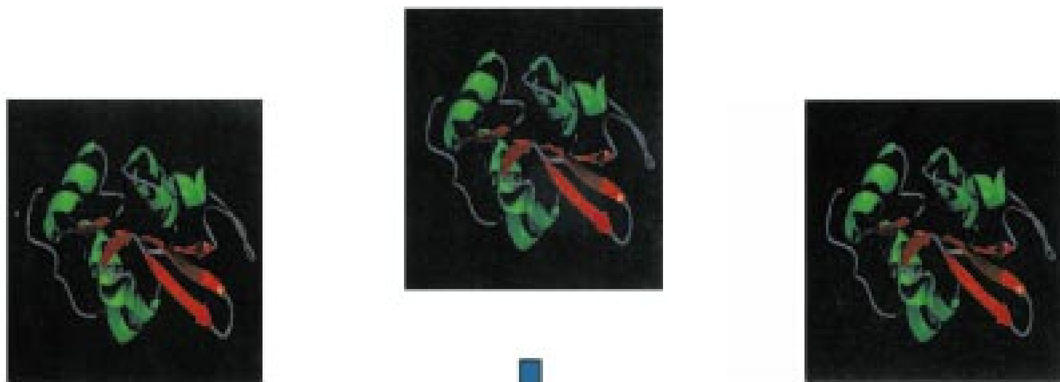
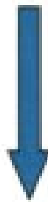
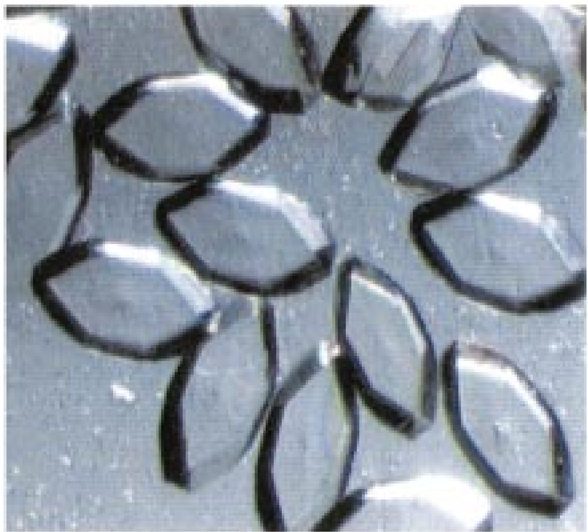
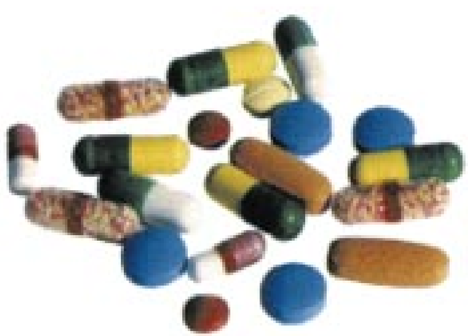


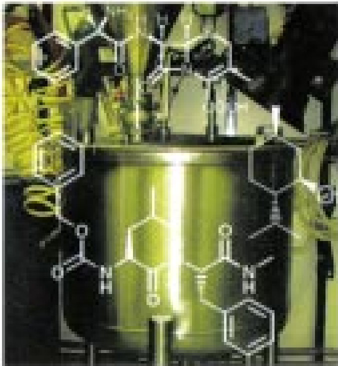




Protein Crystals



Biopharmaceuticals



Biocatalysts

ANGEWANDTE CHEMIE © WILEY-VCH

Protein Crystals as Novel Catalytic Materials

Alexey L. Margolin* and Manuel A. Navia*

In this era of molecular biology, protein crystallization is often considered to be a necessary first step in obtaining structural information through X-ray diffraction analysis. In a different light, protein crystals can also be thought of as materials, whose chemical and physical properties make them broadly attractive and useful across a larger spectrum of disciplines. The full potential of these protein crystalline materials has been severely restricted in practice, however, both by their inherent fragility, and by strongly held skepticism concerning their routine and predictable growth, formulation, and practical application. Fortunately, these problems have turned out to be solvable. A systematic exploration of the biophysics and biochemistry of protein crystallization has shown that one can dependably create new protein crystalline materials more or less at will. In turn, these crystals can be readily strengthened, both chemically and mechanically, to make them suitable for practical commercialization. Today, these novel materials are used as industrial catalysts on a commercial scale, in bioremediation and "green chemistry" applications, and in enantioselective chromatography of pharmaceuticals and fine chemicals. In the near future, their utility will expand, to include the purification of protein drugs, formulation of direct protein therapeutics, and development of adjuvant-less vaccines.

Keywords: biotransformations · drug research · enzyme catalysis · medicinal chemistry · protein crystals

1. Introduction

For most people, protein crystals are strongly associated with structural biology, as a necessary first step—or a necessary evil—in the course of X-ray diffraction experiments. This focus of attention is well justified, given the remarkable advances in our understanding of biological processes at the molecular level that have been brought about by macromolecular crystallography. As of May 15th 2001, there were 12514 structures posted on the Protein Data Bank internet site that were determined by X-ray diffraction analysis.[1] These structures include membrane proteins, enzymes, nucleic acids and protein–nucleic acid complexes, hormones and their receptors, carbohydrates, peptides, and viruses. Many of these structures describe complexes of specific ligands with target proteins of therapeutic interest at atomic resolution. In a number of instances, that information has led to approved drugs on the market—drugs developed through the application of structure-based drug design.[2] Some aspect of this vast and comprehensive panorama is constantly under review in the literature.[3]

One of the earliest practical applications of macromolecular crystallization methods involved the purification of jack bean urease by Sumner in 1926,[4] which was done as a prelude to further biochemical studies of that enzyme.[5] Sumner's work predates the demonstration in 1934 by Bernal and Crowfoot that crystals of the enzyme pepsin could diffract X-rays at atomic resolution.[6] Sumner's crystallization experiments, in turn, were themselves predated by a series of nineteenth century crystallizations on hemoglobins, seed proteins, and other nonenzymatic proteins, as reviewed by Fruton[7] and more recently by McPherson.[8]

Protein crystals have not been widely exploited in the past, apart from their use in structure determination and (to a more limited extent) in protein purification. This neglect may be attributed, in part, to the peculiar conditions under which protein crystals are usually grown and maintained, conditions that are largely incompatible with most practical applications that one might consider. In addition, macromolecular crystals are inherently quite fragile and prone to dissolution or shattering under relatively benign conditions.

Against this backdrop, we sought to create cross-linked enzyme crystals (CLECs)[9] and later, more generally, cross-linked protein crystals (CLPCs) with greatly enhanced

[*] Dr. A. L. Margolin
Altus Biologics Inc.
625 Putnam Avenue, Cambridge, MA 02139 (USA)
Fax: (+1) 617-577-6502
E-mail: margolin@altus.com
Dr. M. A. Navia
The Althexis Company
1365 Main Street, Waltham, MA 02145 (USA)
Fax: (+1) 781-647-5552
E-mail: navia@althexis.com

 © WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2001 1433-7851/01/4012-2205 $ 17.50+.50/0

stability and performance characteristics that would allow for the routine use of such enzymes and other proteins under the harsh conditions found in chemical manufacturing.[10] CLECs have now been used in the synthesis of fine chemicals, chiral intermediates, and peptides. These transformations have now been carried out not only on the lab bench, but also on a commercial scale that involves multiple kilograms of catalyst and thousands of kilograms of product.

In the subsequent course of our investigations, we have expanded our interests to encompass other practical applications for crystals and further improvements in crystallization and cross-linking methods. The applications, discussed in detail below, have included:

1) crystallization as a "routine" purification tool for proteins and other macromolecules,
2) stabilization of protein therapeutics to facilitate drug delivery and extend shelf-life prior to use,
3) use of CLPCs as novel microporous materials to be used as "bioorganic zeolites" and as chiral chromatographic media,
4) environmental applications, including the destruction of nerve agents for bioremediation and civil defense, and
5) biomedical applications including lumenal therapies (see Section 4.4.1), self-adjuvanting vaccines, and biosensors.

What is remarkable in all of these considerations, is the fact that nature provides us with such precisely constructed arrays of macromolecules for a relatively modest effort on our part. As Bernal and Crowfoot state in their historic paper, "the arrangement of atoms inside the protein molecule is...of a perfectly definite kind". The protein molecules in a crystal are "relatively dense globular bodies, perhaps joined together by valency bridges, but in any event separated by relatively large spaces which contain water". The regularity, uniformity, and precision of the crystal lattice—properties which are exploited so effectively in diffraction experiments—imply other properties and effects whose practical application has not yet been as aggressively pursued. By what other means, for example, could one hope to precisely position atomic-scale objects, such as a cofactor or prosthetic group in a protein, in a given orientation in space, using only one's fingers!

2. Beyond Crystallography

2.1. Crystallogenesis: Order from Chaos

The science of crystallogenesis has been extensively and comprehensively reviewed.[11] Under conditions of supersaturation at high protein concentration, or in the presence of reagents or conditions that reduce protein solubility, protein molecules will aggregate. According to the extent to which solution conditions have been suitably manipulated, those

Alexey L. Margolin graduated from Moscow University in 1975 with an M.S. in chemistry. In 1979 he received his doctorate in bioorganic chemistry from the same university. He then continued his research work at the Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry. In 1986 he moved to the Massachusetts Institute of Technology where he worked on enzyme-catalyzed reactions in organic solvents with Professor Alexander Klibanov. From 1988 to 1993 Dr. Margolin was responsible for biocatalysis activities at the Merrell Dow Research Institute. He then moved to Altus Biologics where he spearheaded the research and development of cross-linked enzyme crystals. He is currently Vice President of Science at Altus Biologics and is responsible for all science activities conducted by the company. Dr. Margolin's research interests focus on the field of biocatalysis, the use of enzymes in organic synthesis, and protein stabilization and immobilization. His recent efforts include the development of protein crystals for manufacture, formulation, and delivery of biopharmaceuticals.

A. L. Margolin

M. A. Navia

Manuel A. Navia, born in Havana, Cuba, received his B.A. in physics from New York University in 1967 and his M.S. in biophysics from the University of Chicago in 1969, after which his studies were interrupted by military service. He received his Ph.D. in biophysics in 1974 from the University of Chicago, where he worked with Prof. Paul Sigler on the structure of tRNA. Postdoctoral studies with Dr. David Davies on the structures of an intact antibody and antibody fragments followed at the National Institutes of Health in Bethesda, Maryland. From 1980 to 1989, Dr. Navia worked at Merck Research Laboratories, where he engaged in the structure-based drug design of enzyme inhibitors that resulted in approved and marketed drugs. From 1989 to 1997, he was at Vertex Pharmaceuticals in Cambridge, Massachusetts, where he solved the structures of calcineurin, the IL-1 beta converting enzyme (Caspase-1), and the HIV protease, in support of structure-based drug design programs against immunosuppression, inflammation, and HIV infection. The latter program led to discovery and development of amprenavir, now approved for the control of AIDS. While at Vertex, Dr. Navia was a co-inventor of the CLEC technology and founder of what is now Altus Biologics. In 1997, he cofounded The Althexis Company, in Waltham, Massachusetts, where he serves as Executive Vice President. Althexis is dedicated to the structure-based drug design of novel antibiotics.

early intermolecular associations (Figure 1a–c) will reflect the interactions that will later propagate through the body of the macroscopic crystal ultimately formed (Figure 1d).[12] The global physico-chemical characteristics of such early complexes will differ from those of the resulting crystal; this is reflected, for example, in the different conditions that often are required for the formation of crystal nuclei on the one hand, and crystal growth from those nuclei on the other.

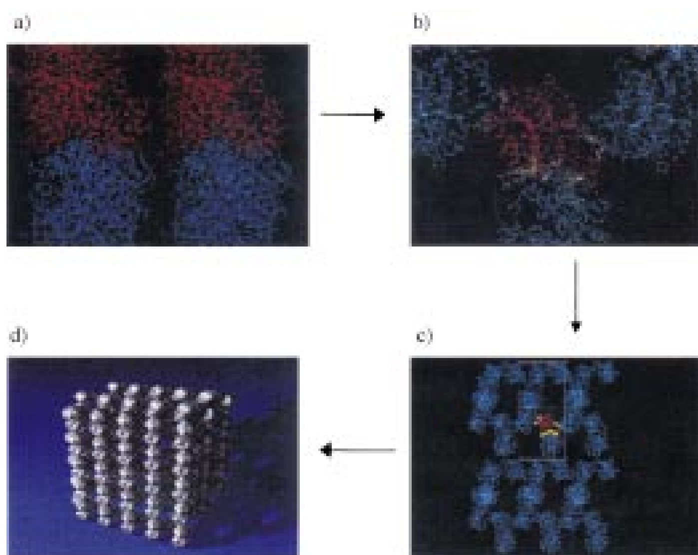


Figure 1. Serine carboxypeptidase II in the $P4_12_12$ crystal form. a) Stereo-image of a contact region.[13] b) Cross-sectional view of an interior molecule (red) in the unit cell. Regions involved in intermolecular contacts are highlighted in yellow, and include the region of extensive contacts shown in (a), as well as two other regions of lesser contact. c) Cross-sectional view of a hypothetical prenuclear complex structure. d) Representation of a crystallization nucleus (see Table 2).

