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Rat liver gap junction protein: Properties and partial sequence

(peptide mapping/membrane proteins/micro-sequence-analysis/aggregation/proteolysis)

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ABSTRACT **Gap junctions, strongly implicated as channels for direct cell-to-cell communication, have been isolated from rat liver in high yield and purity. These gap junction fractions contain few morphologically recognizable contaminants, but NaDodSO$_4$/polyacrylamide gel electrophoresis reveals a number of polypeptides. With the exception of a nonjunctional component of M_r 38,000 and some poorly soluble material, including collagen, all the polypeptides have very similar or identical two-dimensional peptide maps and arise from proteolytic cleavage of the COOH-terminus or aggregation of a M_r 28,000 protein. We report the sequence of the NH$_2$-terminal 52 amino acids of this protein. The polypeptide ($M_r \approx$ 10,000) characteristic of trypsin-treated gap junction preparations is shown to be two distinct polypeptides, both derived from the M_r 28,000 protein.**

An extensive body of evidence implicates the gap junction as the mediator of exchanges of ions and small molecules between cells. Early attempts at characterization of the junctional proteins were carried out on partially proteolyzed fragments (1–4). Fractions of similar purity can now be obtained without the use of proteases, and junctional proteins of M_r 26,000–28,000 are consistently found (5–9). Finbow *et al.* (6) have also obtained independent lines of evidence associating a protein of M_r 26,000 with gap junctions. Besides identifiable contaminants (e.g., uricase, actin, collagen), other polypeptides found have been shown to result from aggregation of this protein (5), or are related to it, perhaps as proteolytic fragments [e.g., M_r 21,000 protein (5)]. We present here direct evidence as to the nature of all the polypeptides detected on gels of gap junction fractions isolated from rat liver. The evidence is based on analysis by two-dimensional peptide mapping and NH$_2$-terminal sequence determination. We conclude that rat liver gap junctions are composed of a single major component of M_r 28,000.

MATERIALS AND METHODS

Isolation of Gap Junctions. After perfusion with warm saline, the livers of 50 young adult rats were homogenized, each in 100 ml of cold isolation buffer (2 mM $NaHCO_3$/0.5 mM $CaCl_2$, pH 7.4) in a Tissuemizer (Tekmar Ultra Turrax, SDT-182EN) at maximum power for 4–5 sec. Plasma membranes were then prepared by an adaptation of the two-phase method described by Finbow *et al.* (6). These fractions were treated by a modification of the Hertzberg and Gilula protocol (7). The salt, Sarkosyl, and sodium carbonate treatments were unaltered, except for doubling the volumes. The washed pellet from the carbonate treatment was suspended in 24 ml of 0.09% Sarkosyl and 54.1% (wt/vol) sucrose. This and all other sucrose solutions were made up in 1 M urea/2 mM $NaHCO_3$ (pH 7.4). Six discontinuous gradients were then formed by successively layering 4 ml of 77.2% (wt/vol) sucrose, 4 ml of the sample, 4 ml of 40.3% (wt/vol) sucrose, and 1–2 ml of 33.8% (wt/vol) sucrose. After centrifugation in a Beckman SW 41 rotor at 38,000 rpm for at least 90 min, the gap junction fraction was collected at the 40.3/54.1% sucrose interface, diluted with 2 mM $NaHCO_3$, and pelleted at 40,000 rpm for 60 min in a Beckman 42.1 rotor. Virtually no gap junctions were found at the 33.8/40.3% (wt/vol) sucrose interface, contrary to the original finding by Hertzberg and Gilula (7), but consistent with a recent modification (10). In some cases, 0.5% phenylmethylsulfonyl fluoride ($PhMeSo_2F$) was included in all solutions to reduce endogenous proteolysis. Gap junctions were also isolated by a slight modification of the technique of Finbow *et al.* (6), using increased collagenase and trypsin concentrations of 1.6 and 0.6 mg/ml, respectively, and a Sarkosyl extraction at neutral pH in 35% less volume.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. The Laemmli discontinuous buffer system (11) was used with running and stacking gels of 15% and 4.5% acrylamide (Bio-Rad, electrophoresis grade), respectively (30:0.8 crosslinking ratio). For analytical purposes the microslab system of Amos (12) was used. Samples were solubilized at room temperature for 30–45 min in 2% NaDodSO$_4$ (Bio-Rad, electrophoresis grade) and 5% (vol/vol) 2-mercaptoethanol in 62.5 mM Tris·HCl (pH 6.8)/0.25 mM $MgCl_2$/10% (vol/vol) glycerol. The relative amounts of protein in the bands on a gel after Coomassie blue staining were quantitated by measuring the area under the peaks from a Joyce–Loebl densitometer scan with a digitizing tablet interfaced to a Tektronix minicomputer (4052) and correcting for the width of the lane.

