ON THE MECHANISM OF ATP-INDUCED SHAPE CHANGES IN HUMAN ERYTHROCYTE MEMBRANES

II. The Role of ATP

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ABSTRACT

In the preceding paper (Sheetz, M. and S. J. Singer. 1977. J. Cell Biol. 73:638-646) it was shown that erythrocyte ghosts undergo pronounced shape changes in the presence of Mg-ATP. The biochemical effects of the action of ATP are herein examined. Phosphorylation by ATP of spectrin component 2 of the erythrocyte membrane is known to occur. We have shown that it is the only membrane protein that is significantly phosphorylated under the conditions where the shape changes are produced. The extent of this phosphorylation rises with increasing ATP concentration, reaching nearly 1 mol phosphoryl group per mole of component 2 at 8 mM ATP. Most of this phosphorylation appears to occur at a single site on the protein molecule, according to cyanogen bromide peptide cleavage experiments. The degree of phosphorylation of component 2 is apparently also regulated by a membrane-bound protein phosphatase. This activity can be demonstrated in erythrocyte ghosts prepared from intact cells prelabeled with [32P]phosphate. In addition to the phosphorylation of component 2, some phosphorylation of lipids, mainly of phosphatidylinositol, is also known to occur. The ghost shape changes are, however, shown to be correlated with the degree of phosphorylation of component 2. In such experiments, the incorporation of exogenous phosphatases into the ghosts reversed the shape changes produced by ATP, or by the membrane-intercalating drug chlorpromazine. The results obtained in this and the preceding paper are consistent with the proposal that the erythrocyte membrane possesses kinase and phosphatase activities which produce phosphorylation and dephosphorylation of a specific site on spectrin component 2 molecules; the steady-state level of this phosphorylation regulates the structural state of the spectrin complex on the cytoplasmic surface of the membrane, which in turn exerts an important control on the shape of the cell.
Obtained from Sigma, cyanogen bromide (CNBr) from Reagents (San Diego, Calif.). Other reagents were obtained from Eastman, and p-nitrophenylphosphate from Calbiochem Sigma Chemical Co., St. Louis, Mo. Strophanthidin was nonlabeled additives (ATP, AMP-PNP, etc.) under the conditions described in the previous paper (31). Thus, the membranes were incubated at 0°C and a hematocrit of 50% for 5 min only. Ghosts were often immediately washed in the 20-fold volume of lysis buffer as specified below. Fresh ghosts were labeled with γ-[32P]ATP under the "shape change conditions" described in the previous paper (31). Thus, the membranes were incubated at 0°C and a hematocrit of 50% for 5 min with, for instance, 0.8 mM γ-[32P]Mg-ATP (sp act 0.1 Ci/mmol and higher) plus one of several other additives (AMP-PNP, NaPi, etc.). Salt was then added to a final concentration of 140 mM KCl, 20 mM NaCl, and 5 mM MgSO4, followed by incubation at 37°C. Reaction was stopped either by dissociation in sodium dodecyl sulfate (SDS) or by a second lysis and washing as specified below. Spectrin 32P-label turnover experiments were carried out with ghosts from intact cells prelabeled with Na[32Pi] (20) or by label turnover experiments were carried out with ghosts from intact cells prelabeled with Na[32Pi] as described above, incubating the ghosts with γ-[32P]ATP under the conditions described in the previous paper (31). Thus, the membranes were incubated at 0°C and a hematocrit of 50% for 5 min only. Ghosts were often immediately washed in the 20-fold volume of lysis buffer as specified below. Fresh ghosts were labeled with γ-[32P]ATP under the "shape change conditions" described in the previous paper (31). Thus, the membranes were incubated at 0°C and a hematocrit of 50% for 5 min with, for instance, 0.8 mM γ-[32P]Mg-ATP (sp act 0.1 Ci/mmol and higher) plus one of several other additives (AMP-PNP, NaPi, etc.). Salt was then added to a final concentration of 140 mM KCl, 20 mM NaCl, and 5 mM MgSO4, followed by incubation at 37°C. Reaction was stopped either by dissociation in sodium dodecyl sulfate (SDS) or by a second lysis and washing as specified below. Spectrin 32P-label turnover experiments were carried out with ghosts from intact cells prelabeled with Na[32Pi] as described above, incubating the ghosts with unlabeled additives (ATP, AMP-PNP, etc.) under the conditions described in the case with labeled ATP.