Prenuclear complexes can be quite small in size, and may consist of as few as five or ten repeating cells. In Figure 1c, we see a model cross-section of what such a prenuclear complex might look like for the enzyme serine carboxypeptidase II in its tetragonal crystal form, which was solved by Liao et al.[13] In turn, Figure 1d shows a much larger crystalline aggregate of approximately thirty unit cells that might represent a nucleus for further crystallization through the regular accretion of proteins under supersaturating crystal growth conditions. Further growth may subsequently lead to a large, macroscopic crystal suitable for X-ray diffraction analysis. Both crystal nucleation and growth occur in supersaturated solutions where the concentration of protein exceeds its equilibrium solubility value. Recently, atomic force microscopy has been used to directly visualize the structure of prenuclear complexes of the protein apoferritin and the transition of such complexes to crystal nuclei.[14] An extension of these studies to a broader sample of macromolecules could greatly enhance our understanding of the critical early steps in crystal formation.

The methods and mechanisms of the formation of protein crystals that are suitable for X-ray diffraction analysis have been extensively reviewed.[15] For the purpose of CLEC preparation, however, batch crystallization is clearly the method of choice. This technique is quite attractive because it is simple and can ultimately be scaled up.[16] Indeed, with this technique we have been able to crystallize more than forty proteins, including glycoproteins, enzymes, antibodies, and hormones, with different molecular weights (1–500 kDa) and from a variety of sources. Some of these crystals are shown in Figure 2.

Controlling the kinetics of crystallization can change the size of the crystals. Normally, faster crystallization processes yield smaller particles. For many chemical applications that require multiple reuse of the catalyst, crystals of 50–150 μm are preferred, since they offer a combination of good filtration properties and high activity. Smaller particles (< 5μm) are better suited for biomedical and other applications, when recycling is not necessary. In some instances, smaller crystals can be aggregated into larger porous particles to enhance their sedimentation properties without compromising substrate/product diffusion characteristics.

2.2. Crystallization as a Means of Purification

Crystallinity has always been associated with purity. Indeed, the aim of the early protein crystallization work was the purification of pure homogeneous proteins from complex extracts. The fact that protein crystals could be grown from relatively crude mixtures was proven by the very first crystallization of a protein, hemoglobin, by Hunefeld in 1840 and later by Reichert and Brown.[17] Other dramatic examples, such as crystallization of ferritin from fresh slices of horse spleen by simply adding divalent ions,[18] crystallization of lysozyme directly from egg white,[19] and crystallization of albumins, plant proteins, and enzymes, have recently been reviewed by McPherson.[20] Many of these crystallizations were conducted on a gram scale and resulted in high protein yields.

The advent and use of X-ray crystallography brought significant changes to the science of protein crystallization. The focus of the protein crystallization had shifted from developing reliable purification processes to preparation of diffraction quality single crystals. Nowadays, the purification of proteins and other macromolecules by crystallization is relatively rare.[21] In an age when chromatography dominates as the principal approach to the purification of proteins and other macromolecules, the crystallization methods pioneered more than a hundred years ago may seem quaint and hopelessly outdated.

Crystallization as a method of purification has also suffered, by extension, from the mystique that surrounds the growth of diffraction-quality macromolecular crystals suitable for X-ray structural analysis. In this regard, one has to be sensitive to the differences between crystals needed for diffraction purposes and those that would otherwise be perfectly suitable for protein purification or for further elaboration into cross-linked protein crystals. The former need to be quite large, if the X-rays scattered from them are to be detected by our instruments. In addition, diffraction-quality crystals must also be suitably ordered and free of internal flaws (such as twinning) in order to provide the level of resolution that

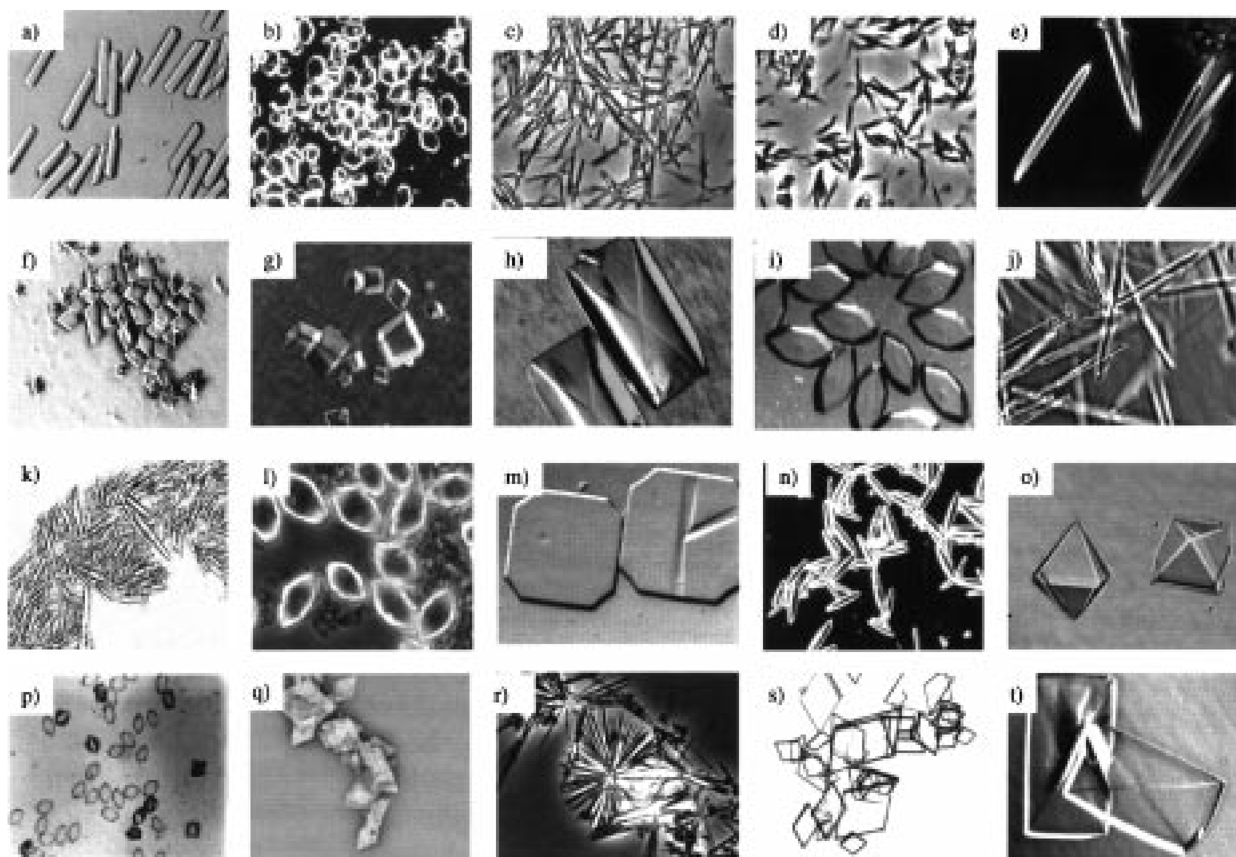


Figure 2. Selected enzyme crystals. a) and b) Lipase from *Pseudomonas cepacia* (enlargement: ×360). c) Alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (×720). d) Glucose oxidase from *Aspergillus niger* (×720). e) Porcine liver esterase (×720). f) and g) Everlase (×360 and ×180, respectively). h) and i) Savinase (×180). j) Subtilisin from *Bacillus licheniformis* (×720). k) Human serum albumin (×720). l) and m) Lipase from *Candida antarctica* (×360 and ×180, respectively). n) Thermolysin from *Bacillus thermoproteolyticus* (×180). o) Urease from jack beans (×360). p) H254R organophosphorus hydrolase (×360). q) Organophosphorus acid anhydrolase from *Alteromonas* sp. (×360). r) Rituximab (×720). s) Lipase from *Candida rugosa* (×360). t) Penicillin acylase from *E. coli* (×180).

would make the resulting structural images biologically relevant. The crystals produced for purification purposes need only be large enough to facilitate their separation from solute and their isolation. The goal in this case is to make the process high yielding, fast, and, ultimately, economical.

These requirements have been met in several large-scale crystallizations of industrial enzymes.[22] Judge et al. have crystallized a model protein ovalbumin out of a mixture containing 2% w/w ovalbumin, 0.167% w/w conalbumin, and 0.167% w/w lysozyme in >99% purity.[23]

High yield and purity has been achieved in bulk crystallization of thermolysin[24] and *Candida rugosa* lipase (CRL).[25] The latter example is instructive. The pure CRL was first obtained by ion-exchange chromatography and a crystallization procedure was developed. This procedure was then applied to crystallization from an impure commercial mixture containing 5–6% w/w CRL and at least three additional enzymes (less than 2% w/w).

These and other examples demonstrate that large-scale protein crystallization is a powerful alternative to classical purification techniques, since it can streamline the purification procedures and yield highly concentrated slurries of pure protein ready for further formulation.

2.3. Cross-Linking

While crystallization of proteins creates a precise spatial arrangement of molecules, subsequent cross-linking inside the crystal "locks" the proteins in the crystalline state outside of the conditions that led to crystallization. Historically, glutaraldehyde has been by far the most popular cross-linking agent for enzyme crystals.[26] Glutaraldehyde is safe, inexpensive, and easy to work with. However, the mechanism of its action is not fully understood.[27] It is clear, however, that the cross-linking is irreversible even in the presence of exogenous amines and cannot be explained by simple Schiff base formation. The ability of glutaraldehyde to form a mixture of oligomers of different lengths and structures[28] makes the use of this cross-linker somewhat unpredictable. The cross-linking conditions (pH, temperature, concentrations, and time) must be carefully optimized (excessive cross-linking may lead to formation of protein precipitates and loss of activity) to ensure both high stability and activity of CLEC catalysts. Nevertheless and despite all the shortcomings, glutaraldehyde-mediated cross-linking yielded enzyme crystals that retained almost full activity toward smaller molecules and were much more stable than the corresponding soluble enzymes.

In addition to glutaraldehyde, we have used several homo- and heterobifunctional agents with spacer arm lengths ranging from 3–30 Å for the cross-linking of enzyme crystals. (Figure 3) Among the cross-linkers used, cleavable agents deserve special attention. In situations when the activity of fully cross-linked crystals toward macromolecular substrates remains low (see Section 3.2), one can design CLPCs that remain intact and stable under one set of conditions, but dissolve with release of activity with time when the environment changes. This can be achieved by varying the degree of cross-linking or by using cleavable cross-linkers.

An interesting example of the latter strategy is shown in Figure 4.[29] First, the crystals of *Candida rugosa* lipase (CRL) were modified by Traut's reagent (Figure 3) in order to introduce additional SH groups. Then the SH groups were cross-linked with a cleavable homobifunctional agent DPDPB (Figure 3). The fully cross-linked crystals of CRL (CRL-CLECs) have very low specific activity ($2.3\,U\,mg^{-1}$) compared to the activity of the native soluble enzyme ($732\,U\,mg^{-1}$) in the olive oil assay. However, after the CRL-CLECs were incubated for one hour with cysteine, the crystals completely dissolved to "release" 85% of the initial activity. The principle of controlled dissolution of CLPCs has been successfully applied in the preparation of subunit vaccines, detergent enzymes, and cosmetics (see Section 4.4.2).

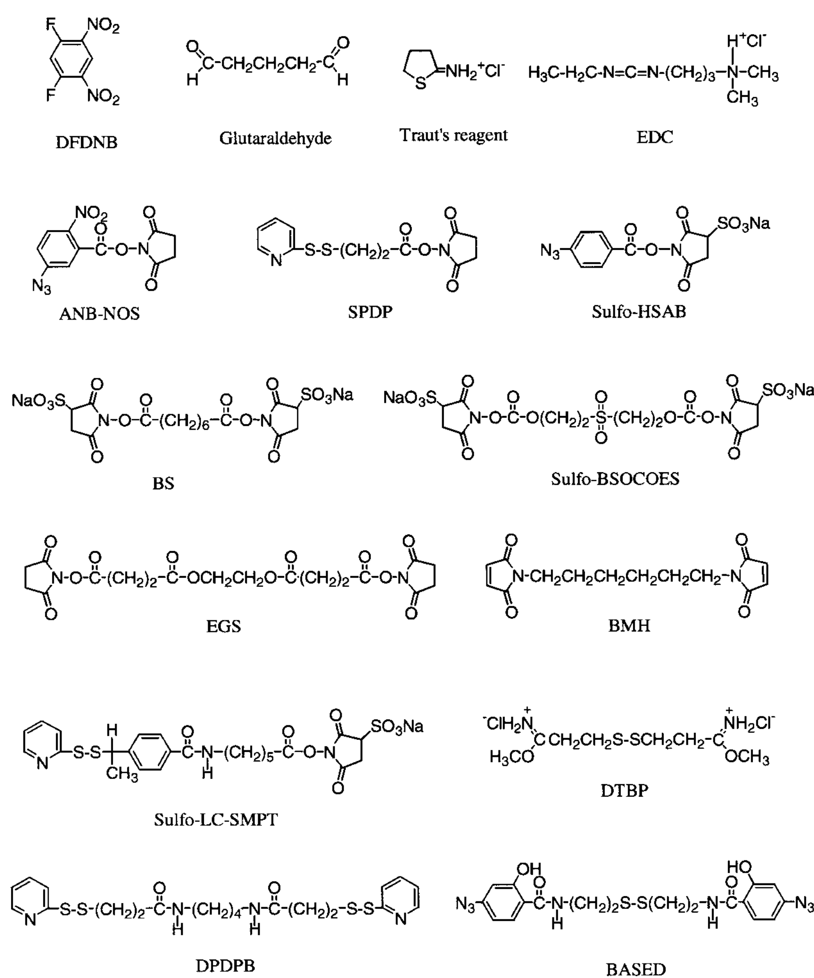


Figure 3. Typical reagents used for cross-linking of protein crystals. DFDNB = 1,5-difluoro-2,4-dinitrobenzene, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, ANB-NOS = *N*-5-azido-2-nitrobenzoyloxysuccinimide, SPDP = *N*-succinimidyl-3-(2-pyridyldithio)propionate, Sulfo-HSAB = *N*-hydroxysulfosuccinimidyl-4-azidobenzoate, BS = bis(sulfosuccinimidyl)suberate, Sulfo-BSOCOES = bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone, EGS = ethyleneglycol bis(succinimidylsuccinate), BMH = bis(maleimidohexane), Sulfo-LC-SMPT = sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate, DTBP = dimethyl-3,3′-dithiobispropionimidate · 2HCl, DPDPB = 1,4-di-[3′-(2′-pyridyldithio)propionamido]butane, BASED = bis-[β-(4-azidosalicylamido)ethyl]disulfide.