Iodination of Gap Junctions. Gap junction fractions were iodinated in the absence of detergents by the chloramine-T method of Greenwood *et al.* (13). The junctions were separated from free iodine by repeated centrifugations at 40,000 rpm for 30 min in a Beckman 42.1 or type 65 rotor. For peptide mapping, the junctional protein was solubilized in 2% NaDodSO$_4$ before iodination. In this case, separation of protein and free iodine was achieved by NaDodSO$_4$/polyacrylamide gel electrophoresis. Quantitation of the radioactivity of a given band on a gel was determined from an autoradiogram (Kodak XR film exposed at −70°C with a Du Pont Cronex Lightning Plus intensifying screen) as described above for Coomassie blue-stained gels.

Peptide Mapping. The method of Elder *et al.* (14) as modified by Takemoto *et al.* (15) was used to obtain tryptic (Sigma type XI) and α-chymotryptic (Worthington) two-dimensional peptide maps. (Only iodinated peptides generated by complete proteolysis are detected in this system.) In addition to gap junction proteins, we have mapped others, including collagen, actin, glycophorin, and the other erythrocyte ghost proteins, and all have produced unique maps. Results from these proteins showed the recovery of radioactivity from the gel slices to be (70 ± 20)%. The errors represent one standard deviation.

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Abbreviation: $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

Sequence Analysis. Analytical grade reagents, deionized glass-distilled water, and acid-cleaned glassware were used throughout the preparation of polypeptides for sequence or amino acid analysis. The polypeptides present in junctional fractions were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and identified by brief Coomassie blue staining and destaining (total time 4 hr). After excision of the bands, the polypeptides were removed from the gel by electrophoretic elution (16) (recovery from gel fragments was ≈75%), exhaustively dialyzed against 0.03% $NaDodSO_4$/50 mM NH_4HCO_3 (pH 7.8), lyophilized twice to remove the NH_4HCO_3, and stored at −20°C. NH_2-terminal amino acid sequence analysis using automated Edman degradation was performed by the method of Hunkapiller and Hood (17) and Johnson *et al.* (18). $NaDodSO_4$/polyacrylamide gel electrophoresis of an aliquot of the sample was used to detect possible degradation immediately prior to sequence analysis. The yield of analyzable peptide compared to the total peptide loaded on the sequenator was ≈35%. Total peptide was estimated from Coomassie blue staining on analytical $NaDodSO_4$/polyacrylamide gel electrophoresis or quantitative amino acid analysis of an aliquot.

RESULTS

Characterization of the gap junction fractions and their proteins

Yield and Purity (Morphological Characterization). Aggregation of junctions with denser contaminants causes losses during the preparation of gap junctions in the absence of added proteases (7). These losses were minimized by shortening the exposure of junctions to Na_2CO_3, including Sarkosyl in the loading layer of the sucrose gradient, and loading the junctions between the two interfaces where most of the material was collected. We now isolate 300–600 μg of gap junctional protein from 400 g (wet weight) of liver. This final junctional fraction contains only minor contamination by amorphous material (Fig. 1*a*). Appearing primarily as flat sheets, the junctions show the typical packing of "connexons" (Fig. 1*b*) described previously (5–8). When gap junctions were prepared from trypsin- and collagenase-treated plasma membranes, ≈150 μg of M_r 10,000 junctional protein could be isolated from 400 g (wet weight) of liver. This final fraction also showed minimal contamination in negative stained specimens and the gap junctions seemed to form vesicles or curved sheets (not shown, but see ref. 2).

