The corrin portion is sandwiched between the two domains of the protein, while the nucleotide tail penetrates into a pocket in the Rossman fold domain (5.1.a).

MetS catalyzes the synthesis of methionine by two successive methyl transfers:

1. CH$_3$-cob(III)alamin + homocysteine → cob(I)alamin + methionine

2. cob(I)alamin + methyltetrahydrofolate → CH$_3$-cob(III)alamin + tetrahydrofolate

5.1.2 CheY

CheY is a single domain protein, assuming a Rossman fold (Figure 5.1 b). CheY is part of a chemotactic signal transduction mechanism in the E. coli bacterium. CheA$_L$, a kinase of the chemotaxis system, phosphorylates CheY which acts as the response regulator. Transiently phosphorylated CheY interacts with the bacterial flagellar motor to cause clockwise rotation of the flagella, creating a distinct motion response dubbed “tumbling”.
MetS and CheY are two enzymes that on a sequence and functional level have nothing in common. The former is a methyltransferase, while the latter is a phosphorylated signal transducer. However, the Rossman-fold domain of MetS bears a strong structural similarity to that of CheY. When aligned, (figure 5.2) several MPCs which have distinct but different functions in both proteins are revealed.

MetS:S804 is an MPC with CheY:D57 (figure 5.3 a). MetS:S804 hydrogen bonds to N3 of dimethylbenzimidazole, which is the same nitrogen that is coordinated to the
cobalt in free methylcobalamin. The structure of MetS shows this bond is important for cofactor binding. CheY:D57 is the residue that is phosphorylated by CheA to activate CheY.

MetS:H759 is an MPC with CheY:F14 (figure 5.3b). MetS:H759 axially α-coordinates the cobalt atom which lies in the corrin ring. (Axial β coordination is performed from the C-terminal domain, which is not aligned with CheY, and is therefore not discussed.)
here). CheY:F14 has been the subject of several mutagenesis studies in Luis Serrano’s group. Serrano et al. have shown that CheY:F14N mutation stabilizes the protein, and accelerates refolding after the protein has been subjected to urea denaturation. The reason that position 14 is a capping position of the \(\alpha\)-helix and the asparagine mutant forms a better N-cap. However, in another study it was shown that the CheY:F14Y mutation activated CheY constitutively, without phosphorylation. Although an accurate functional role for CheY:F14 has not been discovered, it is hypothesized that the proximity to the catalytic residue CheY:D13 renders it sensitive to mutations, which although serve to stabilize it (CheY:F14N, and CheY:F14A have shown to lower the unfolding energy of the protein) probably change the protein’s function, as in the CheY:F14Y mutation. A clue as to CheY:F14’s functional role comes from LIGPLOT, a program which automatically discovers and classifies ligand-protein interactions. CheY:F14 is proposed to form a hydrophobic contact with a free \(\text{SO}_4^{2-}\) anion (figure 5.4). This \(\text{SO}_4^{2-}\) anion is one of three which have been identified in the crystal structure. The \(\text{SO}_4^{2-}\) anion is derived from the crystallization solution, which contains ammonium sulfate. This particular anion, is centrally located in the most accessible region of the active site. Its oxygen atoms are bound to the \(\epsilon\)-amino nitrogen of Lys-109 and \(\text{N}\delta\) of the Asn-59 amide side chain. As \(\text{SO}_4^{2-}\) is physically and chemically similar to \(\text{PO}_4^{3-}\), it may be representative of how a \(\text{PO}_4^{3-}\) would interact with the unactivated CheY in a non-covalent manner. It should be noted that the hydrophobic interaction CheY:F14–
PO$_4^{3-}$, and consequently its supportive role has not been proposed elsewhere except for by the LIGPLOT diagram. However, taken together with the constitutive activation of the CheY:F14Y mutation, and the proximity to the catalytic site, it appears that CheY:F14 does have a functional role having to do with the binding of the free PO$_4^{3-}$ group.

Figure 5.3: Yellow: MetS; blue: CheY; red: cobalamin prosthetic group. (a) (Center) Orange: MetS:S804; cyan: CheY:D57 (unphosphorylated). (b) Orange:MetS:H759; cyan: CheY:F14

Table 5.2 shows all MPCs found in the MetS / CheY pair and, for those for which
Figure 5.4: LIGPLOT proposed interaction between the free $\text{SO}_3^-$ and the catalytic site in CheY. See text for details.

