Proteins in organic solvents

Carla Mattos* and Dagmar Ringe†

Catalysis in organic solvents and the mapping of protein surfaces using multiple solvent crystal structures are two rapidly developing areas of research. Recent advances include the study of protein folding and stability in different solvents, and the demonstration that it is possible to qualitatively rank the affinities of protein binding sites for a given organic solvent using the multiple solvent crystal structures method.

Addresses
*Department of Molecular and Structural Biochemistry, North Carolina State University, 128 Polk Hall, Raleigh, NC 27695-7622, USA; e-mail: carla_mattos@ncsu.edu
†Rosenstiel Basic Medical Sciences Research Center, Brandeis University, 415 South Street, Waltham, MA 02254-9110, USA; e-mail: ringe@binah.cc.brandeis.edu

Current Opinion in Structural Biology 2001, 11:761–764

0959-440X/01/$ – see front matter

Abbreviations
DMF dimethylformamide
DMSO dimethyl sulfoxide
MSCS multiple solvent crystal structures

Introduction

The concept that proteins should remain folded and active in organic solvents is counterintuitive. After all, proteins evolved in an aqueous milieu and have ubiquitously incorporated water in their folding, three-dimensional structure, function and dynamics. Structural studies have implicated water molecules in protein architecture and catalysis, and in the specificity, cooperativity and thermodynamics of ligand binding [1•]. Structural work has gained insight from the study of water–protein interactions through a variety of different methods that stress the uniqueness of water in its properties as a solvent and a key player in biological processes [2]. Yet, it is well known that proteins are stable in some organic solvents and even multicomponent enzymes have been shown to retain catalytic activity, albeit at significantly lower levels than in water [3]. Work on enzymes in organic solvents began in the first part of the twentieth century, but only in the past two decades has the field developed into a robust and powerful area of research [4]. It currently spans the fields of enzymology, synthetic organic chemistry and, most recently, structural biology. This review focuses on the latest advances contributing to the understanding of the structure of proteins in organic solvents. For a focus on the chemistry that can be catalyzed by enzymes in aqueous and nonaqueous media, the reader is referred to a series of review articles that appeared recently in the journal *Nature* [5–7,8•,9–11].

Protein catalysis in organic solvents: structural aspects

It is well known that the catalytic activity of enzymes in organic solvents is far lower than in water [12]. Many of the advances in the past few years have contributed to both the elucidation of the underlying reasons for this difference in activity and the discovery of remedies to overcome the resulting limitations [8•]. From a biochemist's viewpoint, there is such a large divide between the behavior of proteins in aqueous solution and that in organic solvents that the distinctions between the different types of solvents are of no relevance. For the organic chemist using enzymes as biocatalysts, the differences are indeed significant. Clearly, not all organic solvents are equivalent and properties such as solvent hydrophobicity, hydrogen-bonding capacity and miscibility in water have profound influence on the structural integrity and catalytic activity of enzymes.

Proteins in hydrophobic solvents are thought to retain their native structure as a result of kinetic trapping, which results from stronger hydrogen bonding between the protein atoms and a more rigid structure in the absence of water. In hydrophobic water-immiscible solvents, any water that might be present will tend to stay at the protein surface because of the solvophobic and hydrophilic nature of the protein surface [13]. In fact, the addition of even a minute amount of water (1% v/v) is sufficient to drastically increase catalytic activity in these unnatural solvents; this observation is linked to the role that water plays in the structure and dynamics of the protein [14]. Conversely, polar solvents that can easily strip water from the surface of the protein and compete strongly for hydrogen bonds between protein atoms (e.g. dimethyl sulfoxide [DMSO], dimethylformamide [DMF], formamide) usually denature the structure to a largely unfolded state [15•]. Alcohols have some hydrophilic component, but are only moderate competitors for amide hydrogen bonds. They tend to disrupt tertiary structure and leave secondary structure interactions largely undisturbed [16]. Indeed, methanol has gained some attention as a denaturant that increases the concentration of possible folding intermediates and has therefore been used in protein folding studies [16,17] and to study conformational transitions in synthetic peptides [18,19]. These folding studies are conducted over a wide range of water/organic solvent mixtures and take advantage of the fact that proteins in solvents containing different proportions of water and organic solvent exhibit very different behavior from that observed in either water alone or neat organic solvent [20]. Recent protein folding studies in a variety of solvents have shown that the native structure can represent the global minimum in some nonaqueous solvents, such as glycerol, in which the unfolded protein does fold to the native state and the resultant structure is at least as thermodynamically stable as that in water [21,22]. Much of the work on protein structural integrity in organic solvents is motivated by an established correlation between structure and function. In this light, it is noteworthy that, although there is no doubt that there exists a minimum