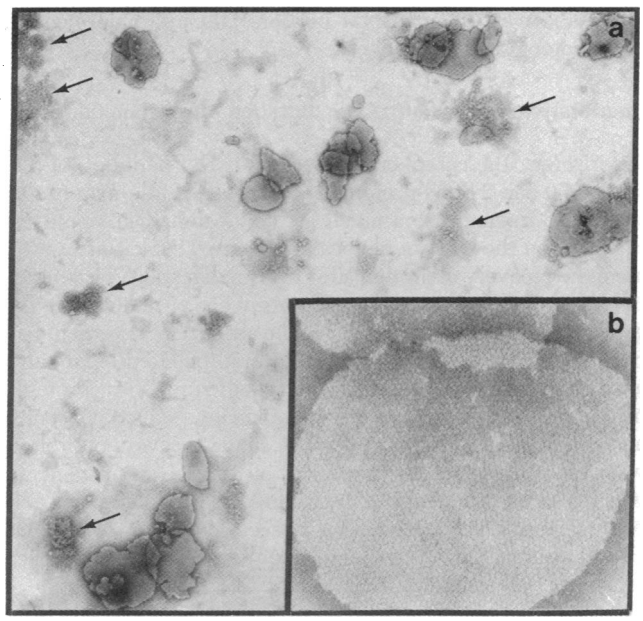

FIG. 1. "Native" gap junction fractions negatively stained with 2% phosphotungstic acid. (*a*) At low magnification (×11,000), the fraction appears highly enriched for gap junctional sheets and shows only minor contamination by amorphous material (arrows). (*b*) At higher magnification (×105,000), the individual gap junction sheets show closely packed connexons in a hexameric array.

Protein Components. When endogenous protease activity is limited by thorough perfusion of the livers before excision and inclusion of $PhMeSO_2F$ in all solutions, the junctional fraction contains a major protein of M_r 28,000 (Fig. 2, lane a). Although not readily identifiable in Fig. 2, lane a, minor and more variable components at M_rs of 50,000, 38,000, 26,000, 24,000, and 21,000 can be clearly seen in fractions loaded at a higher concentration (Fig. 2, lane b). The M_r 38,000 protein is specifically enriched in the lower interface [54.1/77.3% (wt/vol) sucrose] of the final gradient (Fig. 2, lane d), where morphologically and biochemically identifiable gap junctions are sparse. Hence, it is believed to be nonjunctional. The M_r 26,000 and 24,000 polypeptides and the M_r 10,000 polypeptide are likely to be proteolytic degradation products of the M_r 28,000 protein, because they are enriched in preparations in which endogenous proteolysis was more likely (Fig. 2, lanes b and c) or exogenous proteases (trypsin and collagenase) were specifically added (Fig. 2, lane e). *In vitro* trypsin treatment of isolated gap junctions

