INVESTIGATION OF RECRYSTALLIZATION OF COLLAGEN (I) EMPLOYING CONTRACTION AND STATISTICAL CONFORMATION PROPERTIES

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The ability of collagen (I) to recrystallize by cooling after a thermal denaturation was attempted by a method of contraction energy and theoretical statistical conformation analysis. Contraction energy of the native collagen was $1{\cdot}96 \pm 0{\cdot}6$ J/g; that of denaturated collagen after cooling in contracted state 0·036 J/g and in the stretched state $0{\cdot}45 \pm 0{\cdot}1$ J/g. This corresponds to a 1·8 and 22·8% of recrystallization, respectively.

Theoretic calculation of the probable conformation of the whole sequence of α1(I) chain showed that approximately only 60% of sequences have the ability to form spontaneously stretched polypyrrolidine conformation, whereas approximately 34% of the chain was randomized. About 5% of amino acid sequences had a preferred α-helical conformation and terminal peptides, especially the C-tail have a high probability of extended conformation of a β-pleated sheat type. The collagen chain reveals a significant tendency to form reverse turn. Statistical conformation properties are compared with those of some model polypeptides.

Although recrystallization of collagen after a thermal or a chemical denaturation was often attempted, the results led to different conclusions. Reversibility of the transformation collagen--gelatin is in few papers[1,2] taken for relatively high, but this process is sometimes specified as irreversible or partially reversible[3]. As known, the reversibility also depends on further conditions and on the degree of covalent cross-linking of collagen. A favourable effect of mechanic stretching of fibrils on the recrystallization degree was found when investigating[4] recrystallization of benzoquinone-cross linked collagen. The recrystallized collagen had, however, a lower transformation temperature; like results were also reported[5,6]. The decrease of transformation temperature indicated possible differences between the native and the recrystallized conformations. The known fact that the recrystallized collagen is sensitive towards enzymes, which do not act with the native material, *e.g.* pepsin[7], indicates this possibility.

The recrystallization ability of collagen is important not only for understanding the mechanism of biosynthesis and pathology of connective tissue, but also in practical use of denaturated collagen, *e.g.* when determining the degree of covalent cross-linkage employing the kinetic theory of elastic body.

A real renaturation degree of a triple-helix conformation is difficult to evidence. The recrystallization of collagen was studied[8] *via* optical rotation and differential calorimetry (DSC). Nevertheless, the interpretation of results is complicated by the uncertainty concerning specific rotation of collagen at 100% and 0% content of triple helix. On the other hand, calorimetric data cannot be exactly interpreted due to the unknown exact value of hydrogen bonding energy,

a main type of stabilizing interactions involved in the enthalpy of melting collagen. Therefore, experiments reported the recrystallization to proceed between 20 and 147%.

Even greater difficulties were encountered when determining recrystallization of insoluble collagens. Specific optical rotation of soluble collagens is about —275° and that of a well-gelled gelatin 200°. On the contrary, gelatin of the insoluble collagen has an apparently higher degree of ordering after recrystallization than the native collagen, since its specific rotation was found to be 404°, ref.[8]. To rationalize the optical rotation exclusively by the content of collagen-triple--helixes seems to be, therefore, unproper. More recently, the reconstitution of collagen was studied by a wide-angle X-ray diffraction[3]. Films of gelled gelatin showed only a little content of triple-helical structures, which increased by mechanical stretching. The maximum content of collagen-type conformations found in this gelatins was about 25%.

It is generally assumed that the spontaneously formed ordered conformations of polypeptide chains first of all depend on their amino acid sequence. Theories of statistical probability of the formed regular conformations of polypeptides were developed to such a degree that the secondary structure is possible to calculate with an accuracy comparable with experimental procedures. Methods of statistical anticipation of ordered conformations (α-helixes, extended conformations, reverse turns) are based upon the sequence of amino acids and statistical properties of the individual amino acids[9]. A lot of new information concerning collagen has been reported in last years; thus, a complete amino acid sequence of the α1 chain was published[10]. Various genetically different types of collagen were described; the most investigated collagen I is the principal constituent of skin and the only collagen of tendon and bones[11]. Its chains consist of $[\alpha 1(I)]_2\,\alpha 2$, where α1 and α2 are homologous to a some extent. Collagen characteristic of hyaline cartilage belongs to type II; it has three identic chains denominated $[\alpha 1(II)]_3$. Next type collagen III, is a minor, but still significant constituent of skin and possibly the principal collagen of veins, collagen IV is present in the basal membrane; further types of collagen may exist. The sequence of collagens II—IV is not perfectly known. Chain α2, forming 1/3 of collagen I, is known to about 70% and its composition is to a some extent homologous with chain α1(I).

