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Synthesis, physicochemical and biological properties of poly-α-amino acids — the simplest of protein models

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Key words: α-helix, β-pleated sheets, helix-coil transition, synthetic antigenes, genetic code, multiple sclerosis, amino acid reiteration

During the 1950s, linear and multichain poly-α-amino acids were synthesized by polymerization of the corresponding *N*-carboxy-amino acid anhydrides in solution in the presence of suitable catalysts. The resulting homo- and heteropolymers have since been widely employed as simple protein models. Under appropriate conditions, poly-α-amino acids, in the solid state and in solution, were found to acquire conformations of an α-helix and of β-parallel and antiparallel pleated sheets, or to exist as random coils. Their use in experimental and theoretical investigations of helix-coil transitions helped to shed new light on the mechanisms involved in protein denaturation.

Poly-α-amino acids played an important role in the deciphering of the genetic code. In addition, analysis of the antigenicity of poly-α-amino acids led to the clucidation of the factors determining the antigenicity of proteins and peptides.

Interest in the biological and physicochemical characteristics of poly-α-amino acids was recently renewed because of the reported novel findings that some copolymers of amino acids are effective as drugs in multiple sclerosis, and that glutamine repeats and reiteration of other amino acids occur in inherited neurodegenerative diseases. The presence of repeating sequences of amino acids in proteins, and of nucleotides in DNA, raises many interesting questions about their respective roles in determining protein structure and function, and gene performance and regulation.

More than 50 years ago, H. Staudinger, K.H. Meyer and others (see review by Mayer [1]) suggested that synthetic high-molecular-weight compounds might serve as useful models in the study of biopolymers. When I was working at the Hebrew University in Jerusalem during the early 50's, this idea caught my attention. It seemed to me that if I could prepare synthetic high-molecular-weight polypeptides consisting of one or a few amino-acid residues, a study of their properties might contribute to the elucidation of the structure-function relationships in proteins. I thought it was worth a try.

While searching the literature for a suitable monomer, I accidentally came across the work of Leuchs in Abderhalden's Handlexikon [2]. After synthesizing *N*-carboxyglycine anhydride by cyclization of *N*-carbomethoxyglycyl chloride, Leuchs has found that it readily gives

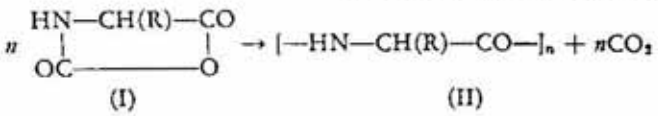
Abbreviations: Cop I, a copolymer of Ala, Glu, Lys, Thyr; DRPLA, dentatorubal-pallidoluysian atrophy; EAE, encephalomyelitis; HD, Huntington disease; MBP, myelin basic protein; MHC, major histocompatibility complex; MS, multiple sclerosis; SBMA, spinal and bulbar muscular atrophy; SCA I, spinocerebral ataxia type I.

off carbon dioxide to yield what he called an anhydroglycine. Suspecting that this product was in fact a linear polyamino acid, I decided to synthesize some additional *N*-carboxy-α-amino acid anhydrides (I), study their polymerization, and characterize the corresponding amino-acid polymers obtained (II) (Scheme 1).

The amine-initiated polymerization of *N*-carboxy-α-amino acid anhydride is presented in Scheme 2.

SYNTHESIS OF LINEAR AND MULTI-CHAIN POLY-α-AMINO ACIDS

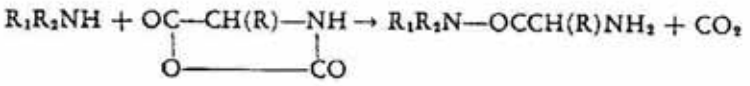
At first, I was particularly interested in preparing a high-molecular-weight water-soluble polyamino acid, and therefore decided to start with the synthesis of poly-L-lysine (VI). This polypeptide was finally obtained by polymerization of ε,*N*-carbobenzyloxy-α,*N*-carboxy-L-lysine anhydride (IV) to yield poly-ε,*N*-carbobenzyloxy-L-lysine (V), and removal of the protecting group by suitable means [3] (see Scheme 3). Removal of the protecting group was extremely difficult and after many attempts I discovered, together with my first Ph.D. student Izhak Grossfeld, that it could be done with PH_4I. At first we assumed that the benzyl groups of the benzyloxycarbonyl residue are reduced by the liberated PH_3; however, since we found ourselves weeping copiously during synthesis we realized that benzyl iodide was being evolved as a result of the HI liberated. These findings led, many years later, to the development of the classical technique for removal of the benzyloxycarbonyl protect-



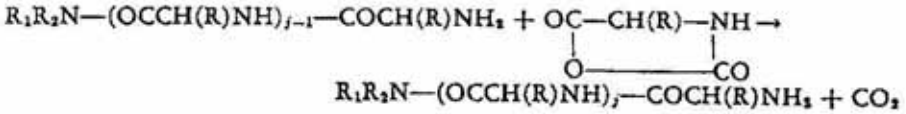
(I) (II)

Scheme 1. Preparation of a linear poly-α-amino acid (II) by polymerization of an N-carboxy-α-amino acid anhydride (I).

