Biochimica et Biophysica Acta, 773 (1984) 237–245
Elsevier

BBA 72126

THE ROLE OF ANKYRIN IN SHAPE AND DEFORMABILITY CHANGE OF HUMAN ERYTHROCYTE GHOSTS

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(Received August 2nd, 1983)
(Revised manuscript received February 29th, 1984)

Key words: Ankyrin; Cell shape; Tryptic digestion; Erythrocyte membrane

Human erythrocyte membranes (ghosts) from acid/citrate/dextrose preserved blood were digested with trypsin (protein/trypsin = 100:1) under hypotonic conditions and then analyzed by SDS-polyacrylamide gel electrophoresis. After digestion for about 20–30 s at 0°C, only ankyrin had disappeared and other bands including spectrin, actin, band 4.1 and band 3 remained intact. This observation was supported by electron micrographs showing that the horizontally disposed, filamentous structure was a little apart from the lipid bilayer and its components were not destroyed. In contrast to intact ghosts, treatment with chlorpromazine, or Mg-ATP did not induce shape change in these trypsin-treated ghosts. The number of transformable cells correlated closely with the amount of remaining ankyrin in the SDS-polyacrylamide gel electrophoresis pattern. Furthermore, the chlorpromazine- and Mg-ATP-induced decreases in viscosity of suspensions of erythrocyte ghosts were also prevented by trypsin treatment for 20–30 s at 0°C. These findings suggest that ankyrin plays an important role in the change in shape and deformability of erythrocyte ghosts. The molecular mechanism of drug-induced shape change and the role of undermembrane structure in regulating erythrocyte shape and deformability are discussed.

Introduction

Human erythrocytes maintain a characteristic biconcave disk shape and change shape reversibly under various conditions [1]. Various amphipathic agents have been employed to change the normal biconcave shape into either a crenated or a cupped shape [2]. Sheetz and Singer [3,4] proposed a bilayer couple hypothesis for the molecular mechanism of shape change induced by amphipathic agents, based on the assumption of hypothetical insertion of the agents into the asymmetric lipid bilayer. Recently Conrad and Singer [5,6] cast some doubt on this theory, using a hygroscopic desorption method. Nakao et al. [7,8] first showed that the shape of the human erythrocyte is dependent on the intracellular ATP content. Later, Sheetz and Singer [9] showed that ghosts prepared from fresh intact cells change shape from crenate to disk after incubation with Mg-ATP at 37°C. Birchmeier and Singer [10] suggested that ATP is used for spectrin phosphorylation, which induces structural change of the spectrin complex on the cytoplasmic surface of the membrane. However, some authors opposed this view and insisted on the importance of lipid metabolism [11,12]. The

Abbreviation: SDS, sodium dodecyl sulfate.

addition of chlorpromazine or an increase in ATP content decreased the viscosity of metabolically depleted erythrocytes, accompanied with the change of shape [13,14]. In relation to the molecular mechanism of the shape change and deformability, the existence of an undermembrane structure of human erythrocyte membrane has been established [15–19] and Tsukita et al. [20,21] clearly visualized this cytoskeletal network by electron microscopy with tannic acid fixation. However, the direct relation between the cytoskeletal network and various functions such as shape and deformability has not been elucidated yet. Recently we showed that ankyrin is necessary for their shape change [22], and that there are two steps in the ATP-dependent shape change of ghosts [23] in preliminary reports. This paper deals with a more precise investigation of the role of the undermembrane structure in shape and deformability change in erythrocyte ghosts.

Materials and Methods

Preparation of erythrocyte membranes (ghosts). Human erythrocytes obtained from acid/citrate/dextrase (NIH formula A) blood (4–6 weeks preserved) were washed three times with physiological saline and hemolyzed by 1 : 20 dilution with 10 mM Tris-HCl buffer (pH 7.4). The ghosts were pelleted at 18 000 rpm for 20 min and were washed twice with the hemolysis buffer at 0°C.

Proteolytic digestion. The pelleted ghosts were resuspended in 10 mM Tris-HCl buffer, or 2 mM Mg-ATP solution (pH 7.4 adjusted with Tris or NaOH) to a protein concentration of 2.5 mg/ml and then trypsin (Boehringer Mannheim) was added to a final concentration of 25 μg/ml and the mixture was incubated at 0°C. After incubation for 0 to 60 seconds, soybean trypsin inhibitor (Sigma type II-s) was added to a concentration of 100 μg/ml and aliquots of the ghost suspensions were withdrawn for analysis by SDS-polyacrylamide gel electrophoresis with 3.3% or 5.6% polyacrylamide disc gel according to the methods of Fairbanks [24]. The osmotic pressure was raised to isotonic by addition of KCl in some experiments.

