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Rat Heart Gap Junctions as Disulfide-Bonded Connexon Multimers: Their Depolymerization and Solubilization in Deoxycholate

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Summary. Unproteolyzed gap junctions isolated from rat heart and liver were analyzed for the presence of inter-subunit disulfide bonds by sodium dodecylsulfate polyacrylamide gel electrophoresis. Rat cardiac junctions contained multiple disulfide bonds connecting the M_r 47,000 subunits of the same connexon and of different connexons. Inter-subunit disulfide bonds were absent in liver junctions. Unproteolyzed rat heart gap junctions were resistant to deoxycholate in their oxidized state, but dissolved readily in the detergent when the disulfide bonds were cleaved with β-mercaptoethanol. Disulfide bonding in proteolyzed cardiac junctions was limited to pairs of M_r 29,500 subunits. These junctions were not soluble in deoxycholate even in the presence of β-mercaptoethanol. These results show that heart and liver junctions differ in their quarternary organization.

Key Words gap junctions · connexons · disulfide bonds · cardiac membranes · solubilization of membrane proteins · cell-to-cell channels

Introduction

In mammalian heart muscle, myocardial cells are electrically coupled through gap junctional membranes. Ultrastructural studies show that the pipe-like channel-containing structures of cardiac gap junctions, like those of liver gap junctions (Makowski, Caspar, Phillips & Goodenough, 1977), consist of two interlocking hexamers called connexons, each of which is made up of six presumably identical polypeptide subunits (Manjunath, Goings & Page, 1984*b*; Manjunath & Page, 1985*a*). We have previously reported that the connexon subunit of rat heart gap junctions (as studied by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)) has a relative molecular weight (M_r) of 44,000–47,000 (Manjunath et al., 1984*b*), a value much larger than the corresponding values of 26,000–28,000 for liver and lens junctions (Brookhuyse, Kuhlmann & Stols, 1976; Alcala et al., 1978; Hertzberg, Morganstern & Gilula, 1978; Goodenough, 1979; Henderson, Eibl & Weber, 1979; Hertzberg, 1980; Nicholson et al., 1981). We have shown that the difference is accounted for by the presence of a M_r 14,500–17,500 cytoplasmic surface component of the cardiac channel protein which is covalently bound to the part of the connexon subunit in the lipid bilayer of the membrane (Manjunath et al., 1984*b*). This component can be visualized electron microscopically both as a fuzzy layer on the cytoplasmic surface of the junction in thin sections and as cytoplasmic surface particles in freeze fractured, deep-etched isolated cardiac gap junctions (Shibata, Manjunath & Page, 1985). The cytoplasmic surface component is cleaved during isolation by an alkaline serine proteinase released from the granules of mast cells present in the heart, a proteolytic reaction that is inhibitable with phenylmethylsulfonyl fluoride (PMSF) (Manjunath, Goings & Page, 1985). Gap junctions isolated from rat liver, which lack this component even when isolated with PMSF, are devoid of cytoplasmic surface fuzz (Manjunath, Goings & Page, 1984*a*) and cytoplasmic surface particles (Shibata et al., 1985). These observations, as well as differences in amino acid sequences (Nicholson et al., 1985), suggest that the protein compositions of cardiac and liver gap junctions differ in important respects.

The systematic study of gap junctional proteins and the further characterization of the differences between heart and liver junctions have been impeded by the lack of a method for solubilizing the connexons and their subunits in the relatively mild detergents used to study other membrane proteins. We have now overcome this problem and have succeeded in solubilizing isolated, unproteolyzed rat heart gap junctions in Na deoxycholate. We demonstrate the existence of multimers of the unproteolyzed rat heart connexons. We show that these structures are stabilized by multiple disulfide bonds between neighboring connexons and between subunits of the same connexon; that disulfide linkages

are present both in the cytoplasmic surface component and in the remaining portion of the connexon within the lipid bilayer and gap; that it is these disulfide linkages which are responsible for the resistance to solubilization in Na deoxycholate; that reduction of the disulfide bonds is a prerequisite for solubilization of rat heart junctions in deoxycholate; and that rat liver gap junctions lack both the inter-connexon and intra-connexon interchain disulfide bridges characteristic of rat heart gap junctions.

An abstract describing these observations has been published (Manjunath & Page, 1985*b*).

Materials and Methods

Preparation of Gap Junctions

Unproteolyzed Rat Heart Gap Junctions

The experimental animals were 300-g Sprague-Dawley rats anesthetized with ether. Unproteolyzed rat heart gap junctions were prepared as previously described (Manjunath et al., 1984*b*; *see* "preparation of gap junctions"), except that the initial homogenization and the subsequent extraction of myofibrillar proteins were done in the presence of solid phenylmethylsulfonyl fluoride (PMSF) rather than previously dissolved PMSF. Solid PMSF was added to ice-cold 1 mM $NaHCO_3$, pH 8.2, in an amount which, if all of it had dissolved, would have given a 1-mM concentration of PMSF. After the addition of PMSF, the solution was stirred for 15 min in the cold before use. Fresh, solid PMSF was also added to the solution used to extract myofibrillar proteins (0.6 M KI, 6 mM $Na_2S_2O_3$, 1 mM $NaHCO_3$, pH 8.2) immediately after suspending the pellets of crude membrane in this medium. This suspension was stirred overnight at 4°C and filtered through six layers of cheesecloth to remove undissolved PMSF. The filtrate was centrifuged at 12,000 rpm for 30 min in a Sorvall Model RC-2B centrifuge equipped with a Sorvall SS-34 rotor; the resultant supernatant was discarded. The pellets were suspended with three strokes of a Dounce homogenizer (pestle A) in 100 ml of 0.6 M KI, 6 mM $Na_2S_2O_3$, 1 mM $NaHCO_3$, pH 8.2, and centrifuged as before. The supernatant was again discarded. The pellets were washed with 5 mM trishydroxymethyl-aminomethane (Tris), pH 9.0, by resuspending and recentrifuging them as before. The pellets thus obtained were further purified with respect to gap junctions exactly as described in Manjunath et al. (1984*b*), except that the procedure was scaled up to handle eight hearts instead of four by doubling the volume of all solutions while keeping concentrations of reagents constant.

Proteolyzed Rat Heart Gap Junctions

The procedure for preparing proteolyzed cardiac gap junctions was identical to that for unproteolyzed cardiac gap junctions (above), except that PMSF was omitted.

Unproteolyzed Rat Liver Gap Junctions

We prepared gap junctions from rat livers by simplifying our previously published method (Manjunath et al., 1984*a*) as follows: The liver from a single adult rat (Sprague-Dawley, of either sex, 300–350 g) was excised and placed in an ice-cold solution (medium A) whose composition was 1 mM $NaHCO_3$, 1 mM PMSF, 0.1 mM *p*-hydroxymercuribenzoate (PHMB), pH 8.2. In this medium, the tissue was first minced and homogenized for 30 sec with a VirTis homogenizer set at maximal speed, then homogenized further for 5–6 sec with a Tissuemizer (Tekmar Instruments, Model SDT 100 EN), also set at maximal speed. The homogenate was made up to a volume of 100 ml with medium A and added to 200 ml of medium A containing 63% sucrose. The resulting suspension was stirred in the cold for 10–15 min and then filtered through six layers of cheesecloth. The filtrate was centrifuged at 11,500 rpm for 20 min in a Sorvall SS-34 rotor. The supernatant was discarded. The pellets were suspended with three strokes of a Dounce homogenizer (pestle A) in 100 ml of 42% sucrose in medium A. The suspension was centrifuged as before, and the supernatant was discarded. The pellets were washed by resuspension and recentrifugation in 300 ml of cold 5 mM Tris, pH 9.0, containing 20 μM leupeptin. Leupeptin was used to inhibit the detergent-activated proteolytic activity present in rat liver plasma membranes (Guenet et al., 1982). The washed pellets were suspended in 30 ml of 5 mM Tris, pH 10, containing 20 μM leupeptin (medium B) at room temperature (22°C). The suspension was mixed with an equal volume of medium B containing 0.6% N-lauryl sarcosine (Sigma, St. Louis, MO). After 10 min of stirring at room temperature, 20-ml aliquots of the suspension were layered on a discontinuous density gradient containing, respectively, 10 ml of 35% sucrose, 0.3% Na deoxycholate in medium B, and 8 ml of 49% sucrose, 0.3% Na deoxycholate in medium B. The tubes were centrifuged for 60 min at 22,000 rpm in a Beckman Model L5-50 centrifuge at 15°C using the Beckman SW 28 rotor. The material floating at the 35%/49% sucrose interface was collected, diluted 1 : 1 with 0.3% deoxycholate in medium B, and centrifuged at 28,000 rpm for 1 hr at 15°C in a SW 28 rotor. The resulting pellets were suspended by brief sonication in 1 ml of medium B containing 0.3% Na deoxycholate. This suspension was layered over 5.8 ml of 35% sucrose, 0.3% deoxycholate in medium B, and centrifuged at 21,000 rpm for 1 hr at 15°C in a Beckman SW 25.1 rotor. The supernatant was removed and replaced with 1 ml of medium B containing 1 mM PMSF and 1% NaN_3, and the tubes were stored overnight at 4°C. During the period of overnight storage, urate oxidase present in the pellet diffused out, leaving behind a membranous pellet that was highly enriched in gap junctions. This pellet was gently washed with distilled water and processed for electron microscopy and SDS-electrophoresis.

