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Band 3-hemoglobin associations

The band 3 tetramer is the oxyhemoglobin binding site

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The associations between the band 3 protein of the human erythrocyte membrane and oxyhemoglobin, in solutions of a nonionic detergent, were studied by sedimentation equilibrium experiments in the analytical ultracentrifuge. The following results were obtained: (i) hemoglobin is bound virtually exclusively to the band 3 tetramer, but not to the monomer or dimer; (ii) the band 3 tetramer can bind up to four hemoglobin tetramers; (iii) unlike the unstable dimers of unmodified band 3, stable dimers crosslinked via S–S-bridges also represent hemoglobin binding sites.

Band 3 protein; Hemoglobin; Protein–protein association; Analytical ultracentrifugation; Erythrocyte membrane

1. INTRODUCTION

Band 3, the main integral protein of the erythrocyte membrane, exists in the membrane in different oligomeric states, most probably as monomers, dimers and tetramers at thermodynamic equilibrium with each other [1–4]. Simultaneously, it is a multifunctional protein, serving as an anion transport pathway and as a binding site for various other proteins [2–6]. The relationships between oligomeric structure and functions of the protein have attracted much attention, but reliable information on these relationships is still scarce. Two fundamental questions have, however, recently been answered: it was shown that it is the band 3 tetramer that acts as the high affinity ankyrin binding site [7,8], whereas anion transport can already be performed by the band 3 monomer [9]. The present paper is concerned with the role of self-association in another function of the band 3 protein, the binding of hemoglobin (Hb).

Erythrocyte membranes, at low ionic strength and pH 6–7, show two classes of Hb binding sites. The sites of higher affinity, approx. 1.0×10^6 per cell, reside on the cytoplasmic domain of band 3; they most probably serve as Hb binding sites also in the intact cell (for reviews see e.g. [4,5,10]). The stoichiometry of the band 3–Hb association has been intensely studied, by using either intact or modified erythrocyte membranes [11–15] or the isolated cytoplasmic domain of band 3 [16–18]. The data obtained with the latter system clearly showed that each band 3 molecule possesses binding sites for two Hb dimers or one Hb tetramer [4,5,10,16]. However, the isolated domain does not show the same monomer/dimer/tetramer association equilibrium that exists in mildly isolated band 3 preparations [2–4,19,20] but forms stable dimers [4,5].

The present paper describes the first investigation on the role of the different band 3 oligomers in Hb binding. It utilizes sedimentation equilibrium experiments to analyze the associations of the two proteins in solution of a nonionic, nondenaturing detergent, nonaethylene glycol lauryl ether ($C_{12}E_9$). Due to technical limitations, we have used the oxygenated form of Hb (oxy-Hb). Oxy-Hb and deoxy-Hb differ in their affinity to band 3 and in their mode of complex formation; however, the structure of the complexes may be similar in both cases [5,10,16,18,21].

2. MATERIALS AND METHODS

2.1. *Materials*

Band 3 protein was solubilized and purified in solutions of $C_{12}E_9$ as described earlier [19]. In this detergent, the band 3 protein can be kept in a monomer/dimer/tetramer association equilibrium for approx. 2 days [19]. Band 3 dimers covalently crosslinked via S–S bridges were prepared according to [22] and purified by gel filtration on Sephacryl S-300 (Pharmacia, Freiburg, Germany). Hb was prepared and purified by gel filtration similar to [16]. Buffer during the latter step and ultracentrifugation was 5 mM sodium phosphate (pH 7.1), 0.5 mM EDTA 0.4% $C_{12}E_9$. Hb concentration was determined spectrophotometrically [16]. The degree of oxygenation of the Hb was >96% [23].

2.2. *Analytical ultracentrifugation*

Sedimentation equilibrium experiments were performed in a Spinco model E ultracentrifuge equipped with monochromator, multiplexer and photoelectric scanner, using 12 mm double sector cells and an

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An-G rotor. Sample volume was 110 μl, rotor speed 10 000 rpm, and rotor temperature 8°C. The concentration distributions of Hb and its complexes with band 3 were recorded at 505 nm and evaluated as described in detail elsewhere [8,24]. In essence, sums of Boltzmann distributions, which represent the different particles expected to be present, are fitted to the data. It was assumed in the calculations that the partial specific volume, $\bar{v}$, of the proteins is 0.746 ml/g [25]. The corresponding value of the 'reduced' molecular weight, M* [8,24], for the band 3 monomer (including bound detergent) is 115 000 [8].