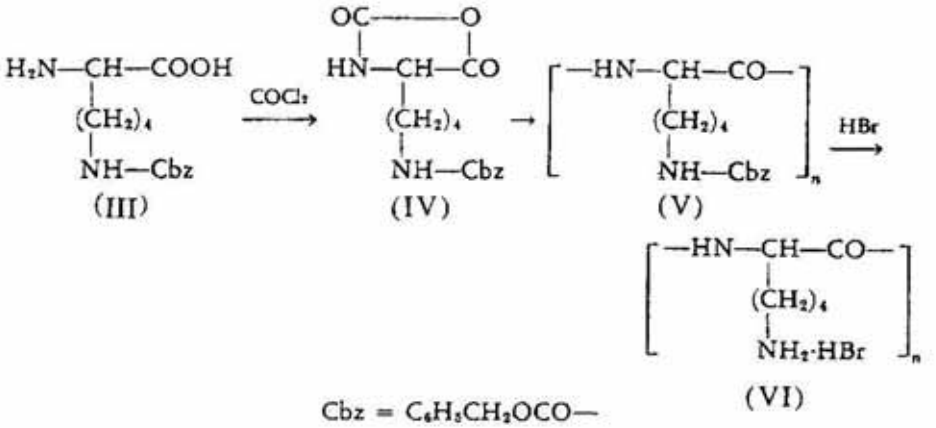
(a) Initiation:



(b) Propagation:



Scheme 2. Amine initiated polymerization of N-carboxy-α-amino acid anhydrides.



(III) (IV) (V) (VI)

Cbz = $C_6H_5CH_2OCO$—

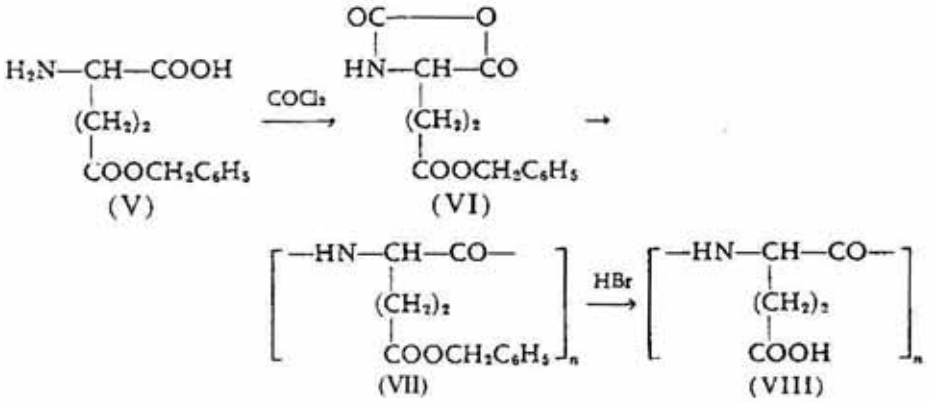
Scheme 3. Synthesis of poly-L-lysine (VI) by the polymerization of ε,N-carbobenzyloxy-α,N-carboxy-L-lysine anhydride (IV) and removal of the protecting Cbz-groups with HBr.

ing groups with HBr in glacial acetic acid by Arieh Berger and Dov Ben Ishai in my laboratory.

