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THE MOLECULAR BASIS OF THE DEFECT IN PHOSPHORYLATION OF SPECTRIN IN HUMAN HEREDITARY SPHEROCYTOSIS

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The molecular basis for the depressed phosphorylation of the smaller polypeptide of spectrin (band 2) in the erythrocytes of patients suffering from hereditary spherocytosis is investigated. Comparison of healthy and spherocytic spectrin polypeptides by controlled proteolysis reveals no abnormality in the degradation patterns or in the sites of phosphorylation. It is concluded that the lesion is a consequence of a defective control of phosphorylation. The defect can be mimicked in healthy cells by the introduction of calcium into the erythrocyte and the possibility that the primary pathological lesion is a deficient control of the calcium content of the erythrocyte is discussed.

Introduction

In an earlier paper [1] it was shown that in the human genetic condition, hereditary spherocytosis, the phosphorylation of the proteins of the erythrocyte membrane was abnormal in patients showing clinical symptoms. The phosphorylation of spectrin (band 2) was diminished relative to syndein (band 2.1). In this communication the factors responsible for the defect in phosphorylation are investigated.

There are many claims that the lesion in hereditary spherocytosis resides in the spectrin polypeptides themselves, e.g. the aggregation of spectrin from the pathological cells has been found to be impaired [2] and, even more pertinently for the present work, the ratio of serine/threonine phosphorylation is changed in the membrane proteins of spherocytics [3] although it is not known whether this change takes place in spectrin. We have therefore explored the possibility that the depressed phosphorylation of band 2 is due to some intrinsic abnormality of the polypeptide by analysing spectrin by controlled proteolysis using chymotrypsin and *Staphylococcus aureus* V8 protease [4]. Secondly, as calcium inhibits the phosphorylation of band 2 [5,6] and as it has been reported that the calcium level of the diseased erythrocyted is greater than normal [7] the possibility that the high calcium affects the phosphorylation of band 2 in spherocytics has been investigated.

Materials

^{125}I as NaI in dilute NaOH and $^{32}PO_4$ as orthophosphate in dilute HCl were purchased from the Radiochemical Centre (Amersham, Bucks., U.K.), enzymes, biochemicals from Sigma Chemical Co. (St. Louis, MO, U.S.A.) with the exception of *Staphylococcus aureus* V8 protease from Miles Laboratories Ltd. (Stoke Poges, Slough, U.K.). Ionophore A23187 was kindly provided by Eli Lilly Co. Chemicals were of analytical grade from B.D.H. Chemicals Ltd. (Poole, Dorset, U.K.).

Spherocytic blood was obtained from patients at the Blood Clinic at the Royal Infirmary, Edinburgh, normal blood from healthy adult volunteers.

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Methods

Extraction and ^{125}I-labelling of spectrin. Spectrin was extracted from white ghosts [8] by addition of 2 vol. of 0.5 mM EDTA (adjusted to pH 7.5 with NaOH) and dialysis of the resulting suspension in 0.5 mM EDTA (pH 7.5) at 4°C overnight. The suspension was centrifuged at 80 000 × *g* for 30 min at 4°C and the supernatant containing the extracted spectrin iodinated by lactoperoxidase catalysis [9]. To 1 ml spectrin solution were added 5 mM glucose, 0.1 μg glucose oxidase (Sigma, Type V), 10 μg lactoperoxidase (Sigma powder), 0.5 μM $K^{127}I$ and 100 μCi of $Na^{125}I$ and the mixture incubated for 1 h at 37°C. The reaction was terminated by the addition of 100 μl of 0.5% $Na_2S_2O_5$ in 0.2 M sodium succinate buffer (pH 5.5) followed by a further 1 ml of the succinate buffer to precipitate the spectrin. After 1 h at 4°C the spectrin was collected by centrifugation at 38 000 × *g* for 20 min, the small pellet washed four times with the succinate buffer, and then dissolved in 1-ml electrophoresis sample buffer [1,10].