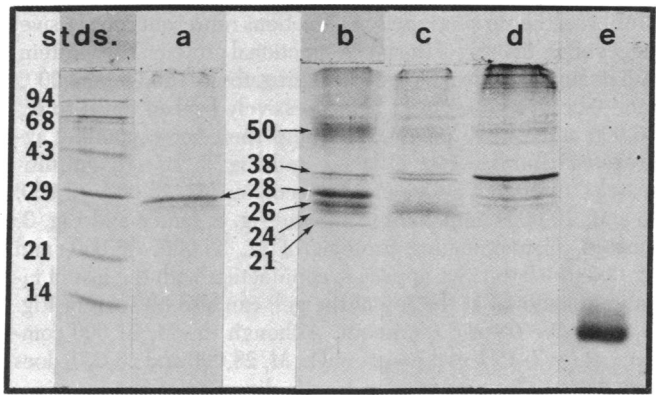

FIG. 2. Coomassie-blue stained $NaDodSO_4$ microslab gel of various gap junction fractions isolated from rat liver. The Bio-Rad low molecular weight standards are shown on the left with their molecular weights in thousands. The estimated molecular weights of the components in lanes a and b are similarly marked. Lane a, a "native" gap junction fraction [i.e., 40.3/54.1% (wt/vol) sucrose interface], prepared in the presence of 0.5% $PhMeSO_2F$ to inhibit proteolysis and run immediately after isolation to reduce aggregation, shows a major band at M_r 28,000 and some very faint minor components. Lane b, a "native" gap junction fraction prepared without specific protease inhibitors contains more obvious components just below the M_r 28,000 protein at M_rs of 26,000 and 24,000. Because the sample was loaded more heavily than in lane a, the minor components are visible (M_r 50,000, 38,000, and 21,000). The relative amount of M_r 50,000 and higher molecular weight (M_r 80,000 and 110,000) material compared to the M_r 28,000 and 26,000 bands was found to increase with concentration of the loaded sample, time in or heating in $NaDodSO_4$, and storage time at −20°C. Lane c, a similar fraction prepared from poorly perfused livers, in which the likelihood of endogenous proteolysis is increased, shows an enrichment for the M_r 24,000 polypeptide compared to lane b. Lane d, the lower interface [54.1/77.3% (wt/vol) sucrose] of the final sucrose gradient of the "native" gap junction isolation also contains junctional bands (see text), but is specifically enriched for the M_r 38,000 protein and contains more insoluble and high molecular weight material contributed in part by the high collagen content of the sample. Lane e, an "enzyme-treated" gap junction fraction prepared from trypsin- and collagenase-treated plasma membranes contains a single diffuse band at M_r 10,000.

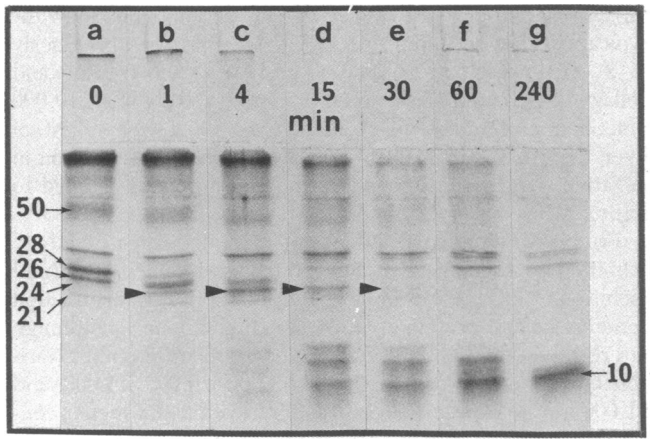

FIG. 3. A purified fraction of "intact" gap junctional sheets (protein ≈ 0.3 $\mu g/\mu l$ in 50 mM $NaHCO_3$, pH 7.8) was subjected to trypsin (Sigma, type XI, 3 $ng/\mu l$) at 37°C in nondenaturing conditions. At various intervals (marked in minutes above each gel lane), samples were removed, added to a 2-fold excess of soybean trypsin inhibitor (Sigma), solubilized for 15 min in regular gel solubilization buffer, and frozen for later analysis by polyacrylamide gel electrophoresis. The M_r 24,000 band is indicated by an arrowhead in each lane where it appears, and the major bands present at 0 time are marked in $M_r \times 10^{-3}$. Lanes a–c and d–g are taken from different gels run on the same experiment.

