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Cross-Linking of Cardiac Gap Junction Connexons by Thiol/Disulfide Exchanges

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Summary. SDS-polyacrylamide gel electrophoresis and immunoblotting were used to investigate inter- and intramolecular disulfide bonds to connexin 43 (the cardiac gap junctional protein) in isolated rat heart gap junctions and in whole heart fractions. In gap junctions isolated in the absence of alkylating agent, connexin 43 molecules are cross-linked by disulfide bonds. The use of iodoacetamide (100 mM) for the first steps of isolation procedure prevents the formation of these artifactual linkages. Investigation of connexin 43 in whole heart fractions by means of antibodies confirms the results obtained with isolated gap junctions; that is, connexin 43 molecules are not interconnected with disulfide bridges. In whole heart fractions treated with alkylating agents, a 38 kD protein, immunologically related to connexin 43, and containing intramolecular disulfide bonds is detected. It is hypothesized that this protein might be a folded form of connexin 43, a precursory form of the molecules embedded in the gap junctions.

Key Words cardiac gap junctions · thiol/disulfide exchanges

Introduction

Recent evidence indicates that gap junction proteins, in spite of the diversity of their molecular weights, belong to the same protein family, the family of "connexins" (Nicholson et al., 1985; Kumar & Gilula, 1986; Paul, 1986; Beyer, Paul & Goodenough, 1987; Nicholson et al., 1987; Paul & Goodenough, 1987; Dupont et al., 1988; Kistler, Christie & Bullivant, 1988). In the plasma membrane, gap junction proteins are assembled into hexameric units, the connexons, in the center of which is located a hydrophilic transmembrane channel (Baker et al., 1983). Connexons of a junctional membrane interlock in the extracellular space with connexons of the opposite junctional membrane to form cell-to-cell channels responsible for direct intercellular communication (Loewenstein, 1981). In their turn, connexons are clustered into hexagonal patterns to form stable membrane structures: the gap junctions (Pitts & Finbow, 1986; Revel, Yancey & Nicholson, 1986; Warner, 1988).

Cardiac gap junctions are constituted from a major protein, connexin 43, having molecular mass of 43 kD (Beyer et al., 1987; Dupont et al., 1988). Comparison of the connexin 43 amino-acid sequence with connexin 32, the liver major junctional protein, shows that these two proteins share two regions of high homology while other regions have little or no homology (Kumar & Gilula, 1986; Paul, 1986; Beyer et al., 1987). Associated with connexin 32 in liver gap junctions, is a protein of M_r 21 kD, the amino-terminal region of which shows 45% identity with the equivalent region of connexin 32 (Nicholson et al., 1987). Cardiac gap junctions, isolated in the presence of PMSF,[1] a serine protease inhibitor, are characterized by the presence of a fuzzy layer on their cytoplasmic surfaces (Manjunath, Goings & Page, 1984*a*). The cleavage of a protease-sensitive M_r 15–17 kD domain, at the carboxy-terminus of connexin 43, has been correlated with the loss of this fuzzy material on the cytoplasmic surfaces (Manjunath et al., 1987). Hepatic gap junctions, isolated in the presence of PMSF, lack the cytoplasmic material and show smooth cytoplasmic surfaces (Hirokawa & Heuser, 1982; Shibata, Manjunath & Page, 1985).

Little is known about the interactions of connexons in the plasma membrane and the role of those *interactions in the stability* of junction structures. Splitting of heart and liver gap junctions with 8 M urea or at low pH (Manjunath et al., 1984*a*,*b*; Zimmer et al., 1987) indicates that hydrogen and hydrophilic bonds are the most important interactions between connexons in the extracellular junctional space (the "gap"). Lipid extraction experi-

[1] *The abbreviations used are:* BSA, bovine serum albumin; EDTA, ethylene diamine tetra-acetic acid; IAA, iodoacetamide; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsufonyl fluoride; SDS, sodium dodecyl sulfate; Tris, trishydroxymethyl-aminomethane.

ments (Henderson, Eibl & Weber, 1979) also indicate a strong association between cholesterol molecules and the junction proteins, suggesting that this association probably plays an important role in the structure and stability of the gap junctions. More recently, Manjunath and Page (1986) showed that cardiac gap junctions could be stabilized by multiple disulfide bonds between neighboring connexons and between subunits of the same connexons. Liver gap junctions could lack both the inter- and intra-connexon disulfide bridges (Manjunath & Page, 1986).

