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Structural basis for the high activation energy of spectrin self-association

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The association of spectrin hetero-dimer ($\alpha\beta$) to the tetramer ($\alpha_2\beta_2$, which predominates in the cell) is marked by an exceptionally high activation energy, so that the reaction does not proceed measurably in the cold. We have tested the hypothesis that this is due to intra-dimer association between the α- and β-chain ends, which must be broken before tetramers can form. Two mutant univalent spectrins with association defects at the α and β ends, respectively, and incapable therefore of intra-dimer bonding, were found to associate rapidly with one another at 4°C. The bimolecular rate constant is greater than for the association of normal dimers by 6 orders of magnitude.

Spectrin; Hereditary elliptocytosis

1. INTRODUCTION

Spectrin, the major structural protein of the red cell membrane cytoskeleton, on which the mechanical properties of the membrane depend, is an elongated molecule, composed of two types of polypeptide chain, α and β. These are laterally associated to form the protomeric hetero-dimer. In the cell the spectrin occurs predominantly as the tetramer, made up of two heterodimers, linked head-to-head. The interconversion between dimer and tetramer is characterised by an unusually large activation energy [1,2]. Thus, whereas at physiological temperature association or dissociation is complete in minutes, at 0–10°C there is no measurable interconversion over a period of days or weeks.

The appearance of the spectrin dimer and tetramer in the electron microscope after shadowing is that of two ropes, loosely coiled around each other along part of their length. The tetramer has twice the extended length of the dimer [3]. A striking feature is that in the dimer the chains appear to be in contact with each other at both their ends; to form the tetramer the chain ends bearing the self-association sites must presumably separate, so as to join up with their partners in the apposing dimer. At the centre of the tetramer, where the dimers are joined, the polypeptide strands are laterally well separated and the associating chains from opposite dimers appear colinear. It was inferred by Shotton et al. [3] that the self-association sites might be internally satisfied in the dimer. This suggests a basis for the high activation energy of the dimer-tetramer conversion: to form a tetramer the internal α-β link of the dimer must first be broken, giving a transition state with no internal association. We have examined this proposition by reference to two univalent spectrin variants, which cannot form intra-dimer bonds.

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2. MATERIALS AND METHODS

Blood samples were obtained from normal subjects and from two patients with forms of hereditary elliptocytosis. Both the latter are characterised by defective self-association of part of the spectrin, as is common in elliptocytic conditions [4]. One is

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of the relatively abundant type with an anomaly in the N-terminal domain of the α-chain, which carries the self-association site; the other has a truncated β-chain, in which the association site is deleted [5]. Spectrin was prepared by routine procedures [1]: red cells were washed with phosphate-buffered isotonic saline and treated with 2 mM diisopropyl fluorophosphate before hypotonic lysis on ice. The washed membranes were extracted by dialysis against 1 mM Tris, 0.1 mM EDTA, pH 8.0, at 4°C. The crude spectrin was fractionated on a Sepharose 4B column and kept in the cold at all times. Normal spectrin, extracted in the cold, contains less than about 5% of the dimer, whereas preparations from the abnormal cells comprised about equal amounts of dimer and tetramer. The dimer fraction, at least in the case of the β-chain variant, was enriched in the abnormal form [5], as estimated by SDS gel electrophoresis [6], in which the variant chain migrates ahead of the normal β-chain. The α-chain variant was examined by partial tryptic digests [7], which revealed that the normal N-terminal fragment (αI domain) of molecular mass 80 kDa, had been largely replaced by one of 74 kDa; this defines the relatively common variant (or group of variants), sp. $\alpha^{1/74}$ [7]. The dimer fraction was enriched in abnormal protein, but because the ratio of the two types of αI fragment generated by the pure variant remained unknown no quantitative evaluation of the proportion of the abnormal spectrin in its mixtures with the normal species was possible.

For the measurement of association rates all samples were adjusted to a total spectrin concentration of 1 mg/ml, using a specific absorbance of $E^{1\%}_{1cm}$ = 10.7 at 280 nm [8]. The solvent was 0.15 M NaCl, 10 mM sodium phosphate, 25 mM Tris, 5 mM EDTA, pH 8.0. Mixtures were incubated at the required temperature for various times; they were then placed on ice and analysed by gel electrophoresis in agarose gels in the cold as described [5]. The gels were evaluated by staining and densitometry.

3. RESULTS

Incubation of normal spectrin dimers at 4°C leads to no measurable formation of tetramer even after about 3 weeks. This is consistent with the measured activation energy of the conversion [1], which leads to a second-order rate constant at 4°C of $3 \times 10^{-3}\ M^{-1} \cdot s^{-1}$. At the protein concentration used here (1 mg/ml), this corresponds to a time for half-reaction of about 5 years. The measured enthalpy of the dimer-tetramer conversion [1] can be used to calculate the equilibrium constant at 4°C, and thus the equilibrium weight proportion of tetramer that would form at the protein concentration in question; these values emerge as $9 \times 10^7\ M^{-1}$ and 0.95, respectively.

The proposition that the high activation barrier for the interconversion is the result of an internal α–β bond in the dimer [3] carries with it the prediction that two complementary univalent spectrins, each lacking one association site, and therefore unable to form an internal bond, should react with one another rapidly and with high avidity. The two mutant proteins constitute such a pair.