A structural or functional role has been determined, notes the role. For example, MetS:P875 and CheY:D38 are both in the N-terminal capping position of their respective aligned $\alpha$-helices. MetS:T808, a ligand binding residue, is aligned with CheY:P61 which forms a hydrogen bond with CheY:M63.

Section 6.2.4 discusses the implications of structurally aligned functional residues, in structurally similar, functionally different proteins.
### Table 5.1: MPCs in MetS and CheY, annotated by function.

| MetS | role | CheY | role |
|------|------|------|------|
| V750 | L9   | L9   |
| H759 | AS   | F14  | LB   |
| V766 | LB   | V21  | LB   |
| L770 | L25  |      |      |
| N774 | G29  |      | α-C-term |
| Y775 | F30  |      |      |
| I777 | V33  |      |      |
| L780 | A36  |      |      |
| P785 | α-Nc | D38  | α-Nc |
| I789 | A42  |      |      |
| A798 | Y51  |      |      |
| D799 | G52  |      |      |
| I801 | V54  |      |      |
| G802 | LB   | I55  | AS   |
| S804 | AS   | D57  | AS   |
| T808 | LB   | P61  | 3-1 Hbond with M63 |
| M821 | I72  |      |      |
| P829 | P82  |      |      |
| L831 | LB   | L84  |      |
| G833 | LB   | V86  | G833/G834 are conserved for steric reasons |
| G834 | LB   | T87  |      |
| Y850 | G102 |      |      |
| Y856 | Y106 | LB   |      |
| N859 | LB   | P110 | Has a cis bond with K109 (active site) |
| A860 | LB   | F111 |      |

Description: LB - ligand binding; AS - active site member; α-C-term: c-terminal acid in an α-helix; α-Nc: N-terminal capping residue. Annotations were determined according to the crystallographer’s papers, the CATH database, and the Protein Mutant Database (www.genome.ad.jp)
Chapter 6

Discussion

This work presents a unique approach to a topic within the framework of the protein folding problem. It is derived from the premise laid out in the central dogma of structural biology: “sequence determines structure”. When faced with the phenomenon of different sequences which adopt the same fold, a question which automatically arises is: “if sequence determines structure, which elements in different sequences cause them to fold similarly?”

The evaluation of structural / functional importance of discreet amino-acid positions is not a trivial task. Indeed, in most cases we cannot know the role and relative importance of a given residue. Only a handful of proteins have been investigated thoroughly using site-directed mutagenesis as to the role, or “non-role” as the case may be, of each and every residue. When considering a target for site-directed mutagenesis, the positions targeted are normally purposefully selected, based on prior knowledge of the protein’s
sequence to function mapping (or structure to function, if available). The reason is that setting up a functional assay for most proteins, and using it to investigate a large amount of mutants is a laborious process. It is also quite superfluous for most research purposes. Labor-intensivity and superfluousness hold even stronger when performing site-directed mutagenesis and setting up an assay to investigate the effects of mutations on structure, which would require the determination of hundreds or even thousands of structures for a given protein.

Therefore, it is advisable to turn to computational methods for location and characterization of critical positions in a protein *en-masse*. Most computational methods base their analysis on evolutionary conservation. The premise being the following: if a position is determined to be conserved, then it has been positively selected, and for a good reason.

### 6.1 STAIRS

Our initial study (Chapter 2) was concerned with Structurally Aligned Identical ResidueS (STAIRS). The premise being that the few aligned residues which are identical between proteins differing in sequences in the whole, are worth investigating as maintainers of structure or function. Even better candidates are the STAIRS which are spatially close, which we named NSTAIRS, (Neighboring STAIRS). Those may play a structural or functional role common to both pair-mates.
We have determined a conservation score for each aligned position. Conservation for each position in the database was determined by performing a multiple alignment using PSI-BLAST. PSI-BLAST was chosen because it collects and aligns distant family members, in an iterative manner. The degree of evolutionary conservation was calculated from the last PSI-BLAST iteration. The reason for that being, that we wanted to look at conservation which exists in aligned distant family members. This is explained as follows: conservation of a given position might be due to evolutionary relatedness of aligned sequences, without actually being important for the protein’s structure. However, positions conserved between distant sequence family members are better candidates for being critical positions, as presumably they are distant enough so that conservation due to evolutionary non-divergence will be sifted out. Therefore we assessed conservation based on a sequence alignment of distantly related sequences.