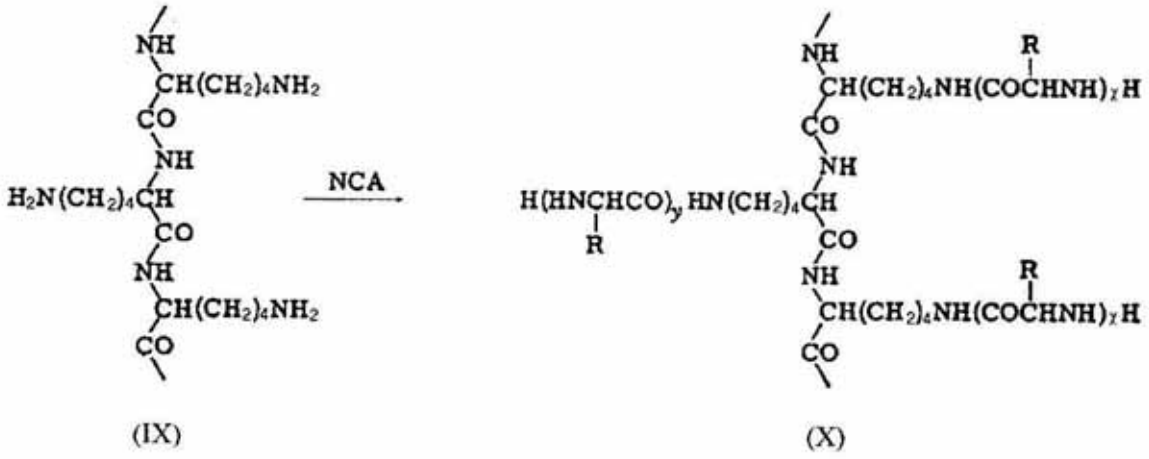
After moving to the Weizmann Institute, I continued to extend my work on polyamino acids as protein models. With my colleagues and students I synthesized a number of other polyamino acids including poly-L-arginine [4], poly-L-histidine [5], poly-L-aspartic acid [6], poly-L-tyrosine [7], poly-L-serine [8], poly-L-cysteine [9], poly-L-proline [10] and poly-L-hydroxyproline [11]. The synthesis of poly-L-glutamate by the polymerization of γ-benzyl-α,*N*-carboxy-L-glutamate anhydride is illustrated in Scheme 4. We also synthesized amino acid copolymers and multichain polyamino acids, i.e. polyamino acids in which a suitable polypeptide backbone, such as polylysine, serves for the attachment of a large number of polypeptide chains, resulting in a branched macromolecule [12] (Scheme 5).

CONFORMATION OF POLY-α-AMINO ACIDS IN THE SOLID STATE AND IN SOLUTION

The availability of high-molecular-weight polyamino acids opened the way to a thorough investigation of their conformation, particularly their secondary structure in the solid state and in solution. In 1951, Max Perutz analyzed the X-ray-diffraction pattern of poly-γ-benzyl-L-glutamate fibers [13] and confirmed the presence of the α-helical polypeptide backbone predicted by L. Pauling and R. Corey. Of par-



Scheme 4. Synthesis of poly-L-glutamic acid (VIII) by the polymerization of γ,benzyl-α,N-carboxy-L-glutamate anhydride (VI), and removal of the benzyl protecting groups of poly-γ,benzyl-L-glutamate (VII) with HBr.



Scheme 5. Synthesis of multichain poly-amino acids (X) by initiating the polymerization of N-carboxy-α-amino acid anhydrides with poly-L-lysine (IX).

ticular interest was the detection of the 1.50 Å meridianal reflection, corresponding to the residue translation of 1.50 Å along the fiber axis, characteristic of the α-helix. Detailed analysis of the X-ray-diffraction pattern of oriented fibers of poly-L-alanine (which acquired an α-helix conformation), was carried out in 1959 by Elliott and Malcolm [14], and their findings were of considerable help to John Kendrew and Max Perutz in their deciphering of the X-ray patterns of myoglobin and hemoglobin. A remarkable observation was that fibers of α-poly-L-alanine undergo a total α→β transformation when stretched in steam.

Polyamino acids synthesized and studied in our laboratory by Arieh Berger, Joseph Kurtz and Jurgen Engel, in particular poly-L-proline [10], polyhydroxy-L-proline [11] and the sequential poly(Pro-Gly-Pro) [15], were useful as model compounds in the elucidation of the three-dimensional structure of the fibrous protein collagen. I was especially intrigued by the finding of J. Kurtz that marked optical mutarotation occurs when our water-insoluble poly-L-proline I, consisting of open right-handed helices with all peptide bonds in the *cis* configuration, is transformed in suitable solvents into the water-soluble poly-L-proline II, consisting of open left-handed helices with all peptide bonds in the *trans*-configuration [16] (Fig. 1). In 1954 G.N. Ramachandran and G. Kartha, and later Alex Rich and Francis Crick, suggested that collagen fibers have a triple-stranded helical conformation. Poly(Pro-Gly-Pro) was the first polymer that both in solution and in the solid phase was shown to form the triple-stranded helical conformation attributed to collagen, and was therefore used by chemists and biologists as a model compound for collagen. A comparison between the X-ray diffraction photographs of fibers of $(Gly\text{-}Pro\text{-}Hypro)_n$ and of collagen is given in Fig. 2.