We demonstrate in this article that disulfide bonds previously identified between connexons of rat heart gap junctions are artifactual linkages formed by oxidation of native thiol groups. Iodoacetamide and N-ethylmaleimide used during the isolation of the junctions prevent the formation of these disulfide bonds. These alkylating agents also allowed us to show the presence of a 38-kD protein, related to connexin 43, in whole heart fractions.

Materials and Methods

Isolation of Cardiac Gap Junctions and Electron Microscopy

In a first set of experiments, gap junctions were isolated from rat hearts as described by Manjunath et al. (1984*a*) and Manjunath and Page (1986) in the presence of 1 mM solid PMSF. In a second set of experiments, gap junctions were isolated in the presence of both solid PMSF (1 mM) and IAA. In this case, 100 mM (final concentration) of IAA were added to the homogenization buffer, then 10 mM (final concentration) were added to all subsequent solutions up to the Sarkosyl step, inclusive.

Pellets of isolated gap junctions were processed for electron microscopy as described by Gros, Nicholson and Revel (1983). Micrographs of ultrathin sections were recorded with a Hitachi H-600 electron microscope at 80 kV.

Preparation of Whole Heart Fractions

Rat whole heart fractions were prepared according to Paul (1985). After dissection, the rat hearts were immediately frozen in Freon 22 and then pulverized with a pestle under liquid nitrogen before being freeze dried. Protein content of these fractions was determined according to Lowry et al. (1951).

Purification and Characterization of Antipeptide Antibodies

The peptide SALGKLLDKVQAY, located at the amino-terminus of connexin 43 (residues 5–17) (Nicholson et al., 1985; Paul, 1986), was synthesized according to Merrifield (1963). Coupling of the peptide to BSA, immunization of rabbits with peptide conjugate, affinity purification and characterization of antipeptide IgGs were described in detail by Dupont et al. (1988).

Analysis of Samples by Polyacrylamide Gel Electrophoresis

Samples (isolated gap junctions or rat whole heart fractions) were analyzed by SDS-PAGE according to Laemmli (1970) with 4.5% stacking and 12.5% separating gels.

Isolated gap junctions were solubilized for 30 min at room temperature in a buffer made of 62.5 mM Tris-HCl (pH 6.8), 40 mM 2-mercaptoethanol, 2% SDS and 10% glycerol. Then alkylation of the sample was performed for 30 min at room temperature by addition of the same volume of the above buffer in which 2-mercaptoethanol was replaced by 120 mM of NEM. In some experiments 2-mercaptoethanol was omitted in the buffer.

For electrophoresis of whole heart fractions, freeze-dried samples containing 100 μg of proteins were dissolved in solubilization buffer (62.5 mM Tris-HCl, pH 6.8; 700 mM 2-mercaptoethanol; 20% SDS; 10 mM EDTA; 5% glycerol, with or without 1 mM solid PMSF); sonicated for 30 sec (MSE Ultrasonic Disintegrator, 100 W model, microprobe, used at maximum power); heated for 5 min at 95°C and then loaded in the gel. In some experiments, 2-mercaptoethanol was omitted or replaced with IAA or NEM (alkylating agents) at final concentrations from 50 to 200 mM. In other experiments, alkylation of the samples was followed by reduction with 700 mM of 2-mercaptoethanol (30 min at room temperature) before SDS-PAGE analysis.

After electrophoresis the gels were either stained with Coomassie brillant blue R-250 or used for immunoblotting.