structural requirement for catalytic activity, the idea that all proteins must be intact relative to the native state for catalysis to occur is not completely general, as partially unfolded subtilisin Carlsburg was recently found to be catalytically active in organic solvents [23].

Efforts to enhance the structural integrity of proteins in organic solvents have resulted in an increased understanding of the factors that favor the native state. Typically, a protein is first lyophilized to remove water before it is transferred to organic solvents for catalysis. In the process of dehydration, some protein denaturation may occur, which is normally reversible upon rehydration. In most organic solvents, refolding is not trivial and the catalytic activity of the enzyme is highly sensitive to its previous history in aqueous solution [24]. It is optimized when the pH of the aqueous solution before lyophilization is at the optimum for enzyme catalysis [13], when salts or other lyoprotectants are present to prevent protein aggregation in the organic solvent [25•] or when the protein is bound to ligands that might help preserve the integrity of the structure during lyophilization [14]. In the presence of salts, the duration of lyophilization and the final water content of the sample are also of importance [13]. Finally, there is a significant dependence of the catalytic activity of enzymes on the steps followed to reach the final organic solution [14,24]. Not surprisingly, the factors shown to be important in preventing protein denaturation during the lyophilization process are coincident with those that optimize protein structure and activity in aqueous solution. These factors also play an important role in maximizing structural integrity in nonaqueous solvents.

Other factors contributing to the decreased activity of proteins in organic solvents are less related to the protein structure and dynamics, and have a thermodynamic basis dominated by the solvent. These include the stabilization of the substrate ground state in the organic solvent and the destabilization of polar transition states. It has long been known that these factors can be controlled by solvent engineering or by mutations in the enzyme that compensate for the deleterious effects of the solvent [26]. Recent studies have been aimed at understanding these effects more thoroughly and have shown that the intrinsic activation of subtilisin Carlsberg is indeed lowest in polar solvents, presumably due to greater transition state stabilization of the transesterification reaction [27••]. There clearly must be a balance between the choice of a hydrophobic solvent for structural integrity, and a polar solvent for transition state stabilization and substrate desolvation effects for catalysis to occur optimally in organic solvents. When possible, this balance can be achieved by adding water to the reaction mixture, but in synthetic pathways, in which water may compete to result in an undesired product, the methodologies need to be continuously improved [28]. In spite of difficulties, the possibilities for the chemical and pharmaceutical industry to use enzymes as catalysts in reactions that have high stereoselectivity and regioselectivity for natural and non-natural substrates, and that are difficult in the absence of the enzyme have driven the scientific community to continuously stretch the possibilities. Progress is constantly being made both in the area of increasing control of enzymatic reactions, for example, enantioselectivity [29], and in expanding the types of biocatalysts and corresponding chemistries available in organic solvents [30–33]. Furthermore, the observation that the stability of DNA in organic solvents appears to follow the same trends observed for proteins opens up new territory with tremendous possibilities [34].