Observation of shape. To observe the effect of chlorpromazine, an equal volume of 0.5 mM chlorpromazine in 0.154 M NaCl was added to the ghost suspensions. To observe the first step of Mg-ATP-dependent shape change, the ghosts in the isotonic KCl soln. containing 2 mM Mg-ATP were brought to 37°C and incubated for 30 min. In order to observe the second step, the ghosts, incubated with 2 mM Mg-ATP at 37°C and washed with 1 mM $MgCl_2$ (18 000 rpm 20 min), were resuspended in 2 mM Mg-ATP solution again at room temperature and isotonicity was restored by addition of KCl. When observation was done under hypotonic conditions, the additions of KCl was omitted. Fixation for light microscopic observation was done according to the procedure of Patel and Fairbanks [25], but under hypotonic conditions we observed them without fixation since it is very difficult to fix ghosts. Each sample was examined under a dark-filed light microscope (Nikon, Model S) and 200 membranes were counted.

Observation of viscosity. The specific viscosity of ghost suspensions was determined by using an Ostwald type viscometer which we had manufactured ourselves. Test solution contained 0.15 ml of packed ghosts in 0.3 ml of the same solution as that used for the observation of shape change. The viscometer was standardized with 0.3 ml of H_2O or the indicated medium at 37 ± 0.005°C in an accurately controlled temperature water bath. Specific viscosity is the relative viscosity minus one.

Electron microscopy. The membrane samples were processed for electron microscopy as previously described [20]. Sections were examined in a Hitachi HU-12 electron microscope operated at 100 kV.

Results

Time-course of trypsin treatment

Ghosts, prepared from acid/citrate/dextrase (ACD) blood with 10 mM Tris-HCl buffer, were resuspended in 10 mM Tris-HCl buffer or 10 mM Tris-HCl containing 2 mM Mg-ATP solution (pH 7.4) and digested with trypsin (protein/trypsin = 100 : 1) at 0°C. It was clear from the time-course of trypsin digestion that ankyrin decreased rapidly to almost 0% after 20–30 s, although spectrin, band 4.1, band 5 and band 3 remained unchanged for at least 40 s at 0°C (Fig. 1). A half of the

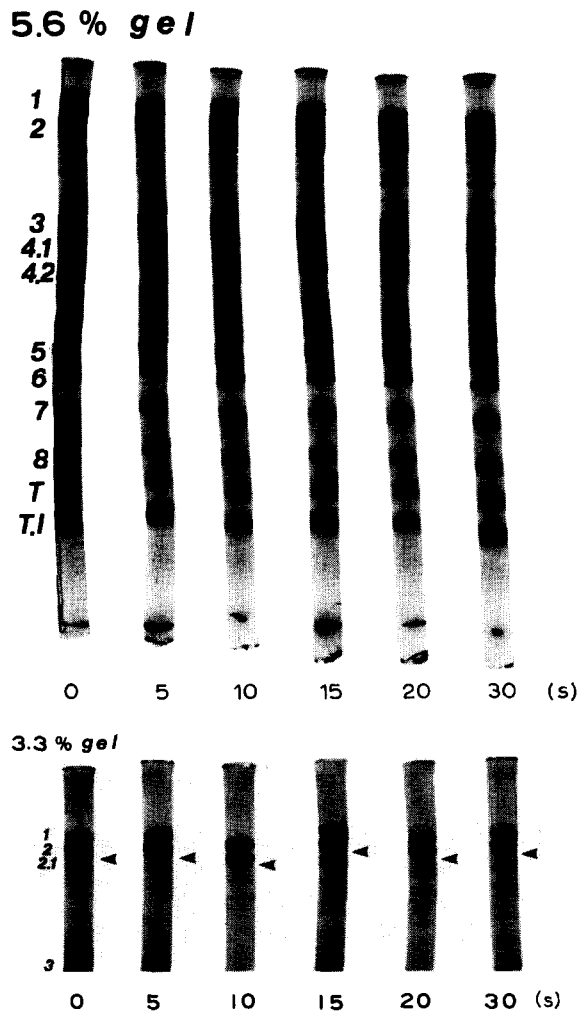

Fig. 1. Time-course of trypsin digestion of human erythrocyte membranes. Experimental conditions are described in Materials and Methods. Samples were subjected to electrophoresis in 3.3% and 5.6% polyacrylamide disc gels by the method of Fairbanks et al. [24]. The data shown are those of a typical experiment taken from five similar series.

ankyrin was lost in about 7–8 s and almost all the ankyrin had disappeared within 20 s. Accompanying with disappearance of ankyrin, new bands in the 2.2 to 2.6 region appeared as shown Fig. 1. After 40 s, band 3 decreased gradually and after 90 s, spectrin began to decrease (not shown). When trypsin inhibitor was previously added to ghost suspensions with trypsin as a control, no change in SDS-polyacrylamide gel electrophoresis pattern or in shape change with Mg-ATP (see below) occurred.