Proteolytic digestion of radioiodinated spectrin [4]. In order to separate bands 1 and 2, 25 μl aliquots of the above spectrin solution were electrophoresed in 4% acrylamide slab gels using the continuous buffer system of Fairbanks et al. [11] for 5 h. After 20–30 min staining in Coomassie blue R250 the gels were destained for 5 min in acetic acid/methanol/water (1 : 1 : 8, v/v), bands 1 and 2 excised and equilibrated in gel electrophoresis sample buffer for 30 min. The gel slices were then inserted into the sample wells of a Laemmli [10] slab gel (11.5% acrylamide running gel, 5% stacking gel) and overlaid with 5–10 μl of protease solution containing varying quantities of enzyme [4]. After an initial period of electrophoresis for 1 h at a constant current of 6 mA (with a slab 1.5 mm thick and 15 cm wide) for proteolysis to occur the resulting peptides were separated over 3.5 h at 35 mA. Two proteases were used, chymotrypsin (Type VII) over a range of 1–20 μg per well and *S. aureus* V8 protease over a range of 0.05–2.0 μg per well. Radioiodinated peptides were detected by autoradiography of dried gels against Kodak X-Omat H film.

The same procedure was used for the proteolysis of the phosphorylated band 2 except that the phosphorylation was brought about by incubation of the cells with radioactive phosphate for 16 h [1].

Results

The structures of purified bands 1 and 2 were compared by Cleveland degradation after lactoperoxidase catalysed radioiodination of the isolated spectrin. Figs. 1 and 2 compare the digestion of band 1 and band 2 with chymotrypsin (Fig. 1) and *S. aureus* V8 (Fig. 2) after autoradiography for 24 h and 4 days. It can be seen that the peptide breakdown patterns from the two high molecular weight spectrin polypeptides are markedly different. This confirms other work emphasising their dissimilarity [12–14] and is contrary to reports of the similarity of the two chains [15,16]. The figures also demonstrate the resolving power of the method in detecting differences between polypeptides.

When isolated band 1 and band 2 polypeptides of diseased cells were examined the iodinated peptide patterns were identical with their normal couterparts suggesting that the depressed phosphorylation of band 2 in the disease is not a consequence of some abnormality in the sequence of either spectrin polypeptide (Figs. 1–3). Alternatively, defective phosphorylation could be a result of one or more of the phosphorylated sites becoming unavailable [17–20]. The nature of the phosphorylation of band 2 was therefore analysed by Cleveland digestion and autoradiography of its radioactive phosphorylated peptides (Fig. 4). Again no abnormality could be detected in pathological cells, all the phosphorylated fragments of normal cells were present. We conclude that the diminished phosphorylation of band 2 is due to some alteration of the rate of phosphorylation or dephosphorylation rather than defects in the polypeptide substrate.

If the high calcium level (see Discussion) is the cause of the abnormal phosphorylation of hereditary spherocytosis then it might be possible to (a) mimic the pathological condition in healthy cells by elevation of calcium concentration and (b) cause diseased cells to revert to normal by removal or lowering of calcium. To attempt the former, healthy cells were incubated with radioactive phosphate [1] in the presence of the ionophore A23187 at 100 μmol per litre cells [21]. If the ionophore was added at the