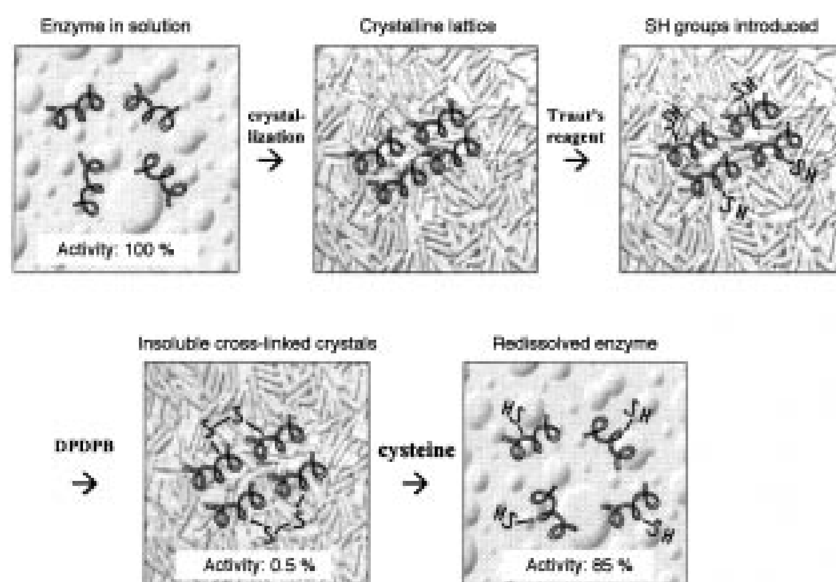


Figure 4. Controlled dissolution of a CLPC that was cross-linked with a cleavable reagent.

3. Properties of Protein Crystals

3.1. Stability

Chemically cross-linked protein crystals are significantly more stable against denaturation by heat, organic solvents, and proteolysis than the corresponding soluble proteins (Table 1).[10a, 30] However, the source of protein stabilization remains unclear: it may be a result of the crystallinity of the material, the chemical modification of proteins, or a combination of both. We have recently addressed the question of the physical stability of powder formulations of proteins and have found that crystalline powders of CRL and glucose oxidase are considerably more stable than amorphous proteins.[31] The crystalline proteins better maintain their native conformation at elevated temperature (see Section 4.4.3) and demonstrate a lower tendency to aggregate. The reason why proteins better maintain their native structure in crystalline

Table 1. Stability of cross-linked enzyme crystals.[a]

Enzyme	Conditions	$t_{1/2}$ (soluble)	$t_{1/2}$ (CLEC)	Ref.
thermolysin	Tris buffer (pH 7.0), 65 °C	6 h	no loss of activity after 5 days	[9]
	50% THF, DMSO, acetone, 25 °C	1.5–8 h	no loss of activity after 5 days	
	pronase	< 5 min	retains > 95% activity after 4 days	
alcalase	acetate buffer (pH 5.7), 60 °C	11 h	no loss of activity after 7 days	[110]
	50% acetone, 40 °C	4 days	no loss of activity after 7 days	
subtilisin	acetate buffer (pH 5.7), 70 °C	< 1 min	> 1hr	[111]
	autodigestion (pH 8.0), 30 °C	20 h	< 1% loss after 20 h	[112]
	acetonitrile, 45 °C	0.35 days	31 days	
selenosubtilisin	50% acetone, 40 °C	< 1 day	no loss of activity after 10 days	[113]
carboxypeptidase A	8 M urea	full inactivation	no inactivation	[114]
Candida rugosa lipase	50% THF, DMF, DMSO	< 1 h	> 150 h	[25]
penicillin acylase	50% *i*PrOH/water, 25 °C	2 h	20% activity loss after 4 days	[115]
rabbit muscle aldolase	phosphate buffer, RT	1–5 days	no loss of activity after 6 months	[116]
lactate dehydrogenase	buffer, 25 °C	2.5 days	< 10% loss of activity after 25 days	[117]
alcohol dehydrogenase	buffer (pH 6–8), 30 °C	< 15 days	no loss of activity after 90 days	[118]
glucose isomerase	45% glucose/fructose solution (pH 7), 77.5 °C	71 min	407 min	[119]

[a] Tris = tris(hydroxymethyl)aminomethane, DMSO = dimethylsulfoxide, THF = tetrahydrofuran, DMF = *N,N*-dimethylformamide.

form lies in the unique properties of the crystalline state. Protein crystals are highly ordered three-dimensional arrays of molecules where the position of each molecule in space is well defined.[32] In contrast, typical amorphous solids do not possess a long-range order and, thus, have physical properties quite different from the crystalline state. The greater free volume and molecular disorder of amorphous materials make them more similar to the liquid state and result in overall lower stability relative to the crystalline state.[33]

When a protein transfers from a solution to a crystalline environment a significant part of its surface becomes inaccessible to solvent. The loss in accessible surface area depends on the size of a protein and varies from roughly 10 to 60%.[34] These buried parts of protein molecules form additional intermolecular contacts. Protein–protein contacts in crystals have been analyzed for hundreds of proteins and many crystal forms.[35] A typical pairwise interface for a monomeric protein has an area of 570 Å^2, involves 10 amino acid residues of each partner, and contains 30 van der Waals contacts.[36] The numbers vary widely according to the protein size and its crystal form, but the buried surface area may reach more than 2000 Å^2 and there may be 100 amino acid residues per contact. These new intermolecular contacts that are formed between proteins on crystallization, whether they present hydrogen bonds, hydrophobic interactions, or salt bridges, will reduce mobility and slow down the unfolding of a protein molecule. Indeed, it is widely accepted that there is only a marginal preference (of roughly 40 kJ mol^{-1}) for the native conformation of a protein over the unfolded one. As such, a single additional hydrophobic interaction (2–26 kJ mol^{-1}) or several polar contacts can increase the half-life of a protein by several orders of magnitude.[37]

It is worth mentioning that the size of the crystal rarely affects the protein stability. This can best be rationalized by considering the relative proportion of "internal" unit cells in the small prenuclear complexes, in a typical crystalline nucleus, in a small crystal suitable for formulation as a CLEC, and finally, in a macroscopic crystal suitable for X-ray diffraction analysis. An internal unit cell would be one that is entirely surrounded by the full complement of intermolecular interactions used to assemble a macroscopic crystal. Other, "external" unit cells would be missing one or more of those interactions; this would be the case for unit cells on the surface of a nascent crystal. Table 2 shows the relative proportion of internal versus external unit cells in the four stages of crystal growth mentioned.

Table 2. The effect of crystal size on the proportion of repeating unit cells in the crystal interior.[a]

Object	Size	Number of unit cells total	Number of unit cells exterior	Interior fraction
unit cell	1nm × 1nm × 1nm	1	1	0%
prenuclear complex	3nm × 3nm × 3nm	27	26	3.7%
crystalline nuclei	5nm × 5nm × 10nm	125	75	40%
small crystal	1µm × 1µm × 2µm	2×10^9	3×10^6	> 99.9999%
large crystal	100µm × 100µm × 200µm	2×10^{15}	3×10^{10}	> 99.9999%

[a] The calculations are based on the tetragonal unit cell of the serine carboxypeptidase II structure (space group: $P4_12_12$; unit cell dimensions: $a = b = 98.6$ Å, $c = 210$ Å, $\alpha = \beta = \gamma = 90°$).[13]

Unit cells in the interior of a crystal are fully engaged in the intermolecular interactions observed through the crystal and are optimally stabilized by those interactions. Once one gets past a modest number of unit cells, most of the protein material in the crystal is internal, and the characteristics of that state would then presumably dominate the observed character. For even the smallest CLECs, the proportion of stabilized unit cells approximates 100%, for all intents and purposes, as is the case for the much larger crystals that are used in macromolecular structure determination by X-ray diffraction.

3.2. Enzyme Activity

The specific intermolecular interactions that account for increased stability of proteins in the crystalline state may also limit the flexibility and "breathing" of protein molecules and dramatically reduce their activity.[38] Three major factors—size of the crystal, size of the substrate, and conformation of the enzyme in the crystal—control enzymatic activity in the crystalline state.

In many cases the full activity of an enzyme crystal cannot be realized because of mass-transfer limitations. The problem of diffusional limitations in the crystalline state has been addressed in several publications.[39] The critical crystal thickness (d_c; the crystal thickness below which diffusional limitations are not significant)[40] depends, as given by Equation (1), on the kinetic parameters $k_{cat.}$ and K_m of the enzyme, the enzyme concentration [E], and the diffusion coefficient of the substrate D_{eff} in the crystal.

$$d_c = (D_{eff}K_m/k_{cat.}[E])^{1/2} \quad (1)$$

The critical thickness for crystals of relatively slow-acting hydrolases was found to be of the order of several microns. For the fast reactions, however, d_c is estimated to be close to the thickness of the unit cell.[41] One has to keep in mind that while even relatively large crystals may be free of diffusion limitations, normally there is a good correlation between the crystal size and reaction rate (Table 3).

Table 3. The effect of particle size on enzyme activity.

Enzyme	Particle size [µm]	CLEC activity [µmol min^{-1} per mg of protein]	Activity[a] [%]
alcalase	40	5	24
	1.2	16	76
penicillin acylase	142	5	21
	57	17	71

[a] Activity relative to the native enzyme.

From a practical standpoint it is important to realize that there are no diffusional limitations as long as the substrate concentration is higher than K_m, which is often the case at the beginning of the enzyme-catalyzed process. As the substrate concentration drops below K_m during the course of the reaction, mass-transfer effects lead to reduced activity of the crystal.[42]

The size of a substrate may dramatically affect the reaction rate. While in principle large molecules, such as casein (M_r 24000), the insulin B chain (M_r 3500[24]), cytochrome c (M_r 12000[43]), and polyethylene glycol (M_r up to 10000[74]), may, at least partially, diffuse through the pores of protein crystals, the reaction rates are normally lower (Table 4).

The third major parameter affecting the activity of crystalline catalysts is the enzyme's conformational flexibility in the crystalline state. In many cases the activity of crystalline enzymes was significantly lower than that of enzymes in solution even when diffusion limitations had been excluded (Table 5). It is possible that in some crystal forms the active sites of enzyme molecules are occluded and, therefore, inaccessible to the substrate. These problems can, in principle, be addressed by producing different crystal forms of the same enzyme.

Lipases present an interesting example in this regard. Lipases are considered to have two important states, defined by the position of the "lid"—a flexible structural element that moves to allow substrate binding to the active site.[44] Although it is well known that lipases exhibit 10^3-10^4-fold increases in activity when their natural substrates, such as lipids, are present as an emulsion,[45] the need for interfacial activation does not seem to be necessary in the hydrolysis of more water-soluble or small synthetic substrates.[12] We prepared CRL-CLECs in both "open"[46] and "closed"[47] crystal forms and compared their activity and enantioselectivity in the resolution of ketoprofen (Figure 5). The activity of the "open" form was almost three times higher than that of the "closed" form. In addition, the "open" form exhibited higher enantioselectivity.

In certain cases the enzyme crystal may even be more active than the same enzyme in solution. For example, CLECs of lipases and subtilisin formulated with surfactants exhibited specific activity in organic solvents higher than that of native enzymes.[48] An even more striking result has recently been achieved in our laboratory. Crystals of CRL cross-linked with sulfo-LC-SMPT (Figure 3) were five to seven times more active than pure native CRL in the olive oil assay.[49] Interestingly, the same modification of the soluble enzyme did not produce an increase in enzyme activity. Apparently, the chemical modification of CRL in a crystal occurs differently from that in solution.

Table 4. Activity of cross-linked enzyme crystals towards large substrates.

Enzyme	Substrate	Particle size [µm]	CLEC activity [µmol min^{-1} per mg of protein]	Activity[a] [%]
subtilisin	azocasein	1.2	9.2	35
thermolysin	oxidized insulin B-chain	35	0.44	4

[a] Activity relative to the native enzyme.

Table 5. Activities of cross-linked enzyme crystals.

Enzyme	Substrate	$k_{cat.}$ ratios[a]	K_m ratios[a]	$k_{cat.}/K_m$ ratios[a]	Ref.
carboxypeptidase A	BzGly-Phe[b]	17	0.11	155	[120]
	BzGly-OPhe[b]	48	0.04	1200	
carboxypeptidase B	BzGly-Arg[b]	17	0.03	570	[121]
	BzGly-OArg[b]	150	0.17	1470	
phosphorylase A	glucose-1-P	179	1.2	149	[122]
phosphorylase B	glucose-1-P	57	1	57	[122]
subtilisin	tosylArg-OMe (TAME)[b]	4.5	2.2	2.1	[111]

[a] Ratios given for native enzyme/CLEC enzyme. [b] Bz = benzyl, tosyl = toluene-4-sulfonyl.

Figure 5. Stereoview of the Cα tracing of *Candida rugosa* lipase (CRL). The serine, histidine, and glutamic acid residues that constitute the catalytic triad for CRL are labeled as S209, H449, and E341 in the figure. The helical lid is depicted in thick lines, with the closed conformer in white lines and the open one in grey. Produced from data supplied in refs. [13] and [14] and the Brookhaven Protein Data Bank (P. Grochulski and M. Cygler, http://www.pdb.bnl.gov/, files "1crl" (open form) and "1trh" (closed form)). Reprinted with permission from ref. [124]. Copyright (1997) Academic Press.