Electron microscopic observation of trypsin-treated ghosts

After tryptic digestion for 30 s at 0°C, electron microscopic observation of thin sections of these ghosts was performed (Fig. 2). As Tsukita et al. [20,21] showed with the aid of tannic acid fixation, a layer of vertical components with granular appearance, and a horizontally disposed, anastomosing meshwork of filamentous structures can be seen in Fig. 2a. After trypsin digestion for 30 min at 0°C, the layer of horizontally disposed, filamentous components was not destroyed but was distant from the lipid bilayer (Fig. 2b). Because vertical granular components are considered to consist mainly of ankyrin, this observation supports our results in SDS-polyacrylamide gel electrophoresis.

No change in shape of ghosts pretreated with trypsin

When ghosts were prepared with 10 mM Tris-HCl buffer (pH 7.4), they were mostly spherocytes and rapidly changed into echinocytes when the tonicity was raised with KCl to isotonic (Fig. 3a) [9]. After addition of chlorpromazine (final concentration 0.25 mM) to these crenated ghosts without trypsin digestion or to ghosts resealed with both trypsin and trypsin inhibitor the shape changed into discocyte form immediately (Fig. 3b).

Once the ghosts had been digested by trypsin, their shape became insensitive to chlorpromazine. The time-course of trypsin digestion and shape changes of ghosts are shown in Fig. 4. After trypsin digestion for 20 s, discocytes were no longer seen and all ghosts remained as echinocytes even if chlorpromazine was added (Fig. 3c). The crenated form remained unchanged for at least several minutes at room temperature after addition of chlorpromazine and then vesicles were pinched off from the surface of the membranes. The number of ghosts which were changed into disc form by chlorpromazine decreased in parallel with the decrease in ankyrin (Fig. 4). Similar shape changes from echinocyte to discocyte were also observed when Mg-ATP was added to ghost suspensions under hypotonic conditions [9,25]. Recently we showed that there are two steps in the Mg-ATP-dependent shape change of the ghosts; the first step proceeds slowly during incubation at 37°C

