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The collagen type I segment long spacing (SLS) and fibrillar forms: Formation by ATP and sulphonated diazo dyes

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ABSTRACT

The collagen type I segment long spacing (SLS) crystallite is a well-ordered rod-like molecular aggregate, ~300 nm in length, which is produced *in vitro* under mildly acidic conditions (pH 2.5–3.5) in the presence of 1 mM ATP. The formation of the SLS crystallite amplifies the inherent linear structural features of individual collagen heterotrimers, due to the punctate linear distribution and summation of the bulkier amino acid side chains along the length of individual collagen heterotrimers. This can be correlated structurally with the 67 nm D-banded collagen fibril that is found *in vivo*, and formed *in vitro*. Although first described many years ago, the range of conditions required for ATP-induced SLS crystallite formation from acid-soluble collagen have not been explored extensively. Consequently, we have addressed biochemical parameters such as the ATP concentration, pH, speed of formation and stability so as to provide a more complete structural understanding of the SLS crystallite. Treatment of collagen type I with 1 mM ATP at neutral and higher pH (6.0–9.0) also induced the formation of D-banded fibrils. Contrary to previous studies, we have shown that the polysulphonated diazo dyes Direct red (Sirius red) and Evans blue, but not Congo red and Methyl blue, can also induce the formation of SLS-like aggregates of collagen, but under markedly different ionic conditions to those employed in the presence of ATP. Specifically, pre-formed D-banded collagen fibrils, prepared in a higher than the usual physiological NaCl concentration (*e.g.* 500 mM NaCl, 20 mM Tris-HCl pH7.4 or x3 PBS), readily form SLS aggregates when treated with 0.1 mM Direct red and Evans blue, but this did not occur at lower NaCl concentrations. These new data are discussed in relation to the anion (Cl^-) and polyanion (phosphate and sulphonate) binding by the collagen heterotrimer and their likely role in collagen fibrillogenesis and SLS formation.

1. Introduction

Fibrous collagen is the prominent protein in connective tissues of all animals, present even in the sponges. Collagen fibres have been studied extensively by transmission electron microscopy since the 1940s, with the linear D-banding structural pattern emerging rapidly from unstained collagen samples as well as from metal shadowing and thin sectioning data. The periodic cross-striations of collagen fibres were indicative of a repeating linear molecular *quazi* crystalline organization. At the molecular level, type I collagen fibrils are constructed from the collagen heterotrimer, containing three left-handed proline-rich polypeptide chains twisted together as a right-handed trimer helix. The *in vitro* assembly of acid-solubilized collagen heterotrimers into fibres under defined biochemical conditions was established by Schmitt et al. (1942) and others, along with another non-fibrillar collagen assembly termed the segment long spacing (SLS) crystallite (Schmitt et al., 1953). Our present study places further biochemical and transmission electron microscopical emphasis upon this collagen SLS crystallite, its structure and formation.

The collagen type I SLS crystallite is a structurally-defined, experimentally-induced macromolecular assembly of collagen heterotrimers, with a length of ~300 nm. SLS crystallites, induced *in vitro* under mildly acidic conditions (pH ~3.0) by the addition of ATP, have been observed for collagen type I from several species and from different types of collagen since their first observation in the 1950s (*eg* Schmitt et al., 1953; Gross et al., 1954; Silver and Trelstad, 1980; Tillet-Barret et al., 1992). SLS crystallites are a rod-like bundle or aggregate of perfectly aligned collagen heterotrimers (all with the same N-/C-terminus polarity) thus possessing a markedly different molecular packing to that found in native and *in vitro*-produced D-banded collagen fibrils, and their thicker multi-fibril

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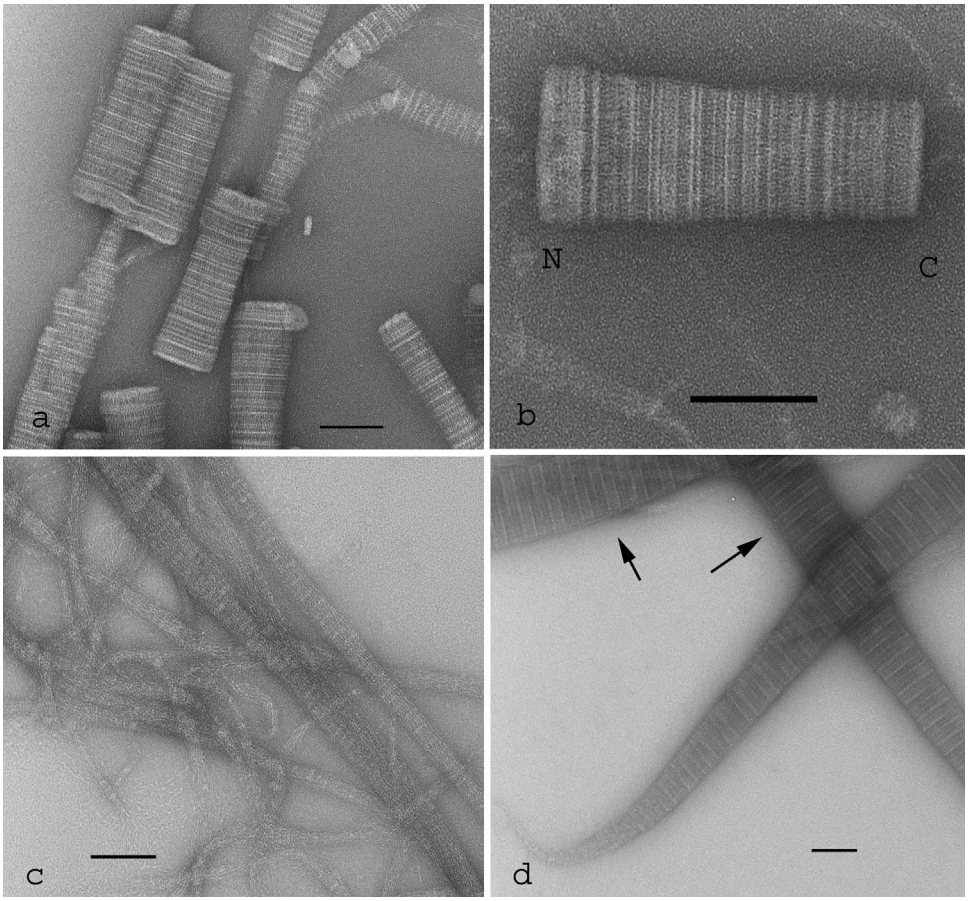