4. Applications

After protein crystals are prepared, they may be formulated in three major ways (Figure 6). First, they may be fully cross-linked by multifunctional reagents to produce CLPCs or CLECs that remain completely insoluble in various media and can be recycled many times. Second, protein crystals may be cross-linked in such a way that they will dissolve in response to a change in the environment, such as a pH, temperature, or concentration change. Finally, protein crystals may be transferred to a different solvent where they remain insoluble without chemical modification, or they can simply be dried. This formulation flexibility allows for several interesting applications. The first applications we will consider are those for CLECs.

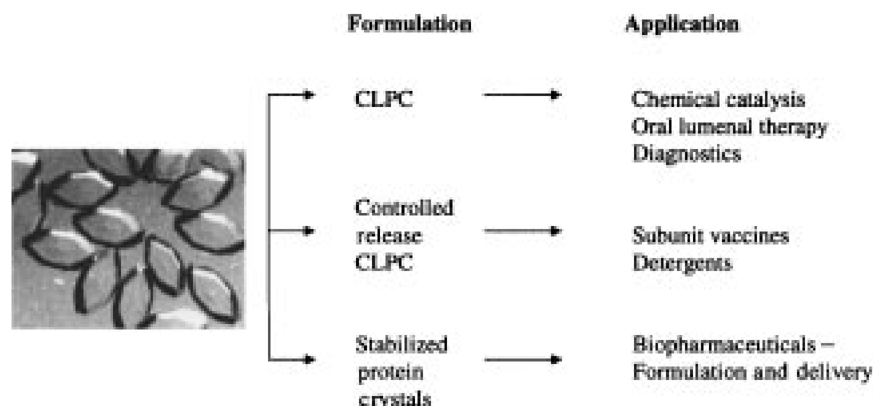


Figure 6. Three major routes for protein crystal formulation and their most important areas of application.

4.1. Synthetic Chemistry

Among the often-cited explanations for poor acceptance of enzymes by the chemical industry, such as high cost, limited substrate specificity, and low enantioselectivity for unnatural synthetic substrates, the main reason is clearly the lack of enzyme stability at elevated temperatures and under process conditions. The well-known strategies for stabilizing enzymes include protein engineering, use of enzymes from extreme environments (extremozymes),[50] immobilization,[51] chemical modification,[52] and CLECs.[30]

One of the advantages of CLEC catalysts is that, in many cases, the enhancement of enzyme stability can be achieved without loss of the specific activity. At the same time, CLEC catalysts may have significantly enhanced stability and productivity in both aqueous and organic media. CLECs remain insoluble throughout the process and can be recycled many times, which increases the productivity of the catalysts. In addition, in some cases the CLEC catalysts can be significantly more enantioselective than the crude mixture of enzymes, an effect that was demonstrated in the resolution of profen drugs.[25]

4.1.1. Resolution of Racemates with Hydrolases

Several substrates for CLEC-catalyzed enantioselective transformations are presented in Figure 7. The protease subtilisin is broadly used in organic synthesis for enantio- and regiospecific reactions, amide bond syntheses, and hydrolyses in both aqueous and organic solvents.[53] The synthetic utility of this catalyst in organic chemistry is limited by two major problems: 1) low stability in water (mostly due to autolysis) and water-miscible organics,[54] and 2) low activity in neat organic solvents. Autolysis not only reduces the life of the catalyst, but it also makes separation and purification of the reaction products time consuming and expensive. This problem is especially serious in the synthesis of peptides and pharmaceuticals, where the requirements for product purity are very high. In turn, the low activity of subtilisin in neat organic solvents makes various processes too slow and economically unfeasible.[55]

Subtilisin-CLECs have shown high enantioselectivity in the resolution of amino acid esters and many organic molecules.

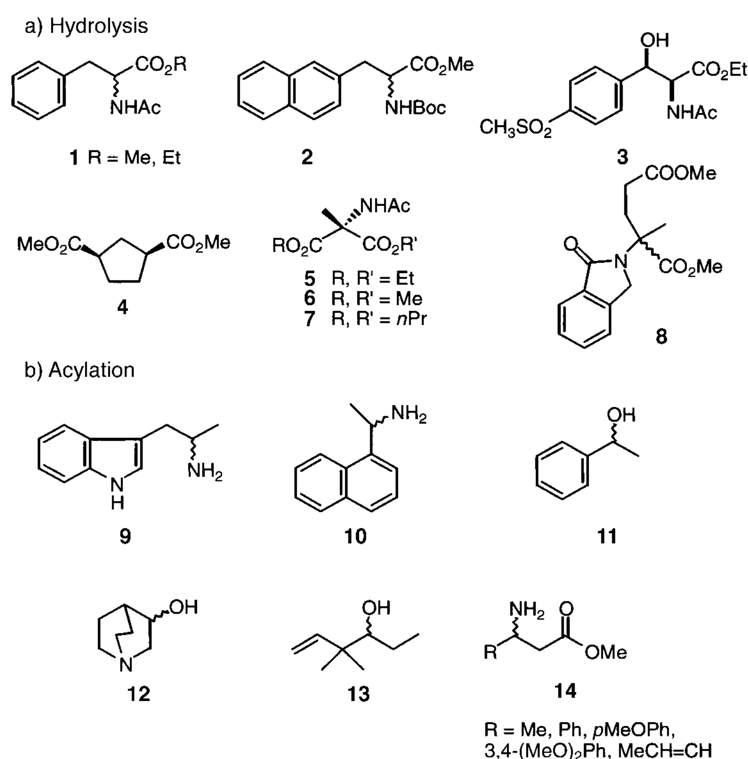


Figure 7. Substrates for CLEC-catalyzed enantioselective hydrolyses (a) and acylations (b). Ac = acetyl, Boc = *tert*-butoxycarbonyl.

Both natural and unnatural amino acids were effectively resolved by this catalyst (Figure 7).[110] The *N*-Acetyl phenylalanine methyl ester (**1**, R = Me) was hydrolyzed to give L-acid product and D-ester residue with enantiomeric excess (*ee*) values of 96% and >99%, respectively. The unnatural amino acids, *N*-Boc-3-(2′-naphthyl)alanine methyl ester (**2**) and *threo*-N-acetyl-β-hydroxy-*p*-methylsulfonylphenylalanine ethyl ester (**3**) were also resolved by this catalyst with high enantioselectivity. The *meso*-diester **4** was enantioselectively hydrolyzed to give the monoester with an *ee* value of 96%. The prochiral diethyl ester **5** was enantioselectively hydrolyzed to give the corresponding monoacid in 97% yield and with 81% *ee*. It is well known that the size of the group near the stereogenic center affects the enantioselectivity in the enzymatic resolution. Therefore, methyl ester **6** and propyl ester **7** were prepared from **5** and hydrolyzed with subtilisin-CLECs. The enantioselectivities for the formation of the corresponding monoacids of **6** and **7** were not significantly different to the result obtained in the hydrolysis of **5**. Another interesting example of enantioselective hydrolysis with the subtilisin-CLEC catalyst was the preparation of analogues of thalidomide (**8**). Both enantiomers were isolated with 95% *ee*.[56]

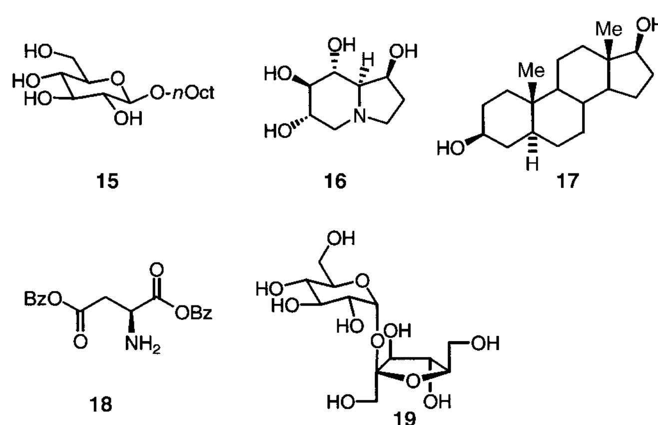
Since the subtilisin-CLECs were active and stable in neat organic solvents, the catalyst was used to perform the resolution of alcohols and amines in such solvents (Figure 7).[110] α-(1-Naphthyl)ethylamine (**10**) was resolved to give corresponding butyramide and remaining amine with *ee* values of 98% and >98%, respectively.[18b] α-Methyltryptamine (**9**) was resolved to give the *R* amine with an *ee* value of >98% at 53% conversion; the enantiomeric excess of the amide product was 94% at 20% conversion. When 1-phenylethanol (**11**) was transesterified by this catalyst, the enantiomeric excesses of the ester product and remaining alcohol were 92% and 40%, respectively, at 30% conversion. The same catalyst was successfully used in enantioselective acylation with vinyl butyrate of racemic 3-quinuclidinol (**12**) in 2-methyl-3-butanol. The *R* enantiomer was obtained in 68% yield and with 96.2% *ee* after crystallization.

The natural products epothilones A and B are structurally very different from taxol but have similar anticancer activity. Significantly, they have been reported to be much more active against cell lines exhibiting multidrug resistance.[57] Taylor et al. have recently published an elegant, formal total synthesis of epothilone A.[58] In this work the authors used the cross-linked crystals of *Pseudomonas* (now *Burkholderia*) *cepacia* lipase (ChiroCLEC-PC) to resolve a key alcohol intermediate **13** by selective acylation with vinyl acetate in *tert*-butyl methyl ether. The enantioselectivity was >20:1 at 47% conversion and efficiently provided gram quantities of the desired *R* alcohol. Since the unreacted *S* alcohol can easily be epimerized by a simple oxidation/reduction sequence and the catalyst reused without significant loss in activity, the method is ideally suited for scale-up.

Among other catalysts, CLECs of penicillin acylase were used for the resolution of racemic β-amino acid esters **14** for the preparation of peptidomimetics. The desired *S* enantiomers were obtained with >95% *ee* in 2% aqueous toluene.[59]

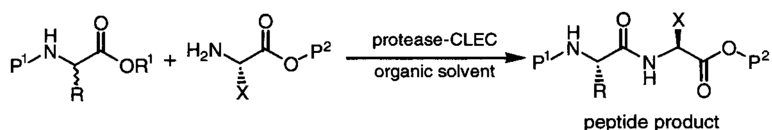
4.1.2. Regioselective Reactions

Subtilisin-CLECs exhibited excellent regioselectivity toward many polyfunctional molecules.[110] The 6-hydroxy group of *O*-*n*-octyl-β-D-glucopyranoside (**15**) was selectively reacted



with *N*-Cbz-Phe-OBz to give the peptido-sugar (Cbz = benzyloxycarbonyl, Bz = benzyl). The 3-hydroxy group of castanospermine (**16**) and 17-hydroxy group of 5α-androstane-3β,17β-diol (**17**) were selectively esterified to give their corresponding monoesters. On the other hand, the α-ester

of aspartic dibenzyl ester (**18**) was selectively hydrolyzed to give the corresponding monoacid in 97% yield. Linhardt and co-workers recently published the synthesis of a series of 1′-*O*-acyl sucrose derivatives **19** with use of the same catalyst.[60] The authors prepared 1′-*O*-lauryl sucrose, 1′-*O*-myristyl sucrose, and 1′-*O*-stearyl sucrose in 80–90% yields. Their method represents a "green" alternative to the organotin chemistry previously used.[61]



Scheme 1. Synthesis of peptides and peptidomimetics catalyzed by protease-CLECs.

4.1.3. Synthesis of Peptides and Peptidomimetics

Several protease-CLECs have been successfully used for the synthesis of peptides and for mild hydrolysis of amino acid and peptide amides.[62, 110] Subtilisin-CLECs effectively catalyzed the synthesis of peptides with > 90% yield by accepting both L- and D-amino acid amides as nucleophiles. Thermolysin-CLECs were efficient in the synthesis of several peptides in 90% EtOH and were used in a multicycle preparation of aspartame in ethyl acetate.[24]

One of the most interesting applications of protease-CLECs is in the synthesis of chiral alkyl amides. Chiral alkyl amides of peptides and amino acids are important building blocks of many marketed and potential pharmaceuticals, such as HIV protease inhibitors, enkephalins, and antibiotics. In chemical procedures, the processes of coupling and resolution are usually separated: First optically pure components are prepared, then the coupling is performed. Despite significant recent advances in both solution-and solid-phase peptide chemistry,[63] the preparation of peptides on large scale often suffers from partial racemization, modest yield, and the difficulties of removing by-products produced during coupling reactions; dicyclohexyl urea, which arises from the commonly used dicyclohexylcarbodiimide (DCC), is a particularly troublesome example.[64, 65]

We found that the use of optically pure substrates was not necessary. The high enantioselectivity of the catalyst toward L-amino acids and *S*-amines resulted in formation of the *S*,*S*-alkylamide regardless of the optical purity of the substrates (Scheme 1). Indeed, optically pure alkylamides were obtained by in situ resolution when *R*,*S*-amines, or even both *R*,*S*-amines and *R*,*S*-amino acids, were used. In the latter case, the diastereomeric excess of product was greater than 98% (Table 6).[62, 110] The same approach was used in the synthesis of single-isomer matrix metalloprotease inhibitors (MMPIs) D1927 and D2163 with thermolysin-CLECs. In these cases, the key molecular structure and a final element of asymmetry were introduced through a PeptiCLEC-TR-mediated amide bond formation between a free carboxylic acid ($R^1 = H$ in Scheme 1) and an amine.[66]

4.1.4. Dehydrogenases

One of the major advantages of enzyme-catalyzed reduction of carbonyl groups in ketones and aldehydes over the use of hydrolases in the synthesis of chiral compounds is the potentially higher product yields. Indeed, by exploiting the prochiral character of carbonyl groups, the formation of racemic intermediates can be avoided.[67]

