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THE INCORPORATION OF ^{32}P INTO SPECTRIN AGGREGATES FOLLOWING INCUBATION OF ERYTHROCYTES IN ^{32}P-LABELLED INORGANIC PHOSPHATE

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Summary

^{32}P was incorporated into spectrin by incubation of fresh erythrocytes with $^{32}P_i$ and glucose. The dimer and tetramer aggregates revealed only covalently-bound incorporation of phosphorus, while a higher aggregate of spectrin revealed both covalent and non-covalent incorporation. The specific activity of the covalently-bound phosphorus in all oligomers was identical, suggesting that the state of association is independent of phosphorylation. The non-covalent incorporation was shown to be due to the association of ATP with this higher aggregate. The nucleotide appears not to be bound directly to spectrin but rather to component 5 (erythrocyte actin) which is also found to be associated with this highly aggregated spectrin structure.

Introduction

Spectrin is one of the principal proteins of the erythrocyte membranes and is believed to play a major role in maintaining the shape and deformability of the membrane [1]. Spectrin is comprised of two polypeptide chains of very high molecular weight which undergo self-association to form oligomeric structures [2—4]. The self-association of spectrin and its possible interaction with other components of the membrane [4—6] may be important in controlling the structure and function of the membrane.

The role of ATP in maintaining erythrocyte shape and deformability has often been explained in terms of a mechanism of complexing calcium and preventing adverse calcium-membrane interaction [7]. However, evidence now suggests that there may be a more specific interaction of the nucleotide with the membrane [8,9]. We have previously observed that substrate depletion of the erythrocyte results in the dissociation of spectrin from a highly aggregated

structure to the lower molecular weight dimer (Dunbar, J.C. and Ralston, G.B., unpublished observations). The lower molecular weight polypeptide chain of spectrin has been shown to be phosphorylated when erythrocyte membranes are incubated with [γ-^{32}P]ATP [10—13]. A possible role for ATP in controlling the membrane structure may be through the control of aggregation of spectrin by phosphorylation [13].

The present investigation was designed to examine the role of spectrin phosphorylation in the self association of the dimer to more highly aggregated states. In order to prevent possible changes in the membrane which may occur on hemolysis and which may alter the pattern of labelling, intact erythrocytes have been incubated with ^{32}P-labelled inorganic phosphate.

Materials and Methods

Fresh human blood, obtained as packed cells from the N.S.W. Blood Transfusion Service, was stored at 4°C and used within 36 h of collection.

High specific activity [^{32}P]orthophosphate (10—25 Ci/mg) in dilute HCl was obtained from the Australian Atomic Energy Commission. The solution was neutralised before use with 1.25 mM sodium carbonate. [^{14}C]Adenosine (549 mCi/mmol) was from the Radiochemical Centre, Amersham.

Preparation of erythrocyte membranes and extraction of water-soluble proteins. Fresh human packed cells were washed three times with cold 0.95% NaCl in 5 mM phosphate buffer, pH 8.0, and the membranes obtained by haemolysis and washing in cold 5 mM phosphate buffer, pH 8.0. Care was taken at all stages to avoid proteolysis [14].

The membranes, diluted 1 : 1 with cold, distilled water, were dialysed against 0.1 mM EDTA, pH 7.5, for 36 h at 2—4°C. The membrane fragments were removed by centrifugation at 35 000 × *g* for 30 min at 4°C, and the supernatant fraction, containing the water-soluble proteins, centrifuged several more times until clear and free of membrane fragments. The dilute protein solution was concentrated by dialysis against Aquacide II.

Gel filtration. The supernatant fractions were analysed by means of gel filtration on a column (65 × 2 cm) of Bio-gel A-15 m agarose beads eluted with a buffer consisting of 0.01 M sodium phosphate/0.10 M NaCl/5 mM EDTA/5 mM mercaptoethanol, pH 7.5 [15].

Spectrin aggregates were further analysed under denaturing conditions on a column of A-15 m agarose beads (44 × 2 cm) eluted with 50 mM Tris/Cl, pH 7.5 containing 1% sodium dodecyl sulphate and 1 mM mercaptoethanol. Chromatography was carried out at 4°C in order to minimise possible proteolysis. Samples in 50 mM Tris/Cl, pH 7.5, were incubated with 1% dodecyl sulphate and 1 mM mercaptoethanol at 100°C for 5 min before application to the column.

