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The complex of band 3 protein of the human erythrocyte membrane and glyceraldehyde-3-phosphate dehydrogenase: stoichiometry and competition by aldolase

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Abstract

The cytoplasmic domain of band 3, the main intrinsic protein of the erythrocyte membrane, possesses binding sites for a variety of other proteins of the membrane and the cytoplasm, including the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aldolase. We have studied the stoichiometry of the complexes of human band 3 protein and GAPDH and the competition by aldolase for the binding sites. In addition, we have tried to verify the existence of mixed band 3/GAPDH/aldolase complexes, which could represent the nucleus of a putative glycolytic multienzyme complex on the erythrocyte membrane. The technique applied was analytical ultracentrifugation, in particular sedimentation equilibrium analysis, on mixtures of detergent-solubilized band 3 and dye-labelled GAPDH, in part of the experiments supplemented by aldolase. The results obtained were analogous to those reported for the binding of hemoglobin, aldolase and band 4.1 to band 3: (1) the predominant or even sole band 3 oligomer forming the binding site is the tetramer. (2) The band 3 tetramer can bind up to four tetramers of GAPDH. (3) The band 3/GAPDH complexes are unstable. (4) Artificially stabilized band 3 dimers also represent GAPDH binding sites. In addition it was found that aldolase competes with GAPDH for binding to the band 3 tetramer, and that ternary complexes of band 3 tetramers, GAPDH and aldolase do exist.

Keywords: Band 3 protein; Glyceraldehyde-3-phosphate dehydrogenase; Aldolase; Erythrocyte membrane; Analytical ultracentrifugation

1. Introduction

The cytoplasmic domain of the anion exchanger of the erythrocyte membrane, band 3, represents a binding site for a variety of other proteins of the erythrocyte membrane and the cytoplasm. Among those are ankyrin (band 2.1), bands 4.1 and 4.2, hemoglobin, and the glycolytic enzymes aldolase (D-fructose-1,6-biphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13), D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) and phosphofructoki-

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $C_{12}E_9$, nonaethyleneglycol lauryl ether; FITC, fluorescein-5-isothiocyanate; FITC–GAPDH, GAPDH covalently labelled by incubation with FITC; M_{eff}, effective molar mass ($M(1-\bar{v}\rho_o)$); M_c, molar mass of a heterologous complex of band 3; M_{ceff}, effective molar complex mass

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nase. These associations have been extensively studied; for reviews see [1–3].

One of the interesting aspects of the band 3/ligand associations listed above is the relationship between ligand binding and the quaternary structure of the band 3 protein. This aspect goes back to the findings that, both in the erythrocyte membrane and after solubilization by mild detergents, band 3 is present as a mixture of different oligomers: dimers and tetramers were detected by a variety of different techniques [3,4], and the presence of monomers, in equilibrium with the dimers and tetramers, was deduced from studies on the solubilized protein [5–7]. The latter results have frequently been challenged [8–10] but were supported, at least in part, by more recent work [11,12]. The heterogeneity of the oligomeric state of band 3 immediately leads to the question for the role of the different oligomers in ligand binding. Our group has been engaged in investigating this problem at length. The method used was sedimentation equilibrium analysis, in an analytical ultracentrifuge, of the detergent-solubilized band 3 protein plus the respective dye-labelled ligand protein. We have found that, with all ligands studied, the band 3 tetramer is the sole or at least the by far predominating ligand binding site: with ankyrin [13,14], hemoglobin [15], aldolase [16], and band 4.1 [17]. In addition, we found that the band 3 tetramer can bind up to four of the respective ligand proteins [15–18], with the exception of ankyrin for which only one binding site exists ([19] and Doelle, F., Mayer, G. and Schubert, D., manuscript in preparation). We have also demonstrated the existence of a ternary complex of the band 3 tetramer, one ankyrin molecule and one to four aldolase tetramers [19]. In the present paper, we have extended our studies to the associations of band 3 with GAPDH.