Solubilization of Rat Cardiac Gap Junctions in Na Deoxycholate

We used two methods to depolymerize the proteins of unproteolyzed rat heart gap junctions and to solubilize them in deoxycholate: reductive solubilization and reduction and alkylation of the junctions followed by solubilization.

Reductive Solubilization. Gap junctional pellets were suspended in 200 μl of 5 mM Tris, pH 10, containing 0.3% deoxycholate and

5% β-mercaptoethanol (vol/vol). The suspension was incubated at room temperature for 30 min. It was then centrifuged at 160,000× *g* for 45 min in a Beckman Airfuge operated at room temperature. The supernatant was separated from the pellet and transferred to a Centricon-30 tube (Amicon, Danvers, MA) containing 300 μl of a solution whose composition was 5 mM Tris (pH 10), 0.3% deoxycholate, and 5% β-mercaptoethanol. This material was then concentrated to 40 μl by centrifugation at room temperature in a Sorvall SS-34 rotor.

Reduction and Alkylation Followed by Solubilization. Gap junctional pellets were suspended in 200 μl of 5 mM Tris, pH 10, containing 5% β-mercaptoethanol. The suspension was incubated at room temperature for 30 min and then centrifuged at room temperature in the Airfuge at 160,000× *g* for 45 min. The supernatant was discarded. The membranous pellet was suspended in 200 μl of 0.1 M iodoacetamide in 5 mM Tris, pH 10. The suspension was incubated at room temperature for 30 min, then stored overnight at 4°C. The next day, the suspension was centrifuged in the Airfuge as already described, and the supernatant was discarded. The pellet was suspended in 0.3% Na deoxycholate in 5 mM Tris, pH 10, and incubated for 30 min at room temperature. The suspension was then centrifuged in the Airfuge as before. The supernatant was separated from the pellet and concentrated to 40 μl with a Centricon-30 microconcentrator equilibrated with 300 μl of 0.3% deoxycholate in 5 mM Tris, pH 10.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

We carried out SDS-PAGE in 1.5 mm thick slab gels with 5% stacking gels and 12.5% separating gels, using Laemmli's discontinuous buffer system (Laemmli, 1970). Gap junctional pellets were dissolved in the sample buffer containing 2.5% SDS and 5% β-mercaptoethanol. The pellets were solubilized for 30 min at room temperature or for 2 min at 100°C. In some experiments β-mercaptoethanol was omitted from the sample buffer. The proteins used as standards were phosphorylase *b* (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,400) (Pharmacia Fine Chemicals, Piscataway, NJ).

To analyze unproteolyzed rat heart gap junctions solubilized in SDS only (i.e., without β-mercaptoethanol to reduce disulfide linkages), we used a 4–30% acrylamide linear gradient gel with a 3% stacking gel. For this experiment, rabbit muscle myosin (heavy chain M_r 200,000) (Bethesda Research Laboratories, Life Technologies, Gaithersburg, MD) and unreduced human IgM (M_r 970,000) (Calbiochem, San Diego, CA) were employed as additional markers.

Protein Estimation

Unproteolyzed rat heart gap junctions were depolymerized with β-mercaptoethanol and alkylated with N-ethyl maleimide. Protein estimation was then carried out using the modified Lowry method of Markwell, Haas, Bieber and Tolbert (1978).

Electron Microscopy of Gap Junctional Pellets

Fixation of membrane pellets with buffered glutaraldehyde, dehydration, embedding, ultramicrotomy, staining with uranium and lead salts, and electron microscopy were done as previously described for isolated gap junctions from rat heart (Manjunath et al., 1984*b*) and liver (Manjunath et al., 1984*a*).

Results

Relationship of Disulfide Linkages to Molecular Weight of Rat Heart Gap Junctional Protein

Figure 1 is an electron micrograph of a thin-sectioned pellet of unproteolyzed rat heart gap junctions isolated in the presence of PMSF. The figure shows that the pellet was highly enriched in gap junctions having the classical seven-layered structure of such junctions. It also illustrates the coating of fuzzy material on the cytoplasmic surfaces (inset) which is characteristic of unproteolyzed cardiac gap junctions (Manjunath et al., 1984*b*). The alternation between heavily stained and poorly stained regions, previously noted (Manjunath, Goings & Page, 1982, 1984*b*), is unexplained. Typically, gap junctional pellets prepared from four rat hearts contained 15–20 μg of protein.

Figure 2 illustrates the results of SDS-PAGE performed on unproteolyzed cardiac gap junctions prepared in the presence of solid PMSF. When the samples were dissolved in 2.5% SDS, 5% β-mercaptoethanol (Fig. 2*a*, lane *B*), the gel showed a prominent major band at M_r 44,000–47,000 and faint bands at M_r 29,500, 31,000, 34,000, 55,000 and >100,000. We have previously shown that the bands at M_r 29,500, 31,000, and 34,000 result from partial proteolysis of the M_r 44,000–47,000 gap junctional polypeptide (Manjunath et al., 1985) and that the M_r 55,000 and >100,000 bands are nonjunctional contaminants (Manjunath et al., 1984*b*). Lane *C* of Fig. 2*a* shows the electrophoretic results for unproteolyzed rat heart gap junctions dissolved in SDS only, i.e., without β-mercaptoethanol. It is evident that without reduction of disulfide linkages most of the SDS-soluble material failed to enter the gel. Moreover, the material that did enter the stacking gel did not enter the 12.5% separating gel, but instead remained as a sharp band at the 5%/12.5% acrylamide interface. The protein of gap junctions solubilized with SDS without β-mercaptoethanol also failed to enter a 4–30% linear gradient gel (Fig.

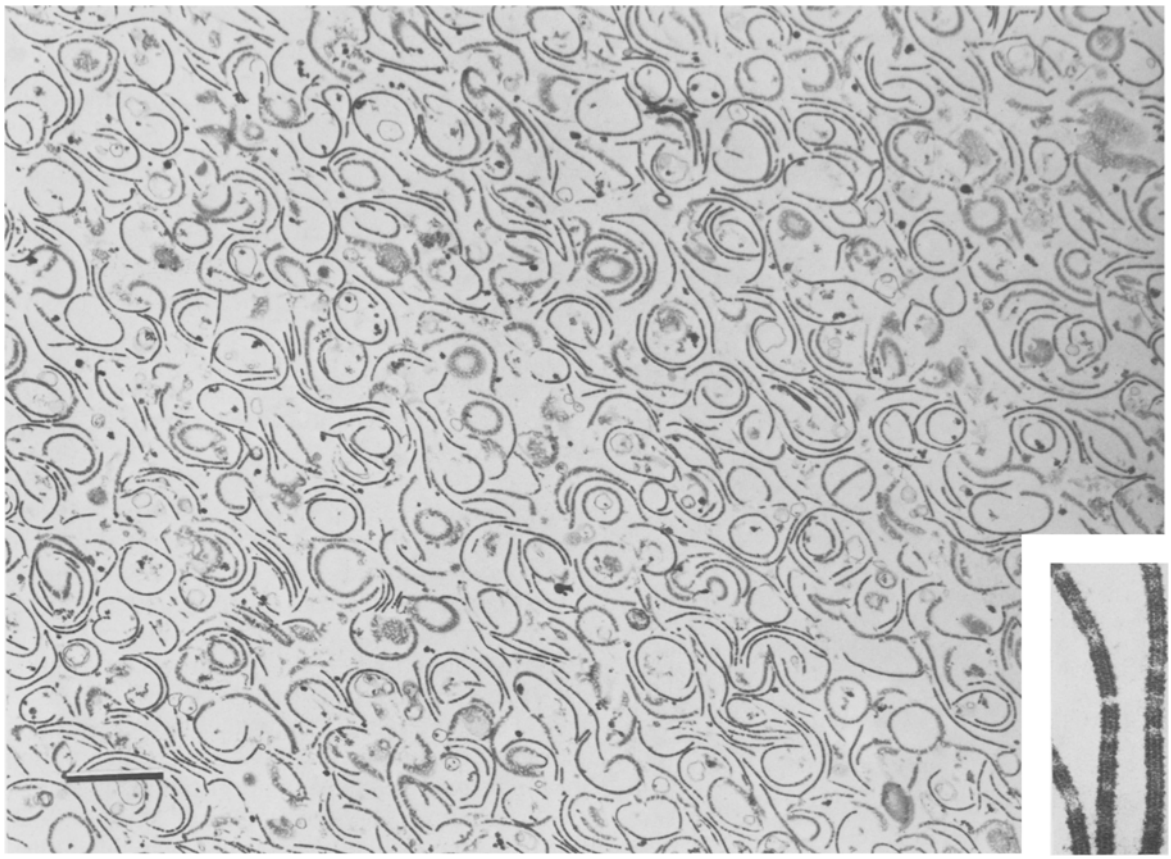

Fig. 1. Electron micrograph of a thin-sectioned pellet of unproteolyzed rat heart gap junctions. Rat heart gap junctions were prepared in the presence of PMSF (1 mM) added directly as a solid. Calibration bar: 1 μm. Inset ($\times 106 \times 10^3$) shows the characteristic fuzzy material covering the cytoplasmic surfaces of the junctions

2*b*, lane *B*) that admits proteins as large as unreduced IgM (M_r 970,000, lane *A*).