Normalized conservation scores ($Z_{ic}$ scores, see Chapter 2/Methods for details) are well correlated between STAIRS and even better between NSTAIRS. This initial finding indicated that examining STAIRS and NSTAIRS as candidates for critical positions is worthwhile.

However, aligned residues may be identical by chance. 39.5% of the STAIRS are not highly conserved. We examined the abundance of STAIRS and NSTAIRS in all the aligned positions, and in aligned positions which are well conserved ($Z_{ic} \geq 1.65$, Chap. 2 / Table 1). STAIRS made up 48.4% of the population of conserved aligned positions, and 75% of the well-conserved STAIRS are NSTAIRS. This finding set the foundation
for the research we conducted and is elaborated upon in Chapter 3. Namely, look for mutual conservation, rather than identity & conservation.

Solvent accessibility is a good initial index for examining location within the protein. We found that the proportions of buried STAIRS was the same as that of the entire aligned residue population (≈50%). However, when examining only conserved STAIRS, the percentage of buried STAIRS was raised dramatically: ≈85%. We have also partitioned the examined positions into hydrophobic (HP) and hydrophilic (HY) residue types. In the entire population, HP residues were more buried than HY types. However, when looking at the well-conserved populations, the differences in the ratio of buried residues partitioned either by hydrophobicity, or according to their STAIRS/NSTAIRS association were not significant. Circa 85% of the residues were buried. This is actually expected, as conserved residues, regardless of physico-chemical traits, are overwhelmingly buried (Cordes et al., 1996).

In this preliminary study we have shown that positions with conserved, identical residues may be explained by burial, participation in secondary structure, and by specific roles pertaining to the functional site in which they reside.

### 6.2 Mutually, Persistently Conserved Positions (MPCs)

A central observation made in the STAIRS study, was that over 50% of the positions with mutually high conservation contain different residues in the two proteins. This has
led us to an extended study, in which we aimed to characterize the mutually conserved residues in SSSD protein pairs, regardless of identity.

6.2.1 Substitution matrices

Our motivations for extracting a substitution matrix from our data were: (1) analysis of the allowed substitutions in MPC positions; (2) comparison of the amino-acid distribution of substitutions to the distributions from which the BLOSUM series has been derived. As shall be explained later, we aimed to determine the difference between substitutions occurring due to evolutionary conservation and critical role, and those conserved due to critical role only.

Specialized substitution matrices have been constructed by several research groups, in order to increase detection sensitivity and alignment reliability, and to study the substitutions within the data-set. For example, several studies have been published concerning the substitutions in transmembrane regions of proteins. The motivation for those studies was that the “generic” substitution matrices are mostly derived from sequences of globular proteins. Since transmembrane regions of a protein are in a distinctly different environment, a specialized substitution matrix would seem more appropriate when analyzing those regions (Persson & Argos, 1994; Jones et al., 1994; Ng et al., 2000). Indeed, the reported detection and correct alignment ability achieved by using those matrices for transmembrane proteins supersedes those of the “generic” matrices. In studies
more relevant to this one, substitution matrices were derived from aligned structures and studied \cite{Prlc2000,Naor1996}. The substitution matrix we have derived from all aligned residues in our database is very similar to the one derived by Prlic \textit{et al} \cite{Prlc2000}.

The MPC-derived matrix exhibits some very interesting traits. The most striking is the high relative entropy (1.015 bits). This relative entropy is comparable to that of BLOSUM85, which is a matrix derived from BLOCKS composed of sequences with at most 85\% identity. The high relative entropy in BLOSUM85 is due to a high rate of synonymous substitutions, which in turn is due to the fact that the BLOCKS fraction from which BLOSUM85 was constructed is composed of closely related sequences, which by definition have a high rate of synonymous substitutions.

The sequences we used to generate the MPC matrix have a very low identity (\textasciitilde 12\%), but the mutually conserved residues we collected had a high rate of synonymous substitutions. In BLOSUM85, the high rate of synonymity is due to evolutionary non-divergence among the composing sequences. In the MPC-derived matrix, that is evidently not the case. The hypothesis offered is that the high rate of synonymity here is due to the irreplaceability of the residues whose substitutions make up the matrix.