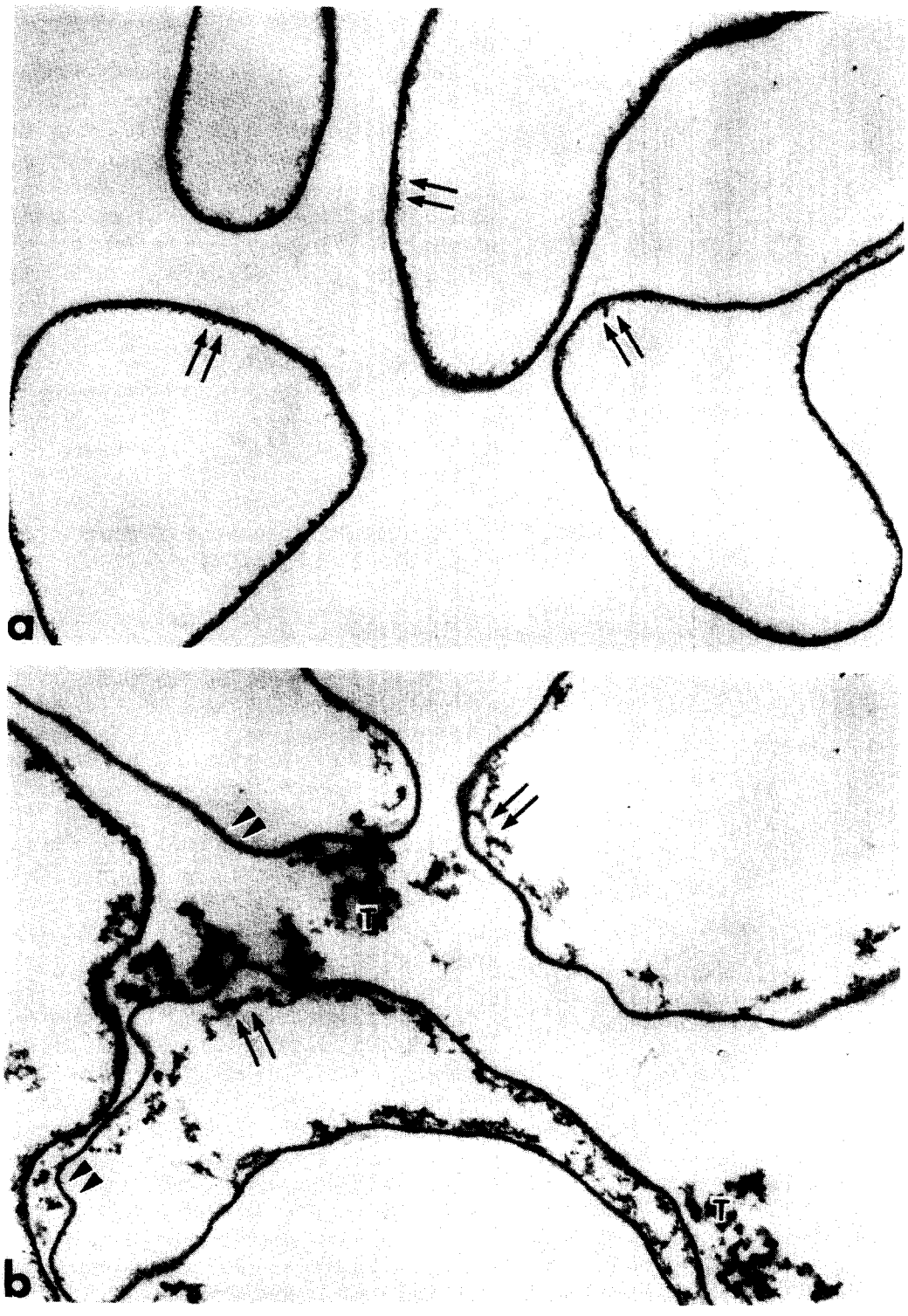

Fig. 2. Thin-section electron micrographs of erythrocyte membranes before (a) and after (b) the trypsin treatment. (a) The cytoskeletal networks underlying the erythrocyte membranes (arrows) are closely associated with the cytoplasmic surfaces of the membranes. (b) In contrast with (a), when the erythrocyte membranes are treated with trypsin (30 s at 0°C), the cytoskeletal networks (arrows) are detached and raised from the membrane proper (arrowheads). T: probable trypsin-trypsin inhibitor complex. Magnification ×40000.