Figure 2*b* also illustrates the effect of various concentrations of β-mercaptoethanol on the SDS-PAGE pattern of unproteolyzed rat heart gap junctions. The results show that β-mercaptoethanol, even at the lowest concentration used (0.01%, lane *C*), caused the appearance of the major band at M_r 47,000 and minor bands at M_r 29,500, 31,000, and 34,000. The intensity of the M_r 47,000 band increased markedly as the β-mercaptoethanol concentration in the sample-dissolving buffer was increased to 0.1% (lane *D*) and 5% (lane *E*), respectively. These results show that unproteolyzed rat heart gap junctions contain 47,000-dalton subunits that are interconnected by extensive disulfide bonds and yield structures with relative molecular weights greater than 1 million when dissolved in SDS.

It is conceivable that the protein-protein disulfide bonds present in isolated rat heart gap junctions might have been formed during the isolation procedure due to oxidation of free-SH groups. To rule out this possibility we prepared rat heart gap junctions in the presence of PMSF (1 mM) and *p*-hydroxymercuribenzoate (PHMB, 0.1 mM). We reasoned that PHMB would block the free-SH groups on the proteins and prevent the formation of protein-protein disulfide bonds during isolation of the gap junctional membranes. Figure 3 shows an electron micrograph of a thin-sectioned rat heart gap junctional pellet prepared with PMSF and PHMB. The pellet contained a large number of gap junctions with fuzzy cytoplasmic surfaces (inset); it showed substantial contamination with amorphous material. Figure 4 shows the SDS-PAGE analysis of rat heart gap junctions prepared with PMSF and PHMB. When the pellet was dissolved in SDS in the presence of 5% β-mercaptoethanol (lane *B*), the gel showed multiple protein bands. The most prominent of these was a diffuse band at M_r 44,000–47,000. When the sample was dissolved in SDS without β-mercap-

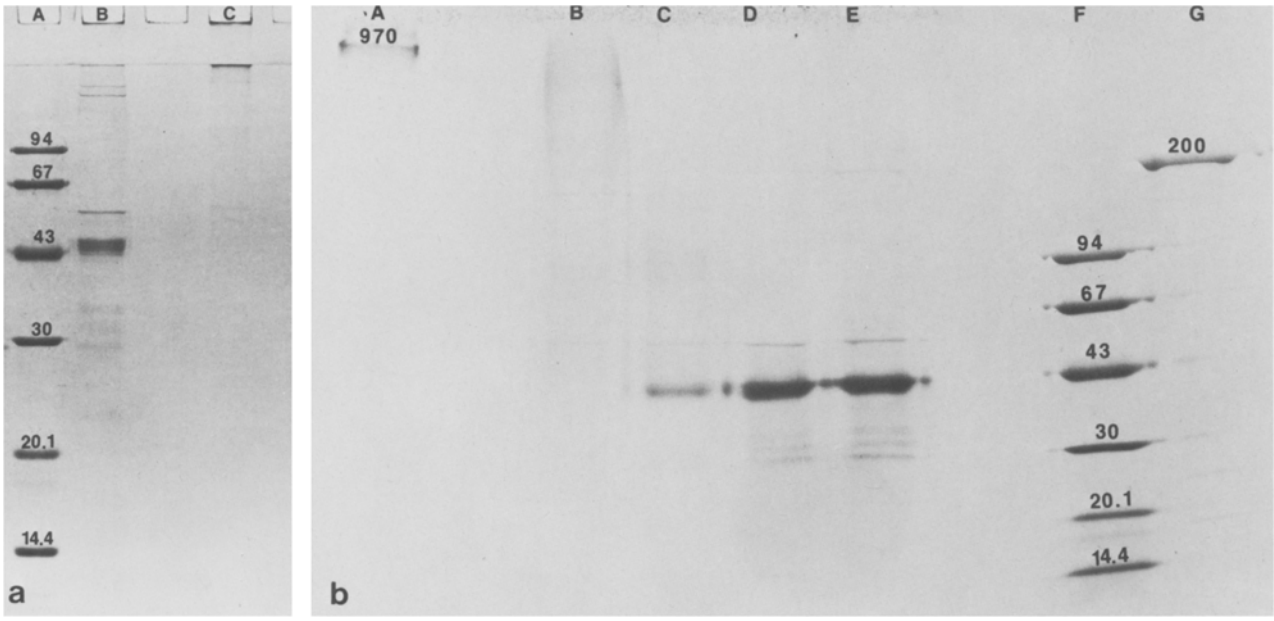

Fig. 2. Effect of β-mercaptoethanol on the SDS-PAGE pattern of unproteolyzed rat heart gap junctions. Gap junctions were prepared in the presence of solid PMSF (1 mM). (*a*) Analysis using 5% stacking and 12.5% separating acrylamide gels. Gap junctional pellets were dissolved at 100°C for 2 min in 2.5% SDS in the presence of 5% β-mercaptoethanol (lane *B*) or in the absence of β-mercaptoethanol (lane *C*). *Lane A:* Reference proteins and molecular weights in kilodaltons. (*b*) Analysis using 3% stacking gel (not shown) and 4–30% linear gradient separating gel. Gap junctional pellets were dissolved at 37°C for 30 min in 2.5% SDS in the absence of β-mercaptoethanol (lane *B*) or in the presence of β-mercaptoethanol at the following concentrations (vol/vol): 0.01% (lane *C*), 0.1% (lane *D*), and 5% (lane *E*). *Lane A:* Unreduced human IgM (M_r 970,000). *Lane F:* Pharmacia low molecular weight kit and molecular weights in kilodaltons. *Lane G:* Rabbit muscle myosin (reduced) and heavy chain M_r in kilodaltons

toethanol (lane *C*), most of the protein was found at the top of the stacking gel and at the interface between the 5% stacking and 12.5% separating gels. The gel also showed that the M_r 44,000–47,000 band and many other major bands were absent. These results suggest that the disulfide bonds connecting the M_r 47,000 subunits are formed before the isolation of gap junctions and are not an artifact of the isolation procedure.

Figure 5 illustrates the effect of reducing disulfide linkages on the SDS-PAGE pattern of *proteolyzed* rat heart gap junctions. We have previously shown that during the proteolytic cleavage of the M_r 14,500–17,500 cytoplasmic surface component which occurs when rat heart gap junctions are isolated without PMSF, the components of the channel protein in the lipid bilayer of the membrane and in the intercellular gap are preserved relatively intact (Manjunath et al., 1984*b*, 1985). When such proteolyzed rat heart gap junctions were dissolved in 2.5% SDS, 5% β-mercaptoethanol, the resulting SDS-PAGE pattern (Fig. 5*a*, lane *B*) showed the single major band at M_r 29,500 which is characteristic for proteolyzed cardiac gap junctions (Manjunath et al., 1984*b*). By contrast, SDS-PAGE of proteolyzed rat heart gap junctions dissolved in 2.5% SDS only (without β-mercaptoethanol) yielded a single major band with an apparent mol wt of 52,000 (Fig. 5*a*, lane *C*). To test whether this increase in molecular weight resulted from heat-induced aggregation of the M_r 29,500 polypeptide, we compared the electrophoretic pattern of proteolyzed junctions dissolved in SDS for 2 min at 100°C (Fig. 5*a*, lane *C*) with that for junctions dissolved for 30 min at room temperature (Fig. 5*b*, lane *B*). The identity of the two patterns indicates that heating the samples in SDS did not produce dimers. Figure 5*c* shows the result of re-electrophoresis of the M_r 52,000 band after reduction (lane *B*) and without reduction (lane *C*) with β-mercaptoethanol. Reduction of the M_r 52,000 band with 5% β-mercaptoethanol followed by re-electrophoresis on a second gel yielded the M_r 29,500 band. This result demonstrates that the M_r 52,000 band is a dimer of the M_r 29,500 peptides in which the monomers are held together by one or more disulfide bonds. (We attribute the apparent discrepancy between the observed M_r 52,000 dimer and the theoretical M_r 59,000 dimer