Multiple solvent crystal structures

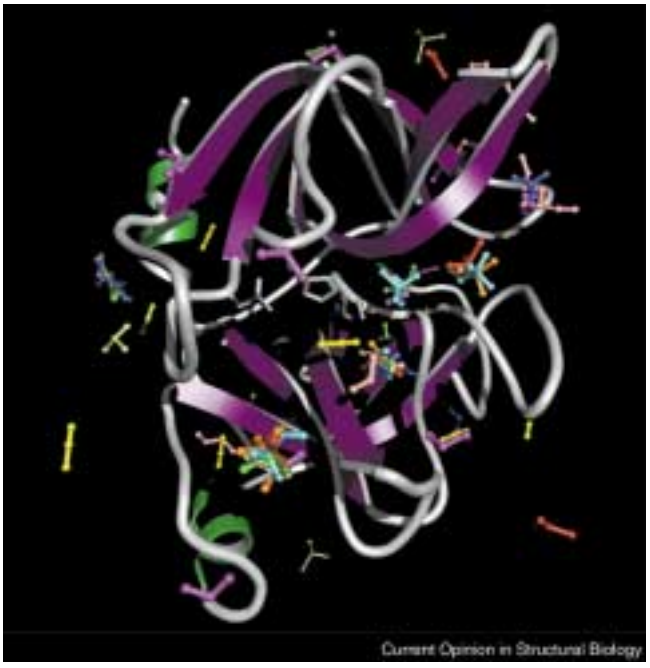
The question of whether the different chemical reactions and selectivity of enzymes in organic solvents resulted from a modified active site geometry provided the initial motivation for solving the crystal structure of an enzyme in organic solvents. The first structure resulted from an interest in observing the features of catalytically active subtilisin Carlsburg in neat acetonitrile [35]. This structure and others that followed established that proteins in several different organic solvents, such as acetonitrile [36], dioxane [37] and hexane [38], are virtually identical to those in aqueous solution. Furthermore, the structure of the acyl enzyme intermediate of subtilisin Carlsburg is the same whether in water or in acetonitrile, establishing that the mechanism of the transesterification reaction in organic solvents must be the same as that of hydrolysis in water [39].

From the seminal work on protein crystal structures in organic solvents arose the idea that organic solvents can be used not only to manipulate the chemistry of enzymes, but also to map binding surfaces of proteins [40]. The features that pointed to this possibility were that the protein structures are unchanged in the organic solvents, with about the same number of water molecules as present in aqueous solution, and that the organic solvents bind only at a few positions, primarily at the active site and in crystal contacts. Elastase was used as a model system to develop the multiple solvent crystal structures (MSCS) method [41]. The full application of the MSCS method to elastase has resulted in the extensive mapping of the protein surface (C Mattos *et al.*, unpublished data). The power of the method consists in superimposing at least five or six structures of a given protein solved in different organic solvents. Elastase was solved in neat acetonitrile and in water mixtures of 95% acetone, 55% DMF, 80% ethanol, 80% isopropanol, 80% 1,2-hexenediol and 40% trifluoroethanol. The superimposed structures after the deletion of water molecules are shown in Figure 1, with the protein represented as a ribbon diagram. The organic solvent molecules are seen to cluster in the active site, delineating the binding pockets for this enzyme at both the P and P′ positions. With one or two exceptions, all other sites found to bind organic solvent molecules are in crystal contacts, where the solvent molecules do not show nearly the same degree of clustering as in the active site. A recent study using isopropanol, acetone and acetonitrile in the MSCS of

thermolysin found these same trends, with greater clustering of solvent molecules in the active site than in the crystal contacts [42]. Sites of protein–protein or protein–peptide interactions are often characterized by small areas of hydrophobic exposed surfaces throughout the binding sites and the organic solvents bind primarily in these areas. Within the sites, each type of molecule binds in a characteristic fashion according to the chemical and steric properties of the particular solvent probe, providing clues to the types of functional groups that might be included in a larger ligand. The use of MSCS has also been expanded to obtain a qualitative assessment of the relative binding affinities of a particular solvent for the various sites it can occupy on the surface of a protein [43••]. To this end, the structure of thermolysin was solved in increasing concentrations of isopropanol: 2%, 5%, 10%, 25%, 60%, 80%, 90% and 100%. Isopropanol molecules appeared at the various binding sites in a way that was additive from one structure to the next, ranking the affinities of the sites for the isopropanol molecule [43••].