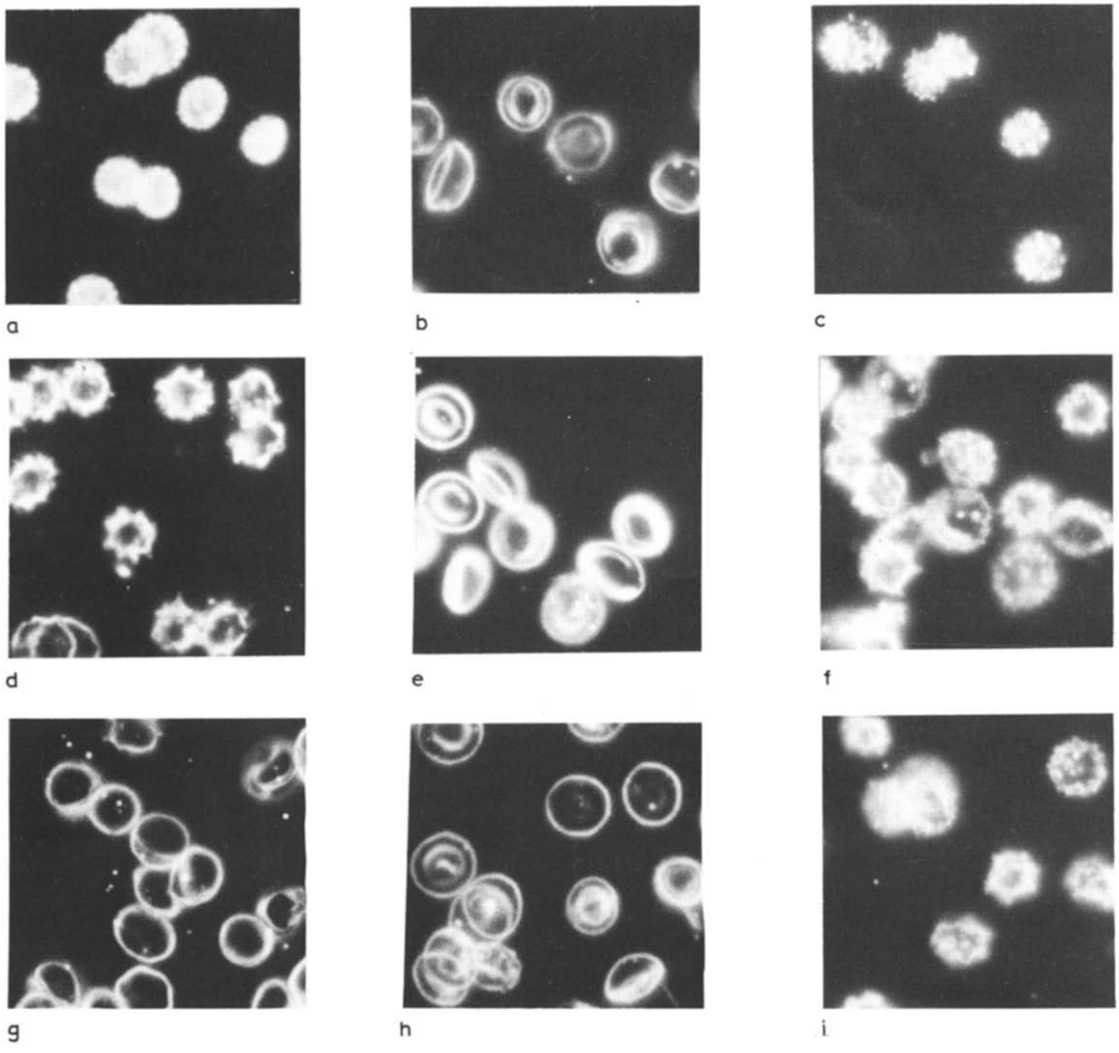

Fig. 3. Shape change of ghosts seen in dark-field light micrographs. The effects of trypsin digestion on chlorpromazine-dependent shape change under isotonic condition (a–c), the first step of Mg-ATP-dependent shape change (d–f) and the second step of Mg-ATP-dependent shape change (g–i) of ghosts under hypotonic conditions. a, control ghosts in 154 mM KCl; b, control ghosts after addition of 0.5 mM chlorpromazine; c, trypsin-treated (30 s at 0°C) ghosts after addition of 0.5 mM chlorpromazine; d, control ghosts in 2 mM Mg-ATP for 0 min; e, control ghosts incubated in 2 mM Mg-ATP for 20 min at 37°C; f, trypsin-treated (30 s at 0°C) ghosts incubated in 2 mM Mg-ATP for 20 min at 37°C; g, control ghosts after preincubation in 2 mM Mg-ATP for 20 min at 37°C and washing with 1 mM $MgCl_2$; h, control ghosts immediately on addition of 2 mM Mg-ATP at room temperature after preincubation in 2 mM Mg-ATP for 20 min at 37°C and washing with 1 mM $MgCl_2$; i, trypsin-treated (30 s at 0°C) ghosts immediately on addition of 2 mM Mg-ATP at room temperature after preincubation in 2 mM Mg-ATP for 20 min at 37°C and washing with 1 mM $MgCl_2$.

for 30 min in the presence of a physiological concentration of Mg-ATP and the second step occurs rapidly on addition of Mg-ATP even at 6°C [23]. The effect of trypsin digestion on each step was examined (Fig. 3, d–i). The number of transformable ghosts paralleled the amount of remaining ankyrin at the first step (Fig. 4). As similar results were also observed at the second step of Mg-ATP-dependent shape change (Fig. 4), ankyrin is also necessary for the ATP-dependent

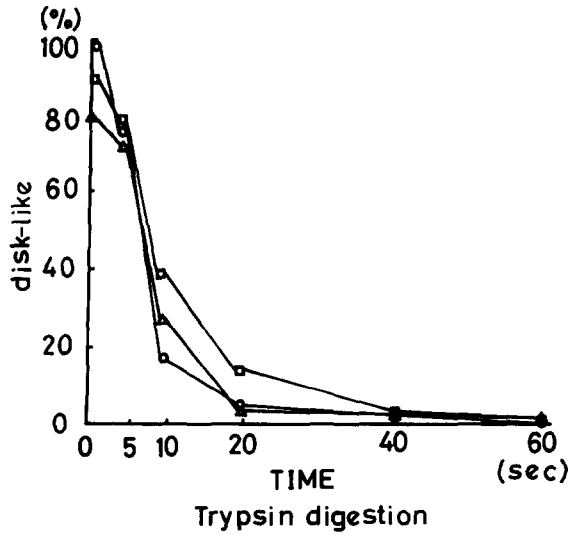

Fig. 4. The effect of trypsin digestion on chlorpromazine-dependent and Mg-ATP-dependent shape change of ghosts. The ghosts were digested with trypsin in 10 mM Tris-HCl buffer at 0°C and the reaction was stopped by addition of trypsin inhibitor at the indicated time, then 0.5 mM chlorpromazine was added (○) after isotonicity had been restored. The ghosts were digested in the presence of 2 mM Mg-ATP at 0°C and trypsin inhibitor was added at the indicated time, then these ghosts were incubated for 30 min at 37°C (△) after isotonicity had been restored. The ghosts, after being preincubated in 2 mM Mg-ATP for 20 min at 37°C and washed with 1 mM $MgCl_2$, were digested with trypsin at 0°C and trypsin inhibitor was added to them at the indicated time, then the shape was observed immediately on addition of 2 mM Mg-ATP at room temperature (□). Each sample was examined by dark-field light microscopy and 200 membranes were counted.

shape change. These changes with Mg-ATP or chlorpromazine were completely prevented in ghosts pretreated with trypsin.

