RECOLLECTIONS, SPECIAL SECTION IN HONOR OF MAX PERUTZ

Proteolytic enzymes past and present: The second golden era*

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I first met Max Perutz when we were both chemistry students at the University of Vienna, where we also shared a passion for skiing. Our love for proteins brought us together again during our professional careers and aroused my admiration for his brilliant scientific ingenuity and achievements. The present autobiographical vignette is contributed as a token of my appreciation of our friendship.

The study of proteolytic enzymes has occupied a central position in protein chemistry from its early beginnings and has remained so throughout the past 60-odd years. The fascination with this group of macromolecules derived in part from the fact that substrates, inhibitors, as well as the catalysts themselves, all were proteins of unknown composition and structure and

hence could not be differentiated from one another chemically. The recognition of their various physiological roles preceded, and had to await, the development of methods for their molecular characterization. Thus, the discovery of trypsin by Kuehne (1876) was soon followed by his observation that this pancreatic protease was initially in an inactive state but was spontaneously converted to the active form and inactivated by heating. This was more than 60 years before the enzyme and its zymogen were crystallized by Northrop and Kunitz (Northrop et al., 1939). Yet Kuehne's observations contained the seeds of the subsequent recognition that enzymes were proteins and that zymogen activation proceeds by proteolytic processing.

Although I have devoted almost all of my professional life to the study of proteins, and particularly of proteolytic enzymes, and have contributed here and there to the miraculous growth of this field, I have never ceased to marvel at the diverse physiological roles of proteolytic enzymes, particularly those that have similar structures and active sites and hence seemed to have evolved from a common ancestor.

In this brief review, I shall first go back to the early days of research on proteases and show in a somewhat anecdotal way where we have come from and how much was achieved with relatively crude experimental tools. This will lead to the first golden era of protein chemistry, which was the springboard for the current, second golden era. A somewhat speculative outlook at the next golden era will conclude this recollection.

Early days

I entered the field of proteins as a physical chemist, or more exactly, as a colloid chemist, a term almost unknown to the current generation. Proteins were large molecules of ill-defined chemical structure. They were selected for study on the basis of availability rather than their inherent chemical or biological interest, e.g., ovalbumin, casein, serum albumin, or hemoglobin. Methods of purification were based on fractional precipitation with ammonium sulfate, with acetone at low temperatures, or by isoelectric precipitation in salt-free solutions. One of the most important pieces of laboratory equipment in those days was the refrigerated Sorvall centrifuge, which we treated with kid gloves.

Because proteins were usually selected without regard to their biological activity, if any, I soon recognized the need to use in my own work proteins with measurable biological functions and

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Hans Neurath received his doctorate in colloid chemistry from the University of Vienna in 1933 and his postdoctoral training in surface chemistry at the University of London. Thereafter, he was a research fellow at the University of Minnesota and at Cornell University in the Department of Chemistry. In 1938, he joined the Department of Biochemistry of the Duke University School of Medicine, where he rose through the ranks to Professor of Physical Biochemistry. In 1950, he became the founding Chairman and Professor of Biochemistry at the University of Washington, School of Medicine, and served in that capacity for the following 25 years. In 1960, Dr. Neurath became the first editor of *Biochemistry*, published by the American Chemical Society, and served in that capacity for 30 years. In 1990, he was appointed Editor-in-Chief of *Protein Science*. He also was an Associate Director in charge of basic research of the Fred Hutchinson Cancer Research Center in Seattle and, subsequently, Director of the German Cancer Research Center in Heidelberg. He is now Professor Emeritus in the Department of Biochemistry at the University of Washington. His research on proteins and proteolytic enzymes has been supported for 46 continuous years by the NIH, whose records list him as the oldest and longest recipient of research grants.

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first selected antibodies for that purpose. The precipitation of antigen-antibody complexes near the equivalence zone was one such measurable parameter. As a novice in this field, I decided to offer a graduate course in immunochemistry when I was an Assistant Professor of Biochemistry at Duke University and, through intense reading, managed to stay ahead of the students by 1 or 2 of the weekly lectures. In my research, I ran across Linus Pauling's proposal that antibody and normal globulins differed in shape rather than in chemical structure and published experimental proof to the contrary. During the war years, I participated in a project to eliminate false-positive reactions in the serological diagnosis of syphilis and devised a test based on the separation of the globulin fraction from human plasma. I soon concluded that, in those days at least, antibodies were not well enough defined to use them as models to study the relation of protein structure to function and, instead, turned my attention to proteolytic enzymes. That was in the early 1940s.