Unlike hydrolytic enzymes, dehydrogenases require cofactors, such as NAD(P)H, flavins, or PQQ, that need to be regenerated in situ (NAD(P)H = nicotinamide adenine dinucleotide (phosphate), reduced form; PQQ = pyrroloquinolinequinone tricarboxylic acid). Unlike other cofactors, nicotinamide cofactors are not tightly bound to dehydrogenases and can easily dissociate into solution. If, however, NADH is cocrystallized with a dehydrogenase, the cofactor remains tightly bound to the enzyme and can be recycled inside the complex by a coupled substrate system. This idea was first introduced by Lee et al.,[68] who demonstrated good activity (26% of that in solution) for crystals of horse liver alcohol dehydrogenase (HLADH) and increased stability of the cross-linked crystals in the presence of zinc salts. By using this system we demonstrated that the HLADH-NAD(H)-CLEC system exhibited high activity (64% of that in solution) without addition of exogenous NADH.[118] This complex was applied to the reduction of additional ketones **20**–**24** (Figure 8). The enantioselectivity and stereochemical preference

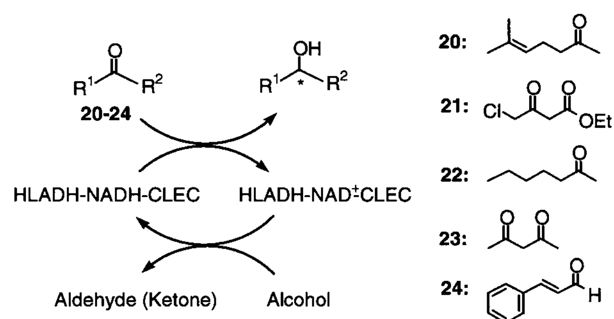


Figure 8. HLADH-catalyzed transformations.

of the HLADH-NAD(H)-CLEC complex were the same as those of the soluble enzyme. Given the fact that the HLADH-NAD(H)-CLEC complex is significantly more stable than

Table 6. Synthesis of peptidomimetics.[a]

Carboxylate	Amine	Product	Solvent	Yield [%]	*de* [%]
(*S*)-Z-PheOBz	(*R*,*S*)-Me-tryptamine	(*S*,*S*)-Z-Phe-Me-tryptamine	MeCN	> 98%	> 98
(*R*,*S*)-Z-PheOBz	(*R*,*S*)-Me-tryptamine	(*S*,*S*)-Z-Phe-Me-tryptamine	MeCN	95	> 98
(*S*)-NAcLeuOMe	(*R*,*S*)-NapEt	(*S*,*S*)-NAcLeuNapEt	3MP	66	91
(*S*,*S*)-Z-AlaSerOMe	(*R*,*S*)-NapEt	(*S*,*S*,*S*)-Z-AlaSerNapEt	MeCN	98	> 98

[a] NapEt = 1-naphthylethylamine, 3MP = 3-methylpyridine.

either HLADH or NADH, we concluded that not only the enzyme but also the cofactor was protected by the crystal environment. Further work will show whether the cofactor will stay tightly bound in the CLEC of other dehydrogenases without dissociation.

A different approach (crystallization of lactate dehydrogenase without a cofactor) was used by Sobolov et al. to prepare L-lactic acid from pyruvic acid in an electrolytic cell.[69] This catalyst maintained constant activity over 25 days and was much less sensitive to pH than the soluble enzyme.

4.1.5. Other Enzymes

In addition to hydrolases and dehydrogenases, several other enzymes, such as glucose isomerase,[70] fructose diphosphate aldolase,[116] and hydroxynitrile lyase[71] have been crystallized, cross-linked, and used as catalysts for chemical transformations. The synthetic potential of the aldolase and hydroxynitrile lyase was demonstrated by the preparation of a series of model compounds.

4.1.6. Productivity

A deciding factor in the evaluation of any catalyst is its productivity—how many kilos of product can one make with one kilo of catalyst? High specific activity and stability of CLEC catalysts in challenging environments, such as in organic solvents, in aqueous–organic mixtures, at elevated temperature, or under high-shear conditions, result in increased catalyst productivity. A good example of the high productivity of CLEC catalysts in organic solvents is the resolution of 1-phenylethanol with vinyl acetate in toluene catalyzed by cross-linked enzyme crystals of *Pseudomonas cepacia* lipase (LPS-CLECs).[72] In this reaction 1 mg of catalyst produces more than 4.6 g of product (with 98.5% *ee*), to give a volumetric productivity of 30 $g L^{-1}$ and a substrate to catalyst ratio of 4600. The high productivity of low molecular weight synthetic catalysts is thought to be their key advantage over high molecular weight enzymes.[73] This example clearly demonstrates that, despite the high molecular weight and quite unusual reaction medium for an enzyme, a CLEC can be highly productive and can compare favorably even with the best synthetic asymmetric catalysts.

In the very important therapeutic class of antibiotics, both 6-aminopenicillinic acid (6-APA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA) are being produced today on a multiton scale using the cross-linked crystal form of penicillin-G amidase (SynthaCLEC-PA). The stability of enzymes in the cross-linked crystal form is again clearly demonstrated by the multicycle pilot reaction run for the hydrolysis of penicillin-G (Figure 9). Other examples of CLEC recycling are presented in Table 7.

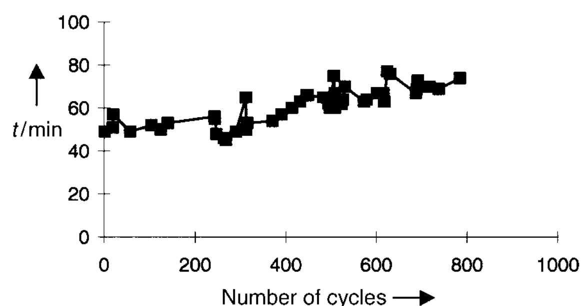


Figure 9. Multicycle hydrolysis of penicillin G. Penicillin G (8%) was hydrolyzed at pH 8 and 28°C with stirring in a 150-mL filtration cell equipped with 10-μm filter. After completion of each cycle the reaction mixture was drained and fresh substrate was added to the cell. A constant pH value was maintained by addition of NH_4OH.

4.2. Protein Crystals as Bioorganic Zeolites

Protein crystals have several common features with inorganic crystalline materials such as zeolites. Both are crystalline microporous materials with a uniform pore-size distribution that is fixed by arrangement of their unit cells.

With the method of macromolecular porosimetry we have estimated the apparent pore sizes and pore-size distributions in solid and soft hydrated porous sorbents directly from size exclusion chromatography (SEC) results. According to this method, CLPCs offer a wide range of pore sizes (15–100 Å), porosities (0.5–0.8), and pore surface areas (800–2000 $m^2 g^{-1}$).[74] The experimental data on the porosities of CLPCs are in good agreement with theoretical estimates of solvent content that are based on available crystallographic information for thermolysin and lipases. The solvent content of typical protein crystals is comprised of solvent-filled channels that take up 30–65% of the total crystal volume.[75] Pore-size calculations based on a cylindrical pore model gave a narrow and symmetrical pore-size distribution for all the CLPCs studied, with a calculated range of pore diameters that differ by less than 10% from the average. One should keep in mind, however, that the channels within protein crystals have quite complicated structures and can hardly be described by a simple cylindrical model. Indeed, as a stereo image of the crystalline lattice of CRL (Figure 10) suggests, a net of

Table 7. Recycling of cross-linked enzyme crystals.

Enzyme	Reaction	Medium	Number of cycles	Reference
subtilisin	resolution of α-methyltryptamine	3-methyl-3-pentanol	7	[110]
selenosubtilisin	reduction of hydroperoxides	aqueous	10	[113]
Candida rugosa lipase	resolution of ketoprophen	50% PEG 1000	18	[25]
alcohol dehydrogenase	reduction of cinnamaldehyde	aqueous	column	[118]
glucose isomerase	production of high-fructose corn syrup	aqueous	column	[123]
hydroxynitrile lyase	synthesis of cyanohydrins	butylether	5	[71]
penicillin acylase	hydrolysis of penicillin G	aqueous	1000	[115]

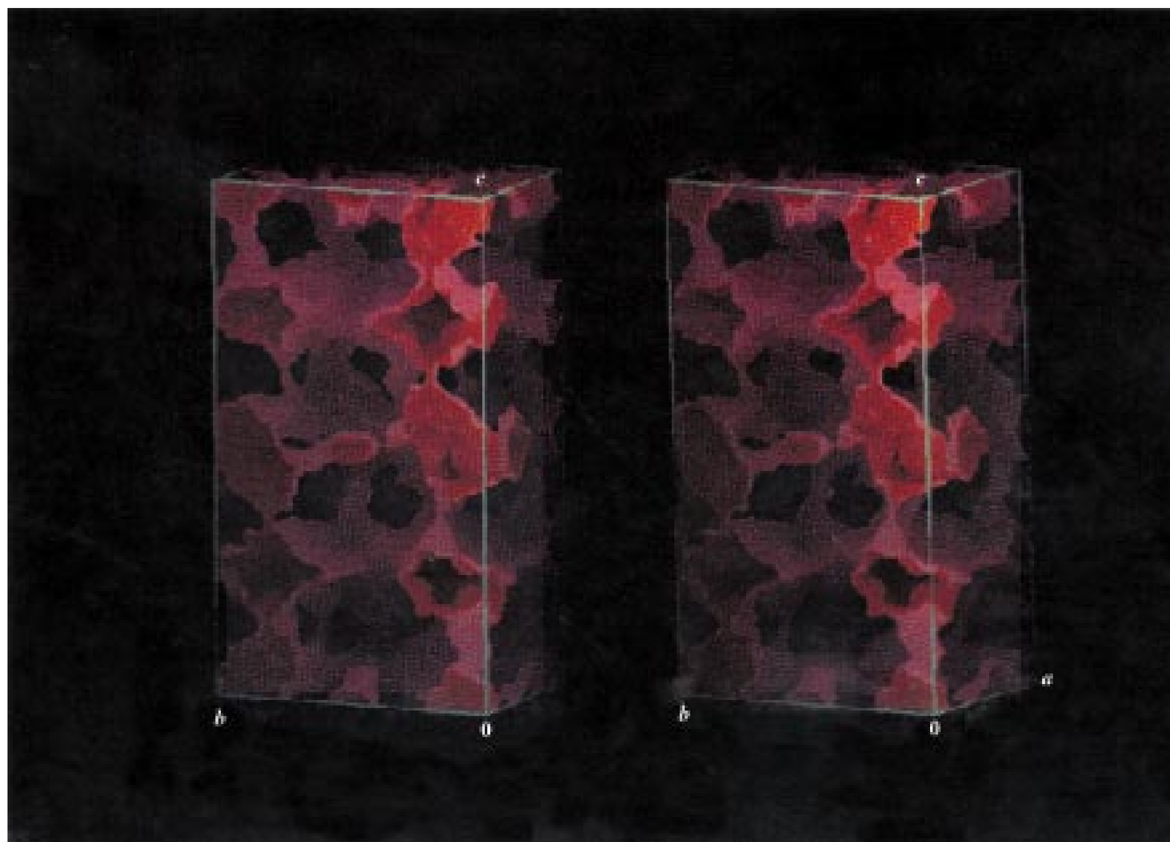


Figure 10. Stereoimage of one unit cell of the crystal lattice of *Candida rugosa* lipase (open form).[46] In this picture, the enzyme molecules that constitute the lattice, have been removed and only the surface of the solvent channels has been contoured, with the solvent side of the channels in orange and the protein side in violet. Reprinted with permission from ref. [74]. Copyright (1998) American Chemical Society.

channels with narrow connecting necks is seen to traverse the crystal unit cell (and hence, the crystalline macroscopic sorbent particles). The channels are predominantly in the direction of the crystallographic *a* axis, with significant cross-channels along the crystallographic *b* axis. Far narrower channels can be seen along the *c* axis.

In a sense, protein crystals represent a distinct and unique class of molecular sieves, with a pore-size range from 20 to more than 100 Å in diameter (Figure 11). The width and linearity of the solvent channels depends in large part on the way the molecules pack in a particular space group. For example, crystals of human superoxide dismutase (Figure 11 F) have wide solvent channels that directly traverse the crystal, whereas molecules of superoxide dismutase from *Xenopus laevis* form crystals with tighter packing and solvent channels that are relatively narrow and convoluted (Figure 11 A).