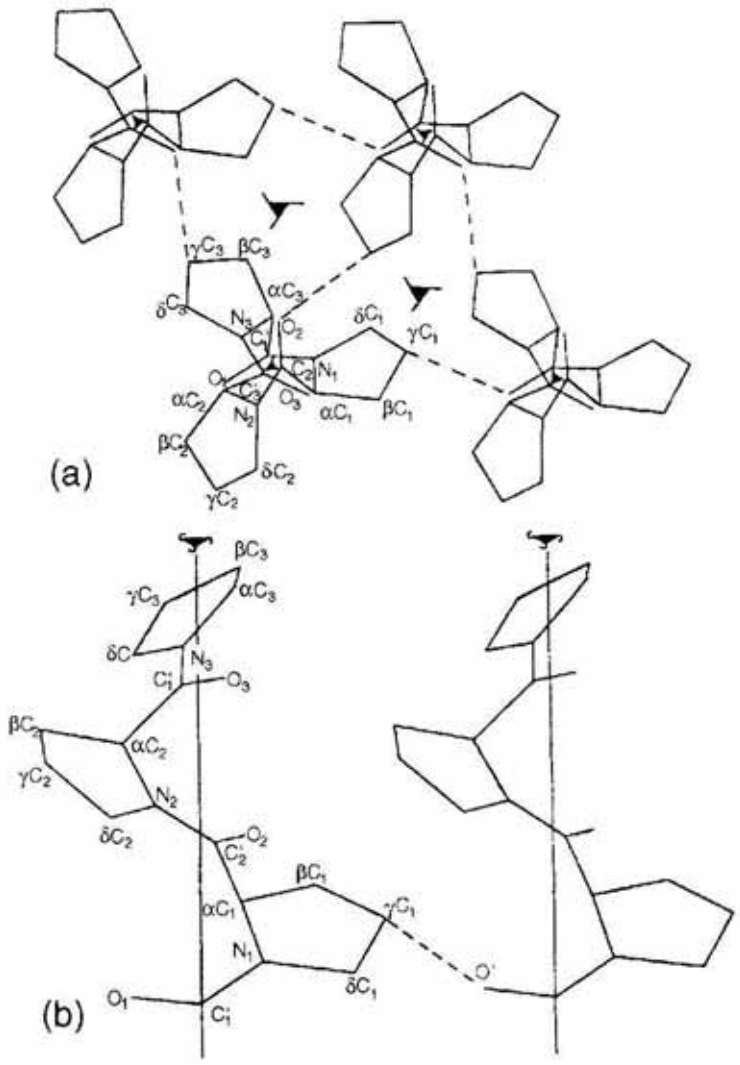


Fig. 1. Conformation of poly-α-L-proline II . (a) Projected on the ab plane. (b) Projected on the ac plane.

A considerable amount of work, both experimental and theoretical, was done on the conformation and conformational transitions of polyamino acids not only in the solid state but also in solution. Studies with solvent systems were initiated experimentally by P. Doty, E. Blout, and their collaborators at Harvard, and theoretically by W. Moffitt and J.G. Kirkwood and by J.A. Schellman (cf. [17, 18]). The existence, in appropriate solvent systems, of regular macromolecular conformations, as well as the existence of the random-coil conformation, was established experi- mentally by Ignacio Tinoco, J.T. Young, Gerald Fasman and Bruno Zimm by hydrodynamic, optical, electrical, and nuclear magnetic resonance methods (cf. [17, 18]). Moreover, conditions were established for attaining a conformational transition between the above macromolecular structures. Taken together, these findings led to a deeper insight