Viscosity of ghost suspensions

Mg-ATP and chlorpromazine acted not only on the shape of ghosts, but also on the viscosity. Fig. 5 shows the results of microscopic observation and ghost viscosity measurement after addition of various concentrations of chlorpromazine. Crenation and stomatocyte formation increased the ghost viscosity in a dose-related fashion as described in the literature [14]. It is clear from Fig. 5 that the change of ghosts to a biconcave shape, as well as that of intact erythrocytes, is accompanied with a decrease in ghost viscosity. On tryptic digestion at 0°C, the decrease in the viscosity of the ghost suspension in the presence of chlorpromazine gradually became less. After digestion for 30 s the viscosity was essentially unchanged when chlorpromazine was added to the ghosts (Fig. 6). Since ghosts which had been digested with trypsin were labile on addition of chlorpromazine as described above, we measured the viscosity of their suspension immediately after the treatment at 37°C. The relative specific viscosity of the ghost suspension decreased gradually during incubation of the suspensions with Mg-ATP, as Quist described [26] (Fig. 7B); there was an increase in the specific viscosity of preincubated ghost suspension after washing with 1 mM $MgCl_2$ and a rapid re-decrease in specific viscosity on addition of 2 mM Mg-ATP at 37°C (Fig. 7A). There seemed to be two steps in ATP-dependent viscosity change, accompanied with shape change (Fig. 7A). We first examined the effect of trypsin digestion on

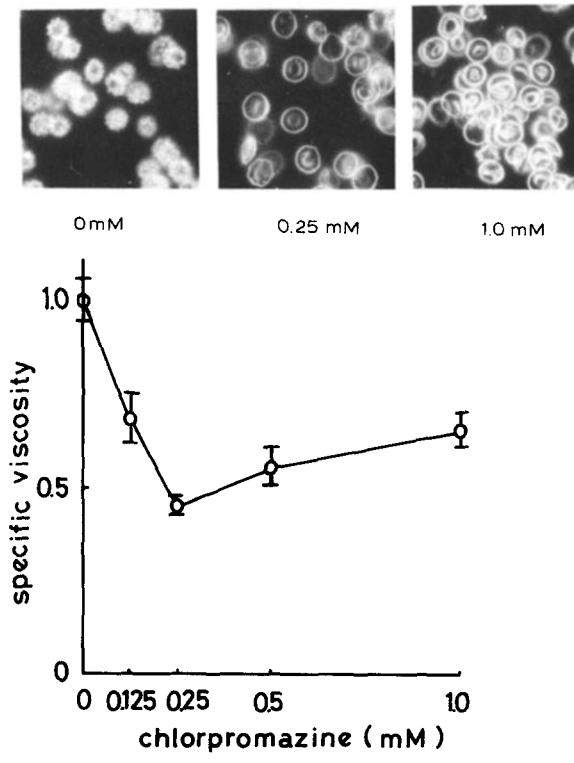

Fig. 5. The effect of chlorpromazine on the specific viscosity of ghost suspensions. After addition of chlorpromazine to ghosts, the specific viscosity was measured with an Ostwald viscometer as described in Materials and Methods and the shape of ghosts was observed with a dark-field light microscope. The average values and standard deviations ($n = 4$) are indicated.

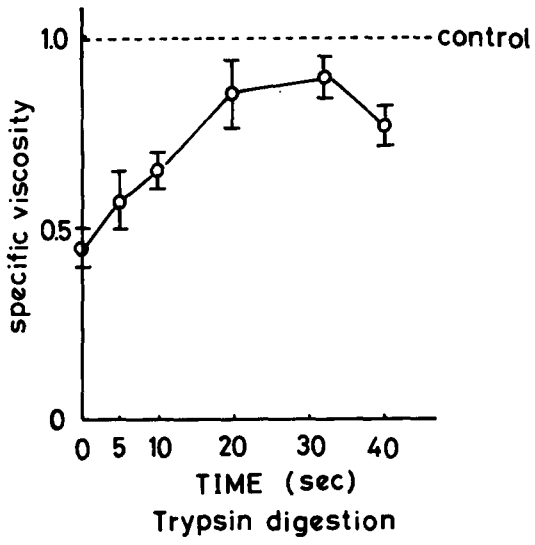

Fig. 6. The effect of trypsin digestion on the chlorpromazine-induced decrease in specific viscosity of ghost suspensions. The ghosts were digested with trypsin in 10 mM Tris-HCl buffer at 0°C and the reaction was stopped by addition of trypsin inhibitor at the indicated times and then isotonicity was restored by adding KCl. Specific viscosity was measured immediately after addition of 0.5 mM chlorpromazine at 37°C. The control level shows the specific viscosity of non-treated ghost suspension in the absence of chlorpromazine. The average values and standard deviations ($n = 4$) are indicated.

