In Vitro Oxidation of Intrinsic Sulfhydryl Groups Yields Polymers of the Two Predominant Polypeptides in the Nuclear Envelope Fraction†

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ABSTRACT: The two quantitatively predominant nuclear nonhistone polypeptides of the avian erythrocyte comprise a significant portion of the nuclear envelope protein. Each of these two polypeptides can be converted almost completely to homogeneous polymeric species via oxidation of intrinsic sulfhydryl groups by *o*-phenanthroline cupric ion complex. Major products are two dimeric species arising from each of the approximately 77 000-dalton pair. The larger of the two polypeptides also yields oligomers greater than dimers. These observations extend our earlier solubility and polypeptide cross-linking evidence which indicated that these polypeptides were involved in an oligomeric, proteinaceous nuclear structure. Ultramicroscopic studies from other laboratories indicate that such a structure may function as a nuclear skeleton. The architectural details of the structure now appear to be accessible at the molecular level.

Ultramicroscopic and chemical studies of macromolecular structures rich in protein, including microtubules (Soifer, 1975; Snyder & McIntosh, 1976), thick and thin filaments (Pollard & Weihing, 1974; Mannherz & Goody, 1976), elements of membranes (Marchesi et al., 1976), ribosomes (Nomura et al., 1974), and chromatin (Van Holde & Isenberg, 1975) allow detailed molecular descriptions of cell structures, and their functions. In contrast, studies of nuclear architecture are more preliminary. Some nuclear nonhistone polypeptides may derive from a proteinaceous structure whose abundance and solubility characteristics enable it to serve as a nuclear skeleton. Ultramicroscopic examination of chromatin-free rat liver nuclear preparations reveal stable fibrous structures. Chemical analysis of these residual fractions indicate a primarily proteinaceous composition. Three polypeptides with molecular weights from 60 000 to 69 000 constitute the major components (Berezney & Coffey, 1974; Aaronson & Blobel, 1975). Nuclear protein fibrillar structures are localized in the inner membrane (Scheer et al., 1976), the fibrous lamina (Aaronson & Blobel, 1975), the lamina and nucleus (Berezney & Coffey, 1974), and, in one study, in virtually every nuclear component (Comings & Okada, 1976). Conflicting views on the primary locus of the fibrous material, which is presumably composed of the three major polypeptides, remain to be resolved. In HeLa cells, but not rodent liver, a fibrous residual structure which resembles the nucleus in size and outline contained 14% DNA. The DNA appears to be essential for morphological integrity. Further, the polypeptide composition is more complex than that reported for rodent liver (Keller & Riley, 1976).

We have characterized an insoluble proteinaceous fraction from avian erythrocyte nuclei. Fractionation of cells and nuclei demonstrates that two polypeptides with molecular weights between 70 000 and 80 000 (herein referred to as "77 000-dalton" polypeptides) are abundant nonhistone nuclear polypeptides and that they occur as the predominant nonhistone polypeptides in the nuclear envelope fraction, in an unsheared chromatin fraction, and in a non-ionic detergent-washed, chromatin-free residual protein fraction (Shelton, 1973; Cobbs & Shelton, 1975; Shelton et al., 1975, 1976). These polypeptides, as well as several others, resist dissolution in non-ionic detergent or salt solutions alone but do dissolve in detergent plus 0.5 M $MgCl_2$ solutions and subsequently precipitate when the $MgCl_2$ is removed by dialysis, indicating strong, protein-protein interactions involving both ionic and hydrophobic binding (Shelton, 1976). Because of these properties, we have proposed that these predominant erythrocyte nuclear polypeptides are involved in an oligomeric protein skeleton. We have directly detected such interaction by reacting chromatin-free nuclear preparations with bifunctional imidoester cross-linking reagents. The 77 000-dalton polypeptides prove to be particularly sensitive to cross-linking and appear to yield polymers as large as trimers or greater (Cochran & Shelton, 1976).

We describe herein further direct evidence of the oligomeric state of the erythrocyte nuclear polypeptides. The location of intrinsic sulfhydryl groups permits probes of polypeptide interactions with great selectivity. Further, the reversibility of disulfide bond formation facilitates identification of monomeric reactants with oligomeric products.