While zeolites are much more stable thermally, protein crystals may offer more control over pore size, porosity, and chemical properties of the pore surface. A distinct advantage of protein crystals over other porous materials is the inherently chiral nature of protein molecules. The L-amino acids that make up proteins create an asymmetric environment that can be exploited in the separation of enantiomers and in catalysis. Furthermore, one can modify the microenvironment of these crystal channels (with respect to both charge and hydrophobicity) by well-known techniques of

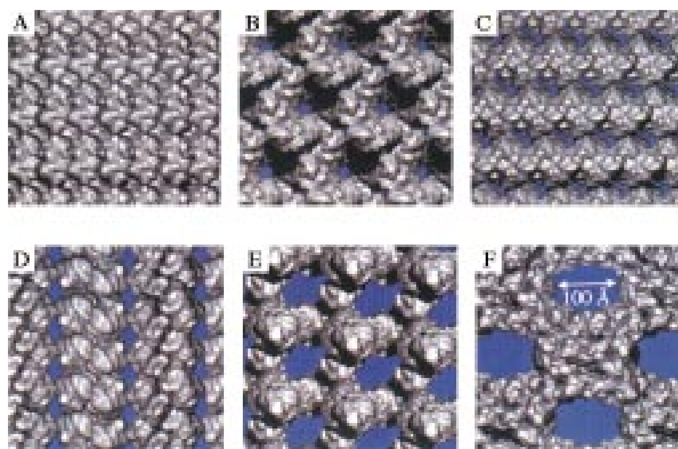


Figure 11. Computer-generated images of six enzyme crystal lattices. The surface representation for each was computed from the electron density that was calculated using coordinates from the corresponding solved crystal structures, which are deposited in the Brookhaven Protein Data Bank.[103] The contouring isovalues were chosen to yield a surface whose enclosed volume approximates that of the molecule. The solvent channels are shown in blue. The corresponding coordinate file name and reference are given for each entry: A) 1XSO, superoxide dismutase (*Xenopus laevis*); B) 8TLN,[104] thermolysin (*Bacillus thermoproteolyticus*); C) 1PNL,[105] penicillin acylase (*Escherichia coli*); D) 1CRL[28] lipase (*Candida rugosa*); E) 1WHS,[106] carboxypeptidase W (wheat germ); F) 1SOS,[107] superoxide dismutase (human recombinant). All structures are shown on the same scale; the bar corresponding to 100 Å establishes the absolute scale of the figure. Reprinted with permission from ref. [74]. Copyright (1998) American Chemical Society.

protein chemistry or by crystallization of modified recombinant proteins expressly designed for this purpose.

In addition to catalysis, one of the most direct and immediate applications of CLPCs is in the area of chromatography.[74] For example, thermolysin-CLECs (rodlike particles, 7 μ in length) provide good separation capability through at least three different mechanisms: size exclusion (Figure 12 A), adsorption (Figure 12 B), and enantiomeric separation (Figure 12 C–D). As discussed above, CLPCs can be considered to be a novel type of bioorganic zeolites. The ability of CLPCs to separate molecules by size may stem from the porous structure of the crystals (Figure 11). In contrast, separation of small molecules by their chemical structure and chirality can be attributed, in turn, to the intrinsic structural diversity and chirality of the protein in the stationary phase. Both thermolysin-CLECs and CPLCs of human serum albumin (HSA-CLPCs) were found to be fairly stable both in column packing (a packing pressure of 1500 psi was applied) and in the chromatography experiments themselves (more than 500 injections were performed without loss of separation efficiency). Furthermore, the repeated changes of eluent from water to 50 % acetonitrile did not influence the efficiency of chiral separations, which also indicates high stability of the stationary phase.

Another interesting example is the purification of xylitol by cross-linked crystals of glucose isomerase.[76] The authors used the principle of affinity chromatography and demonstrated a separation capacity of approximately 1 kg of xylitol per 500 kg of crystals.

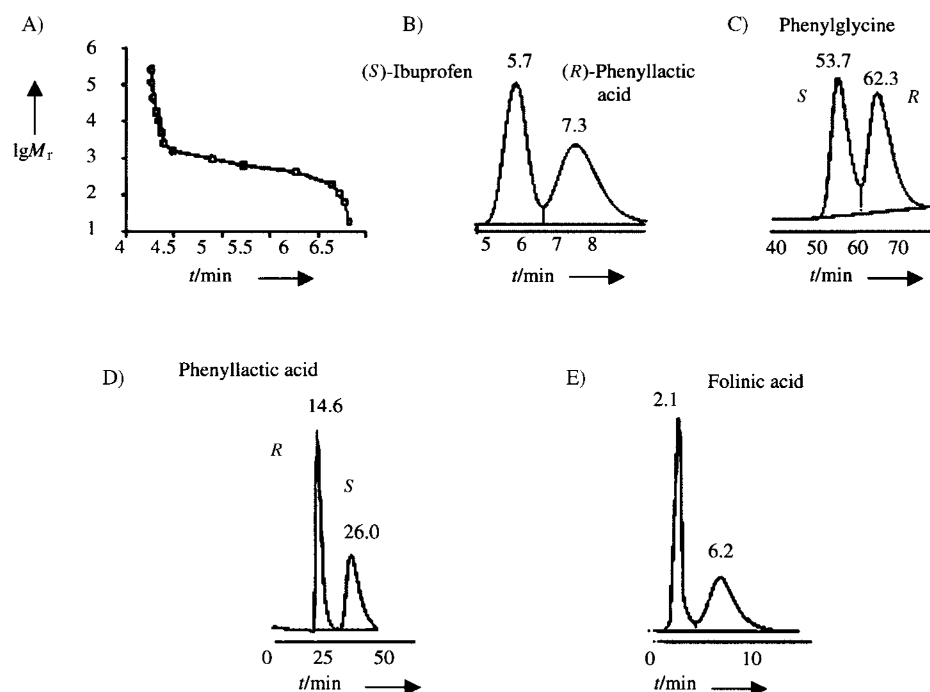


Figure 12. CLPC as a separation medium. Size-exclusion chromatography with PEG samples (M_r = 64–245 000 Da) (A), and chiral separations on thermolysin-CLEC (B–D) and HSA-CLPC (E) packed columns. PEG = polyethylene glycol. Reprinted with permission from ref. [74]. Copyright (1998) American Chemical Society.

4.3. Environmental Catalysis

One potentially significant application of CLEC-catalyzed processes is the detoxification of pesticides and chemical warfare agents. An efficient biocatalyst for this process, organophosphorous hydrolase (OPH) has been identified,[77] but its stability and specific activity may not be adequate for these applications. OPH is a homodimeric zinc metalloenzyme with unusually broad substrate specificity. It is capable of hydrolyzing P–O, P–F, P–S, and P–CN bonds in a variety of insecticides and chemical nerve agents and thus making them nonhazardous. The enzyme's activity varies widely from extremely high for the pesticide paraoxon (P–O bond; $k_{cat.} > 5000\,s^{-1}$) to very low for the chemical warfare agent VX (P–S bond; $k_{cat.} = 0.3\,s^{-1}$).[78] Despite its low activity in the latter reaction, OPH is the only VX detoxifying enzyme identified to date.[79]

Practical applications of OPH in the remediation of chemical warfare agents and other hazardous compounds require enzymes with markedly improved activity and stability in the most challenging media. Given the poor solubility of many of the OPH substrates in water, the high stability of OPH against organic solvents will be particularly desirable.[80] We have demonstrated that OPH-CLECs in fact provide such stability and are quite active against several model substrates.

The active site of OPH consists of a binuclear metal center that can bind any of several metals including Zn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, or Ni^{2+}.[81] The nature of the metal in the active site of the enzyme profoundly affects its catalytic activity.[82] We have demonstrated that metal replacement in the active site of OPH can be successfully accomplished in the CLEC form (Figure 13). The facile metal exchange process supports the notion that CLECs are essentially porous materials with channels traversing the entire body of the crystal to facilitate the transport of small molecules in and out of the crystal. Both Zn^{2+} and Co^{2+} OPH-CLECs were shown to have significant activity towards the common pesticide Demeton-S[83] and the chemical warfare agent VX.[84]

4.4. Biomedical Applications

The combination of purity, high stability, and activity that makes enzyme crystals so useful in many chemical applications provides new opportunities in biomedical fields and diagnostics[125] as well. We will consider three applications that

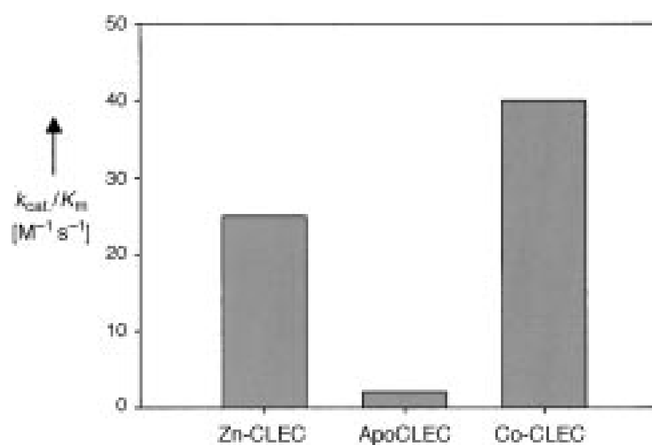


Figure 13. Metal exchange in the active site of OPH-CLECs. Apo-CLEC = the cross-linked enzyme crystals without a metal present.

are based on conventional CLPCs (Figure 6, top), controlled-release CLPCs (Figure 6, middle), and un-cross-linked, stabilized protein crystals (Figure 6, bottom).

4.4.1. Oral Lumenal Therapy

Metabolic or gastrointestinal diseases often result from impaired or missing enzyme or protein function at a critical point in a biochemical pathway. For example, some digestive disorders observed with cystic fibrosis or pancreatitis patients can be traced to improper levels of lipase enzymes in the duodenum. Further examples can be found in the disorders resulting from in-born errors in the metabolism. The amino acid metabolism disorder phenylketonuria (PKU) limits patients to a dietary regime that consists largely of phenylalanine-free protein hydrolysates.[85]

The human gastrointestinal tract presents a challenging environment for proteins due to the acid conditions in the stomach ($pH < 2$) and high concentrations of proteases in the stomach and the gut. For example, less than 1% of lipolytic activity secreted into the duodenum reaches the ileum.[86, 87] It is not surprising that porcine pancreatic lipase, the lipase in currently available commercial preparations, rarely abolishes pancreatic steatorrhea caused by chronic pancreatitis or cystic fibrosis.[88] By virtue of the excellent stability and activity displayed by proteins in the CLEC formulation, such proteins are capable of performing therapeutically beneficial chemistry in the gut lumen without being degraded by extremes of pH or endogenous protease action. Thus, a CLEC therapeutic agent can be orally ingested by a patient and perform the desired therapeutic chemistry in the gut lumen, and then the undegraded CLEC therapeutic agents can be excreted in the stool. Our preliminary results indicate that several lipase-CLECs are at least 100-fold more active and significantly more stable than commercial lipase products. We have also demonstrated the efficacy of certain lipase-CLECs in dogs with pancreatic insufficiency.[89] In principle, lipase-CLECs could lead to more attractive dosing regimens for these conditions that could significantly reduce the total protein pill and greatly improve patient compliance.

4.4.2. Subunit Vaccines

Subunit vaccines that consist of well-characterized molecules are extremely attractive due to their superior safety profile and ease of manufacture through chemical synthesis or recombinant DNA technology. However, the price one has to pay for these advantages is significant: Subunit vaccines are generally poorly immunogenic and in many cases cannot compete with attenuated and inactivated whole virus or pathogen counterparts.[90] To enhance the immune response subunit vaccines universally require improved adjuvants and delivery vehicles.

We recently proposed using CLPCs as antigens and compared the immunogenicity of HSA-CLPCs with that of the soluble protein. Crystalline forms induce and sustain 6–10-fold increases in antibody titer for highly cross-linked crystals and approximately 30-fold increase for lightly cross-linked crystals, relative to the soluble protein, in a study of six months duration in rats.[91] We hypothesized that the depot effect, the particulate structure of the CLPC, and the highly repetitive nature of protein crystals may play roles in the enhanced production of circulating antibodies. Indeed, the highly repetitive structure of a protein crystal results in multiple copies of the antigen presented to the immune system as a part of a relatively large particle (Figure 14). In this respect, CLPCs strikingly resemble polyvalent particulate structures of the hepatitis B surface antigen (HbsAg),[92] *Helicobacter pylori* urease,[93] and virus-like particles (VLP).[94] Several features of CLPCs, such as their remarkable stability, purity, biodegradability, and ease of manufacture make them highly attractive for vaccine formulations. This work paves the way for a systematic study into the effect of protein crystallinity and cross-linking on the enhancement of humoral and cellular responses.

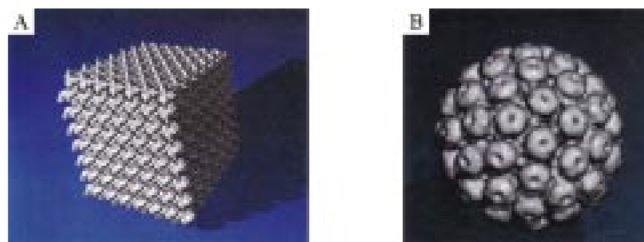


Figure 14. a) Computer-generated image of a three-dimensional HSA crystal. The individual enzyme molecules ($M_r = 66000$) are shown in silver. The crystal has seven unit cells along each edge, hence the dimension of the crystal size is $41.2 \times 62.2 \times 42.5$ nm, and contains 686 HSA molecules in total. The surface representation was computed from the electron density that was calculated using atomic coordinates of the reported structure[108] obtained from the Brookhaven Protein Data Bank. B) Computer-generated image of a virion of polyomavirus (50 nm) containing 360 VP1 molecules.[109] Reprinted with permission from ref. [91]. Copyright (1999) National Academy of Sciences, USA.

4.4.3. Un-Cross-Linked Protein Crystals

Since dry protein crystals can be quite stable even without cross-linking, they can be used as a unique formulation for drug delivery. Indeed, the value of crystallinity is well

understood for small molecules. The majority of small-molecule drugs are produced in crystalline form. This is not surprising since crystallinity normally results in higher storage stability, purity, and reproducibility in terms of a drug's physical, chemical, and therapeutic properties.[33] For macromolecule therapeutics the situation is quite different. Among almost 400 biopharmaceuticals[95] that are either approved or in advanced clinical trials, there is only one product—insulin—that is produced and administered in crystalline form. Yet crystallization of macromolecule pharmaceuticals, particularly proteins, can offer significant advantages:

a) protein crystallization can be used to streamline manufacturing process, as in the case with small-molecule drugs,
b) a crystal is the most concentrated possible form of protein and can be beneficial for drugs, such as antibodies, which require high doses at the delivery site,
c) since the rate of crystal dissolution depends on its morphology, size, and the presence of excipients, crystalline proteins may serve as a convenient carrier-free slow release dosage form (insulin is a good example), and
d) the stability of proteins in crystalline form is higher than that of corresponding soluble or amorphous materials.