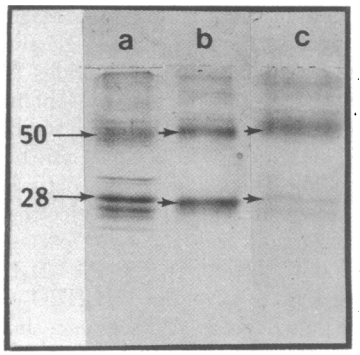

FIG. 4. The protein components of gap junction fractions were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis (lane a), the M_r 28,000 and 50,000 bands were cut out, and the polypeptides were eluted and dialyzed for 3 days against 0.05% $NaDodSO_4$/10 mM sodium thioglycollate/50 mM NH_4HCO_3 (pH 7.8). On reelectrophoresis, the M_r 28,000 protein had apparently aggregated to a dimer (M_r 50,000) (lane b), which comigrates with a band in the original fraction (lane a). Conversely, the isolated M_r 50,000 polypeptide, on reelectrophoresis, had partially dissociated to proteins of M_r 28,000 and 26,000 (lane c). This suggests that this diffuse band of M_r 50,000 is a mixture of dimers of these two proteins, which partially disassociate on standing. Aggregation to higher molecular weight multimers can be seen in all three lanes.

(Fig. 3) supports the idea that these changes in the polypeptide profiles of the various junctional fractions represent progressive stages of proteolysis of the major junctional protein. This protein and its higher molecular weight aggregates (M_r 50,000, 80,000, etc.—see below) seem to be successively broken down to M_r 26,000 and 24,000 polypeptides and their corresponding aggregates (compare Fig. 2, lane c and Fig. 3, lane d) and ultimately, through a series of intermediates (M_r 15,000–12,000) to a M_r 10,000 component (compare Fig. 2, lane e and Fig. 3, lane g). Contaminating proteins of M_r 38,000, 36,000, and 32,000 (the latter two appear in conjunction with the loss of insoluble material at the top of the gel) can also be seen in Fig. 3 to survive trypsin treatment. Although the M_r 21,000 component (≈ 7–15% of the material at M_r 28,000 and 26,000) does not seem to be a product of tryptic digestion of gap junctions (compare lanes b and c in Fig. 2 and see Fig. 3), initial peptide mapping results (not shown) suggest it may also be a degradation product of the M_r 28,000 protein, probably resulting from some non-serine-protease activity (i.e., protease resistant to PhMeSO$_2$F; e.g., cathepsins—see ref. 5) in the liver.

As reported previously for mouse (5), the major M_r 28,000 protein of rat liver gap junctions and its degradation products tend to aggregate on heating or prolonged standing in $NaDodSO_4$ or after extended storage at −20°C. Such preparations show an enhancement of diffuse bands at M_r 50,000 and higher molecular weights (Fig. 2, lanes b and c). This aggregation seems partially reversible, because the isolated M_r 50,000 protein partially dissociates to the M_r 28,000 and 26,000 monomers on standing at room temperature (Fig. 4, lane c). Conversely, the M_r 28,000 protein is seen to aggregate, forming the M_r 50,000 and higher molecular weight multimers (Fig. 4, lane b).

Characteristic patterns of all the polypeptides present in gap junctional fractions have been obtained by two-dimensional mapping of iodinated tryptic and chymotryptic fragments. In both cases the conclusions were the same. Only the chymotryptic maps are illustrated (Fig. 5). All of the polypeptides showed closely related patterns with the exception of the M_r 38,000 protein, which shows no homology (Fig. 5*b*). This supports the conclusion (see above) that this protein is nonjunctional. Of the remaining polypeptides, the M_r 28,000, 26,000, and 50,000 components have nearly identical maps (Fig. 5 *a* and *c*). The same pattern is seen in the higher molecular weight proteins at M_r ≈80,000 and ≈110,000 and even in the material failing to enter the running gel (maps not shown). However, these high molecular weight components show additional peptides, including those characteristic of collagen. The lower molecular weight polypeptides of M_r 24,000 (Fig. 5*d*), 21,000 (not shown), and 10,000 (Fig. 5*e*) retain the hydrophobic peptides present in the M_r 28,000 and 26,000 proteins, but lose several of the most basic and hydrophilic peptides while generating a new hydrophobic one. These results are consistent with the stepwise degradation of the M_r 28,000 junctional protein discussed above and show that the portion of the protein protected from proteolysis is hydrophobic.