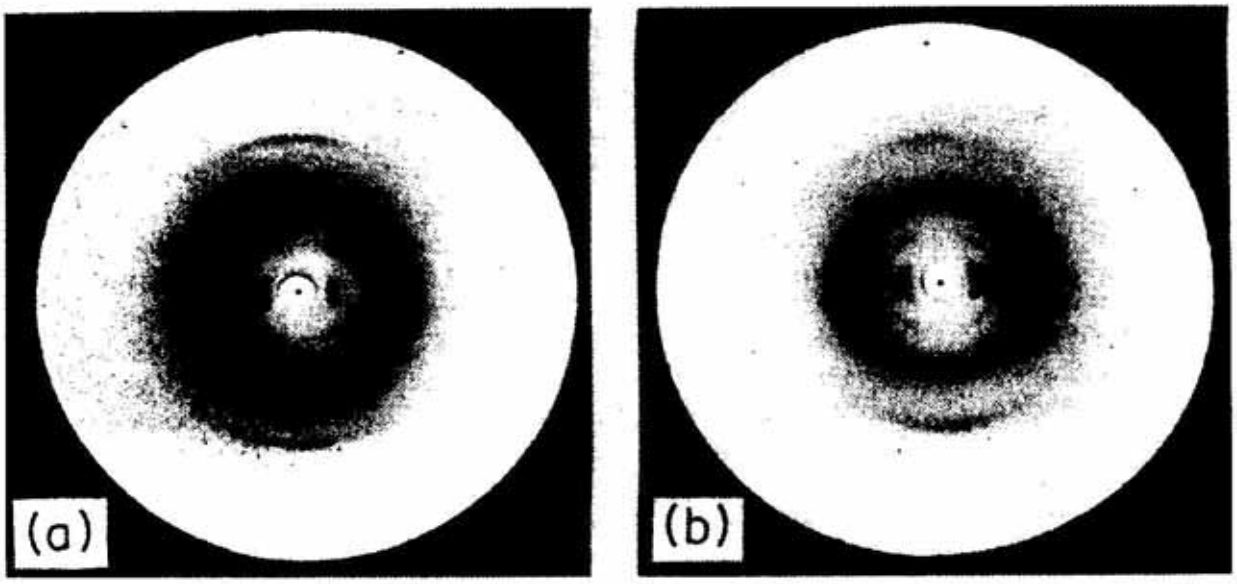


Fig. 2. X-Ray diffraction photographs of (a) Fibers of $(Gly\text{-}Pro\text{-}Hypro)_n$ and (b) Collagen fibers (rat-tail).

into protein denaturation and renaturation. Of particular importance was the finding that the optical rotatory dispersion of poly-α-amino acids in the far ultraviolet (180–260 nm) in helical conformation differs markedly from that in the random coil form. The helical form is characterized by a large positive Cotton effect and the random coil by a smaller negative Cotton effect. The characteristic optical rotatory dispersion of poly-α-L-glutamic acid in its two conformations is given in Fig. 3 [19]. Careful analysis of the above data enables the prediction, in proteins, of the percentage of amino-acid residues in an α-helix or in random coil conformation from the corresponding rotatory dispersion data.

Thus, within some two decades, it was possible to clarify the mechanism and kinetics of polymerization of *N*-carboxy amino acid anhydrides, determine the α-helical conformation of some of the polyamino acids in the solid state and in solution, detect β-parallel and antiparallel pleated sheets of polyamino acids, and induce helix-coil transitions in the solid state and in solution under appropriate conditions. Fruitful collaboration between experimentalists and theoreticians facilitated the successful correlation of the macromolecular conformations of polyamino acids in solution with their hydrodynamic properties, optical properties, dipole moments and nuclear magnetic properties.

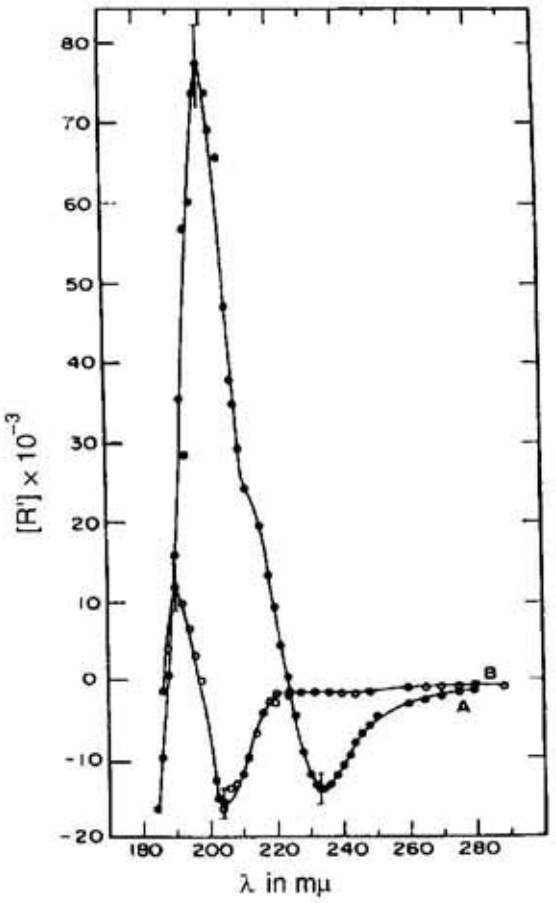


Fig. 3. Ultraviolet rotatory dispersion of the helical and random coil forms of poly-α,L-glutamic acid (λ in mμ = nm). Curve A (●): helical form in water solution, pH 4.3. Curve B (○): random coil form (sodium salt), pH 7.1, in water solution [19].