**Preparation and CNBr-Cleavage of 32P-labeled Spectrin Complex**

Spectrin was isolated according to Marchesi (17) from ghosts that had been labeled as described above by incubation with 8 mM γ-[32P]Mg-ATP. After a second lysis and two washes the packed ghosts were suspended into 20 vol of 0.1 mM EDTA, pH 8.0, at 37°C for 20 min, followed by centrifugation for 60 min at 45,000 rpm. in a Spinco 50 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). During this incubation, spectrin component 2 retained >80% of the radioactivity label as judged by SDS gel electrophoresis. The supernate containing the spectrin complex was then made 10% in formic acid and concentrated by rotary evaporation. Spectrin protein was removed from any small molecular weight 32P-label on a 0.5 x 50 cm column of Sephadex G-100 in 10% formic acid. CNBr-digestion was carried out according to Steers et al. (32) in 70% formic acid for 24 h at room temperature. After concentration by rotary evaporation, small molecular weight products from the CNBr reaction, containing less than 10% of the total radioactivity, were removed on a 0.9 x 50 cm column of Sephadex G-25 in 10% formic acid. The CNBr peptides were lyophilized and analyzed by SDS polyacrylamide electrophoresis.

**SDS Gel Electrophoresis and Autoradiography**

The procedure of Laemmli (15) was used in a slab gel apparatus designed at the Brookhaven National Laboratory (Brookhaven, N.Y.). For the analysis of the erythrocyte membrane proteins, gradient gels from 6 to 10% polyacrylamide were employed. The CNBr peptides of labeled spectrin were electrophoresed on 19% gels. The separation gel was overlayed with a 5% polyacrylamide stacking gel. After staining with Coomassie blue the gel slabs were fixed with 10% trichloracetic acid, 20% methanol, then were washed with 7.5% acetic acid, 10% methanol. The slabs were then dried under reduced pressure and autoradiography was carried out usually for 1-2 days. For measuring radioactivity, parts of the dried gel, e.g., spectrin band 2, were cut out, digested with NCS (Amersham/Searle Corp., Arlington Heights, Ill.) and counted in Toluene-Liquifluor (New England Nuclear, Boston, Mass.) Alternately, gel and autoradiogram were photographed and the negative was scanned with a Joyce-Loebel microdensitometer.

For electrophoresis, samples of erythrocyte ghosts were 1:1 diluted into boiling dissociation buffer composed of 100 mM Tris Cl, 2% SDS, 4 mM EDTA, 2% mercaptoethanol, 20% glycerol, pH 6.8, followed by boiling for 2 min. For experiments with added phosphatases, this buffer was supplied with additional 50 mM NaP, to prevent any dephosphorylation during the dissociation process.

The calculation of the specific radioactivity of the complex which is attached to the membrane. In this paper, we have investigated the biochemical role of the ATP in these phenomena. The ATP could conceivably function as a substrate for an ATPase enzyme, or for a kinase in a phosphorylation reaction. A number of different experiments strongly support the latter view, and are consistent with the proposal that phosphorylation-dephosphorylation reactions involving spectrin component 2 are biochemical events that are critical to the regulation of the shape of the ghost and the intact erythrocyte.

**MATERIALS AND METHODS**

**Reagents**

Acid phosphatase from potatoes, 60 U/mg, and alkaline phosphatase from Escherichia coli, 20 U/mg, both grade I, were purchased from either Boehringer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) or Sigma Chemical Co., St. Louis, Mo. Strophanthidin was obtained from Sigma, cyanogen bromide (CNBr) from Eastman, and p-nitrophosphophosphate from Calbiochem (San Diego, Calif.). Other reagents were obtained from the same sources as indicated in reference 31.

**Phosphorylation of Erythrocyte Ghosts**

[32P]-labeled ghosts were either prepared by incubation of intact erythrocytes with Na[32Pi] (20) or by labeling ghosts with γ-[32P]ATP. Thus, fresh erythrocytes were incubated at a hematocrit of 50% in 146 mM NaCl, 20 mM Tris Cl, pH 7.4, with 5 mM adenosine, 0.2% glucose, and 2.5 mM Na[32Pi] (carrier-free, New England Nuclear, Boston, Mass.) per milliliter for 1 h at 37°C, followed by two washes with a 20-fold volume of the isotonic buffer in the cold.