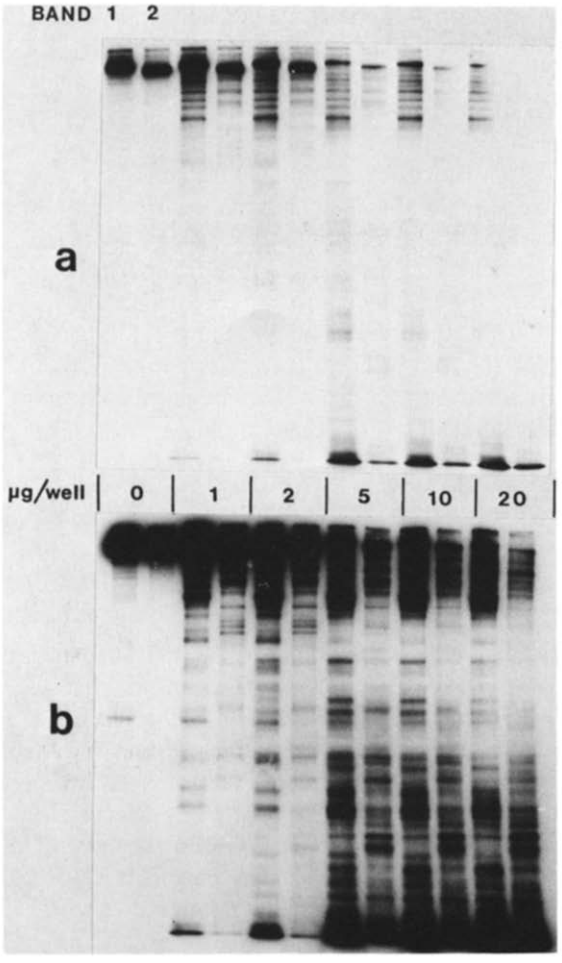

Fig. 1. Autoradiograph of chymotrypsin digestion of bands 1 and 2 from normal cells radioiodinated by lactoperoxidase catalysis. Samples run in pairs, band 1-left, band 2-right. Identical patterns are obtained from spectrin of hereditary spherocytosis (a) 1-day exposure. (b) 4-day exposure.

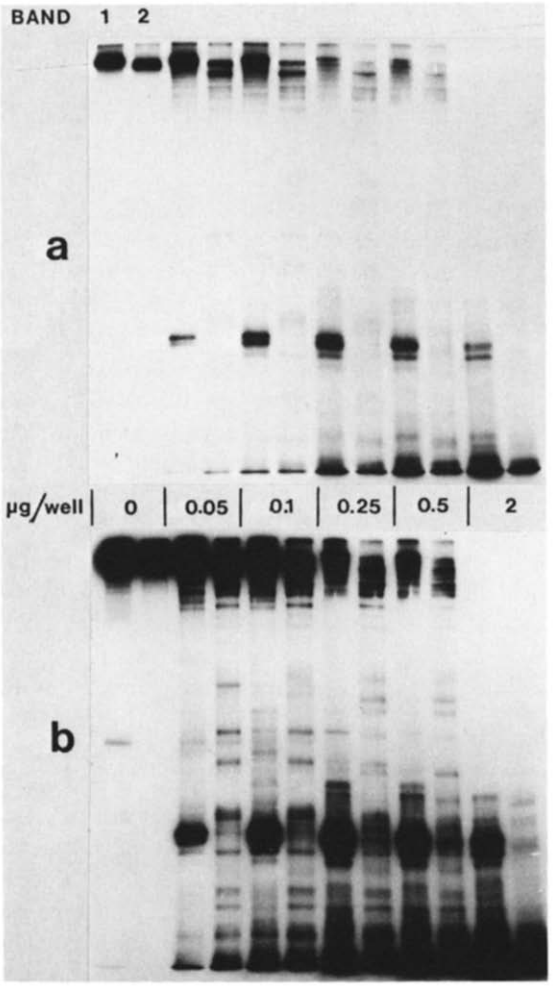

Fig. 2. Autoradiograph of *S. aureus* V8 protease digestion of bands 1 and 2 from normal cells radioiodinated by lactoperoxidase catalysis. Samples run in pairs, band 1-left, band 2-right. The patterns obtained from hereditary spherocytosis spectrin are identical. (a) 1-day exposure. (b) 4-day exposure.

beginning of the incubation, incorporation of phosphate was virtually abolished. The cells were therefore incubated with the phosphate for 6 h before the addition of ionophore and then allowed to incubate for a further 13 h. Compared with control cells there was a pronounced loss of radioactivity from band 2 but incorporation into band 2.1 continued although at a greatly reduced rate (Fig. 5). The ratio of specific activities of bands 2/2.1 consequently fell to the pathological value. The effects of the ionophore were abolished by addition of 0.5 mM EGTA along with the ionophore. There would appear to be sufficient calcium present as impurity in the medium to bring about the effect.

The converse experiment (b) of attempting to remove calcium from diseased cells was only partially successful. EGTA alone and EGTA + ionophore A23187 was added to the incubation. A partial reversion to normality was possible but we have not been able to achieve total restoration of the normal condition. In normal cells prior to splenectomy it is 0.4. EGTA + ionophore elevates the ratio to between 0.6 and 0.9, but not, in our hands, above this value.