Proteolytic enzymes

My introduction to the field was the book by Northrop, Kunitz, and Herriott entitled *Crystalline enzymes* (Northrop et al., 1939). It described the properties and crystallization of pepsin, pepsinogen, pepsin inhibitor, chymotrypsinogen, chymotrypsin, trypsinogen, trypsin, soybean trypsin inhibitor, carboxypeptidase, and other proteins. The main evidence for the purity of any of these proteins was that, in the crystalline state, they obeyed the thermodynamic requirement of constant solubility and, hence, are pure chemical entities. In their preface, these authors wrote: "The problems (of purification) presented formidable technical difficulties, owing partly to the unstable nature of the enzymes and partly to the large quantities of material which it is necessary to handle." When I entered the field of proteolytic enzymes, none of the proteases was commercially available. Worthington Biochemicals had not yet entered the market.

Another concern of mine was, naturally, the measurement and interpretation of enzymatic activity. It was at that time that Max Bergmann and his associates, including such well known names as Joe Fruton, Carl Niemann, Bill Stein, Stanford Moore, Emil Smith, and several others, had synthesized and described the use of peptide substrates to replace the poorly characterized protein substrates, such as hemoglobin and casein, previously in use. Proteolytic activity was expressed in terms of a proteolytic coefficient, which was the first-order rate constant of hydrolysis of these substrates per milligram of enzyme. As a result of my newly acquired familiarity with enzyme kinetics, I questioned the validity of first-order kinetics over the entire range of substrate concentration. I had studied kinetics, starting with the Michaelis-Menten theory (I had met Michaelis in Woods Hole and later received from his widow an inscribed photograph that I still cherish among my memorabilia), followed by Eyring's theory of absolute reaction rates, and Britton Chance's theory of fast reaction kinetics (which I had difficulty understanding).

My entry into the field would not have been possible without the collaboration of graduate students and postdoctoral fellows, among whom I should mention Frank Putnam and George Schwert, serving as postdoctoral fellows early on, together with graduate students who included, among others, Seymour Kaufman and his future wife, Elaine Elkins, then all at Duke University.

Obtaining enzymes in pure form was another matter that, as noted by Northrop et al. (1939), presented formidable difficulties.

I am reminded of several episodes that illustrate this point: for instance, Frank Putnam and I standing in the cold room of the Anatomy Department, using a band saw to make slices from 100-pound lots of frozen bovine pancreas glands, obtained from a slaughterhouse in Chicago. From the collected drip juice, we isolated, in tedious operations, crystalline carboxypeptidase as well as procarboxypeptidase that resisted crystallization. Imagine purifying a protein before affinity or ion exchange chromatography, or before Sephadex had been invented, and without gel electrophoresis for a quick-and-dirty look at the purity of the final product.

In another episode, I was driving a truck, loaded with vats containing 0.25 N sulfuric acid, from the National Cancer Institute in Bethesda to a slaughterhouse in Baltimore, Maryland, to collect fresh bovine pancreas glands from which I prepared, in the laboratories of my close friend Jesse Greenstein at the National Cancer Institute, about 1 g each of crystalline trypsin and chymotrypsinogen.

The thrill of seeing enzyme crystals was no less at that time than it is today, when X-ray crystallographers succeed in crystallizing the protein of their choice, from which they derive its 3-dimensional structure.

Our primary object in those days, as now, was first to characterize the proteases in terms of purity and enzymatic activity, using the best available methods. Purity was tested according to the criteria of Northrop et al. (1939), using mostly Kjeldahl nitrogen analysis for solubility measurements. Later, the newly developed method of Tiselius' moving boundary electrophoresis was also used. Enzymatic activity was measured by following the rate of hydrolysis of synthetic peptide substrates that we synthesized ourselves. The results were interpreted according to Michaelis-Menten kinetics. It was at that time that A.K. Balls described the inactivation of acetylcholine esterase by diisopropyl phosphofluoridate and we discovered that trypsin, chymotrypsin, and carboxypeptidase also have esterase activity, and that carboxypeptidase is inhibited by certain other analogs of peptide substrates.

