

Thermal Stability of the Helical Structure of Type IV Collagen within Basement Membranes In Situ: Determination with a Conformation-dependent Monoclonal Antibody

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ABSTRACT To examine the thermal stability of the helical structure of type IV collagen within basement membranes in situ, we have employed indirect immunofluorescence histochemistry performed at progressively higher temperatures using a conformation-dependent antibody, IV-IA8. We previously observed by competition enzyme-linked immunosorbent assay that, in neutral solution, the helical epitope to which this antibody binds undergoes thermal denaturation over the range of 37–40°C. In the present study, we have reacted unfixed cryostat tissue sections with this antibody at successively higher temperatures. We have operationally defined denaturation as the point at which type IV-specific fluorescence is no longer detectable. Under these conditions, the in situ denaturation temperature of this epitope in most basement membranes is 50–55°C. In capillaries and some other small blood vessels the fluorescent signal is still clearly detectable at 60°C, the highest temperature at which we can confidently use this technique. We conclude that the stability of the helical structure of type IV collagen within a basement membrane is considerably greater than it is in solution, and that conformation-dependent monoclonal antibodies can be useful probes for investigations of molecular structure in situ.

In all collagenous molecules the basic structural form is the triple helix (for reviews, see references 1 and 2). The integrity of this molecular conformation and how it is affected by environmental factors can be studied in solution by circular dichroism (CD)¹ spectroscopy. When this procedure is used to examine the thermal stability of different genetic types of collagen at neutral pH, those from homothermic vertebrates generally have a T_m of 42°C or lower (see, for example, references 3–5), but exceptions do occur (5). Because this is only a few degrees above physiologically normal temperature, it is thought that collagen molecules must be considerably more stable in tissues, possibly due to their assembly into fibrillar structures (6).

The thermal stability of the structure of collagen molecules packed in fibrils in situ cannot, in any simple way, be deter-

mined by methods of measurement such as CD spectroscopy, which must be performed in solution. However, evidence for increased in situ stability has been obtained by examining the temperature-dependent shrinkage of native collagenous connective tissues and reconstituted collagen fibrils (6). This method, however, examines the response of the overall collagenous matrix. It cannot, for example, distinguish among potential differences in the various genetic types of collagen that may be present.

We reasoned that specific and direct probes for molecular structures such as the collagen helix in situ might be monoclonal antibodies directed against conformation-dependent determinants. Most of the anticollagen type-specific monoclonal antibodies that we have produced are directed against epitopes, which are both conformation dependent and located within the helical domain of the molecules. Some have been assayed by competition enzyme-linked immunosorbent assay for their ability to bind in solution to collagen samples that

¹ Abbreviation used in this paper: CD, circular dichroism (spectroscopy).

had been raised to progressively higher temperatures (7; Schmid, T., and T. F. Linsenmayer, manuscript submitted for publication). In general, good agreement has been found between the temperature at which antibody binding is lost and that at which the helical structure is destroyed, as physically measured by CD spectroscopy. This is true for both the shape of the denaturation curves generated and the T_m obtained (see, for example, reference 4).

The approach that we have devised for using such antibodies to probe the thermal stability of the helical structure of collagens in situ involves immunofluorescence histochemistry of unfixed cryostat tissue sections reacted with antibodies at elevated temperatures. We have operationally defined the denaturation temperature as the temperature at which detectable fluorescence, indicative of antibody binding, is lost. The values obtained by this method should represent a minimum value for complete denaturation (see Discussion).

In the present study we have used the antitype IV (basement membrane) collagen antibody, IV-IA8 (7). This antibody was chosen because (a) we know it to be directed against a helical epitope, which in neutral solution loses binding capacity over the narrow temperature range of 37–40°C as determined by competition enzyme-linked immunosorbent assay (7); (b) the 150-nm-long domain to which it binds, termed (F1)₂F2, is entirely triple helical, with no disulfide bonds or other apparent discontinuities in its structure (8, 9); and (c) by rotary shadowing analyses it binds exactly in the middle of this domain (10) and thus is flanked on either side by stretches of at least 75 nm of purely helical structure.