This hypothesis is strengthened by comparing the distances between the distributions making up the MPC-matrix, and the BLOSUM matrices \cite[figure 4]{chapter3}. The distribution of substitutions making up the MPC-matrix differs significantly from those making up the BLOSUM matrices. In comparison, the distribution of substitutions
making up the structurally derived matrix (SDM) is quite similar to that of BLOSUM35. The latter finding is not surprising, when we consider that BLOSUM35 is derived from BLOCKS of sequence alignments with a sequence identity of no more than 35%. The data-sets from which both BLOSUM35 and SDM are derived are aligned sequences exhibiting a low similarity.

An interesting observation regarding the MPC matrix, which was not addressed in Chapter 3, is offered here. The score of Ile/Ile replacement is actually lower than the Ile/Val replacement score ($\text{MPC}[I,I] < \text{MPC}[I,V]$)\(^1\), suggesting that replacement of Ile by Val or vice versa, is superior to Ile’s conservation. The difference between isoleucine and valine rests with the removal of a single methyl group substituting isoleucine’s $C\beta$ atom. It has been shown that Ile$\rightarrow$Val mutations lead to an increase in protein stability, up to 2 kcal M$^{-1}$, due to the deletion of the methyl group (Ventura et al., 2002). It may be hypothesized that the reason the non-synonymous Ile/Val substitution scores higher than the Ile/Ile substitution is because of that. On the other hand, stability is not the only selective factor in a protein’s fitness. Another is foldability: $\phi_{_{3-U}}$, which is measured as the change of stability in the protein’s transition state introduced by a mutation, divided by the change in the stability of the mutated protein’s folded state ($\Delta\Delta G_{3-U}/\Delta\Delta G_{F-U}$). Negative $\phi_{_{3-U}}$ values can result if the introduced mutation stabilizes the folded state, but destabilizes the transition state. The Ile/Val mutants

\(^1\text{MPC}[i,j]$ denotes the log-odds value for the substitution of any amino acid $i$ by amino-acid $j$ in the MPC-derived matrix.
discussed in Ventura et al. have negative $\phi_{4-U}$ values that may be the result of strained interactions. In any case, as the hypothesis that $\text{MPC}[I,I] < \text{MPC}[I,V]$ is due to this phenomenon has not been tested, it is presented simply as an interesting supposal.

### 6.2.2 Cluster analysis

When predicting the location of critical residues, evolutionary conservation is seldom the sole criterion for determining the importance of a given position in a protein. Methods concerned with prediction of critical positions use also structural information in order to: (1) sift and supplement information given by evolutionary conservation; (2) analyze conserved positions, in order to determine why they are conserved. In this study, we have also examined the clustering of MPCs. The term *cluster* is used here in a very specific sense: it is a measurable trait of contacting residues. The quality measured is the inverse of the probability that those residues shall be in contact, given the sequence distance of contacting residues in the protein’s fold. In other words, the less probable it is those residues are in contact, the higher their clustering score. However, as our study’s main concern was discovery rather than prediction, we used the information from structural positioning in order to classify the various roles MPCs may play in defining protein structure.

Spatial proximity of positions provides good augmentative information to evolutionary conservation. Poteete’s studies of critical positions in the T4L have shown that
the critical residues were more evolutionary conserved than the other positions in the protein (Rennell et al., 1991). This was shown to be especially true for positions in the catalytic site, and in those hypothesized to maintain the hydrophobic core. In both cases those are positions which are both conserved and spatially close.

Shakhnovich and Mirny (1999) have published a study where they examined positions which are conserved according to the CoC method, and correlated them with known folding nucleation centers. Those residues were usually in contact, although they were not necessarily close in sequence.

Kannan et al. have developed a clustering-analysis method using a weighted-graph representation of the Cβ atoms. The graph is represented as a Laplacian matrix, and clustering information is derived from the weighted components of the second lowest eigenvalue. In the first study of this series (Kannan & Vishveshwara, 1999) it was observed that many of the clusters were hydrophobic and buried. However, clusters near the active and binding sites were also detected. A similar study was conducted specifically on α/β-barrel proteins (Kannan et al., 2001), where it was shown that clustered residues are often conserved, and predicted to be part of the folding nucleus. Certain clusters were found to be part of the active site, or close to the active site.