the second step. Trypsin was added after preincubation of ghosts with Mg-ATP and washing, and the action was stopped by the addition of trypsin inhibitor. The viscosity of the treated ghosts were not decreased when Mg-ATP was added. The extent of the decrease was reduced according to the reaction time for digestion (Fig. 7A). Next, in order to examine the effect of trypsin digestion on the first step, we measured the viscosity of trypsin-treated ghosts after incubation in the presence of Mg-ATP. When trypsin digestion was carried out for a long time, the ghosts broke down and their viscosity decreased after incubation at 37°C. However, within at least the first 40 seconds of trypsin digestion, ghosts were stable as long as the tonicity of the sodium was isotonic, and their specific viscosity was not changed for 30 min at 37°C, even through ankyrin had completely disappeared (not shown). These data show that the decrease in specific viscosity with Mg-ATP both at the first step and at the second step (Fig. 7B) was prevented by mild trypsin treatment.

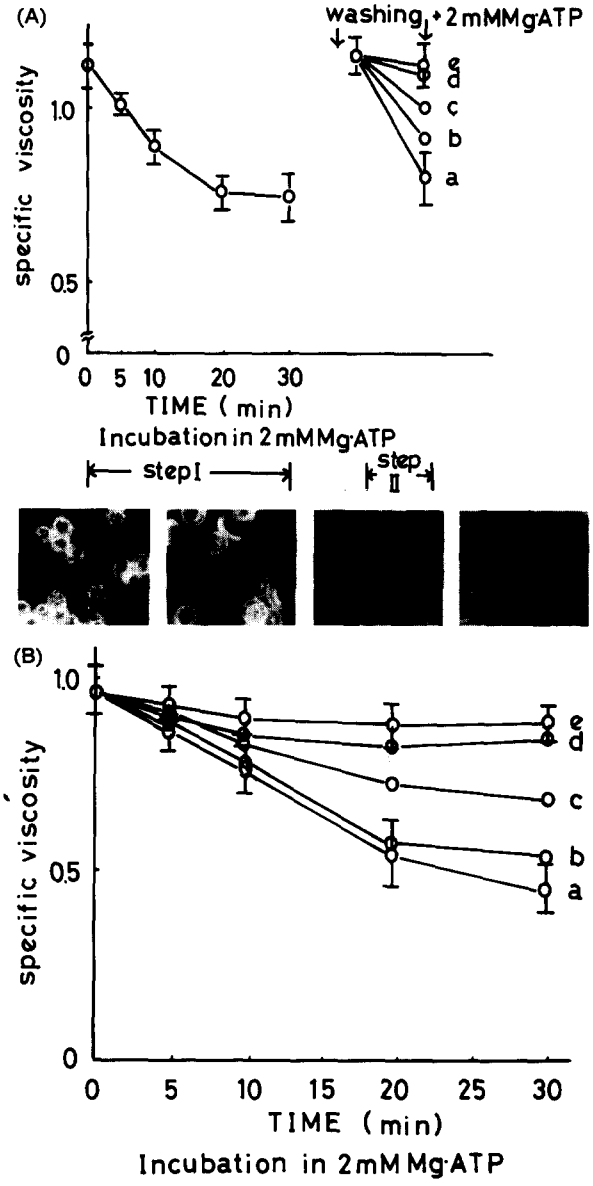

Fig. 7. The effect of trypsin digestion on the two steps of Mg-ATP-dependent decrease in specific viscosity of ghost suspensions. (A) Second step. The ghosts, after being preincubated in 2 mM Mg-ATP for 30 min at 37°C and washed with 1 mM $MgCl_2$, were treated with trypsin as described in Materials and Methods (a, 0 min; b, 5 s; c, 10 s; d, 20 s; e, 30 s). The specific viscosity was measured immediately after addition of 2 mM Mg-ATP at 37°C. Dark-field micrographs show control ghosts in the two steps. The average values and standard deviations ($n = 4$) are indicated. (B) First step. The ghosts were treated with trypsin (a, 0 min; b, 5 s; c, 10 s; d, 15 s; e, 20 s; f, 30 s) in the presence of 2 mM Mg-ATP and incubated at 37°C after isotonicity had been restored by addition of KCl. Specific viscosity was measured at the indicated time. The average values and standard deviations ($n = 4$) are indicated.