Incorporation of radioactivity into erythrocyte membranes. Fresh red cells, washed three times with 10 mM Tris/Cl, pH 8.0, containing 0.95% NaCl, were incubated with 20 mM glucose and [^{32}P]orthophosphate (20 μCi/ml) or [^{14}C]-adenosine (0.5 μCi/ml) at 37°C for a period of 13 h unless otherwise specified. The pH of the incubation mixture showed only a slight decrease to pH 7.4 over a period of 18 h. Following the incubation, the membranes were prepared as previously described.

The incorporation of radioactivity into the membranes and of ^{14}C-label into protein was determined by scintillation counting of samples suspended in a Triton X-100/toluene-based scintillant [16]. Incorporation of ^{32}P into fractions eluted from the agarose columns was determined by means of the Cerenkov radiation, measured in an Isocap liquid scintillation counter, model 300. Specific activity was defined as cpm per mg protein.

Triton X-100 extraction of the membranes. ^{32}P-labelled membranes were extracted with 1% Triton X-100 in 5 mM phosphate buffer, pH 8.0, according to the methhod of Yu et al. [17]. After centrifugation at 35 000 × *g* for 30 min, the residue was dissociated with 1% sodium dodecyl sulphate containing 1 mM mercaptoethanol.

Phosphate estimation. The protein was hydrolysed in 2 M KOH at 90°C for 5 h. After neutralization of the hydrolysate and precipitation of the protein with silicotungstic acid, the inorganic phosphate was measured by the method of Ames [18].

Nucleotide analysis. Samples used for nucleotide analysis were dialysed extensively against distilled water to remove any low molecular weight, ultraviolet absorbing components of the gel filtration buffer.

Protein-bound nucleotide was liberated by treatment with 0.3 M perchloric acid for 20 min at 0°C. Following centrifugation, neutralisation of the supernatant and removal of excess perchlorate with KOH the sample was applied to an AG1-X2 anion exchange resin (1.5 g) and washed through with 20 ml of 20 mM NH_4Cl followed by 40 ml 0.02 M HCl and 20 ml 0.25 M HCl. Fractions from the column were neutralised with NH_4OH, freeze-dried and eluted with distilled water from a Sephadex G-10 column, to remove the NH_4Cl.

Alternatively, the protein solutions were mixed with an equal volume of chloroform/isoamyl alcohol (24 : 1, v/v) and shaken for 30 min at 0°C. After centrifugation at 3000 rev./min for 5 min at 4°C, the upper layer was removed and concentrated by evaporation.

Samples from both procedures were then further analysed by paper chromatography on Whatman 3 MM chromatography paper. The nucleotides were located under ultraviolet light and detected chemically with 0.3% $AgNO_3$ in 50% acetone [19].

Results

^{32}P-Incorporation into water-soluble proteins. Incorporation of ^{32}P into the membrane of fresh cells was slow and reached a maximum near 20 h. Up to 25% of the activity is extracted with the water-soluble proteins. Fractionation of the extracted proteins on a column of Bio-gel A-15 m agarose beads resulted in an elution profile consisting of six major peaks (Fig. 1). Electrophoresis in dodecyl sulphate [14] showed that peaks II and III contained spectrin only and correspond to the tetramer and dimer, respectively [15]. Peak I also consisted predominantly of spectrin. However, densitometry of the staining pattern in the gels of this fraction revealed that, while spectrin accounted for 91% of the protein, two other proteins, component 5 and the fastest migrating component of the 4.5 region [1] were also present to the extent of 7% and 2%, respectively.

