STRUCTURAL STUDIES ON COLLAGEN USING 1-ANILINO NAPHTHALENE-8-SULPHONATE AS A FLUORESCENT PROBE

Synopsis of the Thesis to be submitted to the
UNIVERSITY OF MADRAS
for the Degree of
DOCTOR OF PHILOSOPHY

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Structural studies on Collagen using 1-anilinonaphthalene-8-Sulphonate as a fluorescent probe.

Collagen is a widely occuring fibrous protein which has received much attention due to its wide ranging application. The protein occurs in several parts of the body and has found use as a biomaterial, a leather making protein, a cosmetic aid etc. Therefore, it is not surprising that biosynthetic aspects and the characterisation studies of the protein have been carried out in detail. Each molecule of Collagen is now known to be composed of 3α polypeptide chains. The nature of amino acids as well as sequence in each αchain of the protein are now well established (1), atleast in the case of the protein from a few sources. The specialities of the amino acid sequences and their importance in structural and mechanical stability of the protein have been discussed by earlier workers (2). The molecular architecture as well as biophysical aspects of the protein have been much investigated (1,2). The presence of nonhelical extensions termed telopeptides at the N & C terminal ends and their importance in stabilising the quarternary structure of collagen are well known (2). It is also established that Collagen has in its molecule an assembly of polar (charged) amino acids namely Arginine (Arg), Lysine (Lys), Glutamic acid (Glu) Aspartic acid (Asp) as well as residues with long hydrophobic side chains like Valine (Val), Leusine (Leu), Isoleucine (Ileu), Phenylalanine (Phe) Tyrosine (Tyr) and Methionine (Meth) (3).

Molecules of Collagen in aqueous environment tend to aggregate and the thermodynamics of such a process are influenced by the interaction of the protein with the water molecule (4). The role of water in the stabilization or destabilization of Collagen molecule has been much discussed and

even changes in the X-ray diffraction pattern depending on whether Collagen is hydrated or not, have been noted (2). Furthermore theories exist that tanning is nothing but the removal or replacement of bound water molecules in collagen(5), although changes in the conformational structure of collagen on tanning may also well occur. However, the conformation structure of neither the native collagen nor the tanned collagen has been established. Thus inspite of many detailed structural studies on collagen, there are still many unanswered questions regarding the microenvironment and conformational structure of native and modified collagen. The conventional characterization studies often do not yield precise information on either microenvironments or conformational structure of collagen. Nevertheless there have been suggestions in the literature that fluorescent probe techniques may be developed in the understanding of structural details of proteins and other macromolecules (6). The fluorescence property being an extremely sensitive function of several parameters including reorientation and translation of energy from an excited state to the neighbouring molecules, probes based on fluorescence for investigating conformational structure and microenvironment have now become possible (6). If relaxation of electronic excited state through vibrational energy transfer is rendered more difficult due to decreased polarity, increased viscosity or rigidity of the molecules, an intense fluorescence at short wavelengths may be observed. Therefore, fluorescence probe technique does seem to lend itself as a powerful and sensitive means to assess charged and hydrophobic regions in macromolecules (6).

The fact that molecules like 2-Toluidino Naphthalene 6-Sulfonate (TNS) and Anilino Napththalene Sulfonate (ANS) may exhibit higher dipole moments in the excited state than the ground state makes them particularly suitable as

fluorescent probes. Hence it is not surprising that several biomolecules including α-chymotrypsin(7), κ-casein (8), Rhodopsin (9) and Epimerase (10) have been probed using fluorescent techniques. In the case of collagen, it is obvious that microenvironment in which water is in the assembly of collagen molecules is of interest in the understanding of reactivity of protein including tanning process. In this regard, there has been a brief report of interaction of TNS with collagen leading to fluorescent excited states (11). The fluorescence emission from the TNS-collagen adduct has been used to investigate the hydrophobic regions in collagen. Chiang et al(11) analysed even the topology of collagen using TNS as a fluorescent probe. The authors (11) concluded that there were hydrophobic regions in collagen with which TNS interacted and fluorescence can be used to probe perturbations in microenvironments of collagen molecule. Their studies (11), nevertheless, were limited to native collagen and the importance of charged centres in collagen was not obvious from the previous study. Further in order that a fluorescent probe technique can be relied upon, it is necessary to compare the results obtained by other methods, if possible. In order that the nature of interaction and functional groups involved in the fluorescence enhancement due to dye-collagen complexation is assessed, it is also necessary to modify the protein systematically. In other words, effects of blocking or modification of each class of functional groups may be of immense use. An effort to identify the various types of collagen namely type I, type II & type III using fluorescent probe technique would also be worthwhile. Therefore, on the basis of the critical literature survey carried cut and described in the first chapter of this dissertation the objective and scope of the present work were defined to be

- investigation of the potential use of ANS as fluorescent probe

for studying the detailed structure of native collagen

- investigation of the effects of modification of collagen on ANS-collagen interaction

- preliminary study of the variation in ANS-collagen interaction depending on the heterogeneity and types of the protein and

- finally a critical overview of fluorescent probe techniques for mapping specific sequences and interaction of collagen.

Following the first chapter which would describe the need for the present work, the reasons for the choice of ANS as a fluorescent probe etc. a detailed account of the general experimental procedures and analytical techniques employed would be described in the second chapter.