Fig. 1. Collagen type I interaction with 1 mM ATP at increasing pH, with incubation for 24 h at 22 °C. (a) At low pH (pH 2.5) characteristic 300 nm SLS crystallites readily form, with a tendency to link together as a network. (b) Higher magnification image of a single SLS crystallite, with the heterotrimer C- and N-terminal orientation indicated (following Kobayashi et al., 1985). Note the well-defined asymmetric banding pattern along the SLS crystallite, with respect to heterotrimer orientation. (c) At pH 5.0 the collagen molecules aggregate in a more fibrillar manner, with the creation of loosely associated pre-fibrils and fibrils showing an indication of D-banding. (d) At pH 7.0 the collagen molecules associate to form ~67 nm D-banded fibrils that aggregate as spindle-shaped fibre structures, sometimes termed tactoids. Note that within these tactoids, the banding of individual D-banded fibrils tends to be longitudinally misaligned with respect to one another (arrows) within the fibril bundle, unlike typical D-banded collagen fibres (*cf* Harris and Reiber, 2007; Fig. 5). All images are negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

fibres (Harris and Reiber, 2007; Harris et al., 2013; Starborg et al., 2008). Though SLS crystallites do tend to aggregate, there is no evidence that they are able to form larger structures with truly crystalline order and consequently the use of the term *crystallite* is somewhat controversial. The wealth of early SLS data was reviewed by Serafini-Fracassini (1982) and Kühn (1982), who considered SLS crystallites to be a "powerful tool in collagen research" and emphasized the fact that they possess information on the length of the collagen heterotrimer and longitudinal features of its primary and secondary structure.

Most early electron microscopical studies on collagen SLS crystallites were performed using metal shadowing (Gross et al., 1954; Schmitt et al., 1953) and positive staining with uranyl acetate and phosphotungstic acid (Hodge and Schmitt, 1960; Bruns and Gross, 1970). The available SLS negative stain data, from both cationic uranyl acetate and anionic ammonium molybdate staining, is in general agreement with that from positive staining. These specimen preparation techniques have been applied to both the ~67 nm D-banded collagen fibril and the multiple-banded single ~300 nm SLS crystallite, establishing the presence of a lengthwise asymmetric pattern of protein bands, generally attributed to the punctate linear distribution and mass summation within the thickness of the crystallite, generated by the bulkier amino acid side chains along the length of individual collagen heterotrimers (Bender et al., 1982; Bruns and Gross, 1973; Kobayashi et al., 1992). However, the additional mass contribution from bound ATP (a polyphosphate) was not considered, an aspect that could be addressed in the future by cryo-electron microscopy of unstained collagen specimens. In recent years atomic force microscopy (AFM) has been used (Fujita et al., 1997) to provide a model for the oligomeric sub-structure of the SLS crystallite (Paige and Goh, 2001). Of continuing significance is the relevance of the SLS crystallite to present day molecular models for the ~67 nm D-banded collagen fibril, where a well-defined linear collagen heterotrimer partial overlap (0.4D) and spacing (0.6D) is thought to be responsible for the organized/paracrystalline structure of the *in vivo* native and *in vitro* experimentally-formed collagen fibril.

Published data on ATP-induced collagen SLS crystallites is limited with respect to the concentration of ATP required for their creation, the speed of formation, the pH range over which this occurs and the influence of temperature. Furthermore, other nucleoside triphosphates, inorganic sodium tri(poly)phosphate, fructose 1,6-diphosphate and perdisulphuric acid can also produce SLS crystallites (Bowden et al., 1968), but the detailed influence of these reagents on SLS formation has yet to be studied in detail. Other polysulphates such as heparin, glycosaminoglycan and pentosan also remain to be studied. Thus, a number of unresolved questions remain, particularly in relation to SLS formation under varying biochemical conditions in the presence of ATP, their aggregation and dissociation, aspects that are addressed in the present work.