The mechanism of formation of native triple-helical conformation of collagen seems to be specific. The so-called procollagen peptides, orienting three chains in such a conformation, which enables to form the triple helix are of importance and therefore, the use of statistical methods for this case is not suitable. The denaturated collagen, gelatin, on the other hand, has a conformation close to globular proteins, a random coil. The specific mechanism of procollagen peptides cannot come more into account, since they are split off after the finished proteosynthesis. Conformation of the recrystallized collagen shall be more in agreement with predictions of statistical methods derived from properties of globular proteins. This was the reason why we tried to calculate theoretically the fine structure of the recrystallized α1(I) polypeptide chain according to Robson's and Suzuki's method[9].

Further aim of this project was the use of contraction energy of collagen fibrils for a quantitative determination of the recrystallization degree of tendon collagen. The contraction energy is in relation with the change of conformation entropy of polypeptide crystallization[12] and is not subject to the enthalpy of melting of hydrogen bondings, this being an evident advantage when compared with calorimetric methods already used.

EXPERIMENTAL

Collagen of mature Achilles tendon, obtained immediately after slaughter, was freed from globular proteins by repeated extractions with a 10% NaCl solution. Extraction was repeated five times for 30 min with 500% of fresh solution. Tendons were then wet-separated into individual fibrils 60—100 mm long, 0·5—2 mm in diameter, the samples washed with water, defatted by extraction with acetone, washed and equilibrated in a 10% NaCl solution in which they were stored at 5°C. Prior to measurement each fibril was washed with water till a constant conductance was reached.

Tensometric Device for Measurement of Contraction Energy

Contraction properties were measured with a balancing tensometer described earlier[13,14], which was supplemented by a generator of triangle wave voltage serving as a source of time-depending tension acting on the sample. The force stressing the sample increased linearly with time from zero to a final preadjusted value in the 5—250 g interval. Differences in the sample length were continually recorded with a photoelectric circuit. The tensometer output was connected with an x—y plotter BAK 5T (Aritma, Prague). The obtained records showed dependence of the sample length on the force applied. Although this device is of rather special construction, its properties are comparable with commercial tensometers (*e.g.* Instron *etc.*), the exceptions being the possibility to work in a thermostated liquid medium and at higher sensitivity of the balancing tensometer.

Procedure Employed in Determination of Contraction Energy

Collagen fibril was fixed in the tensometer hangers and placed in a vessel filled with distilled water. The vessel was equipped with a thermostated water-jacket. Temperature of the thermostat medium — ethylene glycol — was either constant or time-programmed. Linear heating was controlled by an electronic circuit, which kept a constant temperature difference between the thermostat medium and water in the vessel of tensometer by means of a couple of thermistors. Water in the vessel was moderately stirred during measurement with a magnetic stirrer. The collagen fibril was heated during determination from 10°C to 70°C at a 5°/min rate at which stretching of the fibril was recorded. At 70°C, what is approximately 10°C over the transformation temperature, heating was stopped, the generator of triangle wave voltage was switched on and a force increasing about 50 g/min was applied to the sample. The deformation curve was recorded, *i.e.* the dependence of the fibril length on the applied force till the fibril stretched to the same length as close before the contraction temperature. The record served for graphic determination of contraction energy as an area below the deformation curve. The energy relates to 1 g of dry collagen divided by dry weight of the sample (w). Contraction energy is defined by equation

$$K = 1/w \int_{l_s}^{l_n} F_{(l)}\, \mathrm{d}l\,,$$

where l_s is the contracted length, l_n the original length of collagen fibril and w the dry weight of collagen in g. The strength is given in N, length in m, so that the dimension of K is J/g.