Plaxco et al. (1998) have coined the term relative contact order to describe the average sequence distance between all pairs of contacting residues normalized by the total sequence length. For all contacting residues indexed $i, j$: 
\[ CO = \frac{1}{L \cdot N} \sum_{i,j}^{N} \Delta S_{i,j} \] (6.1)

Where \( \Delta S_{i,j} \) is the sequence separation, \( N \) is the total number of contacts, and \( L \) is the total number of residues in the protein. CO is used to quantify the mean sequence separation between contacting residues in a chain. In that study, Plaxco et al. have shown that relative contact order is inversely correlated with the folding rate. However, the utility of contact order may be expanded to examine the clustering of chosen positions along the structure. The question asked would be: “given a number of contacting positions, are they expected to be contacting given their sequence separation?” positions which have a low mean sequence separation are also expected to be in contact. However, widely separated positions which are in contact, might indicate a functional site, or a structural stabilizer.

We have developed a novel method for assessing residue clustering. The method’s input is a protein structure, and an indication of the residues to be analyzed. The method reports whether the indicated residues are more clustered than expected for the particular protein being analyzed. A modified version enables us to input two structurally aligned proteins, and to assess the clustering of indicated residues, MPCs in our case.

The method is described in detail in the Materials and Methods section in Chapter 3. Here I will describe its benefits:
1. **Fold Specificity.** The method uses a weighted graph to describe the clustered residues. The weights of the edges are based upon the probability of two residues (graph nodes) being in contact \(d(C_A\beta - C_B\beta) < 7 \, \text{Å}\), for the given protein. We propose this weighting scheme to be better than weighting according to sequence distance, as for different folds there are different probabilities of two residues being in the same sequence distance to be in contact.

2. **Assessment of Statistical Significance.** Given a score, we assess it by a Monte-Carlo procedure. The method is repeated for the protein with randomly selected positions (or, in the case of MPCs, randomly selected aligned positions). In this manner, a distribution of clustering scores for the number of contacting residues is generated. This distribution is generated each time the assessment is performed, so that it too is specific for the protein’s (or aligned pair’s) structure.

Our clustering assessment method uses the structure of the protein to score and assess the distance between residues, normalized by the probability of those positions being in contact. Although we use this method in context with MPC evaluation, it may be used for any type of clustering evaluation of selected positions, within a single protein or within a structural alignment.

Conceivably, this method may be enhanced or modified for a different purpose using a statistical energy function for contacting residues. The edge weight for two contacting residues will be parameterized not only by their sequence distance, but also by a type-
dependent and distance-dependent energy function. Thus, contact between residues is not a "qualitative event" (i.e. they are either "in contact" or "not in contact"). Rather, it is a quantitative event. For example, suppose that in a given protein two contacting residues, Glu and Arg are separated by \( N \) positions along the sequence, and two other contacting residues, Ala and Ile are separated by \( N \) residues along the sequence. The graph edge designating the Glu-Arg contact should be weighted higher than the Ala-Ile contact, as Glu and Arg may form a salt bridge, whereas Ala-Ile will form a hydrophobic, or a VDW bond. An extra refinement could involve the addition of the actual spatial distance between the residues (based on \( C_\beta \) atoms, or on centroids), to determine whether either of the pairs are within an optimal distance for their respective interactions. To summarize, the introduction of an energy function can contribute by refining the weight of the graph’s edge based not only on sequence distance, but also on contacting residue type & spatial distance.

Our clustering-analysis method is a relatively simple one, and derives its rationale from Plaxco et al.’s contact order. However, the computational overhead is relatively large due to the Monte-Carlo method of assessment of statistical significance. It is, however a very precise method, as it checks for statistical significance on a case-by-case basis.

In order to improve the time efficiency of the significance analysis, an analytical method may be developed based on the analysis of residue clustering distributions throughout all protein structures. The clustering score distribution’s parameters can
CHAPTER 6. DISCUSSION

be established empirically in the following manner:

1. Select a group of representative folds (e.g. from SCOP).

2. Establish a distribution of clustering scores for 3, 4, 5,... contacting residues in each fold. The result would be a collection of clustering score distributions, parameterized by fold type and number of residues in cluster.

3. When assessing the significance of an MPC clustering score in a given SSSD protein pair, the clustering score of the MPCs should be compared with that of the appropriate score from the “distribution bank” above. Given a score $S_{MPC,4}$ for 4 MPCs in a TIM-barrel, the score would be compared with the random distribution of scores for 4 residues in a TIM-barrel fold, in order to determine its significance.

6.2.3 Secondary structure analysis

Another level of determining the structural role of examined residues is to assess their distribution within secondary structure elements (SSEs). The distribution of residue types within $\alpha$-helices has been studied extensively by surveying the residue distribution in helices e.g. [Richardson & Richardson, 1988, Aurora & Rose, 1998, Kumar & Bansal, 1998].