MATERIALS AND METHODS

The characterization of monoclonal antibody IV-IA8 has been previously described (7, 10, 11). In all experiments the antibody-containing medium harvested from the hybridoma cultures was used undiluted as the source of antibody.

Unfixed tissues from 17–18-d chick embryos were sectioned at 8 μ m with a cryostat. They were air dried onto albumenized 12-spot multislide slides (Shandon Scientific, Sewickley, PA) and then stored at –20°C until used.

For the thermal denaturation experiments, Coplin jars containing ~75 ml of phosphate-buffered saline (PBS, pH 7.2), complete culture medium (which served as a negative control), or monoclonal antibody-containing medium were heated in a water bath to the desired temperature. The temperature of the solution within the jars fluctuated <1°C as monitored by an immersed thermocouple attached to an electronic digital thermometer (Omega Engineering Inc., Stamford, CT).

Slide preparations were first immersed for 0.5 h in PBS maintained at the desired temperature and were then transferred to a jar of antibody-containing medium and held at the same temperature for an additional 0.5 h. In most experiments a protease inhibitor mixture was included in these solutions. The slides were washed in PBS maintained at the same elevated temperature and then twice at room temperature. Subsequent steps were performed either at room temperature or at 4°C. These involved reacting the sections with a rhodamine-conjugated goat anti-mouse IgG secondary antibody (Cappel Laboratories, Cochranville, PA), washing in several changes of PBS, and mounting in glycerine-PBS (95:5). The sections were then viewed with a Zeiss universal microscope (Carl Zeiss, Inc., New York) equipped for epifluorescence. Photographs were all taken through a \times 25 plan-neofluor objective, using Tri-X-Pan film (Eastman Kodak Co., Rochester, NY) and an exposure time of 1 min. They were printed identically.

The experiments were repeated on most tissues at least four times, duplicate sections being exposed at each temperature. In each experiment, temperatures from 35° to 65°C were examined at 5°C increments. All experiments were examined without previous knowledge of the temperature, and most were evaluated by two individuals.

RESULTS

Control Experiments (Data Not Shown)

Certain experimental parameters such as renaturation of

the epitope were evaluated. If cooling of the thermally treated sections was allowed to occur before they were reacted with the primary antibody, the resulting staining patterns suggested that rapid renaturation of the helical structure had occurred. This was eliminated by reacting the sections in the primary monoclonal antibody at the elevated temperature being tested (see Materials and Methods).

The inherent thermal stability of the IV-IA8 antibody molecule itself was also examined. The antibody could be heated to 60°C and cooled to room temperature without loss of binding capacity. After being raised to 65°C, however, subsequent attempts at staining at room temperature showed the fluorescent signal to be either greatly diminished or completely lost. Thus, the highest temperature at which we could obtain valid data was 60°C.

To confirm that thermally dependent changes in basement membrane staining with IV-IA8 would largely reflect changes in the helical determinant against which this antibody is directed, we used another antitype IV collagen monoclonal antibody (IV-IIB12) (10, 11) that recognizes a more thermally stable epitope. By competition enzyme-linked immunosorbent assay, this epitope persists in the competing type IV collagen after heating to 60°C in solution. When antibody IV-IIB12 was used to stain tissues at progressively elevated temperatures, most basement membranes still exhibited positive fluorescence up to, and including the testable maximum of 60°C. This showed that the epitope remained substantially accessible in tissues heated to 60°C, and that antibody binding can still occur, given an epitope stable at this temperature.

Thermal Denaturation of the Epitope Bound by Antibody IV-IA8

Five different tissues, representing a diverse group of basement membranes, were examined, including anterior lens capsule, Descemet's membrane in the cornea, smooth muscle in the tunica media of large blood vessels, and the various basement membranes present in leg skeletal muscle and in kidney.