Analysis of Samples by Immunoblotting

Immunoblots were carried out using a modification of the procedure of Towbin, Staehelin and Gordon (1979). Gels were placed in transfer buffer containing 0.02% SDS, and the apparatus was assembled. Transfer onto 0.22 μm nitrocellulose paper (Schleicher & Schüell) was carried out for 15 hr at 25 V. After transfer, immunoreactions were carried out as described by Dupont et al. (1988). Replicas were incubated for 8 hr at 4°C with saturation solution, the so-called BLOTTO (40 mM Tris-HCl; 0.1% Tween 20; 4% nonfat dry milk; pH 7.5) (Johnson et al., 1984), then overnight at 4°C with affinity-purified antipeptide IgGs (15 μg/ml in BLOTTO). After four washes in BLOTTO, replicas were incubated for 1 hr at room temperature with biotinylated-goat anti-rabbit F(ab')2 (Jackson Immunoresearch Laboratories) diluted 1 : 1,000 in BLOTTO and incubated for 1 hr at room temperature with peroxidase-labeled streptavidin (Jackson Immunoresearch Laboratories) diluted 1 : 2,500 in BLOTTO containing 0.5 M NaCl. Replicas were washed three times in 40 mM Tris-HCl (pH 7.5), 0.1% Tween 20, 0.5 M NaCl and three more times in the same buffer without Tween 20. Peroxidase activity was detected with a solution made of 0.015% H_2O_2, 0.05% 4-chloronaphtol, 16.5% methanol, 40 mM Tris-HCl (pH 7.5), 0.5 M NaCl.

Results

Figure 1 shows micrographs of rat heart gap junctions isolated either in the presence of PMSF and IAA (Fig. 1*A* and *B*) or in the presence of PMSF