In the study presented in Chapter 3, we have looked at the distribution of MPCs within SSEs in our database. We have aligned the edge and flanking positions of all SSEs ($\alpha$-helices and $\beta$-strands) of a minimal length to each other, and looked at flanking and in-SSE positions for high distributions of MPCs. Chapter 3 Figure 5 describes
our results. In α-helices we found that MPCs are highly abundant in certain flanking positions. Examining the positions where MPCs were found to be abundant has revealed that amino-terminal flanking MPCs were mostly residues characterized as hydrogen-bond acceptors, whereas those flanking the C-terminal were found to be hydrogen-bond donors. We therefore suggest that in this structural context, MPCs are important for determining the helix ends by hydrogen bonding to the backbone atoms.

The residue type distributions in the α-helix termini taken from the entire database were found to be in agreement with previous studies of amino-acid distributions (Richardson & Richardson, 1981). The predominance of MPCs in the flanking regions, and the over-representation of hydrogen-bond acceptors and donors in the N- and C-flanks respectively was quite interesting. To the best of our knowledge no such systematic investigation of conserved residues along helix positions has been carried out. The CKAAPS (Reddy et al., 2001) study has determined the frequency of CKAAPS within α-helices, but did not systematically report their relative frequency along the helix itself on a whole scale database analysis, but only for selected test-cases.

### 6.2.4 Discussion of Case Studies

Two case studies are presented in, in Chapter 3 and in Chapter 5, and are discussed in detail here. In chapter 3 a cluster of MPCs from haloalkane dehalogenase from X. autotrophicus (XADL) and lipase B from C. antarctica (CALB) was analyzed. It was
shown that those clustered MPCs participated in the active site, the backbone scaffolding adjacent to the active site, and in substrate stabilization. XADL and CALB use the same reaction mechanism to catalyze their reactions, although on different substrates, and with different nucleophiles (XADL:D124/CALB:S105). The other MPC position in this cluster which does not have the same residue is XADL:W125/CALB:Q106. Possibly this has to do with substrate specificity, as XADL:W125 is known to play a critical role in XADL’s substrate binding. The other MPCs in this cluster were all glycines, and from the examination of the structures it appears that they serve as a crucial part of the scaffolding for the respective proteins. It is interesting to see that mutual conservation can be used to locate a low common functional denominator, in this case between functionally related proteins.

In chapter 5 a different pair of proteins is analyzed. Functionally, MetS and CheY do not have anything in common. However, several functional residues are conserved as MPCs. Naturally, it is expected that given a similar fold, residues that maintain structural similarity will be MPCs, as they should align within the proteins respective cores, or stabilizing the secondary structures. Those that were found are described in Table 4.1. Discovering functionally maintained residues as MPCs raises interesting biological and bioinformatical possibilities which merit further investigation. Biologically, how common is the phenomenon of functional positions being aligned in proteins which share the same structure but not the same function? If indeed it were found to be common, could it be utilized by structural prediction techniques in order to perform
functional predictions? This could be done as follows:

1. Perform homology modelling, using a template with a known function.

2. Infer from the alignment which residues in the protein with the unknown function may be responsible for function, based on the functional residues in the template protein.

3. Use a knowledge based system i.e. a databank of functional sites with an accompanying search algorithm in order to characterize the function of the protein with the unknown function. For example, PROCAT provides facilities for interrogating a database of 3D enzyme active site templates. PROCAT can be thought of as the 3D equivalent of the 1D templates found in sequence motif databases such as PROSITE and PRINTS. Instead of searching for 1D sequence motifs in a newly derived protein sequence, the PROCAT database allows searching for 3D enzyme active site template motifs in a protein structure [Wallace et al., 1997].

Currently, many studies are concerned with fold prediction, but another predictive challenge is that of function prediction, as enzymatic mechanism determination does not automatically follow from fold prediction [Erlandsen et al., 2000; Todd et al., 1999]. Locating those residues suspected of participating in the active sites, and intelligently predicting a protein’s function based on the residues’ identities, can save much of the effort involved with the structural determination of proteins normally necessary for the elucidation of mechanism of action. Function prediction can also aid in offering worthwhile
alternative hypotheses to current accepted wisdom. A good example is the alternative mechanism offered by Rigden et al. (2000) to the putative nucleotide binding site (NBS) of the R-type plant resistance genes. Rigden et al. have suggested that the NBS domain, which was determined to be so based on sequence motifs only, is actually a phosphorelay domain. This hypothesis was based on threading, sequence analysis and the construction of a molecular model. Although no compelling biochemical evidence has been found to support the phosphorelay hypothesis, it is still accepted as a viable alternative to the NBS hypothesis (Rigden et al., 2000; Fluhr, 2001; Gebhardt & Valkonen, 2001). The MPC analysis presented here may set the foundations for such a predictive scheme.