Similar procedure was employed with both the native and recrystallized collagen. Denaturated samples were recrystallized by cooling to 10°C for 30 min. Cooled were samples in contracted stage and also denaturated samples mechanically elongated to the original length by applying tension of the tensometer generator, which was kept constant during recrystallization.

Procedures Employed for Calculation of Statistical Probability of Conformation of the Recrystallized Collagen

According to the described procedure[9] it is possible to calculate the statistical information on spatial conformation, which is bearing the sequence of amino acid residues, from the statistical properties of the given residue and from directional information transferred through the residue in positions 1 to 8 in both directions from the given residue. Details on statistical calculation and tabulated values of individual amino acids are listed in the original paper.

Calculator Hewlett-Packard was used for calculation of the α1(I) chain; the terminal sections were taken as a constituent of the main chain, so that 16 amino acids of the N terminal peptide, 1011 amino acid residues of the helical section and 25 amino acids of the C terminal peptide were considered a part of a single chain with 1052 amino acid residues. Since the above-mentioned paper[9] does not report the conformation properties of hydroxyproline and hydroxylysine, these amino acids are considered identical with proline and lysine, respectively. Also positions of acid amino acids, the carboxy group of which is not positively amidated (Glx, Asx) were considered as being occupated by glutamic and aspartic acids. The probable effect of these approximations is discussed later on.

RESULTS

Contraction energy values found with native and recrystallized collagens cooled either in contracted or stretched state are listed in Table I. The per cent of recrystallization is the magnitude of contraction energy of collagen related to contraction energy of the native sample.

Results of statistical conformation analysis are plotted in Figs 1–5. Fig. 1 shows the formation probability of extended ordered conformations in a collagen α1(I)

TABLE I
Contraction Energies of Collagen (J/g)

Native material	Recrystallized contracted	Recrystallized stretched
2·15; 1·31;	$8{\cdot}51 \cdot 10^{-2}$	0·49; 0·39;
2·48; 1·16;	$1{\cdot}12 \cdot 10^{-2}$	0·49; 0·61;
1·42; 1·60;	$1{\cdot}09 \cdot 10^{-2}$	0·47; 0·44
2·40; 2·23	$3{\cdot}55 \cdot 10^{-2}$	0·33; 0·36
3·01;		
1·96[a]	$3{\cdot}57 \cdot 10^{-2a}$	0·45[a]
0·595[b]	$3{\cdot}63 \cdot 10^{-2b}$	$8{\cdot}89 \cdot 10^{-2b}$
	1·82[c]	$22{\cdot}8 \pm 4{\cdot}5$[c]

[a] The mean value; [b] standard deviation; [c] % of recrystallization.

chain in individual sequences of amino acid residues along the chain. Positions of amino acids are lettered from the N tail and contain terminal peptides, as well. The statistical probability in decinates[9] is plotted on the ordinate; the negative values indicate the formation of the extended conformation to be unlikely. As long as the negative value is small this conformation cannot be completely excluded. The extended conformation in the employed statistical method is understand to be ordered fine structures with a higher lead of the helix than it is maximally allowed for an intrachain stabilization by regular hydrogen bondings formed between groups of peptide bonds distant by three amino acid residues. Fig. 2 shows results of a similar calculation of statistical probability of an α-helical structure formation. This conformation is untypical for collagen, although most frequent among peptides. Another sterically important property of peptide chains, the tendence to form reverse turn, which is unfavourable for formation of regular tertiary structures, is calculated for collagen in Fig. 3.

Fig. 4 demonstrates the statistical probability of formation of extended conformations in the middle sections of the polypeptides proposed for structural models of collagen. These values hold for amino acid sequences distant by at least 8 residues from the end of the chain.

As shown by statistical analysis, collagen chains have some sections of preferred α-helical conformation and therefore, the difference in probabilities of both conformations was calculated for each amino acid residue and, as long as the results were positive in favour of an α-helix, the differences were plotted in Fig. 5.