The preparations of spectrin from the hereditary elliptocytes contained some 50% of abnormal spectrin in the case of the β-chain variant ($\alpha\beta'$-spectrin), as determined by gel electrophoresis, and an uncertain proportion in the case of the α-chain variant ($\alpha'\beta$). The latter, however, could scarcely have been less than 50%, as judged by the fraction of the smaller tryptic fragment generated by the N-terminal α-chain (αI) domain [7], i.e. 74 kDa/(74 kDa + 80 kDa), which was 0.85 in these preparations, compared with 0.35 for normal spectrin. Both mixtures of abnormal with normal dimers ($\alpha'\beta + \alpha\beta$ and $\alpha\beta' + \alpha\beta$) formed no significant amount of tetramer over a period of several days at 4°C (fig.1). In contrast, when the preparations containing the univalent variants, $\alpha'\beta$ and $\alpha\beta'$, were mixed, tetramer was rapidly formed at 4°C. Because of the time required for gel analysis (~2 h), it was difficult to determine the rate of association with any precision, but as fig.2 shows, the time for half-

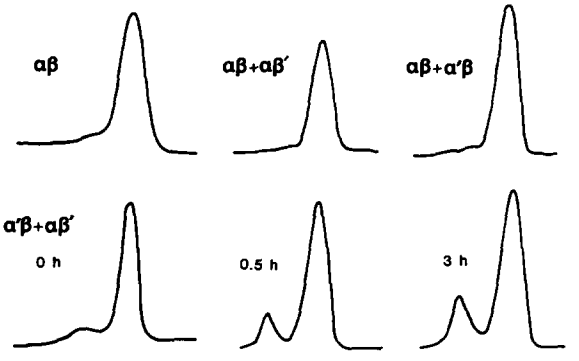

Fig.1. Densitometer traces of stained electrophoretic agarose gels of normal and abnormal spectrin dimers after incubation at 4°C: normal spectrin ($\alpha\beta$), kept at 4°C for 3 days; mixture of normal spectrin ($\alpha\beta$) with mutant spectrin, containing an anomalous α-chain ($\alpha'\beta$) after 1 day at 4°C; mixture of normal spectrin with mutant spectrin, containing anomalous β-chain ($\alpha\beta'$) after 1 day at 4°C; mixture containing both anomalous spectrins (together with normal spectrin), applied to electrophoretic gel immediately after mixing; the same, incubated for 30 min at 4°C before application to electrophoretic gel; the same after incubation for 3 h. Note the appearance of tetramer only in the mixture containing both univalent $\alpha'\beta$ and $\alpha\beta'$ variants.

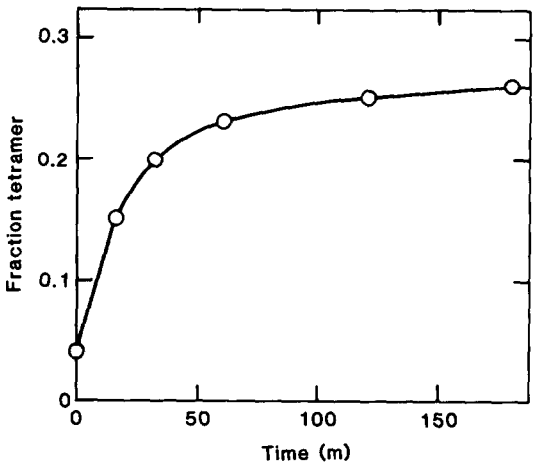

Fig.2. Rate of association of the two univalent spectrins: the fraction of total spectrin in the mixture that has been converted to tetramer is shown as a function of the time of incubation at 4°C before application to the electrophoretic gel for analysis.

reaction was no more than about 15 min. With the assumption that both reactants are present in comparable concentrations of about 0.5 μM, this corresponds to a forward rate constant of the order of 2000 $M^{-1} \cdot s^{-1}$, 6 orders of magnitude greater than that for normal spectrin dimers at the same temperature.

4. DISCUSSION

As the presence of abundant dimer in the cells of subjects with either type of abnormal spectrin shows, both $\alpha'\beta$ and $\alpha\beta'$ species are defective with respect to self-association. The $\alpha\beta'$-spectrin cannot associate with itself, because the relevant site in the β-chain is deleted, but it can form hetero-tetramers, $\alpha\beta \cdot \alpha\beta'$, with normal spectrin [5]. In the case of the $\alpha'\beta$-spectrin, the defect is (presumably) the result of a single-point mutation, and one cannot therefore exclude the possibility that some vestigial self-association capacity may remain. Our results indicate, however, that any internal interaction between the terminal association sites on the α'- and β-chains is insufficient to produce a high activation barrier for the formation of the mixed tetramer, $\alpha'\beta \cdot \alpha\beta'$.

This barrier is preserved in associations in which one partner is normal spectrin. Thus, the N-terminal α-chain fragment, which contains the association site, will bind to the $\alpha\beta$ dimer, but both the forward and reverse reactions are immeasurably slow in the cold [2]. Moreover, the association constant differs by only a factor of two from that for the association of two spectrin dimers [2]. Thus, it would be predicted that the association between the two univalent species, $\alpha'\beta$ and $\alpha\beta'$, in which no internal interactions need to be broken, would be extremely strong. Because of the uncertainty as to the concentration of $\alpha'\beta$-spectrin in the mixtures, we were unable to determine an equilibrium constant, nor were we able to obtain sufficient material for kinetic measurements over a wider temperature range. The present results are largely qualitative; a quantitative thermodynamic and kinetic study awaits the availability of further blood samples.

It may be conjectured finally that the extreme stability of the tetramer of non-erythroid spectrin (fodrin), which cannot be dissociated into dimers under any known conditions, may be the result of the greater rigidity of its constituent chains [9], which could prevent the formation of intramolecular bonding between the chain termini.

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