The third chapter shall describe the experiments carried out in investigating the interaction of ANS with native collagen. It is observed that whereas ANS in water shows only a weak green fluorescence at 525 nm, on interaction with native collagen there is a 100 fold enhancement in the fluorescence intensity of an exited state with energy equivalent to 480 nm when excited at 380 nm. It is true that the native collagen has its own weak emission maximum at 305 nm due to the presence of tyrosine in the molecule. However, neither collagen nor ANS exhibit any fluorescence maxima at 480 nm. Therefore fluorescence probe technique for native collagen using ANS has been examined thoroughly, in this study. Initially the ANS bound collagen was precipitated and free ANS was determined analytically. This investigation was carried for various ANS concentrations ranging from 3×10^{-6}M to 2×10^{-4}M at [collagen] 1×10^{-6}M.

These studies indicated that there are more than one type of association. The biphasic behaviour was analysed using an IBM 370 Computer and a non-linear least squares fit of data to suitable physical models. Number of binding sites and binding constants for two types of associations were obtained. Then using the fluorescence data also, a similar computer analysis was carried out and the same parameters namely number of binding sites and binding constants were obtained. The satisfactory agreement among the values obtained by different means gave credence to the use of fluorimetric data for probing structural details in collagen. The binding processes observed were treated in terms of hydrophobic and charged site interactions and a detailed discussion of data on native collagen is given in the third chapter. The effect of added anions, pH, salt and ionic strength on the fluorescence of collagen-ANS adduct was investigated and these results are described also in the third chapter.

The fourth chapter shall deal with fluorescence studies on modified collagen with a view to understand the role of each type of functional group in the interaction of collagen with ANS. In addition to the effect of chemical modification, the influence of heat denaturation, chemical denaturation and enzymatic treatments involving the removal of telopeptides is also examined. The attempts to identify the ANS binding sites of collagen involved the modification of

- hydrophobic methionine residues by treatment with iodoacetic acid
- esterification of carboxyl groups
- deamination of ε-amino groups of lysine by means of diazotization and
- introduction of aromatic and the hydrophobic groups.

The results obtained from the study of ANS interaction with modified or denatured collagen are presented in chapter 4.

It was observed that the fluorescence intensity of ANS-collagen adduct decreased on both heat and chemical denaturation of collagen. This drop in fluorescence intensity of the adduct of denatured collagen with ANS helped in detecting the influence of urea. Although viscometric(12) and optical rotatory(13) dispersion data revealed that urea treatment brings about significant changes in the hydrogen bonded structures in collagen only at 6 M urea, the more sensitive fluorimetric measurements provide an elegant means to show that even 1 M urea did bring about substantial changes in collagen structure which were not detected earlier. About 50% loss of fluorescence intensity was observed when the nonhelical telopeptide regions of collagen had been removed by enzymatic treatment with pepsin and trypsin. Thus it is estimated that 50% ANS binding sites are in the telopeptide region. The modification of some specific aminoacid residues in the collagen instead of the removal of entire telopeptide regions brought about generally a less dramatic reduction in fluorescence intensity. From the results obtained in this study it has been inferred that it is specific amino acid sequences that were responsible for binding ANS rather than an individual amino acid. The results obtained from the experiments described in chapter 4 would be analysed quantitatively after the computation of ANS binding data using an IBM 370 computer and suitable programmes. Following such quantitative discussions in the fourth chapter a preliminary study on the influence of heterogeneity of collagen in ANS would be included in the fifth chapter.

The overall discu ssion of all the results obtained on this study in the context of structural aspects of collagen would be presented in the last chapter.

The sixth chapter will discuss our results in comparison to the related studies already published. The strength and limitation of the use of ANS as a fluorescent probe and the potential involvement of specific hydrophobic amino acid sequences would be analysed in this chapter. The possibility of involvement of tyrosine, methionine, leucine, isoleucine, and phenylalanine in ANS binding by collagen would be discussed on the basis of our experimental data. The general discussion of the present study shall also find a place in the sixth chapter.

At the end of the sixth chapter, a reference section and a set of appendices would be included. The details of treatment of experimental data in the ANS and collagen equilibration, the various models used and derivations of equations employed would be presented in the appendices. There will be three appendices, the first one dealing with models and treatment of data, the second giving the derivations of algebraic equations used and third elaborating the computer programme used for non-linear least square fit analysis.

Thus it is attempted in the present dissertation to describe the potential use of ANS as a fluorescent probe of the structural details of collagen with respect to microenvironment and conformational aspects.

References

1. G.N.Ramachandran, Ed. Treatise on Collagen 1 (1967) Academic Press

2. G.N.Ramachandran, A.H.Reddi, Biochemistry of Collagen (1976) Plenum Press.

3. Fietzek, J.Mol.Cell Biochem. 8, 141 (1975).

4. M.E.Nimni, in Seminars in Arthritis and Rheumatism, 13, 1 (1983).

5. K.H.Gustavson, Adv. in Ptn.Chem. 5, 353 (1949).

6. Jan Slavik, Biochim.Biophys.Acta 694, 1 (1982).

7. Johnson, J.D., M.A. El-Bayoumi, L.D.Weber and A.Tulinsky, Biochemistry 18, 1292 (1979).

8. R.F.L.Clarke and S.Nakai, Biochim.Biophys.Acta, 257, 61 (1972).

9. U.P.Andley and S.Chakraborthy, Biochemistry 20, 1687 (1981).

10. A.K.Samantha and A.Bhaduri, Ind.J.Biochem.Biophys. 19, 320 (1982).

11. H.C.Chang and A.Lukton, Biopolymers 14, 1651 (1975).

12. K.T.Joseph and G.Chandrakasan, Leath.Sci. 15, 300(1968).

13. A.F.Woodlock and B.S.Harrap, Aust.J.Biol.Sci. 21, 821 (1968).