Most basement membranes, including those of the smooth muscle layers in the media of large blood vessels (Fig. 1, *a–d*), the anterior lens capsule (Fig. 1, *insets a–d*), and Descemet's membrane in the eye (not shown), gave similar results. Positive reactivity with the antibody was present at temperatures up to and including 50°C (see Fig. 1, *a* and *b*, and *insets a* and *b*); most also showed detectable fluorescence at 55°C (Fig. 1 *c* and *inset c*). The only noticeable change was a progressive decrease in the overall fluorescence intensity at each successive temperature increase (see Discussion). At 60°C no fluorescence was detected (Fig. 1 *d* and *inset d*). Thus, in most basement membranes, the helical structure of the epitope recognized by IV-IA8 was stable at 50°C and frequently up to 55°C, but it became denatured by 60°C.

In skeletal muscle (Fig. 2, *a–d*) and kidney (Fig. 2, *insets a–d*), which contain several different cell types with associated basement membranes, the pattern of thermal denaturation was more complex. In skeletal muscle stained at temperatures below 50°C (Fig. 2, *a* and *b*), fluorescence was observed in all of the basement membranes, including those of the endomysium surrounding the individual muscle fibers, those associated with the capillaries intercalated among the muscle fibers (arrowheads), and those present in the small blood vessels (white arrows) in the surrounding perimysial connective tis-