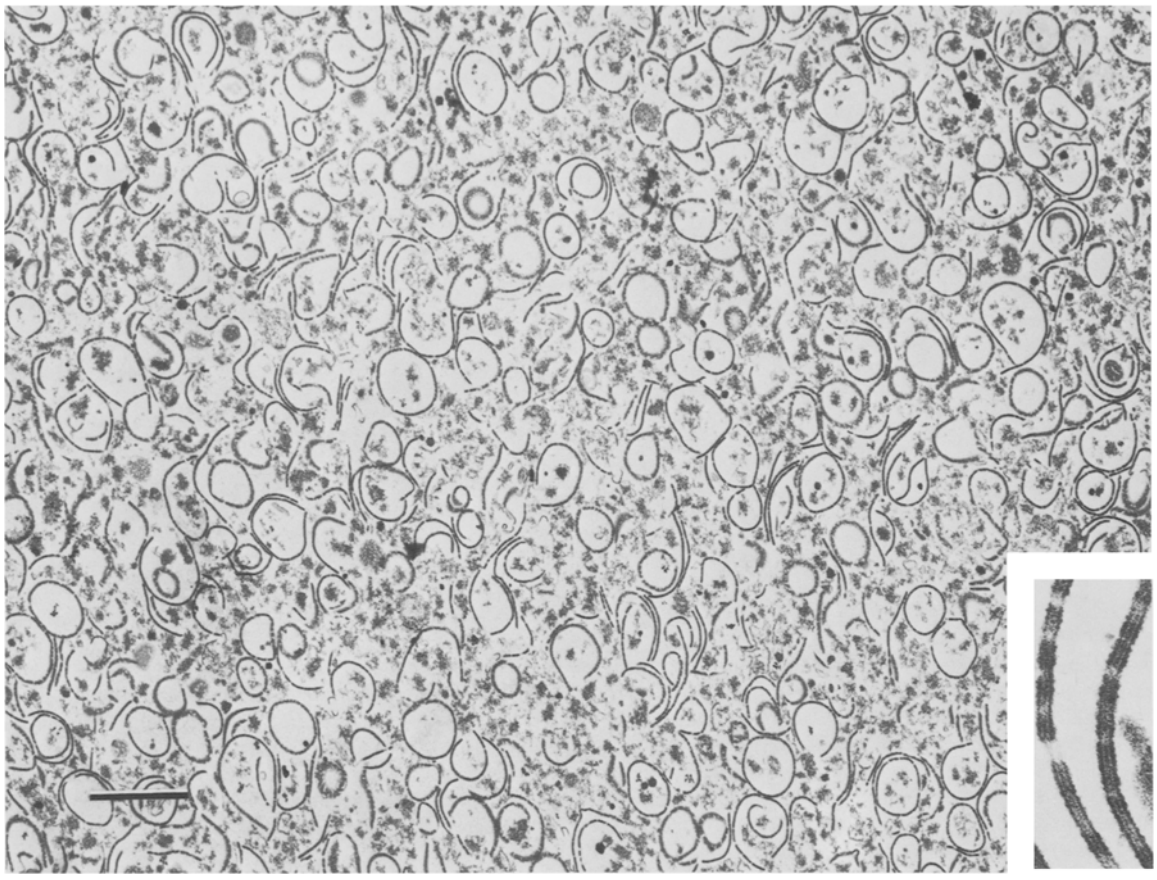

Fig. 3. Electron micrograph of thin-sectioned pellet of rat heart gap junctions prepared in the presence of 1 mM PMSF and 0.1 mM PHMB. The junctions are seen to be mixed with amorphous material. Calibration bar: 1 μm. Inset ($\times 106 \times 10^3$) shows the fuzzy material covering the cytoplasmic surfaces of the junctions

calculated from the M_r 29,500 monomer to increased mobility of peptides in SDS gels in the absence of reducing agents.)

Disulfide Linkages in Liver Gap Junctions

We next looked for evidence of disulfide linkages in rat liver gap junctions that were isolated in the presence of protease inhibitors. Figure 6 is an electron micrograph of thin-sectioned rat liver gap junctions, isolated in the presence of PMSF, para-hydroxymercuribenzoate (PHMB), and leupeptin. The figure shows that the pellet was highly enriched in gap junctions; some contamination with amorphous material was also evident. Although liver junctions showed the characteristic seven-layered structure, they differed from similarly prepared unproteolyzed cardiac gap junctions in lacking a fuzzy coating on their cytoplasmic surfaces (*compare* insets of Figs. 1 and 6).

Figure 7*a* shows the SDS-PAGE patterns of rat liver gap junctions isolated in the presence of PMSF, PHMB, and leupeptin. We have observed that the electrophoretic mobility of proteins in SDS gels is somewhat greater in the absence of β-mercaptoethanol than in its presence. When this difference in mobility is taken into account, the SDS-PAGE patterns for liver junctions solubilized at room temperature in SDS (without β-mercaptoethanol) (Fig. 7*a*, lane *C*) is comparable to that for liver junctions solubilized in 2.5% SDS, 5% β-mercaptoethanol (Fig. 7*a*, lane *B*). Both procedures yielded a major polypeptide of M_r 28,500 and minor bands at M_r 27,000 and 34,500, as well as a diffuse aggregation band at $M_r \sim 47{,}000$. Figure 7*b* shows the SDS-PAGE pattern of rat liver gap junctions prepared in the presence of PMSF and leupeptin but without PHMB. The gel showed essentially the same pattern as in the presence of PHMB (*compare* Fig. 7*a*, lane *B* with Fig. 7*b*, lane *B*), except that the intensity of the M_r 27,000 band was much greater in junctions prepared in the absence of PHMB (Fig. 7*b*, lane *B*). Solubilization of the junc-

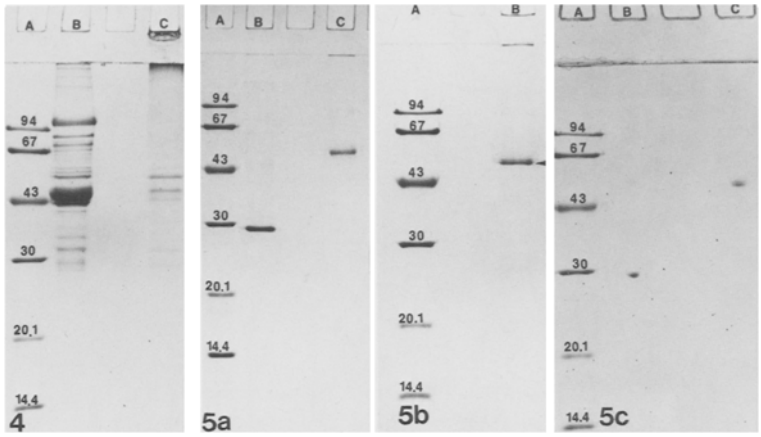

Fig. 4. SDS-PAGE of rat heart gap junctions isolated with PMSF (1 mM) and PHMB (0.1 mM). Gap junctional pellets were dissolved at 100°C for 2 min in 2.5% SDS in the presence of 5% β-mercaptoethanol (lane *B*) or in the absence of β-mercaptoethanol (lane *C*) and electrophoresed on a 5% stacking gel and 12.5% separating gel. *Lane A:* Reference proteins and molecular weights (kD)

Fig. 5. SDS-PAGE analysis of proteolyzed rat heart gap junctions. Proteolyzed gap junctions were prepared in the absence of protease inhibitors as described under Materials and Methods. Electrophoresis was carried out using 5% stacking gels and 12.5% separating gels. (*a*) Gap junctional pellets were dissolved at 100°C for 2 min in 2.5% SDS in the presence of 5% β-mercaptoethanol (lane *B*) or in the absence of β-mercaptoethanol (lane *C*). (*b*) Gap junctional pellets were dissolved at 22°C for 30 min in 2.5% SDS in the absence of β-mercaptoethanol (lane *B*). Arrow indicates the position of the M_r 52,000 band. (*c*) Re-electrophoresis of the M_r 52,000 polypeptide before and after reduction with 5% β-mercaptoethanol. Rat heart gap junctional pellets were dissolved in 2.5% SDS and electrophoresed on a 12.5% SDS gel. The M_r 52,000 band was visualized by staining the gel with 4 M Na acetate (Higgins & Dahmus, 1979). The gel slice containing the band was cut out, washed with water, and cut into two pieces. The pieces were equilibrated for 1 hr at 22°C with 50 μl of sample buffer containing 2.5% SDS, either with 5% β-mercaptoethanol (lane *B*) or without β-mercaptoethanol (lane *C*). The gel pieces were then placed in different wells of a second SDS gel (5% stacking gel and 12.5% separating gel) and electrophoresed along with standard proteins. Lanes *A* in Fig. 5*a–c* represent standard proteins

tions prepared in the absence of PHMB in SDS in the presence of β-mercaptoethanol (Fig. 7*b*, lane *B*), or in the absence of β-mercaptoethanol (Fig. 7*b*, lane *C*) did not alter the SDS-PAGE pattern. These observations rule out the formation of disulfide bonds during isolation of liver junctions in the absence of PHMB. The results also indicate that the inter-chain disulfide bridges that link the subunits of cardiac connexons were absent in liver junctions.