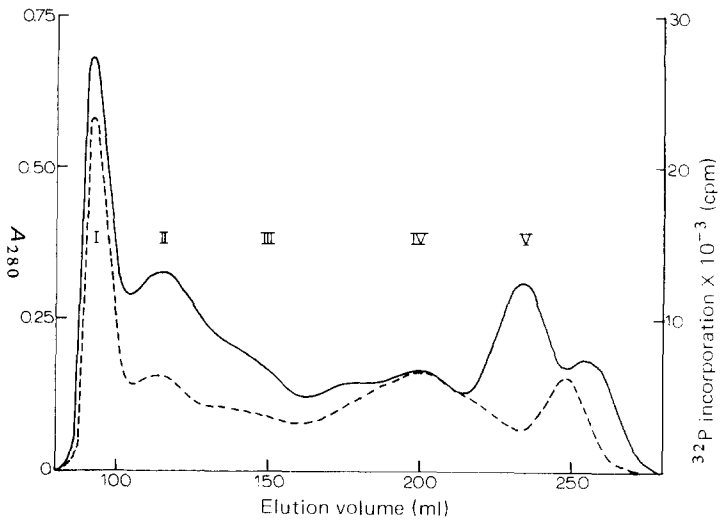

Fig. 1. Distribution of radioactivity into the proteins extracted by low ionic strength from membranes of erythrocytes incubated with $^{32}P_i$ for 13 h. The proteins were fractionated on a Bio-gel A-15 m agarose column eluted with 0.10 M NaCl, 0.01 M sodium phosphate, 5 mM EDTA and 5 mM mercaptoethanol. Protein (———) was estimated by absorbance at 280 nm, and ^{32}P incorporation (- - - - -) was estimated from the Cerenkov radiation.

A broad peak, peak IV, eluting between 170 and 210 ml, contained a number of different proteins, including components 4.5, 5 and 7. Haemoglobin was eluted in peak V.

The peaks of radioactivity corresponded closely with the protein peaks (Fig. 1), and revealed that all spectrin aggregates had been phosphorylated. The broad peak near 200 ml also revealed significant incorporation of radioactivity. A peak of radioactivity also eluted just ahead of the total volume of the column (peak VI), but no protein could be detected under this peak by means of gel electrophoresis.

Phosphorylation of spectrin. The specific activities of the dimer and tetramer oligomers were identical, while that of the peak I protein was considerably higher. The amount by which the specific activity of the peak I fraction exceeded that of the tetramer increased with the time of incubation, as shown in Fig. 2. Chemical analysis of the phosphate content of the tetramer and dimer revealed 3 mol of covalently bound phosphate per mol of spectrin dimer.

Although only covalently-bound phosphorus was detected in the tetramer and dimer oligomers, the peak I protein also revealed the presence of a labelled component which could be dissociated from the protein by treatment with trichloroacetic or sodium dodecyl sulphate. Gel filtration of peak I in the presence of dodecyl sulphate removed the non-covalently-bound label and yielded a pure spectrin fraction with the same specific activity as the tetramer and dimer.

Extraction of ^{32}P-labelled membranes with Triton X-100 resulted in an insoluble residue which consisted predominantly of spectrin and smaller amounts of other polypeptides including components 4.5 and 5. The specific activity of this insoluble residue was at least three times higher than that of the peak I

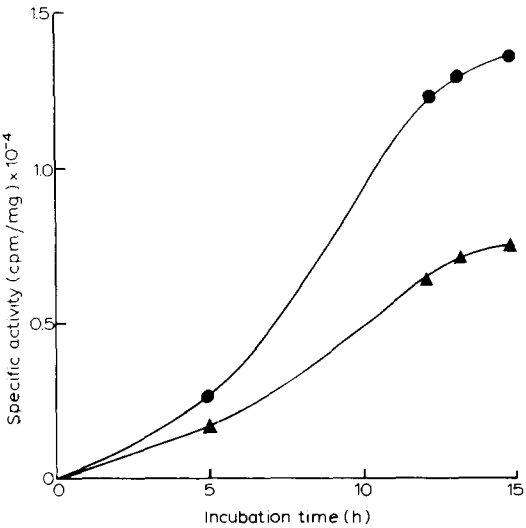

Fig. 2. Time course of incorporation of ^{32}P into spectrin aggregates. The spectrin oligomers, extracted from the membranes of erythrocytes incubated with $^{32}P_i$, were isolated by gel filtration as described in Materials and Methods. The specific activity of the spectrin fractions was calculated from the Cerenkov radiation and the absorbance at 280 nm. ●———●, incorporation into the peak I protein and, ▲———▲, the spectrin tetramer.

material obtained from the same membrane preparation. Phospholipids comprise less than 2% of this insoluble material [17] and would account for only a small part of the total radioactivity. The greater proportion of the incorporation appears to be non-covalent. Dissociation and gel filtration of the residue in the presence of dodecyl sulphate yielded a spectrin peak with a specific activity the same as that obtained for the tetramer and peak I fractions.