Materials and Methods

Nuclei were isolated from chicken erythrocytes after nitrogen cavitation as previously described (Shelton et al., 1976). In some experiments, nuclei, harvested between the first and second wash steps were exposed to 1.0 mM *N*-ethylmaleimide for 5 min at 0–4 °C. A nuclear envelope fraction was isolated by a sequence of pancreatic DNase digestions and salt extraction (steps 1, 2, and 4 of Dwyer & Blobel (1976)). When disulfide bond formation was to be enhanced, nuclear envelope from 0.5 mL of packed erythrocytes was suspended in 0.9 mL of 10% sucrose–10 mM triethanolamine hydrochloride (pH 7.5)–0.1 mM $MgCl_2$ and 0.10 mL of 5.0 mM phenanthroline–1.0 mM cupric sulfate added. The mixture was incubated at room temperature for 20 min (Steck, 1972). Reacted envelope was collected by centrifugation at 27 000*g* for 15 min at 4 °C. Triton X-100 washed nuclear envelope was prepared by suspending envelope from 1.0 mL of packed cells in 1.0 mL of 10% sucrose–10 mM triethanolamine hydrochloride (pH 7.5)–0.1 mM $MgCl_2$ followed by the addition of 0.2 mL of 5% Triton X-100. After mixing, the suspension was held in an ice-water bath for 20 min. Triton-washed material was col-

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lected by centrifugation at 27 000*g* for 20 min at 4 °C. The pellet was stored under 90% ethanol for 18 h at −20 °C.

Protein residues originating from 1.0 mL of packed erythrocytes were dissolved in 1.0 mL of electrophoresis sample buffer without mercaptoethanol (Cochran & Shelton, 1976). When desired, disulfide bonds were reduced by addition of 0.03 volume of 2-mercaptoethanol and overnight incubation at room temperature. Electrophoresis and staining have been previously described (Shelton et al., 1976). Protein was determined by the method of Lowry et al. (1951) as modified by Bensadoun & Weinstein (1976).

The molecular weights of the major cross-linked products were estimated by comparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Shapiro et al., 1967). Mercaptoethanol was omitted from the sample buffer. These gels were 4% monomer and *N,N'*-methylenebis(acrylamide) constituted 2.5% of the total monomer. The molecular weight standards were a series of cross-linked polymers, yielding species with molecular weights of 53 000, 106 000, 159 000 etc., obtained from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y. Electrophoretic mobilities were compared by both a mixed and parallel run.

Results

In the present study we used the methods of Dwyer & Blobel (1976) to prepare the nuclear envelope–residual protein fraction, since this method requires only 1 day and utilizes monovalent salt washes, rather than $MgCl_2$, to dissolve chromatin. This change may favor retention of the nuclear skeleton. Quite similar polypeptide electropherograms are obtained by their method and by our former methods (Shelton et al., 1976).

We have previously investigated polypeptide interactions by use of bifunctional cross-linking reagents (Cochran & Shelton, 1976; Shelton & Cochran, 1977). In extending these studies we detected oligomers in electropherograms of control protein samples which lacked both bifunctional reagents and disulfide reducing agents. These oligomers were sensitive to mercaptoethanol reduction as shown in Figure 1. After mercaptoethanol reduction of the material in gel 1, the band of greater molecular weight at B disappears and the smaller one is greatly reduced. Treatment of the isolated nuclei with the sulfhydryl blocking agent *N*-ethylmaleimide prior to envelope isolation prevented polymerization (Figure 1, examples 3 (no mercaptoethanol) and 4 (with mercaptoethanol)). This result confirmed the nature of the bond involved and demonstrated that in the native state the erythrocyte polypeptides were not disulfide linked. Polymerization appeared to occur during the course of envelope isolation.

The extent of the oxidation reaction occurring during isolation and the specificity for polypeptides, which were presumably oligomeric components of the nuclear protein skeleton, were noteworthy. Phenanthroline cupric complex, a sulfhydryl group oxidant (Kobashi & Horecker, 1967; Kobashi, 1968), was tested to determine whether the polymerization could be enhanced. Addition of the oxidant to a preparation of nuclear envelope resulted in a major depletion of the bands at A and an enhancement of the pair of bands at B as well as the appearance of higher polymers at C (Figure 1, examples 5 and 6).