DISCUSSION

Results of recrystallization of thermally denaturated insoluble collagen of tendon left to gel at a stretching to the original native length, 22·8 ± 4·5%, are in a good agreement with those[3] ascertained by X-ray diffraction (25%). Also the low degree of recrystallization in an unstretched state, 1·8% accords with observations of those authors; nevertheless, no numerical value was given due to a limited resolution of their method. The contraction energy method can obviously be used for the study of recrystallization of collagen, or other crystalline polymers, which lose either by heating or with lyotropic reagents the ordered structure and turn to amorphous coiled conformation. The force needed for stretching the sample bodies to the original length, which is here considered as equal with contraction energy, is proportional to the degree of ordering of the biopolymer prior to transformation. Since collagen is an elastomer over its denaturation temperature[15–19], the force required for deformation is mostly of entropic nature and the contraction energy is in relation with the change of conformation entropy appearing during transformation. Collagen is not an ideal elastomer in the stage of gelatin; it undergoes creeping under tension and its elasticity also depends on the deformation history of the body[19].

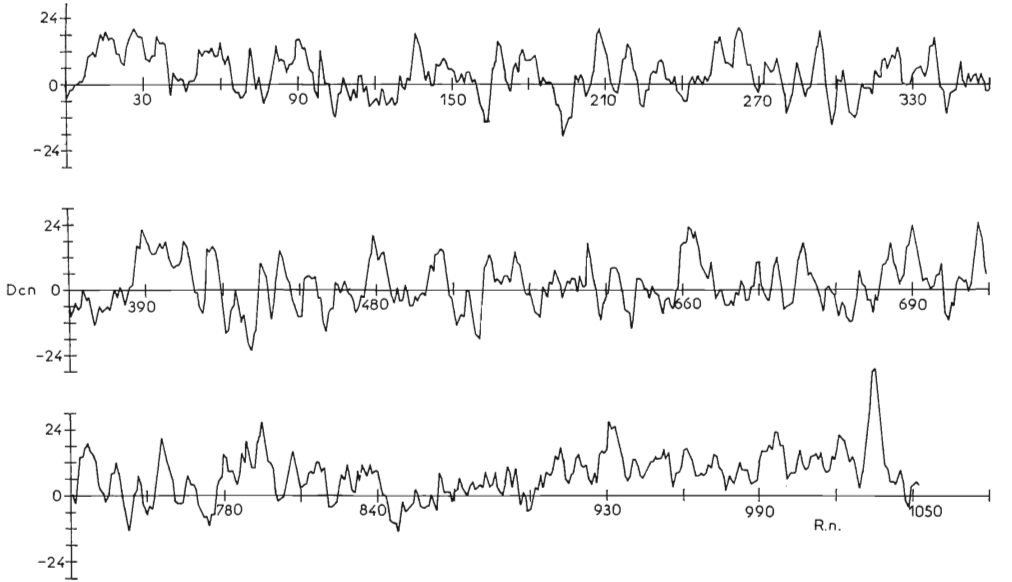

FIG. 1

Probability of Formation of Extended Conformation in α1(I) Chain

Ordinate — the consecutive number of amino acids from the N-side. Terminal peptides are regarded constituents of the main chain. Calculation according to[9]. Dcn = Information decinats, R.n. = Residue number.