MSCS has been developed into a robust method that can be used to locate and characterize protein binding sites. It does not rely on prior knowledge of the biochemical characteristics of the protein or the chemical properties of substrates and inhibitors, and therefore is able to locate binding sites that cannot be identified through kinetic experiments. With the increase in the number of high-quality X-ray protein structures resulting from structural genomics efforts, three major areas have become the focus for structural biologists: the determination of protein function; the identification of the locations and specificities of the sites of protein–protein and/or protein–ligand interactions; and the problem of ligand design aimed at the control of disease. The main focus in using the MSCS method has been in the area of ligand design [41,42,43••]. It is also important to recognize the potential of the MSCS method in more diverse areas of structural biology.

Figure 1



The structure of elastase solved in aqueous solution represented as a ribbon diagram. Crystal structures of elastase obtained in seven different solvents were brought to a common frame of reference by least-squares superposition of the Cα atoms. Protein atoms and water molecules were deleted, and the organic solvents were superimposed on the framework of the reference structure (structure of elastase in aqueous solution). The protein structure is unchanged in the different organic solvents. Elastase contains 240 amino acid residues and is composed of two β-barrel domains, each interacting with an α helix. The two α helices are in green, the β strands are in purple and the coils are in gray. The catalytic triad (Asp108, His60 and Ser203) is shown explicitly. The organic solvent molecules are color coded as follows: red, acetone; yellow, acetonitrile; blue, DMF; green, isopropanol; dark pink, ethanol; light pink, 1,2-hexenediol; cyan, 2,2,2-trifluoroethanol; orange, 2,2,2-trifluoroethanol from a structure of elastase solved in slightly different buffer conditions.

Conclusions

Much of the work done in the past three years to understand and enhance enzyme catalysis in organic solvents has also elucidated the effects of solvents on protein structure. For instance, the study of protein folding in organic solvents, the use of ligands and lyoprotectants to avoid protein denaturation during lyophilization, and the demonstration that the structure of proteins in organic solvents can be controlled by previous history all contribute to understanding structure in organic solvents. Some of these factors are not directly relevant for application in the MSCS method. Other factors, such as the addition of salts or aqueous buffers to the organic solvents into which crystals are transferred for data collection, might aid the stabilization of the protein molecules in the crystals and possibly lead to diffraction at higher resolution.

Although catalysis in organic solvents and MSCS have developed into distinct fields of research, the fundamental principles learned in each are applicable to both fields and ultimately address questions related to structure, dynamics and ligand binding.

Acknowledgements

Carla Mattos is a recipient of the Burroughs Wellcome Fund New Investigator Award in the Basic Pharmacological Sciences.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. • Mattos C, Ringe D: Solvent structure. In *International Tables for Crystallography*, vol F. Edited by Rossmann MG, Arnold E. The Netherlands: Kluwer Academic Publishers; 2001:623-640.

This review provides a summary of the empirical information gathered over the past two decades on the structure of water molecules bound to proteins. The focus is on structures solved by X-ray crystallography, but complementary results obtained using other techniques are also discussed.

2. Bellissent-Funel M-C: *Hydration Processes in Biology*. Amsterdam: IOS Press; 1999.

3. Oppenheim SF, Studts JM, Fox BG, Dordick JS: Aromatic hydroxylation catalyzed by toluene 4-monooxygenase in organic solvent/aqueous buffer mixtures. *Appl Biochem Biotechnol* 2001, 90:187-197.

4. Klibanov AM: Answering the question: 'Why did biocatalysis in organic media not take off in the 1930s?'. *Trends Biotechnol* 2000, 18:85-86.