BIOLOGICAL PROPERTIES OF POLY-α-AMINO ACIDS

Meanwhile, in Rehovot, I concentrated on the study of the biological properties of polyamino acids [20]. To my delight poly-L-lysine, as well as other homopolyamino acids and amino acid copolymers, turned out to be excellent models for investigation of the mechanism of enzymatic protein hydrolysis and transpeptidation. I still remember the excitement with which I followed the rapid hydrolysis of poly-L-lysine by trypsin, using the cumbersome old Van Slyke apparatus. We showed further that the specificity of an enzyme acting on a high-molecular-weight polypeptide is often strikingly different from that observed with low-molecular-weight peptides. Partial hydrolysis of poly-L-lysine yields, as expected, a mixture of lysine oligomers. These were separated chromatographically and investigated immunologically by my former student, Arieh Yaron, in Herb Sober's laboratory at NIH. Penetration of these oligomers into *Escherichia coli* was studied by Charles Gilvarg [21], a visiting scientist at the Weizmann Institute. By using a lysineless mutant of *E. coli* Gilvarg was able to show that all oligomers up to tetralysine, but no higher, can penetrate readily into *E. coli* and permit growth of the lysine auxotroph in their presence.

In our experiments with a prolineless mutant of *E. coli*, Sara Sarid observed that the organism can grow on a synthetic medium in which poly-L-proline is substituted for L-proline. Clearly, the polymer was being hydrolyzed by an unknown enzyme. Further studies by Arieh Yaron on the cleavage of various synthetic proline-containing oligo- and polypeptides led to the identification and characterization of the enzyme aminopeptidase P present in pro- and eukaryotes [22].

POLY-α-AMINO ACID ANTIGENICITY

An important outgrowth of the studies on synthetic polyamino acids was the development in my laboratory of techniques for the preparation of polypeptidyl proteins, i.e., proteins to which polypeptide chains are covalently attached *via* amide bonds to the free amino groups of the protein. The synthesis of polytyrosyl gelatin and the demonstration that it is antigenic, whereas the unmodified protein is not [23], led in 1960 to the preparation by Michael Sela and Ruth Arnon, then in my department, of the first fully synthetic antigen. In this compound, tyrosine and glutamic acid residues are attached to a multipoly-DL-alanyl poly-L-lysine [24]. I vividly remember our immunological experiments, in which guinea pigs injected two or three times with polytyrosyl gelatin went into anaphylactic shock — a most unpleasant experience for the guinea pigs and a sobering demonstration to me of how careful one should be in treating living beings with synthetic or even native polymers. Nevertheless, the way was opened for the fundamental and extensive studies of M. Sela and his co-workers on the chemical and genetic basis of antigenicity [25]. Some of the polypeptidyl enzymes we prepared retained full enzymatic activity. This finding was the basis for our subsequent preparation of a great variety of immobilized enzymes, which are of theoretical and practical interest [26–28].

USE OF POLY-α-AMINO ACIDS IN THE DECIPHERING OF THE GENETIC CODE

Knowledge of the physical and chemical properties of synthetic polypeptides played a decisive role in the work that led in 1961 to the cracking of the genetic code. In their first paper on the subject, Marshall Nirenberg and J.H. Matthei identified the poly-L-phenylalanine produced enzymatically, in a cell-free system in the presence of polyuridylate used as messenger [29], with the poly-L-phenylalanine we had synthesized in Rehovot in 1955. As it happens, Michael Sela was at NIH when M. Nirenberg was working on the code, and he had been able to inform Nirenberg that the normally insoluble poly-L-phenylalanine could be dissolved in acetic acid saturated with HBr. Soon afterwards Nirenberg identified other homo- and heteropolyamino acids in the deciphering of the genetic code: poly A was found to code for poly-L-lysine, poly C for poly-L-proline and poly-G for polyglycine (cf. [30]).