Amino acid analysis of proteins, introduced by Erwin Brand and applied to β -lactoglobulin, required approximately 2 g of starting material. Sanger's method for protein sequencing was published later. It was, in fact, in 1953, when I attended a conference on insulin, sponsored by Eli Lilly and Co., that a participant who had just returned from Cambridge reported this incredible achievement of determining the complete amino acid sequence of both chains of bovine insulin. This feat required 152 peptide fragments. The idea of co-linearity between gene and protein sequences had obviously not yet been born. This brings me to the first golden era of proteolytic enzymes.

First golden era of proteolytic enzymes

The key players in those days included Linderstrom-Lang, Sanger, Stein and Moore, Tiselius, Linus Pauling, Perutz and Kendrew, just to mention a few. This period saw the introduction of the fraction collector, of automated methods of amino acid analysis, column and paper chromatography, Edman degradation, spinning cup sequencers, protein X-ray crystallography, and the tools for protein purification (e.g., DEAE-cellulose, Sephadex). Together they placed protein chemistry on a quan-

titative basis. Due to their sensitivity, these innovations also reduced by 2 or more orders of magnitude the amount of material required for analysis.

Those were the days when Anfinsen's theorem that the 3-dimensional structure was inscribed in the amino acid sequence was unequivocally proven experimentally by Merrifield's de novo chemical synthesis of fully active ribonuclease; when Fred Richards demonstrated that recombination of the residual S-protein of ribonuclease A with its S-peptide, obtained by subtilisin cleavage of ribonuclease, yielded a competent enzyme. Linderstrom-Lang was generally considered the central figure in this period (Richards, 1992). Among his many contributions was the serendipitous discovery that the "spontaneous" conversion of ovalbumin to plakalbumin was actually due to the action of a bacterial enzyme from *Bacillus subtilis*, named subtilisin, which they isolated and crystallized. Think of the far-reaching consequences of this accidental observation in terms of the subtilisin domain in the kexin enzyme in yeast, an essential component of the mating process, and in other regulatory proteases such as those which serve the processing of certain protein hormone precursors. Plakalbumin itself has since been shown to be an important protein for understanding the mode of action of the serine protease inhibitors (serpins).

Pancreatic proteases

After moving from Duke to the University of Washington, my colleagues and I applied the more modern, recently developed methods of protein chemistry to the pancreatic proteases trypsin, chymotrypsin, and carboxypeptidases, and focused in particular on the problem of zymogen activation. Earl Davie was one of my graduate students, Gordon Dixon and Patricia Keller

were among my postdoctoral fellows, as was Ken Walsh, who joined us in 1959. These were golden years, not only of protein research, but also of NIH support. Protein chemistry was clearly in the forefront of biochemical research. Using the newly developed FDNB method of Sanger for end-group and sequence analysis, we elucidated the structure of the activation peptide of trypsinogen, Val (Asp)₄, Lys. This turned out to be an important experimental achievement of long-lasting significance (see below) and in some ways set the stage for proteolytic processing of precursor proteins generally. We also elucidated the activation of chymotrypsinogen and determined the amino acid sequence of the DFP-binding sites of both trypsin and chymotrypsin (Neurath, 1957). Encouraged by these accomplishments, we decided to go the whole way and determined the complete amino acid sequence of trypsinogen, which was Ken Walsh's chosen goal after he joined our laboratory (Walsh & Neurath, 1964).