6.3 Evaluation of PSI-BLAST Alignment Accuracy

PSI-BLAST was developed as a tool extending BLAST to search for distant homologues. PSI-BLAST uses a PSSM generated from any given iteration to extend a search initiated with either a query sequence, or a PSSM generated by other means. PSI-BLAST has risen to a position of prominence among bioinformatics tools, and it is probably the most popular tool for searching for homologues. PSI-BLAST can also be used for fold assignment, by discovering similarity to the query sequence or the subsequent PSSMs in the PDB.

PSI-BLAST’s ability in fold recognition has been assessed by others (Park et al., 1998). However, given a true fold assignment, how good is the actual residue position assign-
ment? The answer to this question is important, as even for a true fold assignment, a bad alignment can mislead homology modeling.

Using the SSSD database as a benchmark, we have assessed PSI-BLAST’s alignment accuracy of distant homologues. The term “alignment accuracy” is a rather vague one, and requires clarification. Assuming we have a gold standard by which we assess the alignment accuracy, how is the questioned alignment to be compared with the gold standard? If the questioned alignment is exactly the same as the gold standard, then we may assign a top score (e.g. 100% accuracy). However, in case the two alignments are not in concord, two separate methods of accuracy assessment exist:

1. Sensitivity:

\[ sens = \frac{N_q \cap s}{N_s} \times 100 \]  \hspace{1cm} (6.2)

2. Specificity:

\[ spec = \frac{N_q \cap s}{N_q} \times 100 \]  \hspace{1cm} (6.3)

Where \( N_q \) is the number of aligned positions in the questioned alignment, \( N_s \) is the number of aligned positions in the gold standard alignment. \( N_q \cap s \) is the number of aligned positions which exist both in the gold standard alignment and in the questioned alignment (the size of the intersecting group between q and s).

So the question “how accurate is a given alignment method?” is forked into two
different questions: (1) “what fraction of truly aligned positions can a method detect out of the total length of the standard alignment?” or “how sensitive is the alignment method?” (2) “what fraction of the aligned positions in a method’s provided alignment are truly aligned?” or “how specific is the alignment method?”

Therefore, no single score can be provided as to the “accuracy” of an alignment method. Rather, when stating alignment accuracy, one must specify in terms of the method’s sensitivity or specificity. In our study, we have found a significant improvement of alignment sensitivity over consecutive PSI-BLAST iterations, past the detection iteration. However, no significant improvement or negative effect was exhibited with regard to specificity. On the one hand, this means that the number of correctly aligned positions does increase over consecutive iterations. On the other hand, the ratio between correctly aligned positions and incorrectly aligned positions remains roughly the same. This observation has interesting implications, both technical and biological. One implication concerns the proper use of PSI-BLAST: given a query sequence, and an interesting target which the user would like to investigate further, it is advisable to continue iterating PSI-BLAST, thus obtaining a more sensitive alignment, one which incorporates more well-aligned positions. Another implication concerns the proportional growth of misaligned positions with that of well-aligned positions. This follows from the observation that over consecutive iterations the sensitivity increases, but the specificity remains the same. Thus in absolute numbers, there are more well-aligned positions as the iterations progress, but in direct proportion to more misaligned positions. Conceiv-
ably, specificity may be improved, by the removal of irrelevant sequences from the PSSM for the next PSI-BLAST iteration. This is normally performed manually, as the choice of sequences to be removed must be made based on sequence annotation and biological knowledge. Automated improvement of homology detections in PSI-BLAST, using the literature associated with a sequence have been performed, although alignment accuracy has not been reported (Chang et al., 2001).

The biological implication may be stated in the following manner: given two proteins whose sequence similarity is undetectable by pairwise alignment, detection by sequence-only considerations is still occasionally feasible. Given enough evolutionary sequence-based information (in the form of PSSMs), this information may be used to obtain alignments as good as those provided by structure prediction programs using structure-based information, such as threaders. Our study’s findings correlate well with the results of another study published concurrently with ours (Sauder et al., 2000). In that study, structural alignments were derived using Combinatorial Extension (CE, Shindyalov & Bourne, 1998) for all superfamily and family-level related proteins in the SCOP database. PSI-BLAST showed a mean sensitivity of 40% after four iterations.