The inactivation of proteins often starts with a reversible unfolding of a protein molecule followed by an irreversible inactivation that leads to protein aggregation[96] and, as a consequence, the loss of biological activity.[97] In order to compare the extent of aggregation in different preparations of glucose oxidase (GO) and CRL, these preparations were incubated at elevated temperatures and reconstituted into aqueous buffers. The insoluble precipitate was filtered out, and the residual soluble protein was investigated by size-exclusion chromatography. The results of these experiments indicate that soluble proteins aggregate quickly by producing poorly soluble precipitates. The comparison of solid formulations indicates that crystalline proteins are less prone to aggregation than their amorphous counterparts. After two days of GO incubation at 50 °C, 73 % of crystalline protein (71 % activity) remains soluble, to give a specific activity for the soluble fraction of 118.6 $U mg^{-1}$. The amorphous formulation yields a slightly higher amount of the soluble protein (76 %) under the same conditions, but only 27 % activity to give a low specific activity of 57.3 $U mg^{-1}$. These data point to significant inactivation of amorphous GO under storage. Unlike the soluble GO, which upon reconstitution predominantly consists of a native dimer form (higher insoluble aggregates were removed by ultrafiltration), solid formulations give a mixture of a native dimer and higher soluble aggregates. However, this intermolecular protein aggregation is more profound in the amorphous formulation (36 % native form) than in the crystalline one (67 % native form). The lower tendency of crystalline formulations to aggregate was further confirmed by an experiment with the much more hydrophobic protein, CRL. Indeed, 91 % of the native CRL form (monomer) is preserved in the crystalline formulation versus only 26 % in the amorphous sample (Figure 15).

One potentially significant application of protein crystals lies in protein drug delivery. Inadequate protein stability during encapsulation, storage, and release is one of the major obstacles for successful formulation of polymeric microspheres.[98] The high stability of the protein and lower tendency to aggregate in crystalline form of the protein crystals, for example, allow them to better survive the harsh conditions of microencupsulation and to yield polymeric microspheres with higher loading (Figure 16).

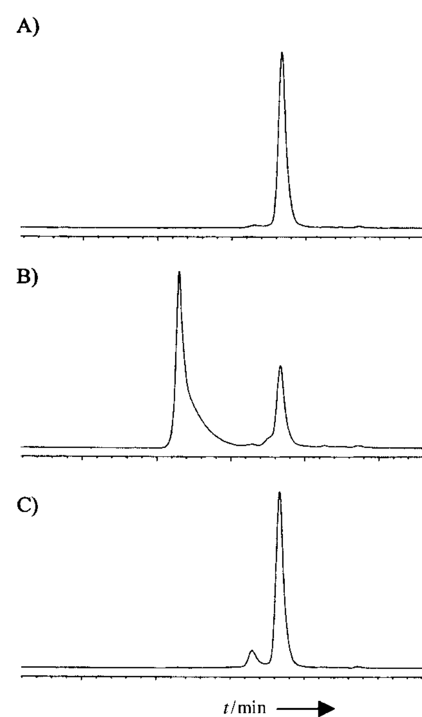


Figure 15. Size-exclusion chromatography of reconstituted CRL formulations. A) Soluble untreated CRL (control). B) Amorphous and C) crystalline CRL formulated with sucrose after 200 days at 40 °C and 75 % RH. The samples were dissolved in phosphate buffered saline (pH 7.4). Reprinted with permission from ref. [31]. Copyright (2001) Wiley Interscience.

Figure 16. Crystals of penicillin acylase incorporated in the PLGA microspheres.

5. Conclusions and Future Prospects

As is the case with many other developments in technology and science, one can readily identify, in retrospect, roots and

precedents for the cross-linked enzyme crystal technology first outlined in 1992,[9] and for the further extensions and new implementations of that concept reported since. With hindsight, there are few, if any, technical reasons why Sumner himself could not have developed some of the abovementioned applications around a jack bean urease in 1926.

One factor that may explain the rapid development and commercialization of CLECs and CLPCs since 1992 is our strongly held view of protein crystals as materials that needed—first and foremost—to be significantly optimized in terms of their underlying chemical and mechanical stability. Having achieved such stability in our cross-linked crystalline materials, we have been free to focus our attention on the introduction of the practical functionality that has proven itself useful in significant real-world applications.

Enzyme catalysts were the logical first step in this development, given that they could bring unprecedented selectivity and specificity to important chemical processes at minimal cost. CLECs have since been developed for a large and diverse panel of uses, as we have discussed in this review.

The theme of stabilized protein crystals as materials extends naturally to their use as novel chromatographic materials that can function repeatedly under the harsh conditions typical of HPLC experiments and that can express the exquisite chirality of their underlying protein matrix. Similarly, the rapid, effective, and economical formulation of protein drugs with unprecedented levels of purity and shelf-life stability is greatly facilitated by this technology, as is the production of vaccines whose immunogenicity is not dependent on the addition of adjuvants and other complicating factors. Additional therapeutic uses for stabilized protein crystalline materials abound and range from protein drug delivery schemes to their use in enzyme replacement therapy.

As we consider the flood of information being produced by the minute through human genome sequencing, we also need to be attuned to the opportunities implicit in the thousands upon thousands of new proteins that will be created for the first time through the expression of that information by proteomics.[99] This information explosion will undoubtedly speed up the development of massively parallel measurement strategies including high-throughput X-ray crystallography.[100] Clearly, the ability to crystallize tens of thousands of proteins quickly and inexpensively may lead to a bottleneck in the whole structural genomics initiative.[101] To this end, advanced methods of robotic automation that are currently being developed will play a key role in monitoring and optimization of crystallization trials.[102] While the major outcome of this effort will be creation of new structural information, the high-throughput crystallization techniques will benefit the crystallization and formulation methodologies reviewed here.

This nascent science of "crystalomics" provides us with a general strategy for putting proteins, both old and new, to work—rapidly and efficiently—in the harsh conditions typical of most practical industrial processes and in therapeutic interventions of unprecedented power and benefit. Future developments will hopefully be at least as exciting as the advances already achieved with these technologies.

We thank our many colleagues at Altus Biologics who played a crucial role in development of the CLEC technology and whose names are listed in the respective references. Additional thanks go to Jim Griffith (Althexis), Kirill Yakovlevsky, and Yi-Fong Wang for their help in the preparation of the manuscript and for useful discussions. We are also grateful to the NIH, NSF, and Department of Defense who generously supported our work through their SBIR programs.

Received: June 27, 2000 [A 416]

[1] H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne, *Nucleic Acids Res.* **2000**, *28*, 235–242.
[2] H. Kubinyi, *Curr. Opin. Drug Disc. Dev.* **1998**, *1*, 4–15.
[3] *Macromolecular Structures* (Eds.: W. A. Hendrickson, K. Wuthrich), Current Biology, London, **1999**.
[4] J. B. Sumner, *J. Biol. Chem.* **1926**, *69*, 435–441.
[5] A. L. Dounce, P. Z. Allen, *Trends Biochem. Sci.* **1988**, *13*, 317–320.
[6] J. D. Bernal, D. Crowfoot, *Nature* **1934**, *133*, 794–795.
[7] J. S. Fruton, *Molecules and Life—Historical Essays on the Interplay of Chemistry and Biology*, Wiley-Interscience, New York, **1972**.
[8] A. McPherson, *Crystallization of Biological Macromolecules*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, **1999**, p. 1.
[9] N. L. St. Clair, M. A. Navia, *J. Am. Chem. Soc.* **1992**, *114*, 7314–7316.
[10] For reviews, see: a) A. L. Margolin, *Trends Biotechnol.* **1996**, *14*, 223–230; b) T. Zelinski, H. Waldmann, *Angew. Chem.* **1997**, *109*, 746–748; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 722–724; c) J. J. Lalonde, *Curr. Opin. Drug Disc. Dev.* **1998**, *1*, 272–277; d) D. Haring, P. Schreir, *Curr. Opin. Chem. Biol.* **1999**, *3*, 35–38.
[11] M. Ries-Kautt, A. Ducruix, *Methods Enzymol.* **1997**, *276*, 23–59.
[12] A. J. Malkin, A. McPherson, *Acta Crystallogr. Sect. D* **1994**, *50*, 385–395.
[13] D. I. Liao, K. Breddam, R. M. Sweet, T. Bullock, S. J. Remington, *Biochemistry* **1992**, *31*, 9796–9812.
[14] S.-T. Yau, P. G. Vekilov, *Nature* **2000**, *406*, 494–497.
[15] P. C. Weber, *Methods Enzymol.* **1997**, *276*, 13–22; *Crystallization of Nucleic Acids and Proteins* (Eds.: A. Ducruix, R. Giege), Oxford University Press, New York, **1999**.
[16] T. S. Lee, J. D. Vaghjiani, G. J. Lye, M. K. Turner, *Enzyme Microbial Technol.* **2000**, *26*, 582–592.
[17] "The Differentiation and Specificity of Corresponding Proteins and Other Vital Substances in Relation to Biological Classification and Organic Evolution": E. T. Reichert, A. P. Brown, *The Crystallography of Hemoglobins*, Carnegie Institution of Washington, Washington, **1909**.
[18] V. Laufberger, *Bull. Soc. Chim. Biol.* **1937**, *19*, 1575; S. Granick, *J. Biol. Chem.* **1941**, *146*, 451.
[19] G. Alderton, H. L. Fevold, *J. Biol. Chem.* **1946**, *164*, 1.
[20] A. McPherson, *Crystallization of Biological Macromolecules*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, **1999**, pp. 6–16 and pp. 235–238.
[21] T. M. Przybycien, *Curr. Opin. Biotechnol.* **1998**, *9*, 164–170.
[22] US Patent 5728559, **1998** [*Chem. Abstr.* **1998**, *122*, 18276]; US Patent 5719048, **1998** [*Chem. Abstr.* **1998**, *122*, 50048].
[23] R. A. Judge, M. R. Johns, E. T. White, *Biotechnol. Bioeng.* **1995**, *48*, 316–323.
[24] R. A. Persichetti, N. L. St. Clair, J. P. Griffith, M. A. Navia, A. L. Margolin, *J. Am. Chem. Soc.* **1995**, *117*, 2732–2737.
[25] J. L. Lalonde, C. Govardhan, N. Khalaf, A. G. Martinez, K. Visuri, A. L. Margolin, *J. Am. Chem. Soc.* **1995**, *117*, 6845–6852.
[26] M. S. Doscher, F. M. Richards, *J. Biol. Chem.* **1963**, *238*, 2399–2406.
[27] D. R. Walt, V. I. Agayn, *Trends Anal. Chem.* **1994**, *13*, 425–430.
[28] T. Tashima, M. Imai, Y. Kuroda, S. Yagi, T. Nakagawa, *J. Org. Chem.* **1991**, *56*, 694–697.
[29] B. Shenoy, A. L. Margolin, unpublished results.
[30] C. Govardhan, A. L. Margolin, *Chem. Ind.* **1995**, 689–693; H. Noritomi, K. Koyama, S. Kato, K. Nagahama, *Biotechnol. Tech.* **1998**,