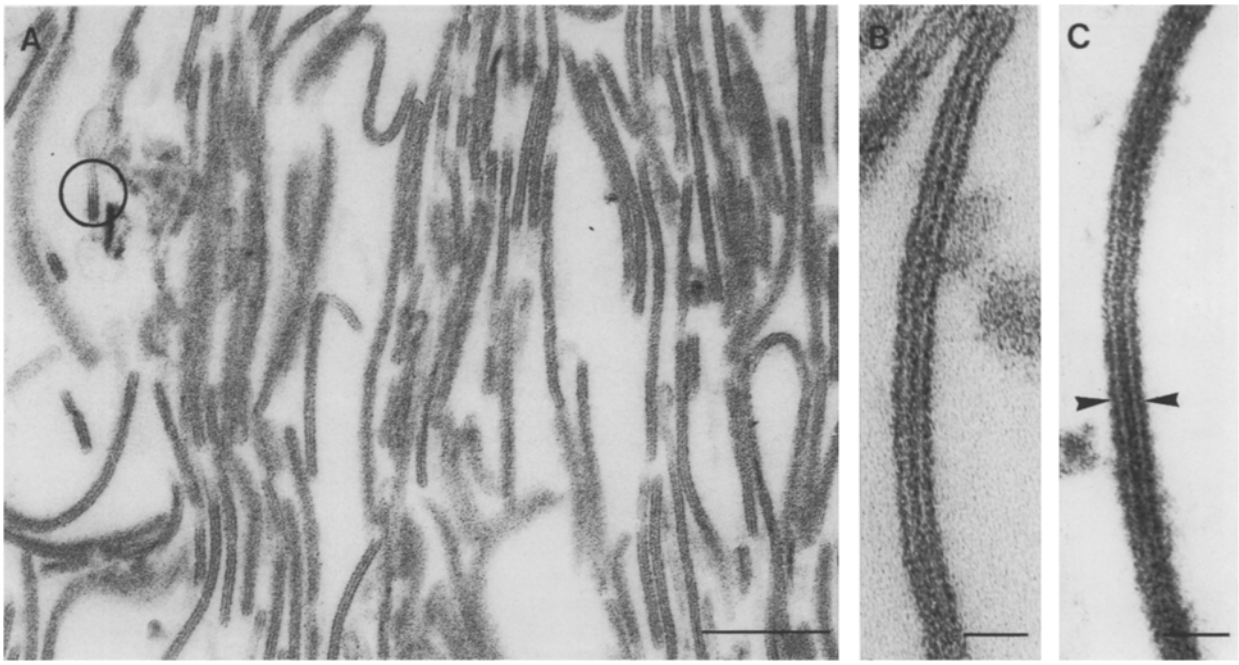

Fig. 1. Ultrathin sections of isolated rat heart gap junctions. (*A*) Section in a pellet of gap junctions isolated in the presence of PMSF (1 mM) and IAA (100 mM). Periodic densities between junctional membranes suggest the presence of connexons (circle). × 84,500. Bar = 0.2 μm. (*B*) High magnification of a gap junction isolated according to the above procedure. × 290,000. Bar = 30 nm. (*C*) High magnification of a gap junction isolated in the presence of PMSF (1 mM). Arrowheads indicate the cytoplasmic fuzzy layers typical of this kind of junction. × 290,000. Bar = 130 nm

only (Fig. 1*C*). For convenience, the first kind of junctions will be termed "PMSF-IAA-gap junctions"; the second kind, "PMSF-gap junctions." A typical fuzzy layer, first described by Manjunath et al. (1984*a*), coats the cytoplasmic surfaces of PMSF-gap junctions (Fig. 1*C*).

Under reducing conditions (40 mM 2 mercaptoethanol in solubilization buffer), SDS-PAGE analysis of both kinds of junctions shows that they contain a major protein of 43 kD, connexin 43 (Fig. 2, lanes *b* and *c*). Under nonreducing conditions (no 2-mercaptoethanol in solubilization buffer), proteins constitutive of PMSF-gap junctions accumulate at the top of separating gels (Fig. 2, lane *d*) while electrophoregrammes of PMSF-IAA-junctions again shows a single band at 43 kD (Fig. 2, lane *e*). Lanes *f* to *i* illustrate the detection of connexin 43 by antipeptide antibodies (Dupont et al., 1988) on immunoreplicas of both PMSF-IAA and PMSF-gap junctions. Connexin 43 is the only protein detected on immunoblots of isolated cardiac gap junctions, however they are prepared. Immunoblots of unreduced PMSF-gap junctions show a major labeling at the top of replicas (Fig. 2, lane *h*).

Proteins of whole heart fractions were separated by SDS-PAGE under reducing (700 mM 2-mercaptoethanol) or nonreducing conditions (no mercaptoethanol) before blotting (Fig. 3). Under reducing conditions, a band at 43 kD, corresponding to connexin 43, is labeled by antipeptide antibodies on immunoreplicas (Fig. 3, lane *a*); under nonreducing conditions, no band is detected (Fig. 3, lane *b*). Addition of IAA (or NEM) in the nonreducing solubilization buffer makes a band appear at 43 kD on immunoreplicas. Intensity of the 43-kD band increases as the concentration of IAA (or NEM) is raised (from 50 to 200 mM) in the buffer, and at the same time a band at 38 kD is detected (Fig. 3, lanes *c* to *e*). Addition of 1 mM PMSF in the buffer used to solubilize whole heart fractions does not change any of the results reported above (*not shown*) and the 38-kD band is always detected in alkylated samples. When samples are alkylated with 100 or 200 mM of IAA and then reduced with 700 mM of 2-mercaptoethanol before SDS-PAGE, only the 43-kD band is detected on immunoblots (Fig. 3, lane *f*).