Erythrocyte ghosts were prepared as described (31) except that incubation in the Tris Cl lysis buffer was carried out for 5 min only. Ghosts were often immediately washed in the 20-fold volume of lysis buffer as specified below. Fresh ghosts were labeled with γ-[32P]ATP under the "shape change conditions" described in the previous paper (31). Thus, the membranes were incubated at 0°C and a hematocrit of 50% for 5 min with, for instance, 0.8 mM γ-[32P]Mg-ATP (sp act 0.1 Ci/mmol and higher) plus one of several other additives (AMP-PNP, NaPi, etc.). Salt was then added to a final concentration of 140 mM KCl, 20 mM NaCl, and 5 mM MgSO4, followed by incubation at 37°C. Reaction was stopped either by dissociation in sodium dodecyl sulfate (SDS) or by a second lysis and washing as specified below. Spectrin 32P-label turnover experiments were carried out with ghosts from intact cells prelabeled with Na[32Pi] as described above, incubating the ghosts with unlabeled additives (ATP, AMP-PNP, etc.) under the conditions described for the case with labeled ATP.
220,000 dalton spectrin band 2 (5) was done as in the previous paper (31) except that ghost protein loaded onto the gel was determined by the Lowry procedure (16), of which 25% represented spectrin bands 1 and 2 (3).

**Determination of 32P-labeled Phospholipids**

The procedures used by Redman (22) were adapted. γ-[32P]ATP-labeled ghosts from a “shape change” experiment were lysed and extensively washed in 10 mM Tris Cl buffer, pH 7.4. Alternatively, washes were carried out with 5% trichloroacetic acid. The packed ghosts (50 µl) were then extracted at room temperature with 3 ml of chloroform-methanol-HCl (200:100:1). To the extract was added 5 ml of 0.1 M HCl, the mixture was thoroughly shaken and the layers were separated by low-speed centrifugation. Aliquots of the organic and the aqueous phases, as well as the entire interfacial layer of protein (washed once), were counted in the NCS-toluene scintillator as described above.

**Preparation of Phosphatases**

The purchased enzymes were dialyzed in the cold against two changes of the 1,000-fold volume of 10 mM Tris Cl, pH 7.4. The activity of alkaline phosphatase was measured in the optical assay with p-nitrophenylphosphate in 0.5 M Tris Cl at pH 8.0 (6). Apophosphatase from the *E. coli* enzyme was made by incubation of the commercial ammonium sulfate suspension with 20 mM EDTA, pH 8.0 (23), for 1 h at room temperature, followed by dialysis against three changes of the 1,000-fold volume of 10 mM Tris Cl, pH 7.4, in the cold. This removal of the metal ion cofactors reduced the specific activity of the enzyme to less than 2% of the initial value. A similar low activity was observed when holoenzyme was assayed in the presence of 10 mM NaP, (23).

Reaction of alkaline phosphatase from *E. coli* with spectrin band 2 was analyzed by the isolated spectrin complex from γ-[32P]ATP-labeled ghosts prepared as described above. The spectrin was concentrated to 2 mg/ml in an Amicon apparatus (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) followed by dialysis against 10 mM Tris Cl, pH 7.4. Alkaline phosphatase holo- and apoenzyme were then added to a final concentration of 0.1 mg/ml, followed by incubation in 140 mM KCl, 20 mM NaCl, and 10 mM Tris Cl, pH 7.4, at 37°C, and analysis of the spectrin label by SDS gel electrophoresis.

**Effect of Phosphatases on the Shape of Erythrocyte Ghosts**

In a shape change experiment similar to the one described in the previous paper (31) except that ghost protein loaded onto the gel was determined by the Lowry procedure (16), of which 25% represented spectrin bands 1 and 2 (3).

In a further experiment, unwashed ghosts were incubated at a hematocrit of 50% for 5 min at 0°C in 10 mM Tris Cl, pH 7.4, with 0.2 mg/ml *E. coli* alkaline phosphatase holo- or apoenzyme. Salt was then added as described, followed by an additional 20-min incubation at 4°C. Chlorpromazine was added at a concentration of 0.4 mM and the temperature raised to 37°C for 2 min. The ghosts were immediately visualized by phase contrast microscopy. In a parallel experiment, ghosts prelabeled with 0.5 mM γ-[32P]ATP were lysed and washed twice. They were incubated with the phosphatases according to the same protocol and were analyzed for the label on component 2 by SDS gel electrophoresis and for label in phospholipids as described above.