3. RESULTS

Under the experimental conditions used by us, sedimentation equilibrium distributions of oxy-Hb alone can be perfectly fitted by the sum of terms which represent the Hb dimer and tetramer, respectively. When band 3 is added to the solutions, however, the quality of the fit decreases substantially: the error sum δ (the sum of the squared residuals) increases by a factor of approx. 100. This indicates that complexes of band 3 and Hb have formed.

3.1. *Band 3 content of the band 3/Hb complexes*

We have first tried to identify those band 3 oligomers which bind Hb. It was utilized that the particle weight of the band 3/Hb complexes is governed by their band 3 content and that, therefore, the influence of their exact Hb content on the concentration distributions will be relatively small. Results derived from three series of fits to the same experimental data are shown in Fig. 1. The figure clearly shows (i) that models taking into account the presence of a high molecular weight complex lead to a considerable decrease in the δ-value, and (ii) that

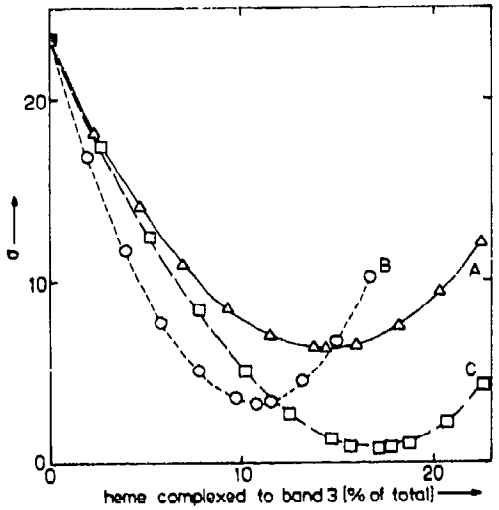

Fig. 1. An analysis of the sedimentation equilibrium data, assuming that the solutions contained the two Hb oligomers plus a fixed amount of complex consisting of one Hb tetramer and one band 3 monomer (A), dimer (B) or tetramer (C); dependency of δ on the relative amount of heme complexed to band 3. The initial concentrations of band 3 and heme were 2 μM and 39 μM, respectively. The association constant corresponding to the minimum of curve (C) is approx. 4.5×10^4 M^{-1}.

by far the best fit is obtained if this complex is assumed to consist of Hb and one band 3 tetramer. In the latter case, the deviations between the experimental and the fitted absorbance data are within the limits of error of the experimental data. This strongly suggests that the band 3 tetramer plays an important role in Hb binding. This conclusion is confirmed and extended by the calculations shown in Fig. 2. In these conclusions, fixed amounts of complexes containing one or two band 3 molecules were constant parameters, whereas the concentration at a fixed radial position (and thus the total amount [9,24]) of the free Hb oligomers and of the complex containing the band 3 tetramer were the fitted parameters. As seen in Fig. 2, any added complex containing band 3 monomers or dimers reduces the quality of the fit. Another line of evidence leading to the same result is obtained from fits which included, as free parameters, the concentrations of all three types of complexes: in these fits, the calculated concentrations of the two smaller complexes were negative, which means that, in the best fit yielding nonnegative concentration values, these complex concentrations will be zero [26]. Thus, it is only the band 3 tetramer, but not the monomer or dimer, which serves as a Hb binding site (at least in the equilibrium situation).

3.2. *Hb content of the complexes*

To determine how much Hb can be bound to the band 3 tetramer, we have varied the band 3 and Hb concentration in the samples, and we have fitted the absorbance-vs.-radius data by using different values for the (average) reduced particle weight, M* [8,24], of the complex. The dependency of the error sum on M*, for the two extreme cases observed, is shown in Fig. 3. It is obvious from Fig. 3 that an optimum value of M* exists which, however, depends on the experimental conditions: at low Hb binding, M* corresponds to a particle containing, besides the band 3 tetramer, one Hb dimer or tetramer, whereas the Hb content increases up to four tetramers at maximum Hb binding.

3.3. *Miscellaneous*

The results described above are based on a large number of different preparations of band 3 and Hb. Contrasting results were, however, obtained when band 3 dimers covalently crosslinked via S–E-bridges [22] were used instead of unmodified band 3 protein. In this case, the data could be perfectly fitted under the assumption that a complex of Hb and one band 3 dimer had formed, whereas fits based on other models of association were of poor quality. Under comparable conditions, the amount of complex found was similar to that formed by unmodified band 3. In contrast to the unstable dimers of unmodified band 3, the stabilized dimer can thus serve as a Hb binding site. This result is consistent with the finding of others that the isolated cytoplasmic frag-