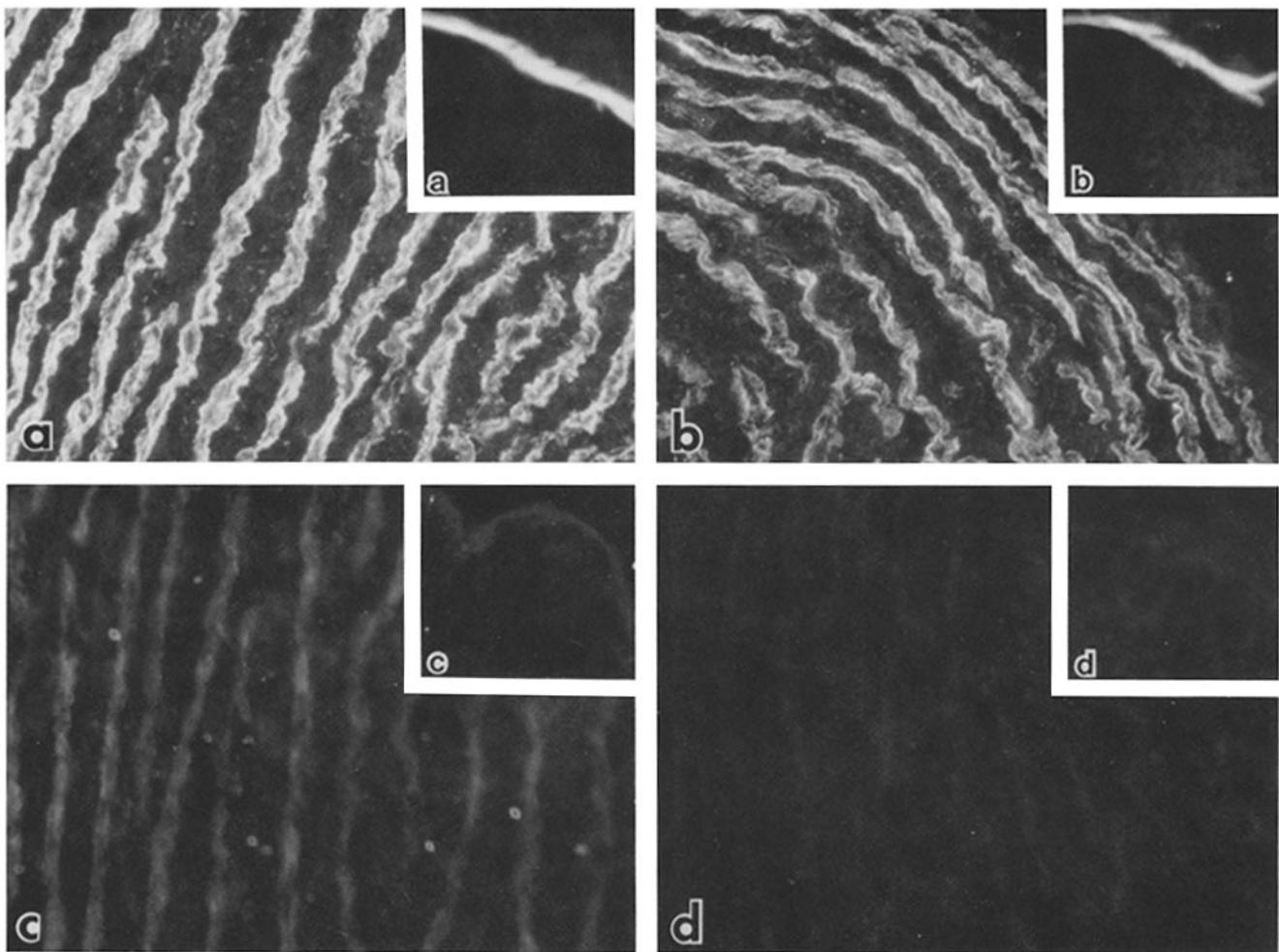


FIGURE 1 Fluorescence micrographs of large blood vessel walls reacted with antibody IV-IA8 at different temperatures. *Insets* show regions of the equatorial zone of the lens capsule reacted at corresponding temperatures. a, 40°C; b, 50°C; c, 55°C; d, 60°. $\times 295$.

sue. In contrast to the intensities of the different basement membranes at 40°C (Fig. 2a), the fluorescence within the endomysium at 50°C (Fig. 2b) was markedly diminished relative to that of the capillaries (arrowheads). This resulted in the capillaries appearing as the more predominant fluorescent structure (see Fig. 2b). At 55°C (Fig. 2c) the staining of the endomysium was patchy and discontinuous; at 60°C (Fig. 2d), except for very faint wisps, endomysial fluorescence was undetectable. In contrast, at both of these higher temperatures, many of the capillaries in the muscle (arrowheads) and some of the small blood vessels embedded in the connective tissue of the surrounding epimysium (white arrows) maintained a capacity to bind antibody.

In the kidney, as in the skeletal muscle, all the basement membranes were positive up to and including 50°C, though again with an overall diminished intensity at the successively higher temperatures (compare the section at 40°C shown as the *inset* in Fig. 2a with that at 50°C, *inset 2b*). The positive-reacting basement membranes included the glomerular basement membrane (asterisk) and that of Bowman's capsule (white arrow) within the renal corpuscle, and the various tubules and ducts (arrowheads) whose precise identification could not be made in these unfixed, unstained sections. At 55°C (*inset* in Fig. 2c) only small wisps of faint, patchy fluorescence could be detected within the tubules and ducts

(arrowheads) and within Bowman's capsule (white arrow). At 60°C, essentially no fluorescence was visible in these structures (*inset* in Fig. 2d). In some, but not all, of the capillary tufts of the glomeruli, however, there was still an easily detectable reaction, even at 60°C (*inset* in Fig. 2d, asterisk).

DISCUSSION

For collagens in general, conformation-dependent antibodies specifically recognize epitopes produced by folding of the component chains into the triple helical structure (12, 13). As demonstrated here, such antibodies can be used to probe this molecular conformation *in situ*. We observed that in most basement membranes, the helical determinant against which antibody IV-IA8 is directed is still intact after being raised to 50–55°C for 0.5 h before reaction with antibody. Some were still intact at 60°C, the testable limit for the assay. This is a considerably higher denaturation temperature than that which we observed for the epitope in neutral solution, as measured by competition enzyme-linked immunosorbent assay using the thermally treated (F1)₂F2 fragment of type IV collagen as the competing antigen (7). In solution, all detectable antibody binding was lost at ~40°C. This comparison seems justified, since, in both cases the preparations were held at the experimental temperature for the same 0.5-h time period. Also, as