**Miscellaneous Methods**

The concentration of ATP during incubation of ghosts was analyzed using γ-[32P]ATP as a tracer, followed by chromatography on polyethyleneimine-coated plastic sheets (Brinkman Instruments, Inc., Westbury, N. Y.) (21). Radioactive spots were visualized by a brief autoradiographic exposure, and this radioactivity was measured by counting excised spots in Toluene-Liquifluor. The Na+K+-ATPase inhibitor strophanthidin was made up as a 10 mM stock solution in dimethylformamide and added to ghosts under shape change conditions in concentrations up to 0.1 mM. Control experiments were run in parallel by addition of the organic solvent only.

**RESULTS**

**Phosphorylation of Erythrocyte Ghosts with Mg-ATP**

Incubation of fresh erythrocyte ghosts with γ-[32P]Mg-ATP under the shape change conditions described in the previous paper (31), at ATP concentrations from 0.4 to 8.0 mM, led to an extensive phosphorylation of spectrin component 2 as judged by SDS gel electrophoresis (Fig. 1). Some labeling of other proteins occurred as well, mainly of a component moving somewhat more slowly than band 3, which did not appear in the Coomassie blue-staining pattern. The total amount of 32P-label distributed over all proteins on the gel other than component 2 was about equal to the amount associated with component 2 itself; clearly, the latter is labeled with a much larger specific activity than any other single protein component. In similar experiments in which SDS-polyacrylamide gel electrophoresis by the procedure of
TABLE I

| Specimen | cpm x 10^{-4} expressed per mg of ghost protein |
|----------|-----------------------------------------------|
| Organic phase* | 50.5                                      |
| Water phase* | 8.3                                      |
| Protein Layer* | 92.9                                      |
| Spectrin component 2† | 39.9                                      |

* Derived from acid-chloroform-methanol extraction (see Materials and Methods).
† Derived from SDS gel electrophoresis.

Fairbanks et al. (3) was used, component 2 was also found to be specifically labeled; in particular, component 2.1 (which is not clearly resolved in the Laemmli gels) was not labeled (M. Sheetz and S. J. Singer, unpublished experiments).

In addition to the protein modification, significant labeling of the phospholipids occurred under the conditions of these experiments (Table I). Such labeling has been observed by others (12, 22), and has been shown under the present conditions to be due primarily to phosphorylation of the inositol moiety of phosphatidylinositol. The amount of this lipid label was similar to that of the label on spectrin component 2.

Labeling of spectrin component 2 under these conditions was rapid initially but reached a plateau after about a 20-min reaction (Fig. 2). This plateau is not due to exhaustion of the Mg-ATP inside the ghosts; >70% of the initial amount of ATP (0.5 mM−10 mM) could be recovered from packed ghosts after the 20-min incubation. A slight excess of Mg-AMP-PNP over the Mg-ATP not only prevented the ATP-induced shape change (not shown) but also decreased the extent of labeling of component 2 (Fig. 2). The plateau level of labeling increased with increasing ATP concentration (Fig. 3), and was nearly 1 mol phospho-

**Figure 1** Phosphorylation of erythrocyte ghosts under "shape change conditions" (Materials and Methods) with 0.5 mM γ-[32P]Mg-ATP for 10 min, as analyzed by SDS-gel electrophoresis. One ghost preparation was loaded onto the gel in two concentrations. The two gels on the left were stained with Coomassie blue. The two gels on the right are autoradiograms of those on the left. The nomenclature of bands is used according to reference 3. The radioactivity at the front of the gel is from labeled phospholipids as well as from some residual ATP (see Table I).
Kinetics of phosphorylation of spectrin band 2 with 0.8 mM γ-[32P]ATP under shape change conditions. The effects of nonlabeled NaPi and AMP-PNP are shown.

**Figure 2**

Dependence of the degree of spectrin band 2 phosphorylation under shape change conditions upon the concentration of γ-[32P]ATP. The values were obtained after 10-min incubation at 37°C and represent near saturation levels for the ATP-concentrations used (Fig. 2 and reference 1).