Identification of the non-covalently-bound phosphorus component. The ultraviolet absorption spectrum of the acid-dissociable label from peak I displayed a maximum near 260 nm and a minimum at 230 nm, suggesting a nucleotide. This component also eluted from an anion exchange column in positions corresponding to adenine nucleotides. However, while the nucleotide obtained from the chloroform/alcohol extraction of peak I was identified as ATP (Fig. 3), the supernatants from the perchlorate precipitation of the protein also yielded ADP, and in some cases AMP, in addition to ATP. Paper chromatography of the eluted nucleotides, using two different solvent systems, and detection with chromogenic reagents specific for purine nucleotides, further confirmed the identity of the adenine nucleotides (Table I). Presumably, the mono- and diphosphates result from the hydrolysis of ATP during the acid precipitation, neutralisation and subsequent analytical procedures.

The 170—210 ml fraction from the agarose column was also associated with a phosphorus-containing component which was acid soluble and which yielded a spectrum similar to that of a nucleotide. Components 4.5 and 5 were common to both this and the peak I fraction. Component 5 has recently been identified as erythrocyte actin [4] and in view of the known ability of actin to bind nucleotide attempts were made to isolate and analyse component 5 for the presence of nucleotide.

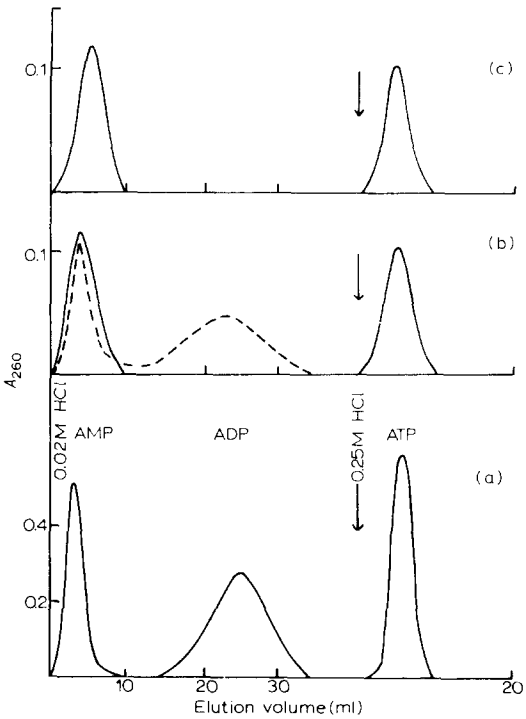

Fig. 3. Chromatography of the non covalently-bound phosphorus components on an anion exchange resin. After application of the sample, the column was washed initially with 20 ml 10 mM NH_4Cl, and then the nucleotides were eluted with 0.02 M HCl followed by 0.25 M HCl. (a), Elution of a mixture of ATP, ADP and AMP; (b), two different preparations of nucleotide obtained from peak I fractions; (c), nucleotide liberated from component 5. The peak of absorbance which elutes near the position corresponding to AMP contained variable amounts of proteins, as revealed by the ultraviolet absorption spectra of these fractions.

TABLE I

R_{AMP} VALUES FOR STANDARD ADENINE NUCLEOTIDES AND NUCLEOTIDES LIBERATED FROM PEAK I

Solvent 1: ascending system (Whatman 3 MM chromatography paper) in isobutyric acid/H_2O/EDTA (660/340/2, v/v) pH 3.7. Solvent 2: 1% ammonium sulphate/isopropyl alcohol (1 : 2, v/v) descending chromatography on Whatman 3 MM paper. Number of experiments is given in parentheses.

Sample	Solvent 1	Solvent 2
Standards		
ATP	0.51	0.61
ADP	0.79	0.71
AMP	1.00	1.00
Cyclic AMP	1.09	1.30
Adenine	1.58	1.50
Peak I nucleotides		
ATP (chloroform/isoamyl alcohol)	0.55 (3)	0.66 (3)
ADP (perchloric acid)	0.82 (2)	0.77 (1)
AMP (perchloric acid)	—	1.02 (1)

Component 5 was purified from the water soluble protein by repeated chromatography on Bio-gel A-15 m agarose eluted with 1 mM sodium phosphate buffer, pH 7.5, containing 0.2 mM dithiothreitol. After the second chromatography, the centre of the peak (K_{av} = 0.55) contained component 5, which was shown to be pure by gel electrophoresis in dodecyl sulphate.

