Human Erythrocyte Band 3 Polymorphism (Band 3 Memphis): Characterization of the Structural Modification (Lys 56 → Glu) by Protein Chemistry Methods

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Band 3 variants occur rather frequently in different populations. Based on sodium dodecyl sulfate (SDS)-polyacrylamide electrophoretic properties, a widespread polymorphism (band 3 Memphis) has been previously described. It corresponds to a protein that has been hypothesized to be elongated in its N-terminal cytoplasmic domain. Band 3 from a heterozygote subject for this polymorphism and that we observed in the mother the presence of an apparently heterozygote subject for this polymorphism and that corresponds to a protein that has been hypothesized to be elongated in its N-terminal cytoplasmic domain. Band 3 from a heterozygote subject for this polymorphism and that has been isolated and its primary structure determined by protein chemistry. Reverse-phase high performance liquid chromatography tryptic peptide mapping showed, as the only difference with controls, that the enzymatic cleavage between the two N-terminal peptides did not occur, yielding a 69 residue-long fragment. Further cleavages of this peptide (cyanogen bromide, V8 protease), amino acid composition, and sequence analyses demonstrated that the lysine at position 56 was replaced by a glutamic acid. Thus, surprisingly, a single amino acid change is responsible for the large difference in the electrophoretic behavior. This result suggests that single amino acid substitutions may similarly be involved in the structural modification of several other protein variants, described as elongated or shortened based only on SDS-polyacrylamide electrophoresis studies. When deletions/insertions were confirmed by sequence analysis, their extent was often different from that expected from electrophoresis.

MATERIALS AND METHODS

Screening blood samples for the band 3 polymorphism was achieved as described by Mueller and Morrison, with minor modifications. Fifty microliters of RBC suspended in 50 μL phosphate-buffered saline (PBS) (pH 7.4) were digested with α-chymotrypsin (1 mg/mL) at 37°C for 1 hour. α-Chymotrypsin cleaves band 3 from outside the membrane of the RBC, yielding two fragments of about 60 Kd and 35 Kd that are the N- and C-terminal parts of the protein, respectively. These fragments remain attached to the membrane during preparation of the ghosts and are visualised by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The cytoplasmic fragments of band 3 were purified as described previously. The cytoplasmic domain of band 3 was cleaved by α-chymotrypsin on inside-out vesicles and isolated by DE 52 cellulose chromatography. The isolated fragment was deionized and freeze-dried.

For peptide mapping, isolated cytoplasmic fragment of band 3 (2 mg) dissolved in 50 mmol/L ammonium bicarbonate buffer (pH 8.8) was digested at room temperature for 18 hours with trypsin (TPCK treated; Merck, Darmstadt, Germany) using an enzyme to substrate weight ratio of 3 p. 100. The peptides were separated by reverse-phase high performance liquid chromatography (RP-HPLC) using a C8 column (Aquapore RP-300; Brownlee Labs, Santa Clara, CA). Peptides were eluted with a flow rate of 1 mL/min, using a 0% to 50% acetonitrile gradient in 0.05% trifluoroacetic acid (TFA) in water. The N-terminal tryptic peptides, isolated by RP-HPLC, were further cleaved by cyanogen bromide as previously described or digested by Staphylococcus aureus strain V8 protease (V8 protease) in 50 mmol/L ammonium bicarbonate buffer (pH 7.8).

To determine their amino acid composition, the peptides (0.2 to 0.5 nmol) were dried and hydrolyzed with hydrochloric acid. Amino acid analyses were performed by RP-HPLC, after conversion into phenylthiocarbamyl (PTC) derivatives. Peptides were digested with cyanogen bromide or V8 protease, and amino acid composition was determined by RP-HPLC.
A chymotryptic cleavage of control RBC. Lane a, erythrocyte membrane proteins from the propositus. Lane b, cytoplasmic domain fragment 1.1. Lane c, cytoplasmic domain of band 3 from control. Lane d, cytoplasmic domain of band 3 from the propositus showing a duplication of the 43-Kd was used to minimize the inner filter effect. The probe was excited within the cytoplasmic domain. To localize the site of the phosphorysm (acrylamide gradient, 5% to 15%). Lane a, erythrocyte membrane proteins. The 80-Kd fragment from band 3 is duplicated (9. Lane c, 1118 YANNOUKAKOS ET AL.

After tryptic digestion, the peptides were separated by electrophoretic pattern. A systematic α-chymotryptic treatment of the RBCs from 60 blood donors, the membrane proteins were submitted to SDS-PAGE. One sample showed two bands of equal intensity in the 60-Kd region (Fig 1), one at the normal position, the second slightly above, similar to the 63-Kd band described by Mueller and Morrison.3

RBCs from this donor, heterozygous for the polymorphism, were further investigated. Sixty milliliters of RBCs yielded 19 mg of band 3 cytoplasmic domain. As shown in Fig 1, a duplication of the 43- and 41-Kd fragments was observed, demonstrating that the structural abnormality lay within the cytoplasmic domain. To localize the site of the modification, a mild trypic digestion was performed on these fragments. This procedure, which gives rise predominantly to a 20-Kd N-terminal fragment, failed to show any further duplicated bands (not shown).

Peptide mapping of the cytoplasmic domain of band 3. After trypic digestion, the peptides were separated by RP-HPLC. This analytical method yields a highly reproducible peptide map.4 The elution pattern showed, in comparison with the control, that the size of the peaks corresponding to peptides T1 (residues 1 through 56) and T2 (residues 57 through 69) was decreased by half and that a supplementary peak, peptide X, was present. It was very hydrophobic, eluting near the position of peptide T9 (Fig 2).