Dephosphorylation of Phosphorylated Component 2

If intact erythrocytes are incubated with Na[32P]i, ghosts can be prepared with component 2 prelabeled (20). Alternatively, ghosts labeled with γ-[32P]ATP can be lysed a second time. With labeled ghosts prepared by either method, incubation at 37°C under shape change conditions but in the absence of ATP resulted in a loss of most of the label from component 2 within 30 min (Fig. 5). Unlabeled ATP incorporated into the ghosts in these experiments significantly inhibited the loss of label. This is a true inhibition and not an artifact due to a dilution of the radioactive label by a phosphorylation by unlabeled ATP, since the de-

1 At large ATP concentrations (>2 mM), after 20-min incubation the ghosts were highly cupped and some endocytosis of the membrane had apparently occurred, as judged by the disappearance of acetylcholinesterase activity (13).
degree of inhibition was independent of the initial amount of label in component 2. NaP\(_1\) and AMP-PNP apparently had no inhibitory effect. Unwashed ghosts (the original cytoplasm 10-fold diluted), as well as hemoglobin-free, extensively washed ghosts (the original cytoplasm 10\(^5\) fold diluted), lost the label on component 2 at equal rates.

When the prelabeled spectrin-complex was isolated and treated with *E. coli* alkaline phosphatase, \(^{32}\)P-label was rapidly removed from component 2 (Fig. 6), without any change in the Coomassie blue-staining pattern. As expected from the known properties of this phosphatase (23), NaP\(_1\) was an effective inhibitor of the release of label. The EDTA-inactivated apoenzyme, as expected, did not appreciably affect the amount of label.

**Effects on the Ghost Shape Changes of Exogenously-Added Phosphatases Incorporated into the Ghosts**

In one set of experiments, potato acid phosphatase was incorporated along with Mg-ATP into the crenated ghosts, and the occurrence of the usual ATP-induced shape changes was monitored by phase optics. The acid phosphatase was chosen because, of several commercial phosphatase preparations we sampled, it had the lowest ratio of ATPase activity to spectrin phosphatase activity under the conditions of our experiments.

In the presence of 0.4 mM ATP, 70–80% of the ghosts changed from the crenated to the disk shape in the usual manner. In the presence of 0.8 mM ATP and the acid phosphatase, this shape change was prevented. In the course of the latter experiment, the ATP concentration had decreased
from 0.8 mM to 0.4 mM according to analyses of the ghost suspension. Therefore, there was more than 0.4 mM ATP present throughout this experiment, yet the presence of the phosphatase inhibited the shape change that the ATP alone would have produced. Correspondingly, there was a 40% lower specific 32p activity in spectrin component 2 after the incubation with 0.8 mM ATP and the phosphatase than in the experiment with 0.4 mM ATP alone. On the other hand, if the initial ATP concentration included with the acid phosphatase was 2 mM, the usual ATP-induced shape change occurred. The latter experiment indicates that the acid phosphatase preparation did not produce a nonspecific inhibition of the shape changes, such as might occur, for example, with a proteolytic contaminant, since the inhibition was reversed if the steady-state level of phosphorylation was sufficiently increased (cf. Fig. 3). These results strongly suggest that the acid phosphatase effect on the ghost shape was due to its dephosphorylating activity.

In a second set of experiments, E. coli alkaline phosphatase was used in the absence of ATP. Because no ATP was involved, the alkaline phosphatase could be used at high concentrations even at 4°C to dephosphorylate component 2 inside ghosts. Fresh erythrocyte ghosts, under the conditions described in Materials and Methods, formed perfect disks after 2 min at 37°C in the presence of 0.4 mM chlorpromazine, whereas they were highly crenated in the absence of the drug (Fig. 7). If, however, the ghosts were preincubated at 4°C for 20 min with active alkaline phosphatase, their crenated shape persisted after the addition of 0.4 mM chlorpromazine. The inclusion in such an experiment of 10 mM NaPi, which substantially inhibits the alkaline phosphatase, resulted in the chlorpromazine-induced change to the disk shape (Fig. 7c), as did the use of the inactive apoenzyme in place of the active alkaline phosphatase (Fig. 7e). These results strongly indicate that it was a dephosphorylation by the alkaline phosphatase that prevented the chlorpromazine-induced change from the crenated to the disk shape.