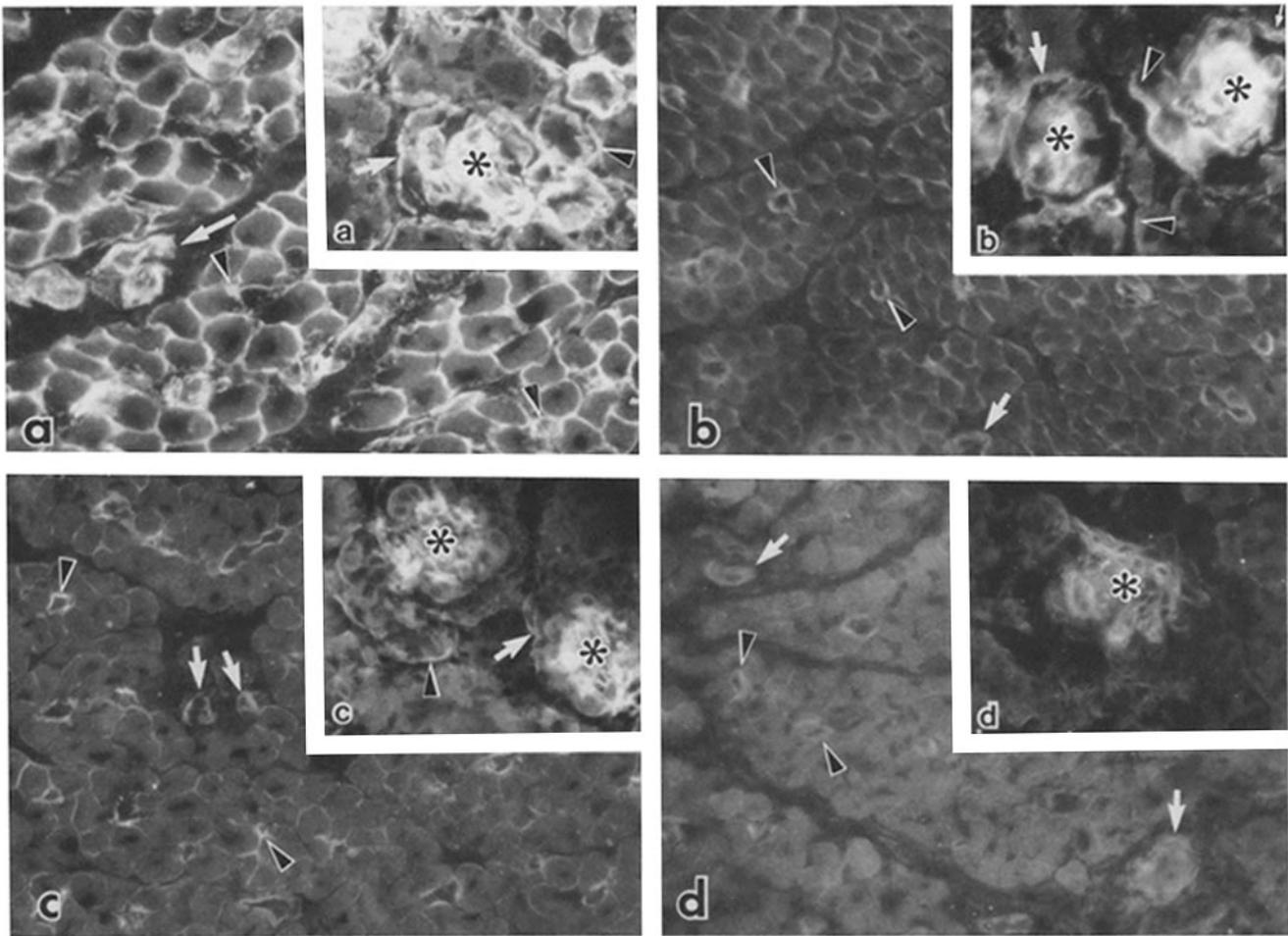


FIGURE 2 Fluorescence micrographs of skeletal muscle reacted with antibody IV-IA8 at different temperatures. In these micrographs, the arrowheads indicate capillaries; white arrows point to small vessels in perimysium. The insets show sections of kidney reacted with IV-IA8 at the corresponding temperature. Here the asterisks show capillary tufts in glomeruli; white arrows indicate Bowman's capsules; arrowheads point out the tubules. In both the figures and insets: a, 40°C; b, 50°C; c, 55°C; d, 60°C. $\times 290$.