Cop I, A COPOLYMER OF Ala, Glu, Lys AND Tyr, AS A POTENTIAL DRUG AGAINST MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system, in which infiltrating lymphocytes, predominantly T-cells and macrophages, cause damage of the myelin sheath. It is thought to be an autoimmune disease, probably associated with an early viral infection. Experimental allergic encephalomyelitis (EAE) serves as the experimental animal model for the autoimmune process in MS. In view of the immunological nature of EAE, attempts have been made to suppress the disease in animals challenged with myelin basic protein (MBP). In guinea pigs, for example, high doses of MBP in incomplete Freund's adjuvant, were shown to be highly effective in preventing EAE, when administered before sensitization, and in suppressing the disease when given after sensitization. These findings led M. Sela and R. Arnon to test the effect on EAE of a basic amino acid copolymer (Cop I), consisting of L-alanine, L-glutamic acid, L-lysine and L-tyrosine (at residue molar ratios of 6.0, 1.9, 4.7, 1.0, respectively), prepared in my laboratory by the polymerization of the corresponding *N*-carboxy-amino acid anhydrides. As expected, the copolymer imitated the immunogenic effects of MBP and could suppress the model disease in guinea pigs (Table 1) when injected at suitable concentrations and time intervals [31]. None of the copolymers tested was found to be toxic or to exhibit any general immunosuppressive activity. These findings prompted Sela and Arnon to test the effect of Cop I on sick monkeys showing signs of EAE, and the encouraging results obtained opened the way for testing the effect of Cop I on MS in humans. Controlled experiments carried out so far have indicated that Cop I represents a potential low-risk drug for the treatment of MS. Analysis of the immunochemical mechanism of EAE suppression by Cop I revealed that Cop I and Cop I-derived peptides can bind to the relevant major histocompatibility complex (MHC) molecules and competitively inhibit the binding of MBP. The microheterogeneity of Cop I might partially account for its success, as it may contain numerous amino-acid sequences that can successfully compete with MBP for class II MHC antigens of many different genetic backgrounds.

PROTEINS WITH GLUTAMINE REPEATS AND REITERATION OF OTHER AMINO ACIDS

Four inherited neurodegenerative diseases are linked to abnormally expanded repeats of glutamine residues near the *N*-termini of the affected proteins. They are: Huntington disease (HD); spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease; spinocerebral ataxia type I (SCAI), and dentatorubral-pallidoluysian atrophy (DRPLA) [32]. All four diseases begin the earlier and become the more severe the longer the glutamine repeats. The repeats tend to lengthen in successive generations of affected individuals, especially in male transmission. These findings prompted M. Perutz and his collaborators [32] to construct molecular models of poly-L-glutamine and study their optical, electron and X-ray diffraction properties. Their data revealed the

Table 1

Guinea pigs with induced experimental allergic encephalomyelitis (EAE) treated with the basic amino acid copolymer, Cop I, consisting of L-alanine, L-glutamic acid, L-lysine and L-tyrosine (at residue molar ratio of 6.0, 1.9, 4.7, 1.0, respectively)

	Guinea pigs with initial attack of EAE	Average day of onset of disease	Guinea pigs with relapses of EAE	Severity of relapses
Control group	10/10 (5 died)	11.4 (severe)	5/5	Very high
Suppression by Cop I	8/8	13.2	6/8	Moderately mild
Prevention Cop I	5/12	89.8	1/12	Very mild

presence of β-sheets strongly held together by hydrogen bonds, suggesting that glutamine repeats may function as polar zippers by joining specific transcription factors bound to separate DNA segments. Lengthening of the repeats may intensify the disease either as a result of increased nonspecific affinity between such factors or by gradual precipitation of the affected proteins in neurons.

Perutz extended the above studies to other polar zippers in proteins with reiterated sequences of polar amino acids, and attempted to predict their role in human disease [33]. *Ascaris* hemoglobin, for example, consists of eight subunits, each containing a C-terminal peptide with the sequence Glu-Glu-Lys-His repeated four times. When plotted on a β-strand, this sequence leads to alternate lysines and glutamates on one side of the strand and alternate glutamates and histidine on the other side, suggestive of a polar zipper that links the subunits together. A computer search of the protein database showed that the same or similar sequences also occur in other proteins. Some contain long repeats of Asp-Arg or Glu-Arg, among them the small nuclear ribonucleo-U1 70-kDa protein, which is an autoantigen in systemic lupus erythematosis. These repeats appear to constitute the dominant epitopes in the autoimmune reaction.

An impressive set of data on codon reiteration was recently published by H. Green and N. Wang [34] (Table 2). In line with the findings discussed above, these data show that hydrophobic amino-acids, and particularly glutamine, account for a large proportion of the longer reiterants. In the genes for these proteins the most common reiterants are those that contain poly(CAG), even out-of-frame or, to a lesser degree, those that contain repeated doublets of CA, AG or GC. A particularly intriguing finding is that the amino-acid sequence