In the same experiment carried out with prelabeled ghosts (Fig. 8), the active alkaline phosphatase removed 85% of the label on component 2 when measured after the chlorpromazine incubation, but little label was removed if 10 mM NaPi was present, or if the apoenzyme was used. An important further finding was that the alkaline phosphatase treatment did not significantly dephosphorylate the labeled lipids under the conditions where the chlorpromazine-induced shape change was inhibited and where most of the label on spectrin component 2 was removed (Table II).

Other Experiments

Strophanthidin added to the resealed ghosts at concentrations as large as 0.1 mM was found to have no effect on the Mg-ATP-induced shape changes. The Ca++ chelator EGTA at a concentration of 0.5 mM included in the ghosts with 2 mM Mg-ATP also had no effect on the usual shape changes.

DISCUSSION

The fact that cleavage of the terminal phosphate bond of ATP is required for the erythrocyte ghost shape changes to occur (31) allows at least two possible roles for the ATP: (a) as a substrate for an ATPase, as with an actomyosin-like contractile activity, or with an ion transport activity; or (b) as a substrate for a protein or lipid kinase, resulting in the phosphorylation of one or more components critical to the shape change.

There is no good evidence in favor of the former possibility. Although the spectrin complex is related to actomyosin (since erythrocyte spectrin [components 1 and 2] cross-reacts antigenically with smooth muscle myosin [29]; component 2 appears to have a site that binds ATP [11]; and erythrocyte component 5 is closely similar to muscle actin [29, 33]) whether significant ATPase activity is associated with the spectrin complex is controversial (14, 25). It is possible that the proper conditions for the expression of such ATPase activity have not yet been found, but for the present we must assume that activity is absent. Furthermore, since the ouabain analogue, strophanthidin (7), has no effect on the ATP-mediated shape changes, the erythrocyte Na, K pump is not implicated. Similarly, since the shape change is inhibited by Ca++ (31) and is unaffected by EGTA in the presence of excess Mg-ATP, no role for a Ca-ATPase pump activity is indicated either.

We have therefore investigated whether the ATP-mediated shape changes result from a phosphorylation process. It is well known that spectrin component 2 is phosphorylated in erythrocyte ghosts treated with ATP, but under most conditions other protein components are phosphorylated as well (1, 26, 34). Furthermore, significant phosphorylation of phosphatidylinositol in erythrocytes has been reported (12, 22). It has also
recently been shown (8, 10) that there is a phosphatase activity associated with erythrocytes which is capable of dephosphorylating $^{32}$P-labeled spectrin. However, the possible significance of these reactions in affecting the shapes of erythrocytes must be established. In the present studies, the observations that shape changes can be induced in erythrocyte ghosts (31) permitted experiments to be carried out directly correlating the extent of phosphorylation of specific membrane components with the shapes of the ghosts. This correlation is discussed in the following sections.

**Characteristics of the Phosphorylation Reaction**

Under conditions where ATP induces the shape changes in the ghosts, the $\gamma$-PO$_4$ groups of ATP that are transferred to the proteins are covalently bound with a remarkably high degree of specificity to spectrin component 2 (Fig. 1). This phosphorylation of component 2 is inhibited by AMP-PNP, but not by phosphate. The kinetics of the phosphorylation (31, and Fig. 2) are consistent with the achievement of a steady state between phosphorylation and dephosphorylation reactions (see below) which is reached by about 20 min at 37°C. The steady-state level of phosphorylation is negligible at ATP concentrations below 0.1 mM and rises steeply above 0.1 mM, reaching nearly 1 mol phosphoryl group per mol component 2 at 8 mM ATP (Fig. 3). The steep rise is probably related to the fact that ATP is an inhibitor of the dephosphorylation reaction (Fig. 5).

Under these conditions, there also occurs a nearly equal amount of labeling of phospholipids as of spectrin component 2; however, as is discussed below, this modification seems not to be correlated with the shape changes in the ghost.

It may well be physiologically significant that in the range of the ATP concentration present in the intact normal human erythrocyte (~2 mM; reference 19), the level of phosphorylation of component 2 in the ghost is close to 0.5 mol phosphoryl group per mol protein. If a single site per component 2 molecule served as the phosphoryl acceptor (see below), and if its complete phosphorylation represented one extreme state of a steady-state reaction while its complete dephosphorylation represented the other extreme, then the physiologically normal steady-state would be intermediate between the extremes. This assumes that the reaction conditions in the intact erythrocyte and in the ghost are equivalent. Furthermore, that normal steady-state level in the ghost is quite sensitive to a change in the concentration of ATP around 2 mM (Fig. 3), which, if these assumptions are correct, could provide an effective regulation mechanism in the intact cell for the extent of phosphorylation and for cell shape.