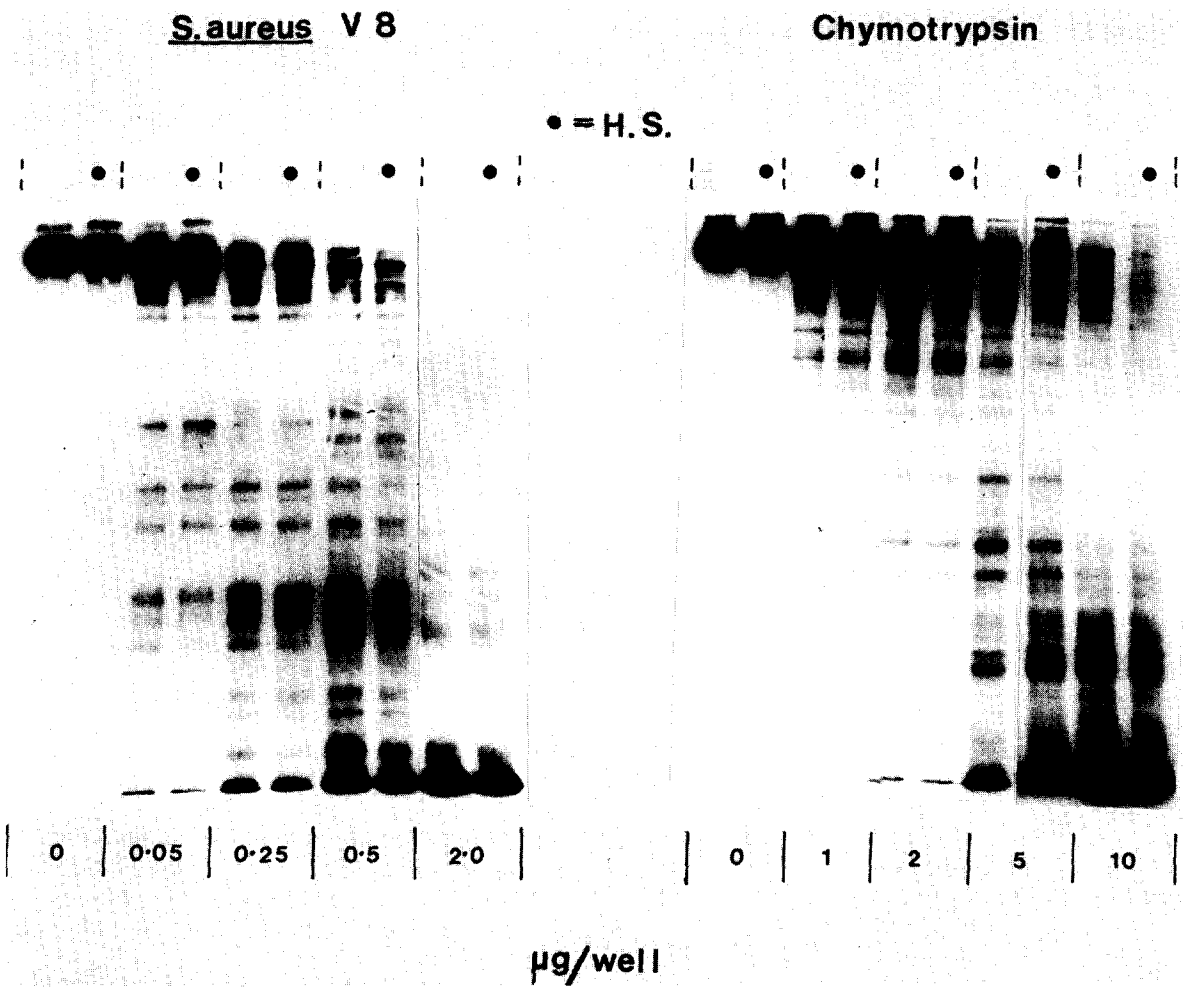

Fig. 3. Autoradiographs comparing the proteolytic digestion of radioiodinated band 2 of normal and cells from hereditary spherocytics produced by chymotrypsin and *S. aureus* protease. No abnormalities are apparent in the diseased blood.