Discussion

PMSF-gap junctions contain multiple disulfide bonds and consequently connexin 43 accumulates at the top of separating gels unless the sample is reduced before electrophoresis. These results con-

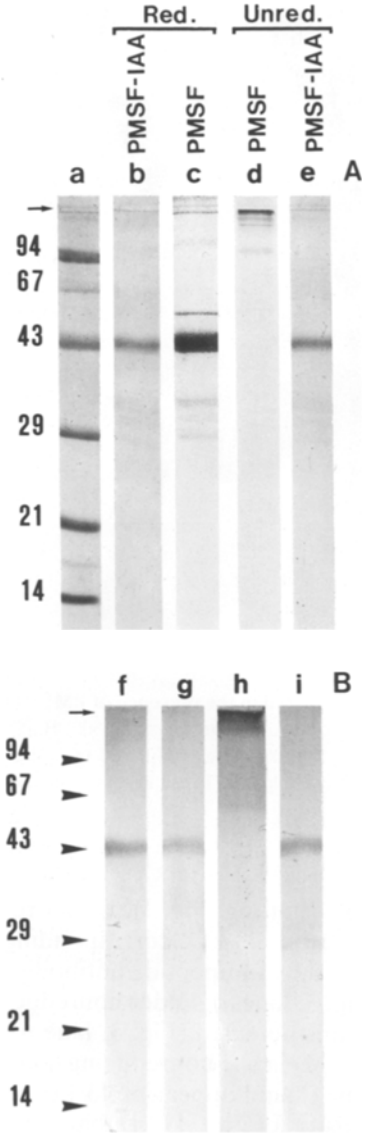

Fig. 2. SDS-PAGE analysis and immunoblotting of isolated rat heart gap junctions. (*A*) Coomassie-blue stained gels. Lane *a*: Molecular weight markers (phosphorylase *a*, M_r 94,000; bovine serum albumin, M_r 67,000; ovalbumin, M_r 43,000; carbonic anhydrase, M_r 29,000; soybean trypsin inhibitor, M_r 21,000; lysozyme, M_r 14,000). Arrow shows the top of separating gels. Lanes *b* and *c*: SDS-PAGE analyses under reducing conditions (40 mM 2-mercaptoethanol in solubilization buffer) of gap junctions isolated either in the presence of PMSF and IAA or in the presence of PMSF only, respectively. The well corresponding to lane *c* was overloaded. Lanes *d* and *e*: SDS-PAGE analyses under unreducing conditions (no 2-mercaptoethanol in solubilization buffer) of gap junctions isolated either in the presence of PMSF only or in the presence of PMSF and IAA, respectively. Comparison of lanes *c* and *d* show that gap junctions isolated in the presence of PMSF contain disulfide bridges preventing connexin 43 from entering into the separating gels. Comparison of lanes *b* and *e* demonstrates that the use of IAA in the isolation procedure prevents the formation of the disulfide bonds. (*B*) Immunoreplicas. Position of the molecular weight markers is indicated by arrowheads. Arrow shows the top of replicas. Replicas *f*, *g*, *h* and *i* correspond to electrophoresis gels *b*, *c*, *d* and *e*, respectively. Connexin 43 was detected by using antipeptide antibodies characterized by Dupont et al. (1988)