Relationship of Disulfide Linkages to Solubility of Cardiac Gap Junctions in Detergent

The experiments presented in Fig. 2 suggested that the presence of disulfide bonds makes cardiac gap junctions resistant to solubilization in detergents. To study this phenomenon further, we examined the effects of cleaving these disulfide bonds on the detergent solubility of rat heart gap junctions. For this purpose we looked at the effect of 0.3% deoxycholate on junctions in which we had reduced the disulfide bonds. We chose this experimental design because our previous studies had shown that mammalian cardiac gap junctions are stable almost indefinitely at this deoxycholate concentration and that this detergent does not disrupt the end-to-end contact between connexons in different membranes (Manjunath et al., 1984*b*).

Figure 8 shows the effect of 0.3% deoxycholate in 5 mM Tris (pH 10) containing 5% β-mercaptoethanol on unproteolyzed rat heart gap junctions. The junctions were suspended in this medium by repeated pipetting and incubated at room temperature for 30 min. The suspension was then centrifuged at room temperature in a Beckman Airfuge at 160,000× *g* for 45 min. We assumed that the resultant supernatant was deoxycholate-soluble material. This supernatant was concentrated to 40 μl. The pellet and the concentrated supernatant were subjected to SDS-PAGE after exposure to 2.5% SDS, 5% β-mercaptoethanol.

When unproteolyzed cardiac gap junctions were treated with 0.3% deoxycholate, 5% β-mercaptoethanol, subsequent centrifugation yielded no detectable pellet. SDS-PAGE of the supernatant (Fig. 8, lane *B*) showed a major band at M_r 44,000–47,000, whereas electrophoresis of the material remaining in the tube yielded only trace amounts of protein (Fig. 8, lane *C*).

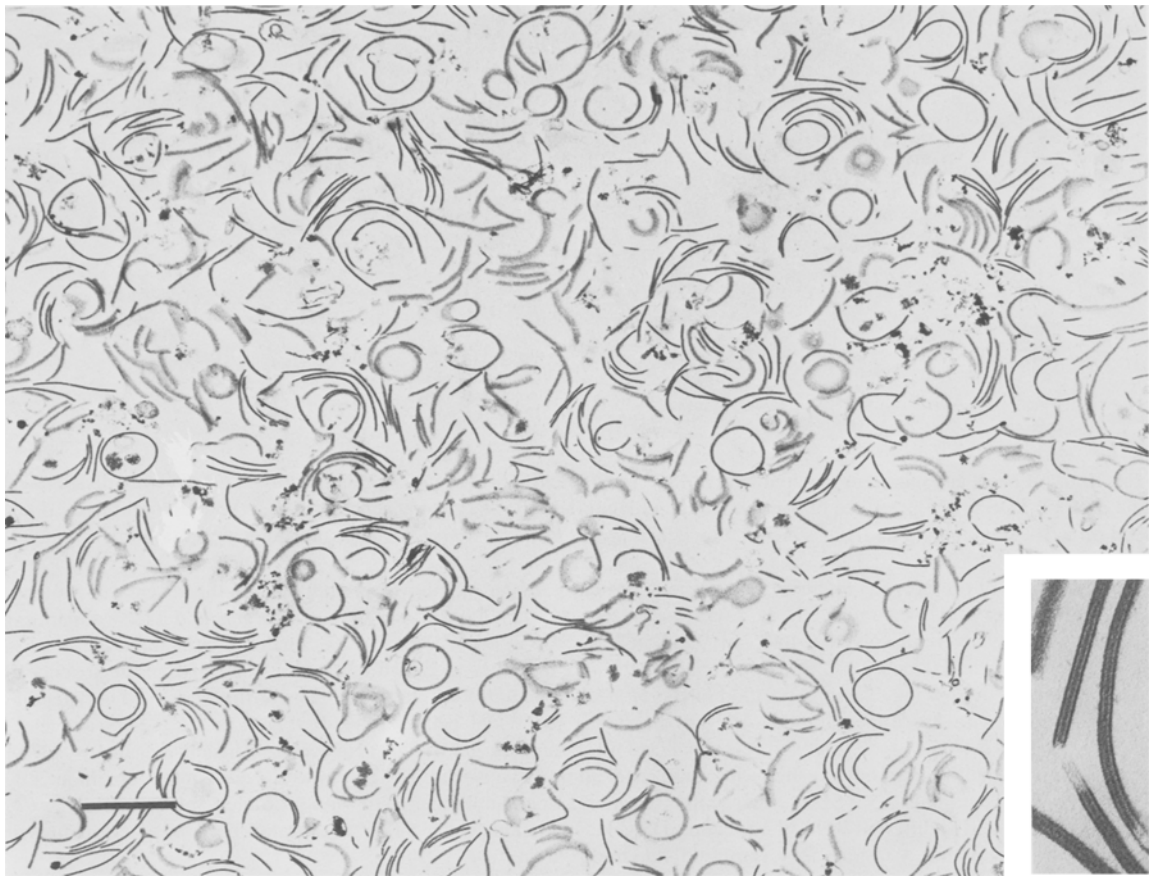

Fig. 6. Electron micrograph of thin-sectioned pellet of unproteolyzed rat liver gap junctions. Rat liver gap junctions were prepared in the presence of PMSF, PHMB, and leupeptin as described under Materials and Methods. Calibration bar: 1 μm. Inset ($\times 106 \times 10^3$) shows the smooth appearance of the cytoplasmic surfaces of the junctions

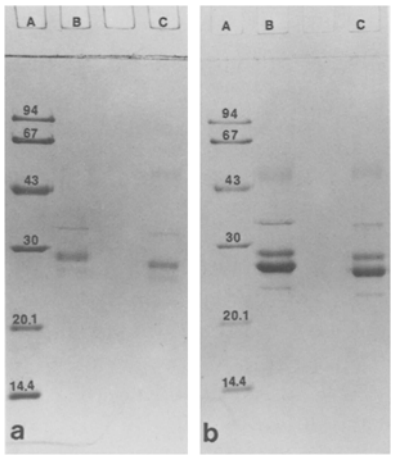

Fig. 7. SDS-PAGE analysis of isolated rat liver gap junctions. (*a*) Rat liver gap junctions were isolated in the presence of PMSF, PHMB, and leupeptin. The junctional pellets were dissolved in 2.5% SDS at 22°C for 30 min in the presence of 5% β-mercaptoethanol (lane *B*) or in the absence of β-mercaptoethanol (lane *C*). (*b*) Rat liver gap junctions were isolated with PMSF and leupeptin but without PHMB. The pellets were dissolved in 2.5% SDS at 22°C for 30 min with 5% β-mercaptoethanol (lane *B*) or without β-mercaptoethanol (lane *C*). Lanes *A* in both *a* and *b* show standard proteins with molecular weights in kilodaltons

Figure 9 illustrates the effect of varying the β-mercaptoethanol concentration on the solubilization of unproteolyzed rat heart gap junctions by deoxycholate. For this purpose, four gap junctional pellets (each pellet prepared from four rat hearts) were extracted for 30 min at room temperature in 0.3% deoxycholate, 5 mM Tris (pH 10) containing progressively higher concentrations (vol/vol) of β-mercaptoethanol as follows: 0.001% (lanes *B* and *C*), 0.01% (lanes *D* and *E*), 0.1% (lanes *F* and *G*) and 1.0% (lanes *H* and *I*). The suspensions were then centrifuged in the Airfuge at 160,000$\times$ *g* for 45 min to separate the pellets from the solubilized supernatants. The pellets and supernatants were treated with 2.5% SDS, 5% β-mercaptoethanol and analyzed by SDS-PAGE. Comparison of lanes *C*, *E*, *G*, and *I* of Fig. 9 indicates that the amount of gap junctional protein solubilized and therefore appearing in the supernate increased as the concentration of β-mercaptoethanol was progressively raised. Solubilization was, however, incomplete, even at a β-mercaptoethanol concentration of 1%; nearly complete solubilization at room temperature was achieved only at a β-mercaptoethanol concentra-