Quantitative analysis of tryptic digestion of gap junctions

Surprisingly, the complexity (total number of peptides) of the M_r 28,000 and 24,000 proteins is conserved in the map of the M_r 10,000 fragment to a much greater extent than would be expected on the basis of molecular weight. This led us to estimate the recovery of protein after trypsin digestion of gap junctions, using several methods. The recovery of radioactivity in gap junction fractions iodinated in the absence of detergent has been measured both in the total material pelleted before and after trypsin treatment and in the specific junctional proteins of these same fractions after separation by $NaDodSO_4$/polyacrylamide gel electrophoresis. The actual recovery of protein was determined directly in a series of parallel experiments in which protein was measured by Coomassie blue staining of gels or by quantitative amino acid analysis of pellets or of proteins eluted from gels. Despite the different errors inherent in each technique, all estimates indicate that 70 ± 15% (mean 67%) of the total junctional protein is recovered in the M_r 10,000 band after trypsin digestion. That two-thirds of the mass is preserved is completely consistent with the number of peptides shown to be conserved when the maps of the M_r 10,000 and 28,000 polypeptides are compared (Fig. 5 *c* and *e*). This suggests that the M_r 10,000 band is composed of two polypeptides, a conclusion supported by sequence analysis (see below).

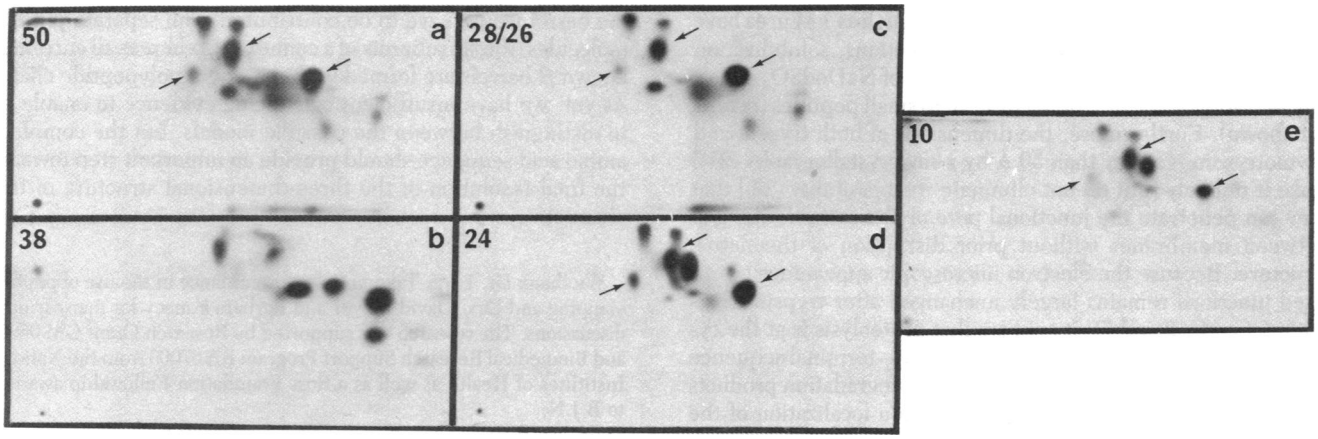

FIG. 5. Characteristic two-dimensional separation of the iodinated chymotryptic peptides of the proteins present in our gap junction fractions. $M_r \times 10^{-3}$ is indicated on each map. In all cases the origin is at the bottom left of the map and separation is achieved on a thin-layer cellulose plate by electrophoresis at pH 1.7 from left to right followed by ascending chromatography in a hydrophobic solvent (see ref. 14 for details). The most basic peptides migrate furthest in the electrophoresis dimension and the more hydrophobic peptides tend to partition to the mobile phase on the chromatography and are found nearest the top of the map. All maps show a great deal of homology amongst the hydrophobic peptides except for that of the M_r 38,000 protein (see text). Three of the peptides conserved in all but the M_r 38,000 protein are marked with arrows to provide a frame of reference, although it should be noted that additional peptides are also conserved. Maps of all polypeptides were repeated at least four times.