pointed out in the beginning of this article, for collagen in solution we have obtained good agreement between denaturation as measured by both CD spectroscopy and by antibody binding (4; Schmid, T., and T. F. Linsenmayer, manuscript submitted for publication), even though for the CD measurements the collagen solution was raised at a considerably more rapid rate, 30°C/h (see also Hayashi et al. [3]). That 0.5 h should be a sufficient time to achieve adequate denaturation is also suggested by the studies of Gross (6) who observed that the temperature-dependent shrinkage of reconstituted collagen fibrils and intact tendons is achieved in the time frame of minutes, or less (Gross, J., unpublished observations).

Inasmuch as $(F1)_2F2$ is a fragment of type IV collagen, albeit a large one, the denaturation temperature that we determined in solution may be an underestimate. Fragments of collagen molecules frequently have a somewhat lower melting temperature than the intact molecule (14). However, using CD spectroscopy, Bächinger et al. (15) have observed that intact type IV procollagen undergoes major helical transitions at 40° and 44°C, values quite close to that which we determined in solution by antibody binding.

At successively higher temperatures we observed a general, progressive decrease in the intensity of fluorescence in all basement membranes, beginning at temperatures well below

those at which the total loss of detectable reactivity occurred (our operational definition of denaturation). This could reflect progressive changes in the stability of the epitope within the collagen molecule, changes in the affinity of the antibody itself, or both. Within the antibody molecules, higher temperatures may decrease the molecular forces involved in formation of the binding site, thus lowering antibody affinity. If so, then the values we have observed for helical denaturation *in situ* should be a minimum. Within a collagen, the helical structure at focal sites along the molecule could become progressively weaker at elevated temperatures that are nevertheless below the temperature at which the entire helix unwinds. Studies employing enzymatic digestions have suggested that focal differences in helical stability may exist along the molecule (16–18).

In the present study we observed that the fluorescent signal persisted at a higher temperature in some basement membranes than in others. This was especially evident in those of capillaries and some other small vessels where stability persisted to 60°C. A similarly high denaturation temperature was observed within the glomerular basement membrane of the kidney. This is consistent with recent observations showing that the type IV collagen within this structure originates in part from synthesis by the capillary endothelial cells (19).

It is intriguing to speculate that the apparent differences in thermal stability of the antitype IV collagen epitope reflect structural differences in the basement membranes of different tissues. Enough variables may be involved, however, that this conclusion must remain tentative. For example, it can be argued that within the vascular basement membranes the persistence of the fluorescence signal at higher temperatures simply reflects the fact that these are among the most intensely reactive ones. Although this may be a contributing factor, it is probably not the sole reason for the noted differences. The anterior lens capsule also reacts intensely, especially in the equatorial zone (7, 20), but the staining within this structure is greatly diminished at 55°C and is undetectable at 60°C. Another possible factor is that during the thermal treatments, other noncollagenous basement membrane components may undergo rearrangements, thus masking or unmasking the antigenic site on the collagen molecule.

At this point we can only speculate on the reasons for the increased stability of the type IV collagen helix in situ, as contrasted to its stability in solution. Several possibilities exist. One is that the helical structure of individual molecules in solution has rotational freedom which becomes severely restricted in situ. In the "network" model for type IV collagen assembly within basement membranes (21, 22), lateral association of molecules, as occurs in collagen fibrils, is probably minimal (however, see reference 23), but both ends of the molecule are covalently bonded to other type IV molecules. This could impart rigidity and stability to the helical structure. In addition, type IV collagen in situ may be complexed with other basement membrane components which confer stability.

Further studies using monoclonal antibodies with specificities for other helical and sequential determinants along the molecule, enzymatic digestions to remove possible stabilizing components, and photometric methods to quantitate the results more precisely, may answer some of these questions.

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