Insolubility in solutions of non-ionic detergent at low ionic strength has been a characteristic of proteins which appear to be involved in the nuclear skeleton and we have demonstrated that the predominant erythrocyte nuclear polypeptides are insoluble in Triton X-100 solution. We therefore sought to confirm that the disulfide cross-linked and the Triton X-100 insoluble polypeptides were identical. This is demonstrated in Figure 2. In this experiment the envelope fraction was subjected to phenanthroline–cupric complex oxidation and then washed with Triton X-100. Upon reduction with mercaptoethanol (panel a) a simple band pattern characteristic of erythrocyte residual nuclear polypeptides was obtained. Most of the protein occurred in the 77 000-dalton band (4 and 5 here). Minor polypeptides are present (bands 1–3, 6, 7). The two polypeptides in band 7 appear to undergo oxidation-mediated cross-linking in contrast to bands 1, 3, and 6. In the "oxidized" state presented in panel b, a pair of major bands appear between bands 1 and 3 and other bands plus unresolved material appear between 1 and the gel top. Only polypeptide bands 4, 5, and 7 are significantly diminished by the oxidation step. Band 2 lies under the polymers and cannot be assessed. Higher resolution of the oxidized material was achieved by extending the electrophoresis time. Only discrete bands were formed until very high molecular weight material occurred. Although molecular weight estimates of unreduced polypeptides via sodium dodecyl sulfate–polyacrylamide gel electrophoresis must be viewed with caution (Griffith, 1972), our value of approximately 160 000 for the new polypeptides at position B, Figure 1, indicates that they are dimers of bands 4 and 5.

The mass distribution in the electropherogram indicates that the oligomers arise predominantly from bands 4 and 5, but cannot reveal the composition of each oligomer with respect to the two monomers. The reversibility of disulfide bond for-

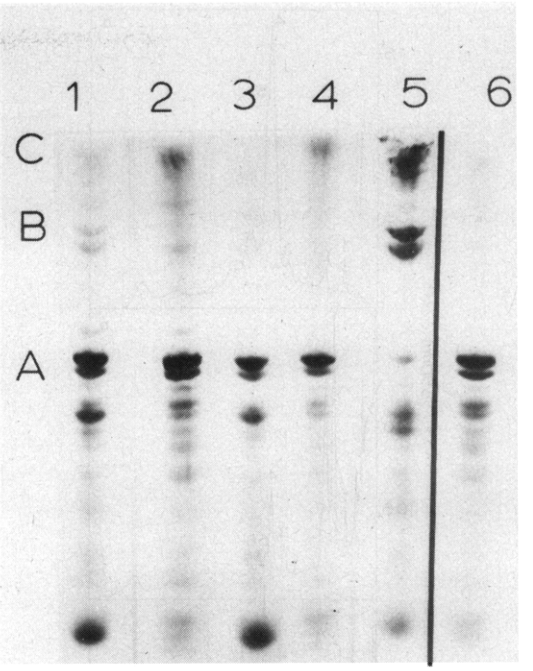

FIGURE 1: Electropherograms of reduced, partially oxidized, and oxidized chicken erythrocyte nuclear envelope polypeptides. The pairs, 1 and 2, 3 and 4, 5 and 6, are each identical except that samples 2, 4, and 6 were reduced overnight with 3% (v/v) 2-mercaptoethanol before electrophoresis. Details of preparation, electrophoresis, and staining are presented in Materials and Methods. 1 and 2 reveal polypeptide cross-linking found after a standard envelope preparation. In 3 and 4 free mercaptan groups were blocked with *N*-ethylmaleimide after nuclei were released from the cells. In 5 and 6 cystine formation was promoted by exposure of isolated nuclear envelope to a solution containing *o*-phenanthroline/cupric sulfate. Protein applied to each gel was 1 and 2, 17.4 μg; 3 and 4, 16.2 μg; and 5 and 6, 24.2 μg. A indicates the position of the 77 000-dalton pair of monomers, B the position of an approximately 160 000-dalton pair of bands, and C indicates high molecular weight polymers.

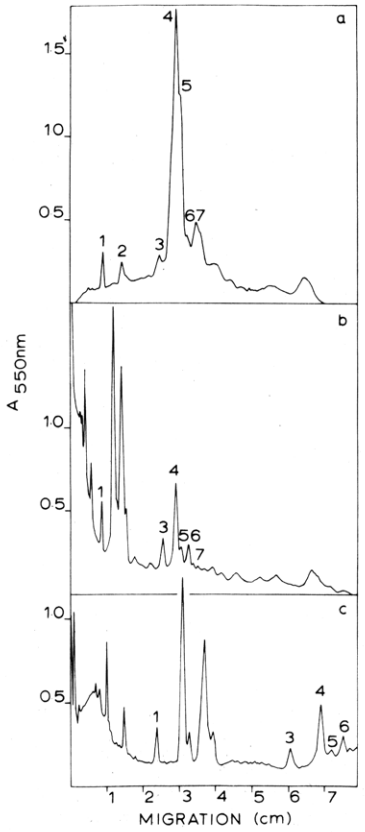

FIGURE 2: Electropherograms of Triton X-100 insoluble, *o*-phenanthroline/cupric sulfate oxidized nuclear envelope polypeptides after (a) mercaptoethanol reduction, (b) no reduction, and (c) extended electrophoresis of the sample in b. Preparation of the samples and the electrophoretic conditions are presented in Materials and Methods. The numbers 1–7 refer to material which appears as distinct bands in the reduced sample. Protein (14.5 μg) was applied to each gel.