5. Walsh C: Enabling the chemistry of life. *Nature* 2001, 409:226-231.

6. Walsh C: Correction: enabling the chemistry of life. *Nature* 2001, 411:824.

7. Koeller KM, Wong CH: Enzymes for chemical synthesis. *Nature* 2001, 409:232-240.

8. • Klibanov AM: Improving enzymes by using them in organic solvents. *Nature* 2001, 409:241-246.
A review article emphasizing the catalytic properties of enzymes in organic solvents. Issues of molecular memory, the solvent dependence of enzyme specificity and the practical applications of enzymes in organic solvents are summarized.

9. Khosla C, Harbury PB: Modular enzymes. *Nature* 2001, 409:247-252.

10. Arnold FH: Combinatorial and computational challenges for biocatalyst design. *Nature* 2001, 409:253-257.

11. Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B: Industrial biocatalysis today and tomorrow. *Nature* 2001, 409:258-268.

12. Klibanov AM: Why are enzymes less active in organic solvents than in water? *Trends Biotechnol* 1997, 15:97-101.

13. Ru MT, Dordick JS, Reimer JA, Clark DS: Optimizing the salt-induced activation of enzymes in organic solvents: effects of lyophilization time and water content. *Biotechnol Bioeng* 1999, 63:233-241.

14. Dai L, Klibanov AM: Striking activation of oxidative enzymes suspended in nonaqueous media. *Proc Natl Acad Sci USA* 1999, 96:9475-9478.

15. • Knubovets T, Osterhout JJ, Klibanov AM: Structure of lysozyme dissolved in neat organic solvents as assessed by NMR and CD spectroscopies. *Biotechnol Bioeng* 1999, 63:242-248.
This article analyzes the structure of lysozyme in five representative neat organic solvents. Two kinds of behavior are observed: the protein unfolds in the solvents that compete effectively for backbone hydrogen bonds (DMF, formamide and DMSO) and retains partial characteristics of the folded state in ethylene glycol and methanol.

16. Babu KR, Moradian A, Douglas DJ: The methanol-induced conformational transitions of beta-lactoglobulin, cytochrome c, and ubiquitin at low pH: a study by electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom* 2001, 12:317-328.

17. Babu KR, Douglas DJ: Methanol-induced conformations of myoglobin at pH 4.0. *Biochemistry* 2000, 39:14702-14710.

18. Das C, Nayak V, Raghothama S, Balaram P: Synthetic protein design: construction of a four-stranded beta-sheet structure and evaluation of its integrity in methanol-water systems. *J Pept Res* 2000, 56:307-317.

19. Awasthi SK, Shankaramma SC, Raghothama S, Balaram P: Solvent-induced beta-hairpin to helix conformational transition in a designed peptide. *Biopolymers* 2001, 58:465-476.

20. Griebenow K, Klibanov AM: On protein denaturation in aqueous-organic mixtures but not in pure organic solvents. *J Am Chem Soc* 1996, 118:11695-11700.

21. Knubovets T, Osterhout JJ, Connolly PJ, Klibanov AM: Structure, thermostability, and conformational flexibility of hen egg-white lysozyme dissolved in glycerol. *Proc Natl Acad Sci USA* 1999, 96:1262-1267.

22. Burova TV, Grinberg NV, Grinberg VY, Rariy RV, Klibanov AM: Calorimetric evidence for a native-like conformation of hen egg-white lysozyme dissolved in glycerol. *Biochim Biophys Acta* 2000, 1478:309-317.

23. Guo Y, Clark DS: Activation of enzymes for nonaqueous biocatalysis by denaturing concentrations of urea. *Biochim Biophys Acta* 2001, 1546:406-411.

24. Ke T, Klibanov AM: On enzyme activity in organic solvents as a function of enzyme history. *Biotechnol Bioeng* 1998, 57:746-750.

25. • Rariy RV, Klibanov AM: Protein refolding in predominantly organic media markedly enhanced by common salts. *Biotechnol Bioeng* 1999, 62:704-710.
The folding of lysozyme was studied in 12 different organic solvents in the absence and presence of 1M LiCl. In all cases, the refolding yield was markedly increased in the presence of the salt, presumably due to its effect in increasing the solubility of the protein in the organic solvents.