Discussion

In an earlier paper [1] it was shown that in patients with hereditary spherocytosis, prior to splenectomy the phosphorylation of band 2 was depressed relative to 2.1 and that the effect was cyclic AMP independent and not due to metabolic depletion. In this paper we report that no abnormality can be detected by controlled proteolysis in the polypeptide sequences of bands 1 and 2 or the sites of phosphorylation of band 2. It may therefore be concluded that the defect involves the control of the phosphorylation of band 2 rather than band 2's structure.

Evidence has been presented in favour of the possibility that the depression of band 2 phosphorylation is a consequence of an elevated level of calcium of erythrocytes of spherocytics. This result is consistent with the inhibitory effect of calcium on phosphorylation [5,6] and the reported elevation of calcium in spherocytic erythrocytes. Increased calcium is particularly marked prior to splenectomy, and in the original reports was associated with a depressed Ca^{2+}-ATPase [7]. These findings have been challenged, but it is not clear whether the attempts to repeat the work were carried out on patients prior to

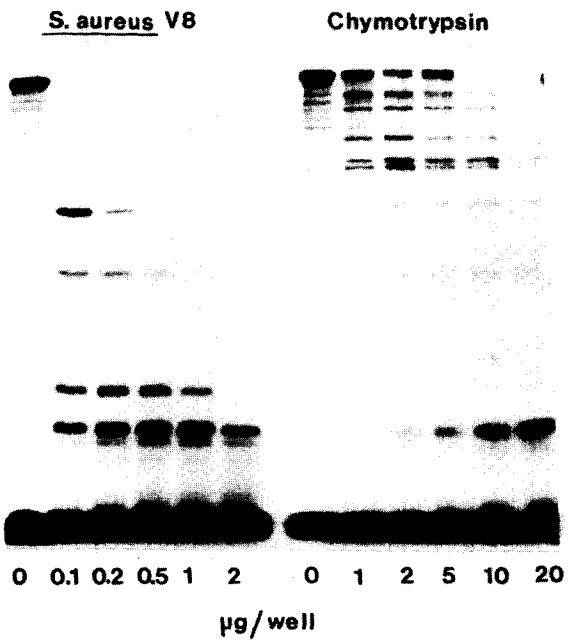

Fig. 4. Autoradiographs of proteolytic degradation of ^{32}P phosphorylated spectrin (band 2) from normal erythrocytes. The patterns are indistinguishable from those obtained from cells from hereditary spherocytic patients.

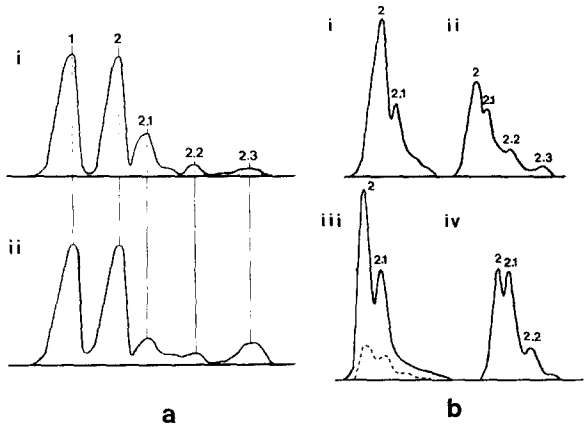

Fig. 5. The effect of ionophore on the incorporation of phosphate into normal erythrocytes. (a) Densitometer scan of Coomassie stained gel: (i) incubated 19 h + radioactive phosphate; (ii) incubated 6 h + phosphate followed by 13 h with A23187 + phosphate. (b) Densitometer scan of autoradiograph of phosphate incorporation: (i) incubated 6 h with radioactive phosphate; (ii) incubated 6 h with phosphate followed by 13 h with A23187 + phosphate; (iii) incubated 19 h with phosphate, compared with condition (ii); (iv) standard hereditary spherocytosis pattern after 19 h incubation. (i) and (ii) are to scale, (iii) and (iv) are to scale, the relationship between the two scales is indicated by the broken line in (iii).

splenectomy [22,23]. Undoubtedly the calcium content, its concentration and distribution, in diseased erythrocytes requires further examination in the light of recent studies on healthy cells [24,27].