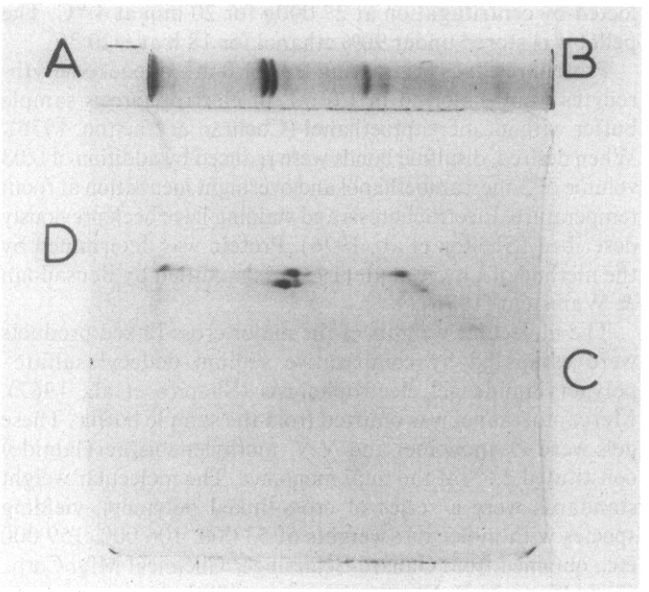

FIGURE 3: Two-dimensional electropherogram of oxidized (1st dimension) and reduced (2nd dimension) Triton X-100 insoluble nuclear envelope polypeptides. Sample (90 μg of oxidized protein) was separated in the first dimension in the buffer of Shapiro et al. (1967). A photograph of a control gel is shown between A and B. To effect reduction the gel was immersed in 100 mL of electrophoresis buffer, containing 5% 2-mercaptoethanol for 45 min at room temperature. The gel, containing reduced polypeptides, was then placed on top of a Pharmacia 4–30% gradient slab gel and electrophoresis in the second dimension performed as previously described (Cobbs & Shelton, 1975). The gel was stained and destained as described in Materials and Methods. A to C indicates the diagonal where polypeptides which contained no interpolypeptide cystine bonds are expected. A horizontal line passing through D and intersecting the A to C diagonal indicates the locus of most polypeptides which were cross-linked prior to reduction.

mation was utilized to determine oligomer compositions. The experimental data are presented in Figure 3. Polypeptides from an oxidized sample were separated in a cylindrical gel (the major pair of dimers are readily apparent in the sample gel shown between A and B in Figure 3). After reduction of disulfide bonds, polypeptides were separated further by electrophoresis in a gradient slab gel (in the direction of B to C in Figure 3). Upon reduction, some material which was too large to enter the first gel (near A in Figure 3) produced a light streak in the second dimension, clearly indicating all polypeptides arising from this zone. Those polypeptides which formed no interpolypeptide disulfide bonds can be seen along a diagonal between A and C, with most of this material nearer C. The region of the slab gel between D and the A to C diagonal yielded the most information about oligomer compositions. Polypeptides from band 4 produced a light streak across this zone with three areas of concentration. These were a spot on the A to C diagonal which reflects monomer, a heavy and a light spot in the dimer region, and several spots at and near the material arising from the top of the one-dimensional gel. Thus the band 4 polypeptide is only partially polymerized and yields both dimeric and higher oligomers. Band 5 polypeptide migrates slightly further than band 4 in the B to C direction. It was located almost entirely in the dimer region and yielded a major and a minor spot. Interestingly band 5 dimer has an apparent molecular weight higher than band 4 dimer, although the monomers are of reverse size. It should be noted that all of the polymers sufficiently small to enter the first dimensional gel are homogeneous because no two spots were found along a single line parallel with the B to C direction. The fainter spots accompanying the two main spots of dimeric material may arise from dimers of sufficiently different conformation to effect migration rate. Such differences could be stabilized by disulfide bonds. Two faint spots in the streak from the origin arise from band 7 polypeptides and indicate that the band 7 oligomer is of large size.