Sequence analysis

The sequence of the 52 residues at the NH_2-terminus of the M_r 28,000 gap junction protein (one-fifth of the protein) has been determined (Fig. 6). After 14 uncharged amino acids at the NH_2-terminus, there is a strongly hydrophobic region of 18 residues (nos. 23–40) interrupted by a single charged amino acid (Arg-32) and flanked at the NH_2 terminus by three basic residues (nos. 15, 16, and 22) and at the COOH terminus by a similarly spaced group of three acidic residues (nos. 41, 46, and 47).

The same NH_2-terminal sequence has been found for the M_r 26,000 and 10,000 products of proteolysis, demonstrating that the COOH terminus of the protein is exposed to proteolytic digestion in the intact structure, whereas the NH_2 terminus is protected. In the case of the M_r 10,000 component (sequence determined to 20 residues), one or two major sequences in addition to that seen for the M_r 28,000 protein can be detected, indicating the presence of at least two polypeptide chains. These additional signals could not be resolved into a unique sequence and probably result from tryptic cleavage at several closely spaced and equally susceptible sites on the original M_r 28,000 protein, which could produce a second M_r 10,000 polypeptide with various NH_2-terminal starting points.

DISCUSSION

We have used a modified version of a published procedure (7) to isolate gap junctions from rat liver in high yield [0.75–1.5 μg of junctional protein per g (wet weight) of liver] and with few copurifying contaminants as judged from negatively stained samples. Nevertheless, as found by others, despite this apparent purity several polypeptides can be detected by $NaDodSO_4$/polyacrylamide gel electrophoresis. Examination of these polypeptides leads to the conclusion that gap junctions are composed of a single major protein of M_r 28,000. All other polypeptides can be attributed to demonstrable contaminants [e.g., collagen, a more dense M_r 38,000 protein, and an alkali-sensitive M_r 34,000 protein uricase (19)] or are derived from the M_r 28,000 protein by proteolysis (compare Fig. 3 with lanes b and c of Fig. 2) or aggregation (Fig. 4 and ref. 5). The two-dimensional peptide mapping system of Elder *et al.* (14) used for this analysis was also found to provide an assay for gap junctions, at least those from rat liver. Differences between gap junctional proteins of different species (15, 20) and tissues (20) have been detected by this system.

The quantitative study of the effect of trypsin on gap junctions has provided some insight into the arrangement of the protein in the membrane. Recoveries of 70% of the junctional protein present in junctions not subjected to proteolysis (major protein M_r 28,000) in the M_r 10,000 fragment of junctions after trypsin digestion, and the presence of more than one polypeptide in this fragment, as detected by sequence analysis, leads us to conclude that the M_r 28,000 protein is initially reduced to a M_r 24,000 polypeptide (Fig. 3, lanes a–d), which is in turn cleaved into two pieces of M_r 10,000 by several steps (Fig. 3, lanes c–g). Several lines of evidence suggest that these two polypeptides are protected from further proteolysis by the surrounding membranes. Peptide mapping and amino acid analysis show the M_r 10,000 tryptic polypeptides to be highly hydrophobic. In ad-

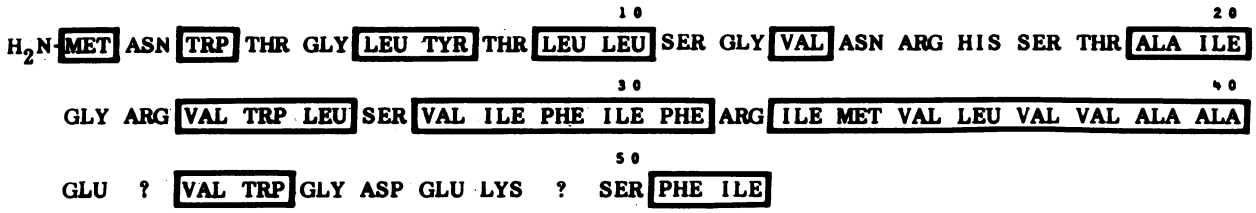

FIG. 6. Sequence of the 52 NH_2-terminal residues of the M_r 28,000 protein of gap junctions. Hydrophobic residues are enclosed in boxes, thus emphasizing the strongly hydrophobic region (residues 23–40) flanked by basic (residues 15, 16, and 22) and acidic (residues 41, 46, and 47) residues. The question marks indicate steps in the sequence at which no unequivocal signal could be distinguished from the background and could indicate a cysteine (undetected by this system) or an amino acid recovered in poor yield.