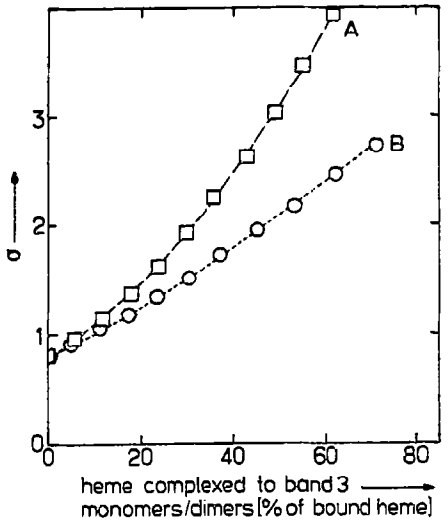

Fig. 2. An extension of the calculations of Fig. 1C: influence of the addition of fixed amounts of complexes containing one band 3 monomer (A) or dimer (B). Protein concentrations: see Fig. 1.

ment of band 3, which also is a stable dimer [4,5], can bind Hb [16–18].

Some of the samples were subjected to band centrifugation in a sucrose gradient [16]. As in the case of the band 3/ankyrin association [7], a band moving ahead of uncomplexed band 3 could not be detected. This shows that, in the buffer used, the complex and its components are linked in an association equilibrium, which is rapidly established on the time-scale of the technique applied.

We have performed some additional sedimentation equilibrium runs on samples to which NaCl in concentrations up to 80 mM had been added, to rule out electrostatic effects on the sedimentation behaviour of the proteins [26]. As expected from published data on the Hb/band 3 association [18], the relative amount of bound Hb decreased strongly with increasing NaCl concentration (e.g. by a factor of 10 at 40 mM NaCl). However, the virtually exclusive association of Hb with the band 3 tetramer remained unaffected by the change in ionic strength.

4. DISCUSSION

The present paper is based on a general principle which is widely used in biomembrane research, namely that solubilization of intrinsic membrane proteins by suitable nonionic detergents will not significantly perturb their secondary and tertiary structure and, as a consequence, their quaternary structure [28,29]. Since protein–protein associations in detergent solutions can be reliably analyzed by analytical ultracentrifugation [24,29], application of this technique to the solubilized proteins should reveal the types of associations present in the intact membrane.

Earlier studies on the stoichiometry of the band 3/Hb

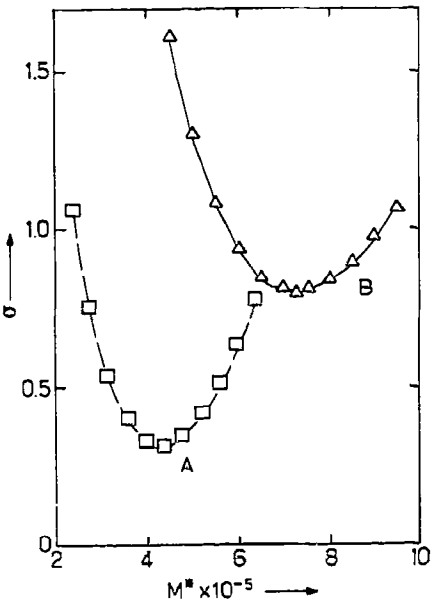

Fig. 3. The influence of the assumed reduced particle weight, M*, on the quality of the fits. The two data sets were obtained at initial concentrations of band 3 and heme of 2.0 μM and 29 μM (A) and 3.4 μM and 75 μM (B), respectively.

association have shown that each band 3 molecule can bind one Hb tetramer [4,5,10,16,21]. This result is, in part, confirmed by the present investigation. However, it is put on an entirely new basis: among the different band 3 oligomers, it is only the tetramer, but not the monomer or dimer, which binds Hb. As in the case of the band 3/ankyrin association [7,8], the band 3 tetramer is thus distinguished among the different band 3 oligomers with respect to the protein's function as a binding site. Possible reasons may be a reduction of the high flexibility of the cytoplasmic domain of band 3 [5] or a conformational change in the Hb binding site following the formation of the tetramer (or the stable dimer).

The results described suggest that binding of Hb to the band 3 tetramer will shift the association equilibrium towards this oligomer. Thus, Hb (as well as ankyrin) may behave as an effector of tetramerization of band 3 in the erythrocyte membrane.

The finding that the band 3 tetramer can bind up to four Hb tetramers may lead to renewed interest in the hypothesis that band 3 may form the nucleus of a multienzyme complex at the inner surface of the erythrocyte membrane [4–6]. Further speculations on this problem should await, however, results on the roles of the different band 3 oligomers in the binding of the glycolytic enzymes.

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