Discussion

The molecular architecture of the nucleus remains obscure despite many advances in molecular biology. The comparatively recent advent of sodium dodecyl sulfate–polyacrylamide gel electrophoresis provided an opportunity for partially resolving the relatively insoluble nuclear nonhistone polypeptides, a significant portion of the nuclear material (Shelton & Allfrey, 1970; Elgin & Bonner, 1970). A large number of related studies have subsequently been published (Stein et al., 1974). However, interpretations of the significance of the isolated polypeptides are frequently clouded by lack of knowledge of native oligomeric arrangements and, indeed, of the cellular localization of the observed polypeptide electrophoretic bands.

We have resolved some of these difficulties in our studies of the avian erythrocyte. The erythrocyte contains primarily two organelles, the plasma membrane and the nucleus (Zentgraf et al., 1971). Also mature erythrocyte nuclei do not divide, are inactive in RNA synthesis (Cameron & Prescott, 1963), and contain a limited amount of nonhistone protein (Dingman & Sporn, 1964). Despite this relative simplicity, an important technical obstacle to utilization of the erythrocyte for nuclear studies has been the difficulty of completely re-

moving the plasma membrane from the nuclear fraction. We have separated these fractions for both goose (Shelton, 1973) and chicken erythrocytes (Cobbs & Shelton, 1975; Shelton et al., 1976). The plasma membrane polypeptide electropherogram resembles that of humans (Shelton, 1973), while the polypeptide electropherograms and the metabolism of phosphoproteins are distinctly different for the erythrocyte plasma membrane and nuclear fractions. Further, we have identified the nuclear envelope as the primary locus of nonhistone protein in this cell (Shelton et al., 1976). These results have been confirmed (Jackson, 1976a,b). The two predominant polypeptides occur as oligomers in the envelope fraction as demonstrated by their selective solubility and differential sensitivity to cross-linking via bifunctional imidoesters (Shelton et al., 1975; Shelton, 1976; Cochran & Shelton, 1976).

The data reported herein reveal that these same polypeptides undergo highly selective cross-linking via disulfide bond formation when the envelope fraction is exposed to oxidizing conditions. Proteins with similar properties may occur in other nuclei. Bornens & Kasper (1973) observed the mercaptoethanol mediated dimer to monomer conversion of a 70 000-dalton polypeptide in the rat liver nuclear membrane. Their polypeptide may be homologous to band 5 in this study. Further, the 70 000- and the 74 000-dalton polypeptides which were identified as almost 22% of the rat liver nuclear membrane fraction protein by Bornens & Kasper (1973), probably correspond to the larger two of the three polypeptides which are enriched in the rodent liver fibrous nuclear structures (see introductory section). Firstly, we find that the largest erythrocyte polypeptide comigrates with the largest rat liver nuclear residual polypeptide in parallel electrophoresis in 4 to 30% gradient polyacrylamide gels (Shelton, K., Cochran, D., & Cobbs, C., unpublished). Secondly, the same erythrocyte nonhistone polypeptides are in several nuclear subfractions including the envelope fraction and a nuclear residual fraction (Shelton et al., 1976). This may well occur with the rat liver nuclear polypeptides. Finally, Jackson (1976a) has shown that the homologous chicken erythrocyte and liver nuclear envelope polypeptides migrate identically when compared in the same electrophoretic system. Thus the phenomena reported herein may indeed apply to the nuclei of other cells.

The variety of the insoluble fibrous structures reported, nuclear lamina, nuclear matrix, or nuclear membrane, is striking. Comings & Okada (1976) have observed both fibrillar and amorphous material in the ". . . inner nuclear membrane, intranuclear matrix and nucleolar matrix, synaptonemal complex, sex vesicle, and probably some cytoplasmic membranes" all of which they propose may consist primarily of the 60 000- to 69 000-dalton polypeptide triplet. In view of the properties of the chicken proteins reported here, it appears that the varying morphological appearances of nuclear proteinaceous residues isolated by different groups may depend in part on the differing solubilities of similar polypeptides as a function of disulfide bond formation. Thus careful attention to oxidation or reduction conditions during isolation may resolve these differences and, further, yield evidence more representative of the native state. In this regard, the tendency of these polypeptides to self-associate and their ability to form disulfide bonds indicate their probable role in the mercaptan-sensitive nuclear gels which have been extensively studied by Dounce (1971).