26. Wangikar PP, Rich JO, Clark DS, Dordick JS: Probing enzymic transition state hydrophobicities. *Biochemistry* 1995, 34:12302-12310.

27. •• Kim J, Clark DS, Dordick JS: Intrinsic effects of solvent polarity on enzymic activation energies. *Biotechnol Bioeng* 2000, 67:112-116.
This paper presents experiments aimed at obtaining a correlation between enzyme activity and the physicochemical properties of the reaction medium. Particular attention is given to desolvation of the substrate and solvent polarity.

28. Klibanov AM, Samokhin GP, Martinek K, Berezin IV: A new approach to preparative enzymatic synthesis. Reprinted from Biotechnology and Bioengineering, Vol. XIX, No. 9, Pages 1351-1361. *Biotechnol Bioeng* 2000, 67:737-747.

29. Ke T, Klibanov AM: Markedly enhancing enzyme enantioselectivity in organic solvents by forming substrate salts. *J Am Chem Soc* 1999, 121:3334-3340.

30. Dai L, Klibanov AM: Peroxidase-catalyzed asymmetric sulfoxidation in organic solvents versus in water. *Biotechnol Bioeng* 2000, 70:353-357.

31. Munir IZ, Dordick JS: Soybean peroxidase as an effective bromination catalyst. *Enzyme Microb Technol* 2000, 26:337-341.

32. Park OJ, Kim DY, Dordick JS: Enzyme-catalyzed synthesis of sugar-containing monomers and linear polymers. *Biotechnol Bioeng* 2000, 70:208-216.

33. Shin JS, Luque S, Klibanov AM: Improving lipase enantioselectivity in organic solvents by forming substrate salts with chiral agents. *Biotechnol Bioeng* 2000, 69:577-583.

34. Bonner G, Klibanov AM: Structural stability of DNA in nonaqueous solvents. *Biotechnol Bioeng* 2000, 68:339-344.

35. Fitzpatrick PA, Ringe D, Klibanov AM: X-ray crystal structure of cross-linked subtilisin Carlsberg in water vs. acetonitrile. *Biochem Biophys Res Commun* 1994, 198:675-681.

36. Allen KN, Bellamacina CR, Ding X, Jeffery CJ, Mattos C, Petsko GA, Ringe D: An experimental approach to mapping the binding surfaces of crystalline proteins. *J Phys Chem* 1996, 100:2605-2611.

37. Schmitke JL, Stern LJ, Klibanov AM: The crystal structure of subtilisin Carlsberg in anhydrous dioxane and its comparison with those in water and acetonitrile. *Proc Natl Acad Sci USA* 1997, 94:4250-4255.

38. Yennawar NH, Yennawar HP, Farber GK: X-ray crystal structure of gamma-chymotrypsin in hexane. *Biochemistry* 1994, 33:7326-7336.

39. Schmitke JL, Stern LJ, Klibanov AM: Comparison of X-ray crystal structures of an acyl-enzyme intermediate of subtilisin Carlsberg formed in anhydrous acetonitrile and in water. *Proc Natl Acad Sci USA* 1998, 95:12918-12923.

40. Mattos C, Ringe D: Locating and characterizing binding sites on proteins. *Nat Biotechnol* 1996, 14:595-599.

41. Ringe D, Mattos C: Analysis of the binding surfaces of proteins. *Med Res Rev* 1999, 19:321-331.

42. English AC, Groom CR, Hubbard RE: Experimental and computational mapping of the binding surface of a crystalline protein. *Protein Eng* 2001, 14:47-59.

43. •• English AC, Done SH, Caves LS, Groom CR, Hubbard RE: Locating interaction sites on proteins: the crystal structure of thermolysin soaked in 2% to 100% isopropanol. *Proteins* 1999, 37:628-640.
A systematic study of the crystal structure of thermolysin in increasing concentrations of isopropanol. The MSCS method is used to qualitatively determine the relative binding affinities of the isopropanol molecule for different sites on the enzyme.