The preliminary evidence (Fig. 4) is that, indeed, the phosphorylation of component 2 occurs predominantly, if not exclusively, at a single site on the molecule. Since component 2 is reported to contain 9 methionyl residues per mol of 220,000 daltons (5), 10 peptide fragments are expected to result from the CNBr cleavage of that molecule. The localization of the major part of the original label to a fragment of 19,000 daltons therefore strongly suggests a high degree of specificity of the phosphorylation reaction. The stability of the label to the conditions of electrophoresis and CNBr cleavage reaction is further indication that a phosphoester and not a phosphoanhydride bond is formed (1). The detailed structure of this phosphorylated fragment is under investigation.

The phosphorylation site must be exposed to the active sites of the enzymes responsible for the membrane-bound endogenous kinase and phosphatase activities, as well as to exogenous phosphatases (see below).

**Figure 7** The effects of chlorpromazine on erythrocyte ghost shape, with or without prior treatment with alkaline phosphatase preparations included in the ghosts, as observed by phase contrast light microscopy. (a) Chlorpromazine alone at 0.4 mM converts the crenated ghosts (which look like those in b) to the disk shape shown. (b) After treatment with the active alkaline phosphatase holoenzyme, however, the addition of the chlorpromazine no longer changes the crenated ghosts to disks. (c) If the active alkaline phosphatase is used in the presence of its inhibitor, 10 mM NaP$_i$, then addition of the chlorpromazine converts the ghosts from the crenated to the disk shape as in (a). (c) 10 mM NaP$_i$ itself has no effect on the chlorpromazine-induced shape change. (e) If the inactive apoenzyme of alkaline phosphatase is used, chlorpromazine addition converts the crenated ghosts to disks as in a.
FIGURE 8 Effect of the phosphatase treatments used in Fig. 7 upon the labeled proteins of γ-[32p]ATP prelabeled ghosts. The SDS-polyacrylamide gel samples (a) through (e) correspond to the same samples in Fig. 7. Two sets of photographs are shown. The set on the left, of Coomassie blue-stained gels, shows that no change in the protein gel patterns has been produced by the various treatments, while the set on the right, of autoradiographs of the gels, shows that only for sample b, in which the active alkaline phosphatase was used and the chlorpromazine-induced shape change was inhibited (Fig. 7b), was a decrease in the specific radioactivity of spectrin component 2 observed. The radioactivity at the front of the gels on the right was from residual ATP and from labeled phospholipids. Separate extraction experiments showed (Table II) that the specific activity of these labeled lipids was not affected by the treatments involved.

Characteristics of the Dephosphorylation Reaction

If a protein kinase activity endogenous to the erythrocyte is responsible for the phosphorylation, one might expect that there also be present an endogenous phosphatase activity so that a steady-state degree of phosphorylation can be achieved. That some such activity exists has been demonstrated by others (8, 10) and in our experiments as shown in Fig. 5, which indicates a decrease in the specific activity of component 2 in ghosts which had been prelabeled with [32p]ATP. This reaction is a relatively slow one at 37°C, and has characteristics different from those previously described. It is of considerable interest that the dephosphorylation is significantly inhibited by ATP at physiologically normal concentrations, but not by AMP-PNP (Fig. 5), and that phosphate itself is not an effective inhibitor. (By contrast, E. coli alkaline phosphatase is markedly inhibited by 10 mM phosphate under these conditions [see Fig. 6 and reference 23]).

Both the protein kinase and phosphatase activities appear to be tightly associated with the erythrocyte membrane, because prolonged washing of the ghosts does not significantly alter them. We have no information about the molecular components involved in these activities. This membrane-bound phosphatase activity appears to be distinct from the cytoplasmically located activity previously described (8) which shows different inhibition properties.