The ease of polymer stabilization via oxidation is striking. Apparently some interpolypeptide chain sulfur atoms are very closely spaced. Although cystine bonds are absent in the mature erythrocyte nucleus, presumably they could form under physiological conditions. Such in vitro oxidizable sulfur atoms have been observed in the fibrous protein tropomyosin where oxidation has been shown to yield α and $\alpha\beta$ dimers (Lehrer, 1975). The erythrocyte plasma membrane protein spectrin also can be polymerized covalently by cystine bond formation. In the case of spectrin, the 225,000- and 250,000-daltons polypeptides appear to form a series of oligomers (Steck, 1972; Wang & Richards, 1974). Interestingly, spectrin is similar to the 77 000-daltons polypeptides with regard to some solubility properties and a proposed skeletal function (Steck & Yu, 1973; Yu et al., 1973). In contrast to these proteins, the LETS protein occurs in the native state as disulfide stabilized oligomers (Rossow et al., 1977).

Several features indicate that the phenomena reported should provide an excellent system for investigating specific details of nuclear protein architecture. These include the high yield of discrete polymeric bands, the presence of both dimers and higher polymers, and the ease with which the polymers are cleaved to the original monomers. At the simplest level the size changes attendant upon the oxidation–reduction conditions described here permit the isolation of two major nuclear polypeptides with molecular specificity.

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Preparation and Analysis of Seven Major, Topographically Defined Fragments of Band 3, the Predominant Transmembrane Polypeptide of Human Erythrocyte Membranes†

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ABSTRACT: Band 3 is the ~90 000-dalton membrane-spanning polypeptide believed to facilitate anion transport in the human erythrocyte membrane. Previous studies have shown that digestion of this protein while still membrane-bound generates large, topographically defined, overlapping fragments which account for all or nearly all of its mass. We have now purified seven of these fragments, utilizing selective membrane solubilization, gel filtration, and preparative gel electrophoresis in sodium dodecyl sulfate. Amino acid analysis revealed that fragments derived from the outer surface and membrane-spanning regions of band 3 were distinctly hydrophobic, while cytoplasmic surface segments were relatively polar; these compositional data parallel the solubility in aqueous solutions and the mode of membrane association of the various fragments. Digestion of intact cells with chymotrypsin generated a 38 000-dalton outer-surface and a 55 000-dalton transmembrane fragment. The sums of their apparent molecular weights and of their compositions approximated the band 3 polypeptide, suggesting a single site of cleavage at the extracellular face. Chymotryptic digestion at both membrane surfaces produced a 17 000-dalton transmembrane fragment having the most hydrophobic composition. Mild trypsin digestion released a 41 000-dalton segment from the cytoplasmic pole of band 3, which could be further cleaved by trypsin to yield 22 000- and 16 000-dalton subfragments. Compositional analysis showed that the sum of these two subfragments was nearly the equivalent of the 41 000-dalton tryptic fragment. Furthermore, these data indicate the presence of a small lysine-rich segment near the juxtamembrane region of the 41 000-dalton fragment, which was lost upon further cleavage. The missing segment may overlap with the 17 000-dalton chymotryptic core. A 23 000-dalton fragment cleaved by S-cyanylation from the cytoplasmic terminal region of band 3 had nearly the same composition as the 22 000-dalton tryptic fragment, indicating that these two species are coextensive. Furthermore, end-group analysis suggested the presence of blocked amino termini on both the 22 000- and 23 000-dalton fragments, supporting previous reports that the amino terminus of the band 3 polypeptide is located on the cytoplasmic side of the membrane.

Band 3, an ~90 000-dalton glycoprotein, is the predominant polypeptide of the human erythrocyte membrane, comprising approximately 25% of the protein mass (cf. Steck, 1974). Band 3 is believed to be involved in the facilitated diffusion of anions across this membrane (Rothstein et al., 1976; Zaki et al., 1975; Ho and Guidotti, 1975; Wolosin et al., 1977; Ross and McConnell, 1977). Its disposition as an asymmetric membrane-spanning dimer is appropriate to this function (cf. Steck, 1974; Marchesi et al., 1976).

We recently described the generation of 11 large fragments by the proteolytic digestion of band 3 while still membrane bound and by S-cyanylation of this polypeptide in $NaDodSO_4$[1] (Steck et al., 1976) (see Figure 1). We now report on the purification of seven of these fragments (which represent all or nearly all of the mass of band 3) and their complete amino acid

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[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; DFP, diisopropyl phosphofluoridate; PAS, periodic acid–Schiff (stain for glycoproteins).

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