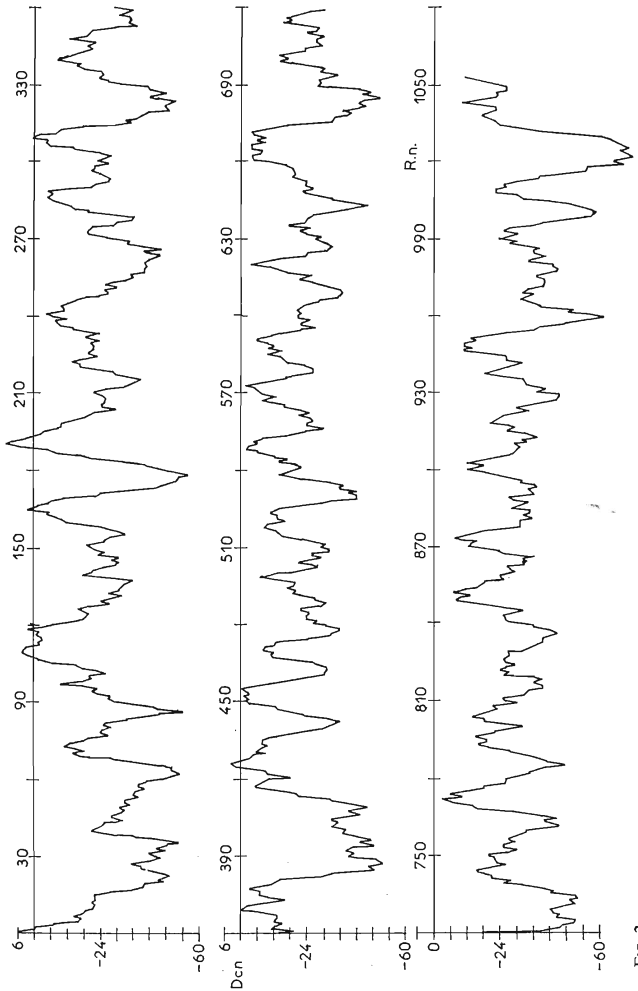

FIG. 2
Probability of Formation of an α-Helix of α1(I) Chain
For specification see Fig. 1. Dcn = Information decinats, R.n. = Residue number.

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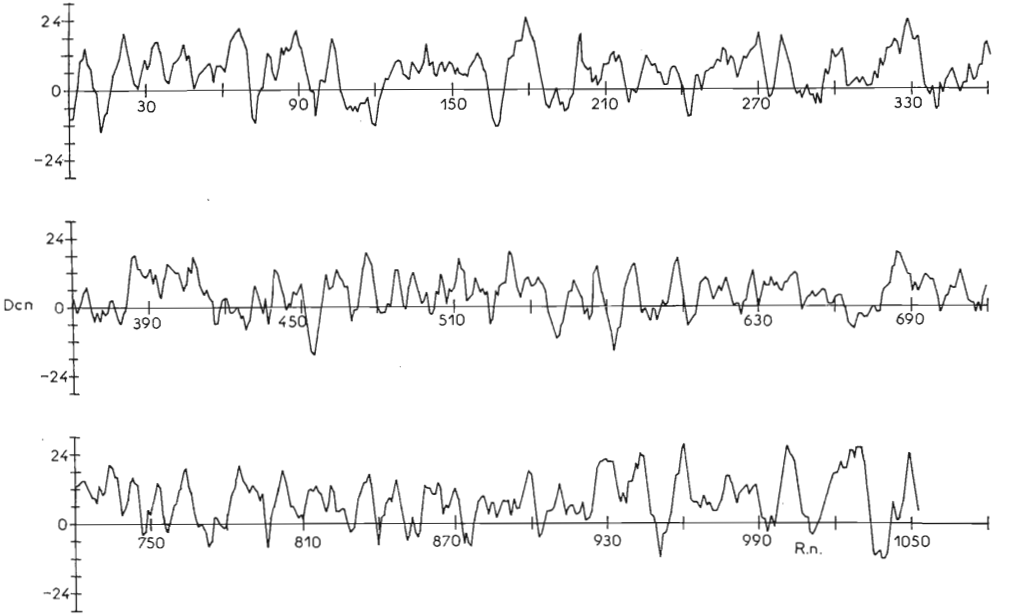

FIG. 3

Probability of Formation of a Reverse Turn in α1(I) Chain

For specification see Fig. 1. Dcn = Information decinats, R.n. = Residue number.

Therefore, the deformation relations were measured in a relatively short time interval. The method is of greater sensitivity and instrumentally more simple than X-ray crystallography. A relatively high scattering is obviously associated with the non-homogeneity of collagen samples. This method avoids difficulties encountered when determining the hydrogen bonding energy, or specific rotation, which are problems of calorimetric and polarization methods discussed in the introductory section.

Statistical conformation analysis of α1(I) chain of collagen offers interesting results. As seen, the greatest part of amino acid sequences, about 60% of the chain length, tends spontaneously to form stretched secondary structures characteristic of collagen. But the situation along the chain alters very substantially and there are many inter-

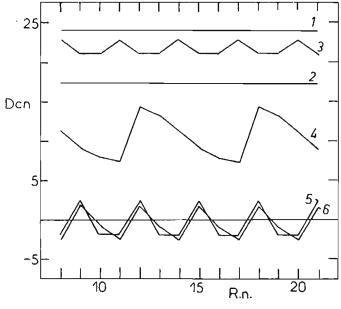

FIG. 4

Probability of Formation of an Extended Conformation in Some Synthetic Polypeptides Proposed as Collagen Models

Ordinate — the consecutive number of amino acid residues. Abscissa — statistical probability (in decinates) determined according to[9]. *1* polyproline II, *2* polyglycine II, *3* $(Gly\text{-}Pro\text{-}Pro)_n$, *4* $(Gly\text{-}Ala\text{-}Pro\text{-}Gly\text{-}Pro\text{-}Pro)_n$, *5* $(Gly\text{-}Pro\text{-}Ala)_n$, *6* $(Gly\text{-}Ala\text{-}Pro)_n$.