Amino acid composition of this supplementary peptide indicated that it was an uncleaved T1-2 peptide but the size of this fragment (69 residue) did not allow a precise determination of the structural abnormality. Peptide X was therefore cleaved by cyanogen bromide. The fragments expected from such an uncleaved T1-2 peptide, corresponding to sequences 2 through 11, 12 through 31, and 32 through 66, were obtained (Fig 3). The amino acid composition of peptide 32 through 66 showed that it contained no lysine residues but possibly a supplementary glutamic acid residue. In addition, sequence analysis from residue 12 to residue 54 was normal. V8 protease, which cleaves specifically at the C-terminal of glutamic residues at pH 7.8 in ammonium bicarbonate buffer,11 was therefore used for further characterization of the structural modification. The peak containing peptide X yielded all the fragments expected for the replacement of lysine 56 by a glutamic acid residue (Figs 3 and 4), and those resulting from the contaminating T9 peptide (sequences 117 through 125 and 126 through 138). Amino acid analysis and microsequencing of peptide 41 through 56 confirmed the Lys 56 → Glu substitution (Fig 5).

Analysis of all the other trypic peptides of the cytoplasmic fragment of band 3 protein failed to show any additional abnormality: their elution pattern and amino acid composition were identical to the control.

Binding of BADS to membranes. The affinity for BADS of membranes containing the band 3 variant was compared with that of control membranes. As shown on Fig 6, there was no difference in the BADS binding curve of the variant compared with the control. The affinities measured were kd equal to 1.3 μmol/L and 1.1 μmol/L for control and variant ghosts, respectively. The specific binding of BADS to band 3 was ascertained by pretreating the RBCs with 10 μmol/L DIDS, which binds specifically and irreversibly to band 3. This pretreatment completely inhibited BADS binding to the ghosts in both preparations.

DISCUSSION

A polymorphism of band 3 has been previously described on the basis of the electrophoretic pattern of membrane proteins after enzymatic digestion of band 3 from outside the RBC.3 It was named band 3 Memphis and was found with high frequency, from 5% to 20%, in the various populations studied by Ranney et al6 (Whites, African Americans, American Indians, and Filipinos). No epidemiologic study has been yet conducted in European populations. This polymorphism may include several variants. For instance, based on the reactivity of band 3 with stilbene disulfonates, two variants were distinguished.4 These two variants were not associated with hematologic abnormalities and the anion transport activities were within the normal range.3 The most frequent variant seems to have a normal reactivity with DIDS.5

The electrophoretic pattern suggested an increased molecular weight of the variant, which was interpreted as the incorporation of an additional segment in the cytoplasmic...
Fig 2. Elution pattern of the tryptic digest of the cytoplasmic domain of band 3 from an individual heterozygous for band 3 Memphis (upper panel) and for the control (lower panel). Peptide nomenclature is according to Yannoukakos et al. Peptides T1 (*) and T2 (*) were reduced by 50% with respect to the control. A supplementary peak X (indicated by the arrow) was eluted near peptide T9. The rest of the profile was identical to that observed in controls.

Fig 3. Primary structure of peptide T1-2 of band 3 Memphis. The fragments resulting from cyanogen bromide cleavage (↓) or from protease V8 digestion (↑) that have been identified are indicated by horizontal arrows. The part of the peptide that has been sequenced is underlined. It was sequenced in three steps: 12 through 30, 32 through 54 from cyanogen bromide (CNBr) fragments, and 41 through 56 after V8 protease cleavage. At position 56 (*) a glutamic acid was found instead of a lysine.

Fig 4. Elution pattern of the peptides resulting from protease V8 cleavage of peak X from band 3 Memphis. Sequences from 1 to 69 result from cleavage of peptide T1-2; the amino acid substitution was found in peptide 41 through 56. The two peptides 117 through 125 and 128 through 138 result from contaminating T9.

domain. Actually, the molecular characterization reported here shows only a single amino acid variation, at position 56, where a glutamic acid replaces the usual lysine residue. How such a subtle change in the protein structure may be responsible for a retardation in the electrophoretic migration in SDS-polyacrylamide gel is still unclear. Changes in side chain hydrophobicity or in the protein conformation as induced by a proline residue have been shown to modify the electrophoretic mobility. Altering the SDS binding to the protein is responsible for the change in mobility. As SDS binds principally to basic residues, the substitution of a lysyl residue for a glutamic acid should decrease SDS binding and may be responsible for the decreased mobility observed, either through the induced modification of charge or through a difference in conformation of the SDS-protein complex. It may be pointed out that the altered migration is observed for the 60- and 43-Kd but no longer for the 20-Kd fragment.

The first report of the primary structure of human band 3, deduced from the cDNA sequence analysis by Tanner et
al,13 described a glutamic acid at position 56. The protein structure analysis of the cytoplasmic domain of the protein, performed on samples from several individuals,14 as well as that of the 20-Kd N-terminal fragment15 differed from that of Tanner et al at this residue. Further, the cDNA sequence of human band 3 reported by Lux et al predicted a lysine residue at position 56.16 It may be suggested that the cDNA studied by Tanner et al carried the “Memphis” polymorphism.

The present band 3 variant is the first whose structural modification is characterized at the amino acid level. It is very likely to correspond to the widespread “Memphis” polymorphism with normal reactivity to DIDS; since the beginning of this work, we found this electrophoretic polymorphism in another subject and structural analysis showed the same mutation. Nevertheless, it cannot be excluded that other amino acid substitutions may result in a similar electrophoretic pattern.

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