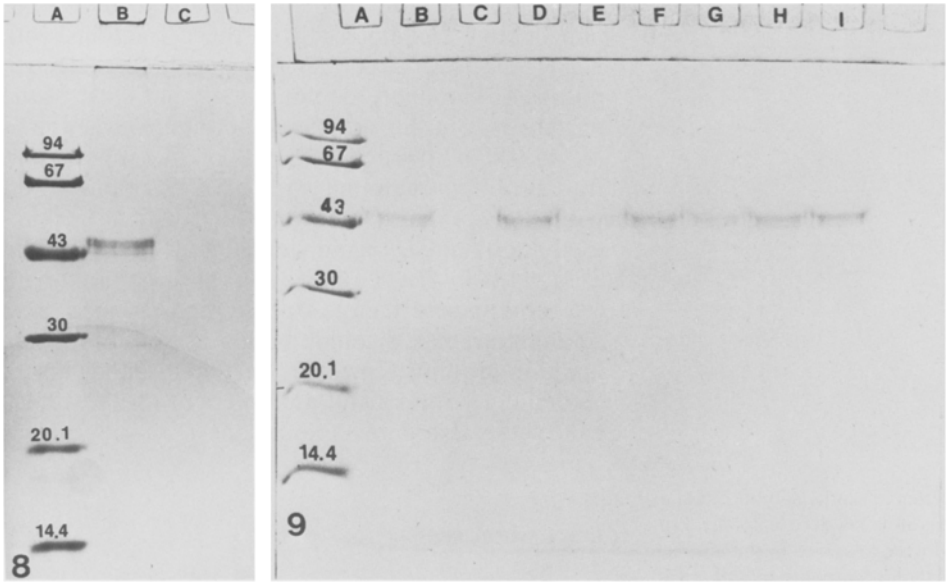

Fig. 8. Solubilization of unproteolyzed rat heart gap junctions in 0.3% deoxycholate in the presence of 5% β-mercaptoethanol. Unproteolyzed gap junctions were prepared from four rat hearts in the presence of PMSF as described under Materials and Methods. The final pellet was incubated at room temperature for 30 min in 200 μl of 5 mM Tris, pH 10, containing 0.3% Na deoxycholate and 5% (vol/vol) β-mercaptoethanol. After the incubation the suspension was centrifuged in a Beckman Airfuge at 160,000× *g* for 45 min to separate the soluble supernatant from the insoluble pellet. The supernatant fraction was concentrated to 40 μl using a Centricon-30 microconcentrator. Both the pellet and the supernatant proteins were solubilized at 100°C for 2 min in 2.5% SDS, 5% β-mercaptoethanol and electrophoresed on a 12.5% acrylamide gel. *Lane A:* Standard proteins. *Lane B:* Deoxycholate-soluble fraction. *Lane C:* Deoxycholate-insoluble fraction

Fig. 9. Effect of β-mercaptoethanol concentration on the solubilization of unproteolyzed rat heart gap junctions in deoxycholate. Unproteolyzed rat heart gap junctional pellets were incubated at room temperature for 30 min in 200 μl of 5 mM Tris, pH 10, containing 0.3% deoxycholate, in the presence of the following concentrations of β-mercaptoethanol (vol/vol): 0.001% (lanes *B, C*); 0.01% (lanes *D, E*); 0.1% (lanes *F, G*); 1% (lanes *H, I*). After the incubation the soluble and insoluble fractions were separated and electrophoresed as in Fig. 8. *Lane A:* Standard proteins. *Lanes B, D, F, and H:* Deoxycholate-insoluble fractions. *Lanes C, E, G, and I:* Deoxycholate-soluble fractions

tion of 5% (*compare* Fig. 9, lanes *H* and *I*, with Fig. 8, lanes *B* and *C*).

The experiments on proteolyzed rat heart gap junctions shown in Fig. 5 indicate the presence of disulfide bridges linking subunits of M_r 29,500. These experiments do not, however, distinguish dimers formed by disulfide bonds between subunits of the same connexon from dimers formed by disulfide bonds between subunits of adjacent connexons (i.e., between subunits of adjacent hexameric half-channel assemblies). We have previously shown that the two connexons of the same cell-to-cell channel are linked across the intercellular gap by hydrogen bonds, not covalent bonds; thus there are no disulfide linkages across the gap (Manjunath et al., 1984*b*). Since the ultrastructure of proteolyzed rat heart gap junctions is not changed by deoxycholate, and since deoxycholate does not solubilize such junctions, we examined whether this structural stability and resistance to solubilization result from the presence of residual inter-connexon disulfide bridges between proteolyzed connexons on the same side of the junction. For this purpose we exposed proteolyzed rat heart gap junctions for 30 min at room temperature to 0.3% deoxycholate in 5 mM Tris (pH 10) containing 5% β-mercaptoethanol. The insoluble material was then collected by centrifugation in the Airfuge. The resultant pellet and supernatant fractions were analyzed by SDS-PAGE (Fig. 10). Figure 10 shows that almost all of the M_r 29,500 protein was present in the pellet (lane *C*) and that the supernatant (lane *B*) contained only trace amounts of protein; i.e., reduction of disulfide bonds failed to solubilize proteolyzed rat heart gap junctions. Since the resistance of proteolyzed junctions to solubilization by deoxycholate is not due to disulfide bridges between connexons, it is presumably related to the greater hydrophobicity of proteolyzed junctions as compared to the unproteolyzed junctions.

To understand the role of disulfide linkages in unproteolyzed rat heart gap junctions, we performed an experiment in which the reduction of disulfide bridges and the solubilization of the junctions by detergent were carried out separately and sequentially (Fig. 11). In order to stabilize the

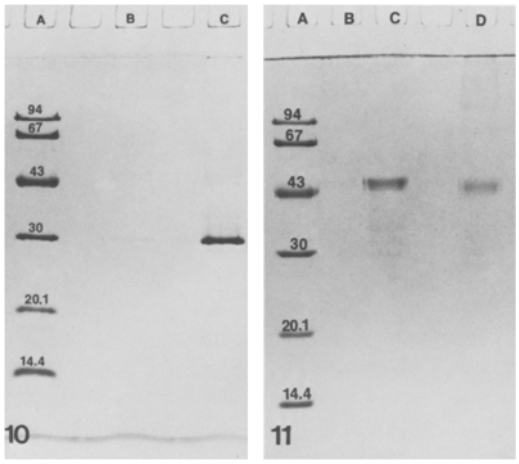

Fig. 10. Effect of 5% β-mercaptoethanol on the solubilization of proteolyzed rat heart gap junctions in 0.3% deoxycholate. Proteolyzed rat cardiac gap junctions were prepared in the absence of protease inhibitors as described under Materials and Methods. The final pellet was incubated at room temperature for 30 min in 5 mM Tris, pH 10, containing 0.3% deoxycholate and 5% β-mercaptoethanol. After the incubation the soluble and insoluble fractions were prepared and electrophoresed as described in Fig. 8. *Lane A:* Standard proteins. *Lane B:* Deoxycholate-soluble material. *Lane C:* Deoxycholate-insoluble material

Fig. 11. Solubilization of unproteolyzed rat heart gap junctions in 0.3% deoxycholate following reduction and alkylation in the absence of detergent. Unproteolyzed rat heart gap junctions prepared in presence of PMSF were reduced with 5% β-mercaptoethanol and alkylated with 0.1 M iodoacetamide in the absence of deoxycholate as described under Experimental Procedures. The membranes were then incubated in 5 mM Tris, pH 10, containing 0.3% deoxycholate at room temperature for 30 min. After incubation the soluble and insoluble fractions were prepared by Airfuge centrifugation as described before (Fig. 8). *Lane A:* Standard proteins. *Lane B:* Deoxycholate-insoluble material solubilized in 2.5% SDS, 5% β-mercaptoethanol before electrophoresis. *Lane C:* Deoxycholate-soluble material solubilized in 2.5% SDS, 5% β-mercaptoethanol before electrophoresis. *Lane D:* Deoxycholate-soluble material solubilized in 2.5% SDS without β-mercaptoethanol before electrophoresis

highly reactive SH groups newly generated by reduction with β-mercaptoethanol, we alkylated them with iodoacetamide. The procedure was to reduce unproteolyzed rat heart gap junctions by incubating them for 30 min at room temperature in 5% β-mercaptoethanol in 5 mM Tris (pH 10), to collect the reduced membranes by centrifugation in the Airfuge and to suspend them immediately in 0.1 M iodoacetamide in 5 mM Tris (pH 10), to expose the resultant reduced and alkylated membranes at room temperature for 30 min to 0.3% deoxycholate in 5 mM Tris, and to centrifuge them in the Airfuge to separate the soluble and insoluble fractions.

Figure 11 shows the SDS-PAGE patterns of the pellet (lane *B*) and the supernatant (lane *C*) obtained after treating each of these fractions with 2.5% SDS, 5% β-mercaptoethanol before electrophoresis. The figure demonstrates that most of the protein was in the soluble (supernatant) fraction at M_r 44,000–47,000. SDS-PAGE of the supernatant fraction after treatment with 2.5% SDS only (i.e., without reducing it a second time with β-mercaptoethanol) (lane *D*) again yielded most of the protein at M_r 44,000–47,000, indicating that treatment with 5% β-mercaptoethanol at pH 10 had reduced most of the interchain disulfide bonds present in the gap junction protein. However, very faint bands corresponding to dimers and multimers of the M_r 47,000 band were also detectable in lane *D*.