Dcn = Information decinats, R.n. = Residue number.

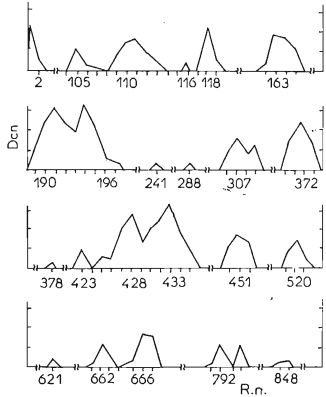

FIG. 5

Sections of α1(I) Collagen Chain in Which α-Helixes are Statistically More Probable than Stretched Polypyrrolidine Conformations

Abscissa — difference between probability of an α-helix and extended conformation (in decinates). Ordinate — the consecutive number of amino acid residues from the N-side. For other details see Fig. 1.

Dcn = Information decinats, R.n. = Residue number.

vals, covering totally 34% of the chain length, in which the extended conformation of an individual chain cannot be obtained because of the negative value of statistical probability. Native collagen has to have these sequences stabilized at a tertiary level, so that the destruction of triple-helical native structure during denaturation can display a considerable measure of irreversibility in those positions. Typical of these sections is that they are relatively short, formed mostly of 10 amino acid residues. As seen in Fig. 1, sequences considerably tending to extended conformations are cumulated in terminal sections of the helical part of chain.

Probability to form an α-helical conformation is little with most sequences of collagen; nonetheless, there are some positions in which such a secondary structure is even preferred. Comparison of statistical probabilities (Fig. 5) reveals the existence of about 15 intervals covering totally approximately 5·3% positions of amino acids with a higher statistical probability of existence of α-helixes. A relatively great part of collagen chain, covering about 34% of sequences does not tend to originate spotaneously ordered conformations. Further sections have their value of probability low only and a great part of sequences tend to reverse turns as seen in Fig. 3. Obviously, these sections hardly undergo spontaneous ordering into an exteded conformation of native collagen, so that the conformation analysis accords with observation that the degree of denaturation of collagen is relatively small after thermal denaturation.

Conformation properties of terminal peptides are also of interest. At the beginning, the N-terminal peptide has a short interval inclining to an α-helix, which tends quite soon to a formation of an extended conformation. The C-terminal peptide can be characterized as a sequence with a very significant tendence to form an extended conformation. Values of statistical probability in decinates, *e.g.* of sequences 6^c to 9^c are as follows: +32, 2 + 45, 2 + 46, 9 + 38, 6; what is the maximum value of the whole collagen chain. Such high probability values virtually exclude the possibility the terminal chain not to be in an ordered extended conformation. This fact alters the assumption that the terminal peptides of collagen are not ordered sections. It is obvious that in the absence of glycine the extended conformation cannot be triple-helical, characteristic of collagen, but rather of β-pleated sheat.

The calculated conformation values relate to an α1(I) chain, which was not hydroxylated, *i.e.* to a chain synthesized by the cell and which was not covalently cross linked. Intercellular hydroxylation mediated by prolylhydroxylase leads to origination of hydroxyproline, the statistical conformation properties of which are not known in detail. Its properties will probably be, from the conformation point of view, close to proline; moreover, it has the possibility to contribute to the stability through formation of a hydrogen bonding. As known, hydroxylation leads to an enhancement of stability of the tertiary structure of collagen[20-22]. Further circumstances, influencing the structural behaviour of the real collagen are the nonhomogeneity on molecular level; collagen of type I is formed of polypeptide chains only to 2/3,

the statistical analysis of which is given here. The remaining 1/3 is formed by an α2 chain, the amino acid sequence of which is so far not fully cleared. It is possible that between other collagen types, II, III and IV, greater sequence differences will appear, what can be reflected by the higher ability to recrystallize the native structure. Collagen of tendon used in experimental section was predominantly of type I, ref.[23]; some experts suppose this type to be the only one present in tendons and bones[11].