Examination of this protein also revealed the presence of an adenine nucleotide. Chromatographic analysis indicated that the nucleotide is ATP (Fig. 3).

Incorporation of [^{14}C]adenosine into erythrocyte membranes. Red cells incubated with [^{14}C]adenosine incorporated label into the membrane. More than 70% of the radioactivity was extracted with the water-soluble proteins. Fractionation of the spectrin aggregates revealed that only one of the aggregates (the peak I protein) contained the label. The radioactive label, however, was readily removed by acid precipitation of the protein.

Discussion

Two types of incorporation of phosphorus into spectrin could be distinguished. One appears to be covalent incorporation since the label remains bound to spectrin after treatment of the protein with trichloroacetic acid or with dodecyl sulphate. Presumably, this covalent incorporation of ^{32}P into spectrin represents the phosphorylation of serine and threonine residues by [γ-^{32}P]ATP [11] produced rapidly on incubation of erythrocytes in solutions containing $^{32}P_i$ and glucose [20].

3 mol phosphate were found to be covalently bound per mol of spectrin dimer. Although higher than previously reported values [10,13,21], this estimate represents total phosphate content and not simply the amount incorporated after brief incubation of membranes with labelled ATP.

The incorporation of covalently-bound ^{32}P into spectrin was identical for all oligomeric states, including the highly aggregated protein eluting in peak I. It would appear then, that the aggregation state of spectrin is independent of the degree of phosphorylation of the polypeptide chain.

The second type of incorporation, apparently non-covalent association of ATP, was observed in only one of the spectrin oligomers isolated by gel filtration; the highly aggregated peak I fraction. The highly associated spectrin · actin complex remaining after Triton extraction of the membranes also revealed significant non-covalent incorporation. The presence in both this and the peak I protein suggests that the nucleotide plays an important role in the maintenance of these highly aggregated structures.

We have demonstrated that component 5 is itself associated with the nucleotide. In view of the failure to detect this type of incorporation with the tetramer and dimer, it is likely that the nucleotide in the aggregated structures is associated not directly with the spectrin polypeptides, but rather, indirectly through component 5. Further support for this suggestion comes from the finding that the concentrations of component 5 and the nucleotide in peak I are equimolar, consistent with the known nucleotide-binding capacity of muscle actin.

Graham et al [22] have also observed that after incubation of membranes with [γ-^{32}P]ATP, a component with the electrophoretic mobility of ATP was

tightly, although not covalently, bound in the water-soluble protein extract. Haley and Hoffman [23] have shown that both the lower molecular weight chain of spectrin and component 5 are photoaffinity labelled with a photoreactive analogue of ATP. Since we have found that pure spectrin seems not to be associated with the nucleotide, it is conceivable that the close proximity of the two proteins in the membrane could result in the indirect labelling of spectrin.

The molar ratio between spectrin and component 5 in peak I is 2 : 1, the same proportion as is found in the membrane. This ratio was constant for a number of different preparations and suggests a constant stoichiometry between spectrin and component 5. The finding of ATP in the peak I complex is strong presumptive evidence that the actin is not in the fibrous form, but rather that monomeric actin participates in the cross-linking of spectrin. This is consistent with the view of Tilney and Detmers [4] that actin in the erythrocyte membrane is not fibrous. (The aggregates of component 5 observed in the 170—210 ml fraction presumably arise from manipulation of the salt concentration during extraction and gel filtration rather than representing an in vivo association state.)

An interaction between spectrin and component 5 has been postulated by a number of workers [4,5] on the basis of an interaction in vitro between spectrin and muscle actin. However, as yet there is little direct evidence that spectrin and component 5 are in fact bound together in vivo, although the coelution of spectrin and component 5 in gel filtration has been suggested as evidence for their association in the water-soluble extracts [5].

It is apparent that there is a complex role for ATP in the maintenance of the structure of the erythrocyte membrane. Variations in the intracellular concentration of ATP would be expected not only to modify spectrin through changes in its phosphorylation, but may also be expected to alter the association between spectrin and component 5.

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