Discussion

Several investigators have described the effect of trypsin digestion on erythrocyte membranes, but under conditions quite different from those of the present experiment. The trypsin-substrate ratio, and the time and temperature of digestion were much milder in the present experiment. Accompanying with disappearance of ankyrin, formation of new polypeptides in the band 2.2 to 2.6 region was observed. These new polypeptides were considered to be not functional species, because the ghosts treated with trypsin did not change their shapes. However, we can not decide whether natural 2.2 to 2.6 peptides without trypsin treatment are functional species or not. According to Steck et al. [27], splitting of band 3 was faster than that of spectrin, as we confirmed in this report, although Triplett and Carraway [28] claimed that trypsin split spectrin more easily than band 3 in isotonic solutions. However, the most remarkable finding in the present work is that ankyrin was digested much faster than spectrin and band 3. Neither Steck et al. nor Triplett and Carraway observed the effect of trypsin on ankyrin in the hypotonic state. It is generally accepted that ankyrin is very sensitive to protease, and the difficulty in its purification is due to this characteristic [29,30]. Further we checked on our results in SDS-polyacrylamide gel electrophoresis by electron microscopic observation. After trypsin digestion for 30 s at 0°C, the layer of horizontally disposed, filamentous components which looked similar to that in intact ghosts was distant from the lipid bilayer. Therefore we consider that among major cytoskeletal proteins, only ankyrin was digested under our conditions. A faintly amount of amorphous substances newly appeared outside the ghosts in Fig. 2b (marked as T). This structure may be trypsin-trypsin inhibitor complex.

For the molecular mechanism of drug-induced shape change of erythrocyte, Sheetz and Singer [3,4] proposed the lipid bilayer couple hypothesis, which is based on the insertion of the drug into a lipid leaflet. However, there are some exceptions, such as sodium dodecyl sulfate or alkyltrimethylammonium, both of which are stomatocytegenic [31]. Recently Conrad and Singer [5,6] cast some doubt on this interpretation on the basis of hygroscopic desorption data. On the other hand, Birchmeier and Singer [10] found that dephosphorylation of spectrin by alkaline phosphatase treatment prevented the chlorpromazine-induced change from crenated to disk shape and suggested that the phosphorylation of spectrin (band 2) is critical to the drug-induced shape change, although they did not examine the effect of re-phosphorylation on these shape changes. However, chlorpromazine can induce stomatocytes even in erythrocytes extensively depleted of ATP [32]. Because chlorpromazine did not induce shape change of ghosts in which only ankyrin was digested, we also doubt this lipid bilayer hypothesis and suggest that ankyrin is necessary for the drug-induced shape change of ghosts.

Calmodulin is known to interact with spectrin [33], and a variety of calmodulin inhibitors including chlorpromazine induce stomatocytes or cupping [34]. Some authors [34] have proposed that calmodulin is required to maintain the normal biconcave shape. There is some possibility that calmodulin may play a role in the shape change of ghosts. Nevertheless, we consider this to be very unlikely, because almost all calmodulin is in the membrane-free hemolysate [35].

Recently we found that there are two steps in the ATP-dependent shape change of human erythrocyte ghosts [23]; the first step proceeds slowly during incubation at 37°C for 20 min in the presence of physiological concentrations of Mg-ATP and the second step occurs rapidly after addition of Mg-ATP even at 6°C. We examined the effect of trypsin digestion on these two steps and found that both steps were inhibited by mild trypsin treatment. Although the number of transformable ghosts paralleled the amount of remaining ankyrin in the first step, it must be considered that not only ankyrin but also a tiny amount of an enzyme which cannot be observed in SDS-polyacrylamide gel electrophoresis might be digested by trypsin. Since the second step was rapid after addition of Mg-ATP even at 6°C, as was the effect of chlorpromazine, we consider that covalent modification of the enzyme by some enzymatic reaction was not involved in the second step [23]. Therefore ankyrin is necessary for the ATP-dependent shape change of ghosts.

Quist [26] reported that the specific viscosity of

ghost suspension decreased in the presence of 1 mM Mg-ATP at 22°C for 20 min when the specific viscosity was determined using a Cannon-Manning semimicroviscometer, and that the changes in viscosity are sensitive indexes of Mg-ATP dependent shape change. Although we determined the specific viscosity in the presence of 2 mM Mg-ATP at 37°C using an Ostwald viscometer, our results agreed quite well with those of Quist, showing that the specific viscosity of ghost suspension decreased gradually during incubation with Mg-ATP. Further, we examined the effect of trypsin digestion on the chlorpromazine-induced and ATP-induced shape changes of ghosts, and the results obtained were consistent with the morphological changes described above. Ankyrin is not only necessary for shape change, but may also play an important role in maintaining erythrocyte deformability, probably because ankyrin connects the flexible but deformable undercoat membrane structure with the lipid bilayer.

Acknowledgments

A part of the work was supported by the grant in aid for Scientific Research (58105010, 56370002) from the Ministry of Education, Science and Culture of Japan.

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