There is also considerable circumstantial evidence for an elevation of calcium in hereditary spherocytosis erythrocytes. The complex pattern of minor polypeptides that characterises the diseased erythrocyte can be mimicked in normal cells by the addition of calcium [28,29] and we (Rennie, C.M. and Maddy, A.H., unpublished results) find that the K^+ content of erythrocytes from spherocytics prior to splenectomy is diminished in a manner consistent with a Gardos effect being actuated by calcium. It is also pertinent to recall that in sickled erythrocytes, where the calcium is elevated and the Ca^{2+}-ATPase depressed [30], the phosphorylation of band 2 is also diminished [31].

It is not at present possible to conclude unequivocally whether the fall in phosphorylation arises from a decreased phosphorylation, an increased dephosphorylation, or a combination of several factors. The balance of evidence suggests that enhancement of dephosphorylation is the predominent effect. (1) The membrane [32] and cytosolic [33] cyclic AMP independent kinases are not inhibited by calcium. An indirect effect of calcium via the generation of ADP has been considered by others and rejected [34]. (2) Introduction of calcium into the erythrocytes by ionophore not only abolishes further incorporation which could result from depletion of ATP without any effect on the kinases per se, but causes an actual loss of previously bound phosphate. (3) Study of dephosphorylation of labelled membranes is of only limited relevance for several reasons, but in a set of experiments (not shown), when ghosts prepared from phosphorylated cells were incubated in the presence and absence of calcium radioactivity was lost from both of the band 2 and 2.1 polypeptides at roughly equivalent rates and the loss from both peptides was equally stimulated by calcium (see also Refs. 34, 35).

A calcium stimulated loss of phosphate is, therefore, at least possible under certain conditions, but now the further question of whether the loss could be due to increased protease rather than increased

phosphatase activity arises. As it is known that erythrocytes have a calcium activated protease [28] there is a prima facie reason for suspecting the former, but the evidence, although not fully conclusive, favours a phosphatase. (1) Band 2.1 is more prone to proteolysis than band 2 [36] yet in the disease the phosphate is lost preferentially from band 2. When ionophore is added to erythrocytes there is a pronounced proteolysis of 2.1 to 2.2 and 2.3 but 2 is unaffected. (2) The amount of phosphate in band 2 relative to 2.1 (i.e. 2.1 + 2.2 + 2.3) falls with time yet the mobility (molecular weight) of 2 remains constant. (3) When radioactive ghosts are incubated to measure the loss of phosphate, although there is a pronounced conversion of 2.1 to 2.2 and 2.3 there is no detectable change in the amount or position of the Coomassie blue staining of band 2. (The previously reported calcium inhibition of erythrocyte phosphatase is not of particular relevance in the present context as the inhibition is only operative at calcium concentrations above 10^{-3} M while the concentration in the erythrocyte is in the order of 10^{-7} M [38]).

These arguments would be weakened if all the phosphate binding sites of band 2 are confined to a terminal peptide so small that its loss is not detected by the SDS-PAGE. As the four phosphate binding sites are distributed along the terminal 10 000-dalton peptide [38] a loss of all phosphorylated sites would be detected in our gels and a partial loss too small to be detected by a decrease in molecular weight, involving only some of the phosphorylated peptides is inconsistent with the identical breakup patterns of phosphorylated normal and pathological spectrin.

The defective phosphorylation is closely related to the clinical status of the patient and is remedied by splenectomy. It is, therefore unlikely to be the primary lesion. The defective control of calcium concentration in the diseased cells is probably anterior to the defective phosphorylation in the chain of events that leads to the demise of the erythrocyte but calcium levels are also partially, if not fully, restored to normality after removal of the spleen. However, abnormal cell shape persists after removal of the spleen. While the biochemical observations at present available may explain the demise of the cell the primary genetic defect remains obscure.

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