dition, whereas protein denaturing agents such as 1 M urea have no effect on the trypsin sensitivity of junctions, solubilization of the membrane with low concentrations of $NaDodSO_4$ allows trypsin to digest the junctional protein to small peptides (results not shown). Furthermore, the dimensions of both trypsin and chymotrypsin [greater than 50 Å by x-ray crystallography (21)] make it unlikely [but do not eliminate the possibility (22)] that they can penetrate the junctional pore or the extracellular gap between membranes without prior disruption of the native structure. Because the electron microscopic appearance of isolated junctions remains largely unchanged after trypsin treatment (cf. refs. 2 and 7), it appears that proteolysis is at the cytoplasmic face. The conservation of the NH_2-terminal sequence of the M_r 28,000 protein in its proteolytic degradation products (M_r 26,000 and 10,000) is consistent with a localization of the COOH terminal of the junctional protein at the cytoplasmic face. In addition, the existence of two major portions of the molecule, both apparently protected by the surrounding membrane yet joined by a region accessible to proteases, suggests that the junctional protein crosses the membrane more than once.

This possibility is so far consistent with the sequence of the M_r 28,000 protein. In the NH_2-terminal one-fifth of the molecule, we have identified a highly hydrophobic stretch of 18 residues, bracketed by basic and acidic amino acids (NH_2- and COOH-terminal, respectively), attributes seen in the transmembrane portions of some other proteins (23–27). However, the secondary structure of transmembrane spans of the gap junction protein is open to speculation. In α-helical conformation, $\approx$23 residues would be required to span the 35 to 40-Å hydrophobic core of the membrane, which in the hydrophobic sequence identified here would require two charged residues (Arg-22 and Arg-32 or Arg-32 and Glu-41) to be buried in a hydrophobic environment. Alternatively, if this hydrophobic region were in β-pleated-sheet conformation, only Arg-32 need be buried in the lipid environment ($\approx$15 residues span the membrane). Both of these alternatives are possible, because ionic bonding between adjacent transmembrane strands could neutralize the charges buried in the lipid (e.g., see ref. 28) or, specifically in the case of the gap junction, the charges could be located in the aqueous channel. Present evidence suggests that the β-sheet conformation is more likely. The Chou and Fasman paradigm (29) applied to the gap junctional protein strongly predicts the hydrophobic region (residues 23–38) to be in a β-pleated-sheet conformation (data not shown). It must be noted, however, that this predictive system, which is based on data from soluble proteins, fails to make consistently correct predictions when applied to at least two membrane proteins of known secondary structure [e.g., *Escherichia coli* protein I (30) and bacteriorhodopsin (our observation)], recent results from x-ray diffraction of liver gap junctions (31) also indicate the presence of β-pleated-sheet structure within the hydrophobic portion of the lipid bilayer. If the protein is in this conformation, it is likely to form a β-barrel structure in order to internally satisfy its hydrogen bonding capacity in a hydrophobic environment. The diameter of such a structure ($\approx$15 Å) is consistent with it representing the wall of the aqueous pore of a gap junction. If such were the case, the polypeptide strands composing the barrel would have to be contributed from separate protein molecules (the six subunits of a connexon), whereas all currently known β-barrels are formed within a single polypeptide chain. As yet, we have insufficient convincing evidence to enable us to distinguish between the possible models, but the complete amino acid sequence should provide an important step towards the final resolution of the three-dimensional structure of this channel.

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