Discussion

Before discussing specific aspects of this work, it is useful to summarize the most significant new observations made in this paper. We have shown that (i) the channel proteins of unproteolyzed rat heart gap junctions exist as multimers ($M_r > 10^6$) whose subunits (M_r 44,000–47,000) are linked by extensive disulfide bonds between subunits of the same connexon and between subunits of adjacent connexons; (ii) the protein of unproteolyzed rat heart gap junctions can be solubilized in Na deoxycholate after reducing the disulfide bonds with β-mercaptoethanol; (iii) solubilization of rat heart gap junctions in deoxycholate also requires the hydrophilic groups present in the M_r 14,500–17,500 cytoplasmic surface component; (iv) after proteolytic cleavage of their cytoplasmic surface component, rat heart gap junctions are no longer soluble in deoxycholate even after reduction of the remaining disulfide bonds; (v) unless reduced with β-mercaptoethanol, such proteolyzed rat heart gap junctions exist as dimers (M_r 52,000) of the M_r 29,500 monomer; (vi) unproteolyzed rat liver gap junctions lack interchain disulfide bonds and their subunits (~ M_r 28,500) are held together by noncovalent interactions only; and (vii) the new observations (i–iii) were made possible by a methodological advance in our procedure for isolating gap junctions (the use of solid PMSF), which reduced contamination of rat heart gap junctions with nonjunctional proteins.

Disulfide Linkages in Isolated Rat Heart Gap Junctions

Analysis of the SDS-PAGE patterns for unproteolyzed rat heart gap junctions isolated in presence of PMSF and reduced with β-mercaptoethanol

showed that the entry of the protein into the gel and the appearance of the M_r 44,000–47,000 band of the connexon subunit require reduction of disulfide linkages; the unreduced, unproteolyzed gap junctional protein is therefore a multimer held together by disulfide bonds. Moreover, the finding that the unreduced protein from unproteolyzed rat heart gap junctions solubilized with SDS without β-mercaptoethanol fails to enter 4–30% gradient gels (which admit the M_r ~ one million standard protein) suggests that the multimers have relative molecular weights exceeding one million. Our previous studies (Manjunath et al., 1984*a*,*b*) have shown that the two connexons originating from the two cells whose plasma membranes form the gap junction interlock in the gap via hydrogen bonds that can be broken with 8 M urea, not by covalent bonds; hence disulfide bonds are not involved in linking the two connexons across the gap to form the cell-to-cell channel. We are therefore justified in assuming that each of the above-described SDS-solubilized multimers of the gap junctional M_r 44,000–47,000 subunit which we have detected in gradient gels was made up of disulfide-bonded subunits derived from one or the other plasma membrane forming the junction and not from both plasma membranes. Based on this assumption, and taking into account that each connexon is made up of six identical subunits of M_r 47,000, we obtained a M_r of 282,000 for the assembled connexon. From this we calculated that no fewer than three connexons must be thus linked by disulfide bridges to yield a structure large enough to be excluded by 4% acrylamide.

Proteolyzed rat heart gap junctions whose subunit mol wt is 29,500 lack the M_r 14,500–17,500 cytoplasmic surface component (Manjunath et al., 1984*b*). SDS-PAGE of proteolyzed heart junctions in which disulfide bonds have not been reduced with β-mercaptoethanol yields a single band of M_r 52,000. Reduction of the disulfide linkages in this band with β-mercaptoethanol, followed by re-electrophoresis, converts it into the M_r 29,500 polypeptide characteristic of proteolyzed rat heart gap junctions. The apparent discrepancy between an M_r of 52,000 for the dimer and of 29,500 for the monomer is accounted for by the increased mobility of unreduced peptides in SDS gels (*also see* Goodenough, 1974).

Disulfide linkages in proteolyzed rat heart gap junctions could be present in the lipid bilayer, in the interstitial gap, or in both of these locations. The finding that the proteolyzed subunits occur as dimers suggests that disulfide bridges in the lipid bilayer and gap are restricted to pairs of subunits. Figure 12 is a diagram illustrating an *en face* view of three of the many possible disulfide bond distributions that are compatible with the finding of a dimer after SDS-PAGE of unreduced, proteolyzed junctions. The three arrangements shown have in common the simplest assumption that each proteolyzed subunit is connected by a single disulfide bond (represented by the dotted line) to one other subunit; the possibility that some or all of the symbols might represent two or more disulfide bonds in parallel cannot be ruled out without additional data.

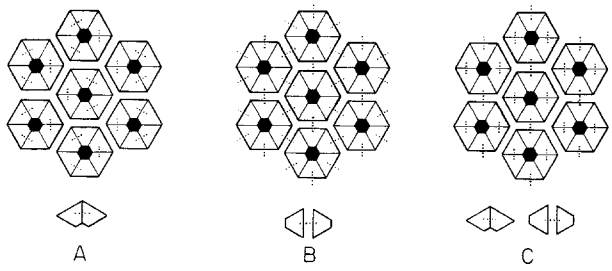

Fig. 12. Schematic diagram of an *en face* view of proteolyzed rat cardiac gap junctions showing the possible positions of disulfide bonds. *A–C* all show rosettes of seven connexons represented by the seven hexagons. The central aqueous channel is represented by the small black hexagon in the center of each large hexagon. The channel is delineated by the smaller of the parallel sides of the six trapezoids which represent the six subunits of the connexon. The disulfide bonds connecting the junctional subunits are represented by dotted lines. The structures shown under each connexon rosette represent the disulfide-bonded pairs of subunits that would be released when the connexons are dissolved in SDS. (*A*) Only intra-connexon disulfide bonds are present. (*B*) Only inter-connexon disulfide bonds are present. (*C*) Both inter-connexon and intra-connexon bonds are present

Each of the arrangements in Fig. 12*A–C* shows rosettes of seven connexons represented by seven hexagons. Each connexon (hexagon) is made up of six subunits represented by trapezoids. The smaller of the parallel sides of the six trapezoids delineate the black hexagon at the center of the connexon which represents the cell-to-cell channel cut in face view at some point in the lipid bilayer or in the intercellular gap. Figure 12*A* shows three disulfide bridges per connexon, connecting subunits of the same connexon; i.e., all disulfide bonds are located within the same connexon. In Fig. 12*B* each connexon is connected by one disulfide bridge to each of its six neighboring connexons, an arrangement whereby disulfide linkages are located between connexons, not within them. Figure 12*C* shows one of many possible arrangements that contain disulfide bridges both between subunits of the same connexon (intra-connexon linkages) and between subunits of adjacent connexons (interconnexon linkages).

That multimers made up of three or more unproteolyzed connexons are stable in the presence of

SDS suggests that all unproteolyzed subunits of the connexon are linked by intra-connexon disulfide bonds and that each connexon is linked to one or more neighboring connexons by inter-connexon disulfide bonds. Thus, unlike the example of Fig. 12*A*, at least five intra-connexon disulfide bonds would be required to prevent dissociation of the connexon subunits by SDS; and, for the simplest case of a "trimer" of three connexons, a connexon would be linked to one other connexon by one inter-connexon disulfide bond or to two other connexons by two inter-connexon disulfide bonds. The minimal number of intra-connexon disulfide bonds per connexon present in the cytoplasmic surface component of the connexon corresponding to Fig. 12 would be five minus the number present in the connexons whose cytoplasmic surface component has been lost by proteolysis; i.e., 2 for Fig. 12*A*, 5 for Fig. 12*B*, and 3 for Fig. 12*C*. Moreover, to form a connexon trimer that is not dissociated by SDS, the cytoplasmic surface component of the unproteolyzed connexons corresponding to the arrangement in Fig. 12*A* would have to have a minimum of either one or two inter-connexon disulfide bridges per connexon.

Absence of Intra-Connexon and Inter-Connexon Disulfide Linkages in Rat Liver Gap Junctions

The SDS-PAGE patterns of rat liver gap junctions are similar regardless of whether the junctions are dissolved in SDS with β-mercaptoethanol or without β-mercaptoethanol. Rat liver junctions thus appear to lack both inter-connexon and intra-connexon inter-chain disulfide linkages. However, our data permit no inferences about the presence or absence of disulfide linkages within individual subunits of either liver or heart gap junctions. The small decrease in mobility of the liver gap junctional protein in the gel when β-mercaptoethanol was present is probably due to the fully extended form of the gap junctional protein in its reduced state. We have observed a similar retardation in mobility of all of the standard proteins except phosphorylase *b* and carbonic anhydrase (data not presented). Our finding that interchain disulfide bonds are absent in hepatic junctions agrees with published findings for gap junctions from rat (Hertzberg & Gilula, 1979) and mouse liver (Henderson et al., 1979), both isolated without added proteases. Goodenough's earlier description of interchain disulfide bonds linking the "connexin A and B" subunits of mouse liver gap junctions isolated with "collagenase" and hyaluronidase (Goodenough, 1974) is difficult to interpret because of the multiple proteolytic enzymes present in the impure collagenase. These enzymes might have converted disulfide bonds within a polypeptide chain into interchain disulfide bonds by proteolysis. Like liver gap junctions, bovine MIP-26 also appears to lack inter-subunit disulfide bonds (Bok, Dockstader & Horwitz, 1982).