The statistical work-up does not consider the effect of covalent cross linkage what is the case with mature collagens. As a result of imperfect separation of chains during thermal transformation, this might influence the conformation properties of adjacent sequences. Thus, those regions, having inherently a greater ability of renaturation will probably be mostly affected, since the most part of cross links is formed in terminal sections.

Theoretical conformational analysis can also lead to a judgment on suitability of some synthetic polypeptides proposed for structural models of collagen. As evident from Fig. 4, homopolymers *e.g.* polyglycine or polyproline in *trans* conformation have a high positive value of statistical probability for all residues in an extended conformation, so that their chains can be embodied in a collagen-like helix individually, without further stabilization at a tertiary level. This is in accordance with their behaviour, because, as known[24], they form in crystalline stage collagen-like but not triple helixes. Similar statistical properties displays the polypeptide $(Gly\text{-}Pro\text{-}Pro)_n$ as well, which really forms collagen-like helixes of triple-helical nature with regard to the presence of glycine in each third position[24–26].

Statistical conformation properties of polytripeptides $(Gly\text{-}Ala\text{-}Pro)_n$ and $(Gly\text{-}Pro\text{-}Ala)_n$ proposed as models[27,28], are very close to collagen. As seen from the conformational statistics, the alanine residue markedly destabilizes the polypyrrolidine helix, so that sections preferring extended conformation and those in which the extended conformation is unstable are alternating along the chain. Such a situation is also encountered in the inside part of collagen chain. Similarly as with collagen, the extended conformation can be here stabilized only through tertiary interactions in a triple helix. As found[29–31], the polymer $(Gly\text{-}Ala\text{-}Pro)_n$ does not exist in aqueous solution in a collagen-like conformation, whereas $(Gly\text{-}Pro\text{-}Ala)_n$ does form such triple-helical conformations in water[31]. The conformation statistics (Fig. 4) reveals, in agreement with this behaviour, a little higher values of the maximum probability of extended conformation and a smaller maximum of negative value with the individual sequences $(Gly\text{-}Pro\text{-}Ala)_n$ in comparison with $(Gly\text{-}Ala\text{-}Pro)_n$ values, even though the mean values of both triplets are equal. A like accordance between the conformation statistical analysis and behaviour of polypeptides is seen with polypeptides $(Gly\text{-}Ala\text{-}Pro\text{-}Gly\text{-}Pro\text{-}Pro)_n$. Such a collagen model forms triple-helical structures very close to collagen[32]. Since the sequence -Gly-Ala-Pro- does not form collagen-type triple helixes, the presence of triplets -Gly-Pro-Pro- must considerably affect the conformation properties in favour of the extended conformation. Fig. 4 backs this

fact; nevertheless, statistical properties are, compared with collagen, markedly more favourable, so that this model would satisfy only some regions of the collagen chain.

The substantial conclusion from results of statistical conformation analysis evidences the fact that the individual α1(I) chain of collagen evidently cannot exist in the native conformation. The stable conformation is attained due to intermolecular interactions in the tertiary structure of the triple helix. Such a triple-helical conformation has a higher degree of order and consequently, a lower entropy; as a result, the reversibility of transformation collagen-gelatin is exclusively the question of mechanism of the ordering process. Some action, having a character of information transfer between substructures, has to come into effect in order the renaturation to be complete. A mechanical stretching of collagen fibrils, also leading to lowering of the conformational entropy, favourizes renaturation. The information transfer in biosynthesis of collagen may be obviously mediated by a specific cell mechanism in which an important role is played by procollagen peptides. On the other hand it is possible that mature collagen is unable to renaturate the native conformation after a thermal denaturation due to a loss of information mechanism. Spontaneously formed conformations may be, therefore, collagen-like triple helical only in a low extent, the remaining ones are either extended conformations of polypyrrolidine type, or statistically randomized with a possible small portion of α-helical sections, or β-pleated sheat in the C terminal peptide.

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