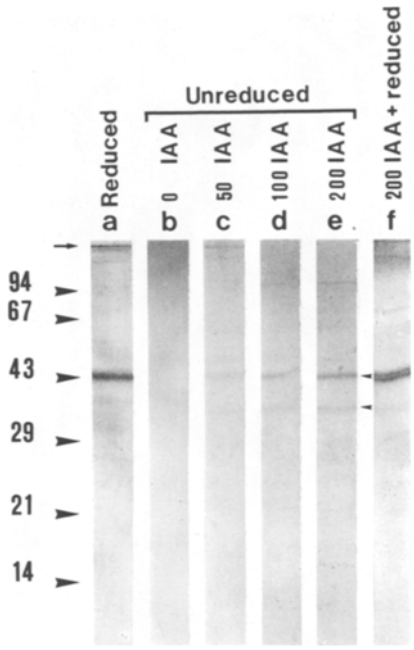

Fig. 3. Detection by immunoblotting of cardiac gap junction proteins in whole heart fractions treated or untreated with IAA. Position of molecular weight markers is indicated on the left by arrowheads. Arrow shows the top of replicas. Lanes *a* to *f* represent replicas of rat whole heart fractions treated with antipeptide antibodies specific to connexin 43. Lane *a*: Sample solubilized under reducing conditions (700 mM 2-mercaptoethanol), before electrophoresis and blotting. Labeling corresponds to connexin 43 (*see* Dupont et al., 1988). Lane *b*: Sample solubilized under nonreducing conditions (no 2-mercaptoethanol). Absence of labeling indicates that connexin 43 molecules are crosslinked by disulfide bonds. Lanes *c* to *e*: Samples solubilized under nonreducing conditions and alkylated with 50 mM IAA (lane *c*), 100 mM IAA (lane *d*) and 200 mM IAA (lane *e*). Alkylation of the samples makes connexin 43 soluble and reveals a 38-kD protein immunologically related to connexin 43 and containing disulfide bonds (arrows on the right). The same results are obtained when IAA is replaced with NEM at the same concentrations. Lane *f*: Sample solubilized under nonreducing conditions and alkylated with 200 mM IAA then reduced with 700 mM 2-mercaptoethanol. Note that the 38-kD band does not appear

firm those previously published by Manjunath and Page (1986). When IAA is added to the solutions used for the first steps of the isolation procedure, connexin 43 migrates into gels without preliminary reduction of gap junctions. IAA, as alkylating agent, blocks free thiol groups and prevents artifactual thiol/disulfide exchanges (Freedman, 1984). These results concern isolated gap junctions that have been submitted to a long and complex preparation procedure; can they be found again with native gap junctions? The use of antipeptide antibodies specific for connexin 43 (Dupont et al., 1988) allows us to answer affirmatively. Immunoblottings of untreated whole heart fractions show that connexin 43

does not enter into electrophoresis gels without a preliminary treatment of the samples with a reducing agent. When IAA (or NEM) is added to the solubilization buffer of whole heart fractions: (i) connexin 43 is detected on immunoreplicas, and (ii) intensity of labeling increases as the concentrations of IAA (or NEM) are raised (from 50 to 200 mM) in the solubilization buffer. These results confirm those previously obtained with isolated gap junctions and demonstrate the artifactual formation of disulfide bonds between connexin 43 molecules. This is in agreement with what is known about reducing properties of cytoplasm which may contain a high concentration (0.5 to 10 mM) of glutathione (Kosower & Kosower, 1978) and especially in heart (Guarnieri, Flamigni and Rossoni-Caldarera, 1979), preventing the formation of multiple disulfide bridges. Conversely, disulfide bonds occur very frequently in extracellular proteins and most classes of extracellular proteins contain disulfide bonds (Freedman, 1984).

A rather puzzling finding is the detection of a band at 38 kD onto immunoreplicas of whole heart fractions treated with alkylating agents. Intensity of this band parallels that of connexin 43 band; i.e., it increases with the raising of IAA (or NEM) concentration in solubilization buffer. The features of this 38-kD protein are the following: (i) it is immunologically related to connexin 43; (ii) it is not detected on replicas of isolated gap junctions (PMSF- or IAA-PMSF-gap junctions); (iii) its labeling depends on alkylating treatment of whole heart fractions; (iv) it does not seem to be a proteolytic product of connexin 43 since reduction of alkylated samples makes it disappear. A reasonable hypothesis to explain the presence of this 38-kD protein is to assume that it represents a folded form of connexin 43 with intramolecular disulfide bonds. Since this protein is not detected in isolated gap junctions (with the techniques used) one can speculate that it represents a precursory form of connexin 43 molecule located either in the nonjunctional membrane (at the periphery of gap junctions?) or in the endoplasmic reticulum or both. The connexin 43 amino-acid sequence, deduced from cDNA, predicts that it contains nine cysteinyl residues (Beyer et al., 1987), but those among them which are involved in intramolecular disulfide bonds are not known.

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