Correlation of the State of Phosphorylation of Spectrin Component 2 and the Shape of the Ghost

If the hypothesis is correct that the erythrocyte ghost shape is the result of a steady state maintained by competing phosphorylation and dephosphorylation reactions, the shape should change predictably upon alteration of that steady-state. It was previously shown (31) that under certain conditions (e.g., in the presence of Ca++) where the ATP-mediated phosphorylation of component 2

| Label | Component 2* | Phospholipid† |
|-------|------------|--------------|
|       | No enzyme | With enzyme | No enzyme | With enzyme |
|       | % of initial value |
| After 20 min at 4°C | 97 | 35 | 85 | 85 |
| After additional 2 min at 37°C | 87 | 15 | 86 | 83 |

* From SDS-gel electrophoresis.
† From chloroform-methanol extraction.
did not occur, the change in shape did not occur either. Similarly, if the ATP concentration was less than 0.2 mM, or if AMP-PNP was present in excess over ATP, the degree of phosphorylation of component 2 was low and the shape change did not occur.

Further correlative evidence has been obtained in two different experiments in which exogenously added phosphatases were used. In one such experiment, the presence of an acid phosphatase enzyme preparation along with ATP inside the ghost inhibited the shape change that the ATP alone would have produced. If, however, larger ATP concentrations were included with the phosphatase, the shape changes were again observed. These results are therefore consistent with the hypothesis that competing phosphorylation-dephosphorylation reactions determine the ghost shape.

In a second type of experiment, an E. coli alkaline phosphatase was used because its inactive apoenzyme could readily be prepared to serve as an effective control. The ghosts were lysed and were converted from the crenated to disk shape, not with ATP, however, but with the drug chlorpromazine. According to the bilayer couple hypothesis (27), chlorpromazine and other positively-charged amphipathic molecules concentrate in the lipid in the inner half layer of the membrane, and therefore increase the surface area of that inner half relative to the outer half; as a result, they convert the intact normal erythrocyte to a cup shape (2). In the present experiment, the same mechanism is invoked to explain the conversion by chlorpromazine of crenated ghosts to the disk shape (Fig. 7). Furthermore, according to the hypothesis made in our previous papers (30, 31), dephosphorylation of the spectrin complex results in a crenation of the erythrocyte membrane because the spectrin complex is thereby depolymerized; this depolymerization produces a decrease in the surface area of the inner half layer of the membrane relative to the outer half, and the membrane crenates. This is the opposite of the effect attributed to chlorpromazine: therefore, the two effects on the cell shape are compensatory, and, if they occur to equal extents, they cancel each other out. Evidence has been presented elsewhere (28) supporting the compensatory nature of the effects of ATP depletion, on the one hand, and the addition of chlorpromazine, on the other, upon the shape of the intact erythrocyte.

Accordingly, we anticipated from these considerations that if the alkaline phosphatase produced a dephosphorylation of the ghosts in the absence of ATP, the ghosts should remain crenated when an appropriate amount of chlorpromazine was added which, by itself, would convert the ghosts to the disk shape. That is exactly what happened (Fig. 7). If the same experiment was carried out with the alkaline phosphatase holoenzyme in the presence of its inhibitor Pi, or with the inactive apoenzyme, then the addition of chlorpromazine converted the ghosts from the crenated to the disk shape as it did with no enzyme addition. These results therefore demonstrate that the persistence of the crenated shape in the experiment with the active alkaline phosphatase and added chlorpromazine was a specific result of a dephosphorylation action of a membrane-bound protein kinase, using ATP as substrate. The dephosphorylation appears also to be mediated by a membrane-bound enzyme. Evidence presented in the preceding paper (31) suggests that the phosphorylation reaction leads to a structural change involving spectrin, and presumably dephosphorylation leads to its reversal. It was suggested that this structural change is some kind of a polymerization involving the spectrin complex. In this view, such a polymerization-depolymerization process occurring on the cytoplasmic face of the membrane is an important controlling element with respect to the shape of the ghost and the intact cell.

That phosphorylation-dephosphorylation reactions might control a polymerization-depolymerization process is not without precedent in biochemistry. There is, for example, the well-known interconversion of phosphorylase b and phospho-
rylase a (4). The phosphorylation of dimeric phosphorylase b by ATP and phosphorylase kinase leads to the polymerization to the tetrameric phosphorylase a. A single phospho-group is introduced into a seryl residue per monomer unit. The action of phosphorylase phosphatase removes the phosphoryl groups and results in depolymerization to the dimer. It is thought, in this case, that the phosphorylation produces a conformational change in the monomer unit that leads to polymerization (9). Whether there is more than a superficial analogy between the behavior of the spectrin system and the phosphorylase system is an interesting subject of further study.

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