Indeed, this feature of PSI-BLAST has been investigated in the CASP3 and CASP4 meetings, using several algorithms that were based upon PSI-BLAST (Moult et al., 2001). One study in CASP3 used a PSSM generated by the query after four PSI-BLAST iterations through the nr database. This PSSM was used to search PDB for a suitable homologue, or for fold recognition as the case may apply (Dunbrack-Jr., 1999). As the
number of queries evaluated was very small, the study did not supply a mean alignment
accuracy score. Also, some of the queries had sequence similarity with PDB entries,
and some did not, so that the evaluation of alignment quality for those two different
categories should be done differently. However, alignment specificity (sensitivity was
not given) was shown to be \(~\)60\% for those queries with a low sequence identity with
the PDB entries. We have shown an alignment specificity of 54.9 \(\pm\) 2.1\% in our study.

Another study (Koretke et al., 2001) has evaluated the use of a method relying heav-
ily on PSI-BLAST for fold recognition, SENSER. SENSER attempts to predict a fold
by initially collecting low e-value hits from a PSI-BLAST run of the query sequence.
(The e-value is described in 1.4.4). In this case low e-value hits are sequences with low,
but significant similarity to the query sequence (< 25\% sequence identity, according to
Koretke et al.)

In the next step, the search is expanded using sequences from the low e-value
collection. Essentially, a transitive search is performed using low percent identity,
but statistically significant similar sequences. Sequences are aligned using HMMer
(http://hmmer.wustl.edu/), a Hidden-Markov-Model based alignment method. A study
conducted on 15 remote homologues yielded an alignment accuracy of 45\% (Koretke et al., 2002).
In this study, alignment sensitivity only is described.

Another concern is the standard by which alignment accuracy is assessed. Typically,
when assessing an alignment method, the standard referred to is the structural align-
ment. However, structural alignments themselves vary (Godzik, 1996), as discussed in
1.4.2 Therefore, the gold standard may vary, depending on which structural alignment algorithm is used. As stated in the Introduction, we chose the DALI alignment, and verified that it indeed is in good agreement with SSAP.

6.4 Conclusions

6.4.1 Critical Residues

When I have started my doctoral studies, the field of critical residue location by bioinformatical sequence and structure consideration was very much in its infancy. So was the study of SSSD proteins. In the past three years we have seen a sharp rise in the number and diversity of papers discussing both topics. This is due to several reasons:

1. A steep rise in the number of structures available for study. Additionally, a very steep rise in the number of sequences available for PSSM collation, thus creating more informative PSSMs, commented upon in Koretke et al., (2001).

2. With the increase in the number of solved structures, SSSD proteins have been shown to be a common phenomenon.

3. Sequence-based fold prediction methods have taken a hold in fold prediction, especially with the introduction of PSI-BLAST, but other methods (e.g. intermediate sequence search) (Li et al., 2000) as well. Taken together with item (2), there has
been a growing interest in the prediction of protein function based on remote homology. Thus, creating good alignments, rather than just predicting the fold, has become an important issue.

4. The availability of structural alignment programs, in a usable manner, opened up this field (among others) to people other than the authors of those programs.

With the advent of structural genomics, I am convinced that many interesting structural, functional, and evolutionary connections between different sequence families populating the same fold will be revealed. There is also an applicable value for knowing the location and role of critical residues, for protein engineering and design. For these reasons, the detection and annotation of critical residues is important, and there is a need for the construction of sensitive computational methods to do so.

### 6.4.2 Alignment accuracy

The use of sequence-based predictors will play a major role in the computational augmentation of the information collected by the crystallographers. PSI-BLAST, by most accounts, will continue to dominate this area for a while. Therefore, it is necessary to know how well PSI-BLAST performs in remote homologue alignments, and what steps should be taken in order to obtain a good alignment. We have studied this problem using our own restrictive database. Others have offered similar analyses (Koretke et al., 2001; Sauder et al., 2000), although we have managed to establish the flux in sensitivity and
specificity over consecutive iterations. This information can prove to be very useful, both for remote-based homology modeling, and as a general guideline for the use of PSI-BLAST, complementing those already published, (e.g. Jones & Swindells, 2002).
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