We have previously reported that the ultrastructure and subunit molecular weights of gap junctions isolated from rat hearts differ from those isolated from rat liver (Manjunath et al., 1984*a*,*b*; Shibata et al., 1985). Our new finding—the presence of inter-connexon disulfide bridges in unproteolyzed rat heart junctions and their absence in unproteolyzed rat liver junctions—suggests a further difference in the quarternary structure of rat heart and liver gap junctional proteins. The two different proteins do, however, have some homologous amino acid sequences. For example, Nicholson et al. (1985) have reported that their preliminary amino acid sequencing data show 43% homology between the first 32 amino acids in the amino terminal region of rat liver and rat heart gap junctions. Similarly, Hertzberg and Skibbens (1984) recently found that antibodies produced against a M_r 27,000 gap junctional peptide from rat liver bind to membrane proteins from rat heart as determined by immunofluorescence microscopy and immunoblot analysis. The injection of these antibodies against liver gap junctional protein into freshly dissociated myocardial cells also blocked the function of the cell-to-cell channels between such cells (Hertzberg, Spray & Bennett, 1985).

Solubilization of Cardiac Gap Junctions

Resistance to solubilization in detergents has been found to be a characteristic property of gap junctions isolated from liver, heart, and lens. While this resistance has been a serious obstacle to the study of the protein chemistry of gap junctional channels, it has been useful for separating gap junctions from other membranes. To date, gap junctions isolated from liver and heart have been solubilizable only in SDS; gap junctions isolated from lens have been solubilized in the non-ionic detergents octylglucoside (Horwitz & Bok, 1983) and octylpolyoxyethylene (Girsch & Peracchia, 1985). In our previous work on heart and liver gap junctions (Manjunath et al., 1982, 1984*a*,*b*) we introduced the use of Na deoxycholate for keeping gap junctional membranes from aggregating during sucrose density gradient centrifugation.

In this paper we show that unproteolyzed rat heart gap junctions are prevented from dissolving in deoxycholate by the extensive disulfide linkages be-

tween connexons. Even SDS, when used alone, fails to dissociate unproteolyzed rat heart gap junctions into single connexons (calculated M_r approximately 282,000) or into single subunits of the connexon (M_r about 47,000). It is necessary to reduce the inter-connexon and intra-connexon disulfide bonds before or during exposure to SDS to achieve such dissociation. Once the disulfide bridges between connexons are reduced, the junctions readily dissolve at room temperature in Na deoxycholate, a much milder detergent than SDS. Furthermore, the cleavage of disulfide bonds between subunits of the same connexon may produce conformational changes in the connexon that favor its solubilization in deoxycholate. Moreover, unlike SDS, deoxycholate preserves the interactions (hydrogen bonds) by which connexons interlock in the "gap" to form cell-to-cell channels (Manjunath et al., 1984*a*), i.e., it does not produce hemijunctions.

Since multimers of three or more connexons linked by disulfide bonds are very large, it is pertinent to define what we mean by "solubilization." In this study we have separated the detergent soluble proteins from insoluble residues by centrifugation for 45 min at 160,000× *g* in a medium of low density (5 mM Tris, pH 10, containing 0.3% deoxycholate). These conditions meet accepted standards for the solubilization of membrane proteins (*see* Hjelmeland & Chrambach, 1984). We then analyzed the soluble and insoluble fractions by SDS electrophoresis to determine the degree of solubilization and the molecular weights of the protein species solubilized.

Solubilization of depolymerized rat heart gap junctional protein by Na deoxycholate is selective in two respects. First, this detergent does not solubilize many of the nongap junctional proteins present in the gap junction-enriched membrane pellet even after reduction with β-mercaptoethanol. Secondly, even after reduction with β-mercaptoethanol, Na deoxycholate does not solubilize proteolyzed rat heart gap junctions that have lost their cytoplasmic surface components due to the action of the alkaline serine protease from mast cell granules (Manjunath et al., 1984*b*, 1985). We interpret this finding to mean that the cytoplasmic surface component makes the unproteolyzed cardiac gap junctional channels relatively hydrophilic and soluble, whereas the proteolytic product remaining after cleavage of this component is more hydrophobic and therefore less soluble. Partially proteolyzed rat heart gap junctions are, however, soluble in deoxycholate after reduction with β-mercaptoethanol, as evidenced by the presence of faint bands at M_r 29,500, 31,000, and 34,000 in the deoxycholate-soluble fraction (Fig. 11).

Functional Implications of the Sulfhydryl Groups in Cardiac Gap Junctions

The myocardial cells of rat ventricles undergo frequently repeated and severe mechanical perturbations with each contraction and relaxation cycle of the heart. At the same time, relatively large and frequently repeated currents (carried chiefly by potassium ions) must flow rapidly through the cell-to-cell channels of cardiac gap junctions to permit the propagation of the action potential throughout the heart. By contrast, the hepatocytes of rat liver are not normally subjected to comparable mechanical stress and the currents that flow through their gap junctional channels are small and infrequent compared to those in the heart. On the basis of these differences, we speculate that in cardiac gap junctions one role of the extensive sulfhydryl bonding between connexons and between subunits of the same connexon may be to stabilize the connexon arrays in the plane of the plasma membrane. We point out that gap junctions between ventricular myocytes in adult mammalian hearts tend to be oriented parallel to the long axis of the cells (Page & McCallister, 1973), so that the cell-to-cell channels are oriented at right angles to this axis. The perturbing forces associated with contraction or passive stretch of the cells are thus in the direction of the plane of the membrane, not in the direction that would lead to a pulling apart at the gap. For a general discussion of considerations important for the mechanical stability of gap junctions, *see* Loewenstein (1981).

The disulfide bonds between cardiac connexons may also play a role in the disassembly of the array of gap junctional channels in the junctional plaques. The first step in disassembling this array might be the separation of the connections between channels by cleavage of their inter-connexon disulfide bonds. The channels could be separated by reducing the inter-connexon disulfide bonds and by the formation of mixed disulfides of the junctional protein and a low molecular weight thiol. Mannervik and Axelsson (1980) have identified a cytoplasmic thioltransferase in rat liver that catalyzes the reversible formation of mixed disulfides of proteins and thiols of low molecular weight.

Reactions involving disulfide bonds may also be implicated in regulating the permeability of cardiac gap junctional channels. The activity of several biologically important proteins is modulated by the formation of mixed disulfides through thiol-disulfide interchange (Kosower & Kosower, 1978; Mannervik & Axelsson, 1980; Klein & Namboodiri, 1982). Recently Klein and Namboodiri (1982) and Gilbert (1982) have proposed that cAMP may change cellu-

lar metabolism by altering the thiol/disulfide ratio in the cytoplasm; the change in this ratio in turn modulates the activity of key metabolic enzymes through the thiol-disulfide interchange reaction. Several observations suggest that a similar mechanism may regulate the permeability of cardiac gap junctional channels: (i) most of the sulfhydryl groups of rat heart gap junctional protein are in the cytoplasmic surface component. These groups are therefore readily accessible to reduction or thiol-disulfide exchange requiring cytoplasmic enzymes and/or thiols; (ii) the formation of mixed disulfides by proteins and low molecular weight thiols, recently demonstrated in cultured rat heart cells, has been shown to vary with the glutathione/glutathione disulfide ratio of the cells (Grimm, Collison, Fisher & Thomas, 1985); and (iii) cAMP enhances gap junctional communication in several types of cultured mammalian cells (Azarnia, Dahl & Loewenstein, 1981; Flagg-Newton & Loewenstein, 1981; Radu, Dahl & Loewenstein, 1982; Wiener & Loewenstein, 1983), as well as between cardiac cells (De Mello, 1984). The enhancement of junctional communication between cardiac cells does not appear to involve changes in cytoplasmic pH or ionized calcium activity (De Mello, 1984).

Methodological Considerations

The experiments described in this paper required both a marked increase in the purity of the gap junctional preparation and the ability to scale up the isolation procedure. These improvements were made possible by a relatively minor change in our published isolation method for unproteolyzed cardiac gap junctions (Manjunath et al., 1984*b*), the substitution of solid PMSF for an ethanolic solution of PMSF. In this way we were able to avoid precipitation of contaminating actomyosin polymers and to dispense with the sucrose-KI gradient centrifugation previously needed to remove these contaminants (Manjunath et al., 1985).

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