It so happened that, at that time, Brian Hartley from Cambridge, UK, joined our laboratory for a 2-year visit with his own sample of bovine chymotrypsinogen, with the intent of determining the amino acid sequence of his protein (Fig. 1). The 2 projects developed side by side and, after Hartley's return to Cambridge, he finished his project. Both publications appeared within the same year and clearly demonstrated sequence homology between trypsin and chymotrypsin, including identity of functionally significant residues of the 2 enzymes. Fortunately, the same type of experimental error was committed in each. In trypsin, aspartic acid 177 in the primary substrate-binding pocket was erroneously identified as asparagine, and the same error occurred in Hartley's analysis of chymotrypsin, where aspartic acid 102 of the charge relay system was erroneously reported as asparagine. This was not surprising because there was

Fig. 1. Group picture of members of the Department of Biochemistry, University of Washington, and of my research group, taken in June 1959. From left to right: Third row: Unidentified (U), Ed Fischer, Ed Krebs, U, Don Hanahan. Second row: Robert Labbe, Ken Walsh, Eric Stein, Joe Kraut, U, Phil Wilcox, Frank Huennekens, Hans Brockerhoff. First row: U, Pat Keller, Al Meister, Hans Neurath, Brian Hartley, Takashi Murachi, Josephine Junge.

no good method available in those days for accurately discriminating between the free acid and its amide. Had these errors not been detected, the difference in specificity between chymotrypsin and trypsin would not have been traced to differences in the composition of the primary substrate-binding pocket, nor would the charge relay system have been discovered in the X-ray structure of chymotrypsin by David Blow. In his recent book on *Protein Structure*, Max Perutz (Perutz, 1992) states that the catalytic mechanism of the serine proteases, based on the charge relay system, is one of the best studied mechanisms in biochemistry.

Our preoccupation with pancreatic serine proteases took us on a side tour of phylogeny, which included the isolation and characterization of trypsinogen of the dog fish and the African lungfish, and prompted us to acquire a cow, placed in the Veterinary School of Washington State University, in order to study the protein composition of pancreatic zymogen granules and the rates of incorporation of radioactive amino acids into pancreatic proteases.

These early studies have been a watershed for many subsequent developments of current interest. They have established experimental proof for the concept of structural homology among the serine proteases and their evolution (Neurath et al., 1970a). The fact that the X-ray structure of elastase agreed completely with that previously predicted on the basis of sequence homology with chymotrypsin was the most convincing validation of the concept of structural homology of mammalian serine proteases. It gave rise to the concept of protease families, among which Hartley recognized 4 main classes, to which new ones are being added, e.g., the metalloendopeptidases, which include the astacins, thermolysin, and the matrix metalloproteinases. Sequence analysis showed that thrombin resembles trypsin (Magnusson, 1970) and that a trypsin-like structure is the catalytic domain of the blood coagulation proteases. Direct evidence for the structural relationship of pancreatic trypsin to the blood coagulation proteases was obtained in our laboratory by sequence analysis of the heavy chain of bovine coagulation factor X₁, which demonstrated sequence homology of the catalytic domain with pancreatic serine proteases (Titani et al., 1975). The importance of serine proteases in other biological systems, as in mast cells or certain viruses, has further enhanced interest in both their varied structures and functions.

The question as to how the proteases themselves are regulated has continued to be a focal point in protease research. One's view of proteases somehow depends on one's purpose. Proteases are either an object of study for their own sake or a nuisance to be eradicated. One is either thrilled by their activation or by their inactivation. Because my own interests in this problem have centered around zymogen activation by precursor processing, rather than protease-inhibitor interaction, I shall include in this account of the first golden era some brief comments on this topic.

Zymogen activation

With a few exceptions, zymogen activation requires primary peptide bond cleavage to separate the "pro" region from the catalytic domain. For a long time, the sole function of the "pro" piece was believed to be to suppress enzymatic activity; more recently, it has been found that the "pro" piece can also have a function of its own. Thus, in the case of carboxypeptidases A, B, and Y, subtilisin, and α -lytic protease, besides acting as

an intramolecular inhibitor domain, it also serves as an intramolecular chaperone to assure the correct folding of the enzyme *in vivo*.

Early on, our attention was focused on the question of how the primary event of proteolytic cleavage of the zymogen relates to the generation of catalytic activity. The nature of the secondary intramolecular rearrangement required for the expression of enzymatic activity proved to be a difficult problem. Our original observation that trypsinogen and chymotrypsinogen have inherent catalytic activity was soon thereafter extended by Huber and Bode to a detailed comparison of the electron density maps of zymogen/enzyme pairs of certain pancreatic proteases (trypsin, chymotrypsin, pancreatic carboxypeptidases). This produced a detailed description of the ensuing conformational changes that accompany these zymogen-enzyme conversions. Today, we know that the activation mechanism is generally characteristic for each protease family but with subtle variations even among members of the same family.