Table 2
Uninterrupted reiterants of ≥ 10 codons

Reiterant AA.	Codon	Access No.	Gene	Species
Ala	$(GCA)_{11}$	M98269	antho-RFamide neuropeptide	*A. elegantissima*
Asn	$(AAC)_{20}$	X16523	AAC-rich mRNA (pl.K330)	*D. discoideum*
	$(AAT)_{16}$	S55235	cAR3=cAMP receptor subtype 3	*D. discoideum*
	$(AAT)_{14}$	M87278	adenyl cyclase germination protein	*D. discoideum*
Asp	$(GAT)_{14}$	M60052	histidine-rich calcium binding protein	*Homo sapiens*
Gln	$(CAA)_{11}$	L19349	hydroxymethylglutaryl CoA reductase	*D. discoideum*
	$(CAA)_{20}$	M17826	SSN6 or CYC8	*S. cerevisiae*
	$(CAA)_{15}$	M60807	merozoite surface antigen I	*P. falciparum*
	$(CAG)_{21}$	L12392	Huntington's disease gene	*Homo sapiens*
	$(CAG)_{19}$	Y00489	ventral prostate glucocorticoid receptor	*Rattus rattus*
	$(CAG)_{19}$	M55654	TATA-box binding protein (TBP)	*Homo sapiens*
Glu	$(GAA)_{10}$	J03998	glutamic acid-rich protein	*P. falciparum*
	$(GAG)_{12}$	J05080	histidine-rich calcium-binding protein	*O. cuniculus*
Gly	$(GGA)_{10}$	M18289	E1B large T-antigen	*Adenovirus 41*
	$(GGC)_{20}$	M23263	androgen receptor	*Homo sapiens*
His	$(CAT)_{11}$	S55234	cAMP receptor subtype 2	*D. discoideum*
Ser	$(AGC)_{10}$	X15898	sporozite antigen	*Eimeria tenella*
	$(AGC)_{10}$	M88749	vitellogenin	*I. unicuspus*
	$(AGT)_{11}$	M31431	attachment protein	*Myco. genitalium*
Thr	$(ACA)_{17}$	M66619	aminocyclopropane carboxylate synthesis	*D. caryophyllus*

of the mastermind gene (*mam*) in *Drosophila virilis* has a region (residue 106–161) containing 44 glutamine residues of which 31 are encoded by CAG and 13 by CAA. All of the remaining 12 amino-acid residues are histidines (encoded by CAC or CAT). These interrupt the glutamine codons at ten locations and are probably the result of single nucleotide substitutions in either CAG or CAA codons. Reiterated hydrophobic or basic residues hardly appear in proteins containing 20 or more residues, suggesting that they are poorly tolerated in proteins.

The mechanism of reiteration is still unclear. It should be mentioned, however, that nucleotide reiterations have also been observed in regions of DNA which do not code for proteins; their role in the regulation of cellular behavior has yet to be elucidated.

It is worth noting that in vertebrates there are specialized telomeric structures, located at the ends of eukaryotic chromosomes, that appear to function in chromosome protection, positioning and replication. These telomers consist of hundreds or thousands of tandem repeats of the sequence TTAGGG [35]. In all normal somatic cells examined to date, terminal restriction fragment analysis has shown that with each cell division the chromosomes lose about 50 to 200 nucleotides of telomeric sequence. This finding led to the suggestion that the shortening of telomers functions as a mitotic clock by which cells count and ultimately limit their division. Remarkably, all immortal cells examined to date show no net loss of telomeric length with cell division, suggesting that maintenance of telomers is required in order for cells to escape from replicative senescence.

CONCLUDING REMARKS

It is now more than 40 years since linear and multichain poly-α-amino acids were first synthesized by polymerization of the corresponding *N*-carboxy-α-amino acid anhydrides in the solid state or in solution. The resulting linear homo- and hetero- polymers were employed as simple protein models to verify the presence of the main secondary conformations of proteins, namely the α-helix and the parallel and antiparallel β-sheets. They were also used in experimental and theoretical investigations of helix-coil and helix-β strand transitions in the solid state and in solution, thus helping to shed new light on the mechanisms involved in protein denaturation.

In addition, poly-α-amino acids were used to elucidate the mode of action of proteolytic enzymes on synthetic macromolecular substrates and to detect new proteolytic enzymes. Some of them played an important role in the deciphering of the genetic code. Analysis of the antigenicity of poly-α-amino acids led to the elucidation of the factors determining the antigenicity of proteins and peptides.

There has been renewed interest in the biological and physicochemical characteristics of poly-α-amino acids because of the recent finding that some copolymers of amino acids are effective as drugs in multiple sclerosis, and that glutamine repeats and reiteration of other amino acids occur in inherited neurodegenerative diseases. The presence of repeating sequences of amino acid in proteins and of nucleotides in DNA raises many interesting questions about their respective roles in determining protein structure and function, and gene performance and regulation. In seeking answers, the poly-α-amino acids, as the simplest protein models, can once again be expected to play a useful role.

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