12, 467–469; C. Govardhan, *Curr. Opin. Biotechnol.* **1999**, *10*, 331–335.
[31] B. Shenoy, Yi Wang, W. Shan, A. L. Margolin, *Biotechnol. Bioeng.* **2001**, *73*, 358–369.
[32] A. McPherson, *Preparation and Analysis of Protein Crystals*, Krieger, Malabar, FL, **1989**.
[33] B. C. Hancock, G. Zografi, *J. Pharm. Sci.* **1997**, *86*, 1–12.
[34] S. A. Islam, D. L. Weaver, *Proteins Struct. Funct. Genet.* **1990**, *8*, 1–5.
[35] C. Jelsch, S. Longhi, C. Cambillau, *Proteins Struct. Funct. Genet.* **1998**, *31*, 320–333.
[36] J. Janin, F. Rodier, *Proteins Struct. Funct. Genet.* **1995**, *23*, 580–587.
[37] R. M. Daniel, *Enzyme Microbial Technol.* **1996**, *19*, 74–79.
[38] A. Mozzarelli, G. L. Rossi, *Annu. Rev. Biomol. Struct.* **1996**, *25*, 343–365.
[39] M. S. Doscher, F. M. Richards, *J. Biol. Chem.* **1963**, *238*, 2399–2406; M. W. Makinen, A. L. Fink, *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 301–343.
[40] L. A. A. Sluyterman, M. J. M. de Graaf, *Biochim. Biophys. Acta* **1969**, *171*, 277–287.
[41] E. M. Westbrook, P. B. Sigler, *J. Biol. Chem.* **1984**, *259*, 9090–9095.
[42] W. Tischer, V. Kasche, *Trends Biotechnol.* **1999**, *17*, 326–335, and references therein.
[43] M. Tegoni, A. Mozzarelli, G. L. Rossi, F. Labeyrie, *J. Biol. Chem.* **1983**, *258*, 5424–5427.
[44] B. Rubin, *Nat. Struct. Biol.* **1994**, *1*, 568–572.
[45] C. Chapus, M. Semeriva, C. Bovier-Lapierre, P. Desnuelle, *Biochemistry* **1976**, *15*, 4980–4987.
[46] P. Grochulski, Y. Li, J. D. Schrag, F. Bouthillier, P. Smith, D. Harrison, B. Rubin, M. Cygler, *J. Biol. Chem.* **1993**, *268*, 12843–12847.
[47] P. Grochulski, Y. Li, J. D. Schrag, M. Cygler, *Protein Sci.* **1994**, 3, 82–91.
[48] N. Khalaf, C. Govardhan, J. J. Lalonde, R. A. Persichetti, Y. F. Wang, A. L. Margolin, *J. Am. Chem. Soc.* **1996**, *118*, 5494–5495.
[49] B. Shenoy, A. L. Margolin, unpublished results.
[50] G. Sellek, J. Chaudhuri, *Enzyme Microbial Technol.* **1999**, *24*, 471–482.
[51] P. Rasor in *Chiral Catalyst Immobilization and Recycling* (Eds.: D. E. De Vos, I. F. J. Vankelecom, P. A. Jacobs), Wiley-VCH, Weinheim, **2000**, pp. 97–122.
[52] V. Mozhaev, *Trends Biotechnol.* **1993**, *11*, 88–95.
[53] a) *Enzyme Catalysis in Organic Synthesis* (Eds.: K. Drauz, H. Waldmann), VCH, New York, **1995**; b) P. Sears, M. Schuster, P. Wang, K. Witte, C.-H. Wong, *J. Am. Chem. Soc.* **1994**, *116*, 6521–6530.
[54] C.-H. Wong, M. Schuster, P. Wang, P. Sears, *J. Am. Chem. Soc.* **1993**, *115*, 5893.
[55] J. L. Schmitke, C. R. Wescott, A. M. Klibanov, *J. Am. Chem. Soc.* **1996**, *118*, 3360–3365; Y.-L. Khmelnitsky, S. H. Welch, D. S. Clark, J. S. Dordick, *J. Am. Chem. Soc.* **1994**, *116*, 2647–2648.
[56] J. H. Shah, G. M. Swartz, A. E. Papathanassiu, A. M. Treston, W. E. Fogler, J. W. Madsen, S. J. Green, *J. Med. Chem.* **1999**, *42*, 3012–3017.
[57] P. Giannakakou, R. Gussio, E. Nogales, K. H. Downing, D. Zaharevitz, B. Bollbuck, G. Poy, D. Sackett, K. C. Nicolaou, T. Fojo, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 2904–2909; K. C. Nicolaou, D. Hepworth, N. P. King, M. R. V. Finlay, R. Scarpelli, M. M. A. Pereira, B. Bollbuck, A. Bigot, B. Werschkun, N. Winssinger, *Chem. Eur. J.* **2000**, *6*, 3116–3148.
[58] R. E. Taylor, G. M. Galvin, K. A. Hilfiker, Y. Chen, *J. Org. Chem.* **1998**, *63*, 9580–9583.
[59] D. Roche, K. Prasad, O. Repic, *Tetrahedron Lett.* **1999**, *40*, 3665–3668.
[60] T. Polat, H. G. Bazin, R. J. Linhardt, *J. Carbohydr. Chem.* **1997**, *16*, 1319–1325.
[61] I. R. Vlahov, P. I. Vlahov, R. J. Linhardt, *J. Carbohydr. Chem.* **1997**, *16*, 1.
[62] Y. F. Wang, K. Yakovlevsky, A. L. Margolin, *Tetrahedron Lett.* **1996**, *37*, 5317–5320.
[63] E. Saxon, J. I. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 2141–2143; T. Inui, M. Nakao, H. Nishio, Y. Nishiuchi, S. Kojima, T. Muramatsu, T. Kimura, *J. Pept. Res.* **2000**, *55*, 384–397.
[64] a) M. Bodanszky, J. C. Tolle, *Int. J. Pept. Protein Res.* **1977**, *10*, 380–384; b) J. P. Greenstein, M. Winitz, *Chemistry of the Amino Acids*, *Vol. 2*, Krieger, **1986**, pp. 1016–1024, and references therein.
[65] G. Wendlber, *Methoden Org. Chem. (Houben-Weyl)*, *6th ed.*, Vol. 12/2, **1974**.
[66] A. D. Baxter, J. B. Bird, R. Bannister, R. Bhogal, D. T. Manallack, R. W. Watson, D. A. Owen, J. Montana, J. Henshilwood, R. C. Jackson in *Matrix Metalloproteinase Inhibitors in Cancer Therapy* (Eds.: N. J Clendeninn, K. Appelt), Humana Press, Totowa, NJ, **1999**, pp. 193–221.
[67] *Enzyme Catalysis in Organic Synthesis*, *Vol. 2* (Eds.: K. Drauz, H. Waldmann), VCH, Weinheim, **1995**, pp. 595–665.
[68] K. M. Lee, M. Blaghen, J.-P. Samama, J.-F. Biellmann, *Bioorg. Chem.* **1986**, *14*, 202.
[69] S. B. Sobolov, M. D. Leonida, A. Bartoszko-Malik, K. I. Voivodov, F. McKinney, J. Kim, A. J. Fry, *J. Org. Chem.* **1996**, *61*, 2125–2128.
[70] K. Visuri, Eur. Pat. 0341503, **1989**.
[71] D. Costes, E. Wehtje, P. Adlercreutz, *J. Mol. Catal. B* **2001**, *11*, 607–612.
[72] N. Khalaf, C. P. Govardhan, J. J. Lalonde, R. A. Persichetti, Y. F. Wang, A. L. Margolin, *J. Am. Chem. Soc.* **1996**, *118*, 5494–5495.
[73] E. N. Jacobsen, N. S. Finney, *Chem. Biol.* **1994**, *1*, 85–90.
[74] L. Z. Vilenchik, J. P. Griffith, N. L. St. Clair, M. A. Navia, A. L. Margolin, *J. Am. Chem. Soc.* **1998**, *120*, 4290–4294.
[75] B. W. Matthews, *J. Mol. Biol.* **1968**, *33*, 491–497.
[76] a) O. Pastinen, K. Visuri, M. Leisola, *Biotechnol. Tech.* **1998**, *12*, 557–560; b) O. Pastinen, J. Jokela, T. Eerikainen, T. Schwabe, M. Leisola, *Enzyme Microbial Technol.* **2000**, *26*, 550–558.
[77] D. P. Dumas, H. D. Durst, W. G. Landis, F. M. Raushel, J. R. Wild, *Arch. Biochem. Biophys.* **1990**, *277*, 155–159.
[78] K. Lai, J. K. Grimsley, B. D. Kulhmann, L. Scapozza, S. P. Harvey, J. J. DeFrank, J. E. Kolakowsky, J. R. Wild, *Chimia* **1996**, *50*, 430.
[79] V. K. Rastogi, J. J. DeFrank, T.-C. Cheng, J. R. Wild, *Biochem. Biophys. Res. Commun.* **1997**, *241*, 294.
[80] J. K. Grimsley, J. M. Scholtz, C. N. Pace, J. R. Wild, *Biochemistry* **1997**, *36*, 14366.
[81] G. A. Omburo, J. M. Kuo, L. S. Mullins, F. M. Raushel, *J. Biol. Chem.* **1992**, *267*, 13278–13283.
[82] J. M. Kuo, M. Y. Chae, F. M. Raushel, *Biochemistry* **1997**, *36*, 1982–1988.
[83] F. C. G. Hoskin, J. E. Walker, R. Stote, *Chem. Biol. Interact.* **1999**, *119–120*, 439–444.
[84] T.-c. Cheng, V. K. Rastogi, J. J. DeFrank, C. Govardhan, K. Yakovievsky, A. L. Margolin, J. K. Grimsley, J. R. Wild, unpublished results.
[85] C. Sarkissian, Z. Shao, F. Blain, R. Peevers, H. Su, R. Heft, T. M. S. Chang, C. Scriver, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 2339–2344.
[86] P. Layer, V. L. Go, E. P. DiMagno, *Am. J. Physiol.* **1986**, *251*, G475–G480.
[87] P. Layer, G. Holtmann, *Int. J. Pancreatol.* **1994**, *15*, 1–11.
[88] E. P. DiMagno, J.-R. Malagelada, V. L. W. Go, C. G. Moertel, *N. Engl. J. Med.* **1977**, *296*, 1318–1322.
[89] A. L. Margolin, B. C. Shenoy, R. J. Sandberg, E. P. DiMagno, unpublished results.
[90] M. J. Newman, M. F. Powell in *Vaccine Design: The Subunit and Adjuvant Approach* (Eds.: M. F. Powell, M. J. Newman), Plenum, New York, **1995**, pp. 1–42.
[91] N. L. St. Clair, B. Shenoy, L. D. Jacob, A. L. Margolin, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9469–9474.
[92] W. J. McAleer, E. B. Buynak, R. Z. Maigetter, D. E. Wampler, W. J. Miller, M. R. Hilleman, *Nature* **1984**, *307*, 178–180.
[93] J. W. Austin, P. Doig, M. Stewart, T. J. Trust, *J. Bacteriol.* **1992**, *174*, 7470–7473.
[94] S. E. Adams, A. S. Kingsman in *Vaccine Design: The Subunit and Adjuvant Approach* (Eds.: M. F. Powell, M. J. Newman), Plenum, New York, **1995**, pp. 769–786.
[95] A. M. Taylor, *Chem. Eng. News.* **1998**, *76*(32), 19–31.
[96] J. L. Cleland, M. F. Powell, S. J. Shire, *Crit. Rev. Ther. Drug Carrier Syst.* **1993**, *10*, 307–377.
[97] V. V. Mozhaev, *Trends Biotechnol.* **1993**, *11*, 88–95.
[98] S. P. Schwendeman, M. Cardamone, A. M. Klibanov, R. Langer, M. R. Brandon in *Microparticulate Systems for the Delivery of Proteins and Vaccines* (Eds.: S. Cohen, H. Bernstein), Marcel Dekker, New York, **1996**, pp. 1–49.
[99] N. L. Anderson, A. D. Matheson, S. Steiner, *Curr. Opin. Biotechnol.* **2000**, *11*, 408–412.

[100] E. Eisenstein, G. L. Gilliland, O. Herzberg, J. Moult, J. Orban, R. J. Poljak, L. Banerjei, D. Richardson, A. J. Howard, *Curr. Opin. Biotechnol.* **2000**, *1*, 25–30.
[101] S. K. Burley, S. C. Almo, J. B. Bonanno, M. Capel, M. R. Chance, T. Gaasterland, D. Lin, A. Sali, F. W. Studier, S. Swaminathan, *Nat. Genet.* **1999**, *2*, 151–157.
[102] E. Wilson, *Chem. Eng. News* **2000**, *78*(27), 27.
[103] F. C. Bernstein, T. F. Koetzle, G. J. B. Williams, E. F. Meyer, Jr., M. D. Brice, J. R. Rodgers, O. Kennard, T. Shimanouchi, M. Tasumi, *J. Mol. Biol*. **1977**, *112*, 535–542.
[104] D. R. Holland, D. E. Tronrud, H. W. Pley, K. M. Flaherty, W. Stark, J. N. Jansonius, D. B. McKay, B. W. Matthews, *Biochemistry* **1992**, *31*, 11310–11316.
[105] H. J. Duggleby, S. P. Tolley, C. P. Hill, E. J. Dodson, G. Dodson, P. C. E. Moody, *Nature* **1995**, *373*, 264–268.
[106] D.-I. Liao, S. J. Remington, *J. Biol. Chem*. **1990**, *265*, 6528–6531.
[107] H. E. Parce, R. A. Hallewell, J. A. Tainer, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 6109.
[108] X. M. He, D. C. Carter, *Nature* **1992**, *358*, 209–215.
[109] J. P. Griffith, D. L. Griffith, I. Rayment, W. T. Murakami, D. L. Caspar, *Nature* **1992**, *355*, 652–654.
[110] Y. F. Wang, K. Yakovlevsky, B. Zhang, A. L. Margolin, *J. Org. Chem.* **1997**, *62*, 3488–3495, and references therein.
[111] E. Tuchsen, M. Ottesen, *Carlsberg Res. Commun*. **1977**, *42*, 407–420.
[112] H. Noritomi, K. Koyama, S. Kato, K. Nagahama, *Biotechnol. Tech.* **1998**, *12*, 467–469.
[113] D. Haring, E. Schuler, P. Schreier, *Proc. 216th ACS Meeting* (Boston, MA) **1998**.
[114] F. A. Quiocho in *Methods in Enzymology*, *Vol. 44* (Ed.: K. Mosbach), Academic Press, New York, **1976**.
[115] J. J. Lalonde, unpublished results.
[116] S. B. Sobolov, A. Bartoszko-Malik, T. R. Oeschger, M. M. Montelbano, *Tetrahedron Lett.* **1994**, *42*, 7751–7754.
[117] S. B. Sobolov, M. D. Leonida, A. Bartoszko-Malik, K. I. Voivodov, F. McKinney, J. Kim, A. J. Fry, *J. Org. Chem*. **1996**, *61*, 2125–2128.
[118] N. L. St. Clair, Y. F. Wang, A. L. Margolin, *Angew. Chem.* **2000**, *112*, 388–391; *Angew. Chem. Int. Ed.* **2000**, *39*, 380–383.
[119] K. Visuri, O. Pastinen, X. Wu, K. Makinen, M. Leisola, *Biotechnol. Bioeng*. **1999**, *64*, 377–380.
[120] C. A. Spilburg, J. L. Bethune, B. L. Vallee, *Biochemistry* **1977**, *16*, 1142–1150.
[121] G. M. Alter, D. L. Leussing, H. Neurath, B. L. Vallee, *Biochemistry* **1977**, *16*, 3663–3668.
[122] P. J. Kasvinsky, N. B. Madsen, *J. Biol. Chem*. **1976**, *251*, 6852–6859.
[123] K. Visuri, US Patent No. 4699882, **1987** [*Chem. Abstr.* **1987**, *104*, 87145].
[124] J. J. Lalonde, M. A. Navia, A. L. Margolin in *Methods in Enzymol.* **1997**, *286*, 443–464.
[125] J. L. de Mattos, L. V. Lukachova, L. Gorton, T. Laurell, A. A. Karyakin, *Talanta* **2001**, *54/5*, 963–974.