Carboxypeptidase

My recollection of the golden era would not be complete without mentioning our interest in yet another protease family, i.e., the Zn metalloexopeptidases as represented by pancreatic carboxypeptidases A and B.

The question of the physiological roles of carboxypeptidase has never received the attention that would be commensurate with the preoccupation of protein chemists and enzymologists with the structure and activity of this enzyme. Carboxypeptidase was the first protease recognized to contain Zn as part of its active site. This unique feature justified the exploration of its molecular properties and, in retrospect, makes a good story.

A number of investigators contributed to its exploration. At a Federation meeting in Atlantic City, New Jersey (where else in those days?), I met Bert Vallee of Harvard University, whose name had been familiar to me from the literature and through mutual friends. He had read our paper in which we disputed a report by Emil Smith that Mg may be a cofactor of catalysis by carboxypeptidase, and we began a collaborative reinvestigation of this problem, using Vallee's advanced methods for the measurement of trace quantities of metals in biological systems. He visited our laboratory in conjunction with the annual Rodeo in Ellensburg, Washington, where he participated in the parade on horseback. This visit also marked the beginning of a long-term collaborative study of the chemical properties of carboxypeptidase in our laboratory and of the functional role of Zn at Harvard. At a meeting of the Faraday Society in Oxford in 1955, the conflicting claims of the nature of the intrinsic metal led to a spirited debate among Bert Vallee, Emil Smith, and myself, which produced more heat than light.

We later engaged in a more ambitious undertaking to determine in our laboratory the complete amino acid sequence of carboxypeptidase A and the nature of the activation process. Ralph Bradshaw, Ken Walsh, Philip Petra, and several other associates were the main actors in this undertaking (Bradshaw et al., 1969; Neurath et al., 1970b). It was at that time that Bill Lipscomb entered the field and initiated his X-ray structure analysis on material supplied by us. Once again, carboxypeptidase became a controversial subject. Bill Lipscomb, Bert Vallee, and we reported our respective data at a meeting of the Royal Society in London in 1969, but, in this case, more light was produced

than heat. The final result was the complete elucidation of the 3-dimensional structure of the enzyme, of enzyme-inhibitor complexes, as well as the nature and location of the metal and its ligands under various experimental conditions. That conference was an important milestone on the road to the exploration of the structure/function relationship of proteases and other enzymes.

I shall now make a giant leap from the first to the second, present golden era of research on proteolytic enzymes and stare into the crystal ball of future developments.

The second golden era

It is difficult to pinpoint the beginning of the second golden era. Perhaps it arrived when protein chemists learned to clone their own proteins. It coincides with the emergence of the concepts and methods of molecular and cell biology on one hand, and the spectacular advances of structural biology by the methods of protein crystallography and NMR spectroscopy, on the other. In addition, application of the most advanced technologies of mass spectroscopy and organic chemistry have extended enormously the reach of protein chemistry into the most intimate molecular details of intracellular protein components present in minuscule amounts. We are now able to probe the structure of proteins and peptides in pico- and femtomole quantities, rather than micromoles, i.e., a diminution by 6–9 orders of magnitude, and to complete the sequence analysis of a protein, directly or via the corresponding cDNA, in days rather than years. We are capable of finding proteins by looking for their respective DNAs and expressing the protein at will in any desired quantity. By using an expanded, man-made genetic code, we can even express proteins containing unnatural amino acids. We can modify native proteins at predetermined sites, not by the tedious use of site-specific chemical reagents, but by genetic engineering, and create new combining sites for high-affinity ligands of novel design. We are finding that many proteins, particularly the “regulated” ones, are tandem arrays of domains, each performing specific functions that may respond to intercellular signaling. We can imitate nature by recombining domains in order to generate novel, multifunctional proteins. We can even endow antibodies with tailor-made enzymatic activities.

We have become smarter, but we have not outsmarted nature; we have been humbled by the recognition that our initial concepts were incomplete, if not naive. For example, we thought that a single amino acid replacement at the primary substrate-binding site would be sufficient to interconvert trypsin into chymotrypsin, or vice versa. Not so, because we have learned that concomitant replacements at additional sites are required to accomplish such interconversions.

We certainly have come a long way since Anson and Mirsky (1931) described the reversible *in vitro* denaturation of trypsin and other proteins. We have acquired detailed knowledge of the intermediate steps in the folding and unfolding of certain proteins, such as bovine pancreatic trypsin inhibitor, which has become a model for understanding the folding process *in vivo* and is probably the most celebrated protease inhibitor molecule. By the same token, trypsin might be declared the serine protease of the 20th century and thrombin the most celebrated trypsin-like protease. We may finally solve the question of what makes proteins fold uniquely *in vivo* and elucidate the mechanism of action of the accompanying chaperones.

We have found that protease inhibitors generally have a less pronounced specificity than the proteases that they inhibit. However, no one has yet been able to convince us that we can replace these enzyme proteins by man-made cyclic peptides, the most recent such claims notwithstanding (Matthews et al., 1994). Nor have we been able to significantly improve the catalytic power of naturally occurring enzymes. We have learned, however, to take advantage of nature's inventions to design more effective inhibitors of proteases or to endow digestive proteases, again by genetic engineering, with regulatory functions.

Structural biology has become a booming enterprise, and X-ray crystallography and/or NMR spectroscopy produce many new 3-dimensional structures of proteins. These structures provide detailed visualization of protein molecules; they are aesthetically pleasing and adorn the pages and covers of scientific journals, including the present one. In the first golden era, protein sequencing led the parade of protein science, and protein crystallography and NMR spectroscopy are leading it today. NMR allows us to see proteins that do not crystallize and, in conjunction with computer modeling, invites us to speculate about their flexibility and dynamics. Mass spectroscopy enables us to weigh proteins and protein fragments to within 1 mass unit and to identify a gamut of posttranslational modifications.

The next golden era

One can well imagine that, in the foreseeable future, we shall hear about man-made vaccines and protein hormones containing protease-resistant peptide bond analogs; about specific, target-directed membrane-bound proteases and their biological functions, such as calpain, enterokinase, and hepsin. We may find newer ways to regulate the activity of proteases, analogous to the metal-switch recently described by several laboratories. We should also have a more complete understanding of the astonishing degree of organization of the proteasomes; about the preferred physiological substrates of mast cell proteases, and about the structure and physiological role of the prostate-specific antigen, a chymotrypsin-like serine protease bound to an anti-chymotrypsin inhibitor. We need to intensify research on the detailed structure and physiological roles of viral proteases, such as human immunodeficiency virus (HIV), that open the door to rational drug design. In this connection, it is worthy of note that the search for an inhibitor of HIV-1 protease has benefited enormously by the recognition that the enzyme is a member of the aspartate protease family, similar in structure to pepsin, one of the earliest role models of proteolytic enzymes. As a result, the search has produced some 2,000 entries in the database of the structure of this protease, in combination with various ligands and synthetic inhibitors. By the same token, the ongoing studies of the processing enzyme interleukin 1- β converting enzyme (ICE), which is a cysteine protease, will bring us closer to an understanding of its role in apoptosis. The time will come when we shall place a stretch of DNA in a machine and have its sequence, the corresponding protein sequence, and its 3-dimensional structure all appear on the screen a few minutes later.

The next golden era that we are about to enter should witness closer cooperation and interdependence of academia and industry in the exploitation of basic research, including research on proteases. The differences in philosophies and goals that have separated these camps in the past will need to be reconciled if the new partnership is to succeed. Universities cannot abandon

the traditional freedom of scientific inquiry and industry cannot sacrifice restricted disclosure for protection of their investments. It is reassuring that both universities and industry are now attracting outstanding scientists to their programs so that, at conferences and symposia, one can hardly tell one from the other. The next golden era will require a better mutual understanding and closer ties of universities, industry, and federally supported research organizations if we are to reap the full benefits of the advances and discoveries to be expected in both basic and applied research.

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