The significance of the band 3/GAPDH association (as well as that of the association of band 3 with the other glycolytic enzymes) has been disputed for many years. There was ample proof that it existed at ionic strengths far below the physiological range, and many details of it were carefully investigated (see, e.g., [1,20–25]). On the other hand, its relevance for the situation in vivo was repeatedly questioned [26–30]. The criticism was based on the finding that, in equilibrium binding studies in vitro, no binding was observed at ionic strengths around 150 mM and pH values above 7.0. Other experiments, however, seemed to support the existence of the band 3/GAPDH association in vivo [22,31,32]. More recent results by Rogalski et al., applying immunolabelling of fixed, intact human erythrocytes [33], apparently have settled the question in favor of the biological relevance of the association.

The binding sites for GAPDH and for other ligand proteins of band 3, including aldolase, differ at least in part [1,23,24,34–36]. We therefore found it worthwhile to study, by the same methodology as before [15–17,37], the stoichiometry also of the band 3/GAPDH association. The main emphasis of the present study is, however, on the investigation of the mixed band 3/GAPDH/aldolase system, with the primary aim of finding out whether ternary complexes of the three proteins do exist. This aspect is of basic importance in any discussion of the possible existence and physiological role of a glycolytic enzyme complex associated with the human erythrocyte membrane [1,3,38–41].

2. Materials and methods

2.1. Materials

Band 3 protein from human erythrocytes was solubilized and purified applying the non-ionic detergent nonaethyleneglycol lauryl ether ($C_{12}E_9$) ('Thesit') as described earlier [6,17]. Transfer into the buffer used during ultracentrifugation was performed in the gel filtration step. Most of the material was prepared in thoroughly degassed buffers and immediately used for the ultracentrifuge study. In these samples the protein was a mixture of monomers, dimers and tetramers ('state a') [6,17]. In the ultracentrifuge cell, initial band 3 concentrations around 200 μg/ml led to a distribution (monomer:dimer:tetramer, averaged over the sample volume) of approximately 25:60:15% (w/w) (present data and [17]). Other samples were stored for 2–3 days in non-degassed buffers and in the absence of reducing agents; they contained a mixture of non-covalent stable dimers and some stable tetramers of band 3 ('state b') [6,16]. Stable band 3 dimers were also prepared by covalently crosslinking the purified protein via disulfide bridges, applying $CuSO_4$/*o*-phenanthroline as cata-

lyst [20,42] ('state c'). For most applications 'state b' and 'state c' dimers were separated from stable tetramers by gel filtration on Sephacryl S-300 [16].

GAPDH and aldolase, both from rabbit muscle, were purchased (as ammonium sulfate precipitates) from Boehringer Mannheim. They were dialyzed overnight against the required buffer, after having been diluted by the same buffer to a concentration of 0.5–1.5 mg/ml. For dye-labelling of GAPDH, the buffer was 50 mM Na_2CO_3/$NaHCO_3$ (pH 9.5), 50 mM NaCl, 1 mM EDTA, 0.1% $C_{12}E_9$. To this sample, a nearly saturated solution (approximately 14 mg/ml) of fluorescein-5-isothiocyanate (FITC; Roth, Karlsruhe, Germany) in 0.5 M Na_2CO_3/$NaHCO_3$ (pH 9.5) was added, up to a FITC/enzyme weight ratio of 0.5–1.0. The mixture was incubated, under gentle agitation, for 3 h at room temperature. Afterwards, unreacted dye was removed by dialysis (overnight) against the buffer used in the ultracentrifuge experiments. Except for the labelling procedure, temperature was 3–5°C.

The concentrations of the proteins were determined photometrically, using published extinction coefficients $A_{1\,cm}$ (280 nm) for solutions containing 1 mg protein per ml: 1.00 for band 3 [5], 1.03 for GAPDH [43], and 0.938 for aldolase [44]. With FITC-labelled GAPDH, the measured absorbance was corrected for the contribution of the dye (which amounted, at 280 nm, to approximately 25% of the absorbance at 497 nm). The figure used for the molar extinction coefficient of FITC was 73 000 M^{-1} cm^{-1} [45]. The absorbance ratio A_{497}/A_{280} of the FITC–GAPDH was in the range of 0.9–1.7. From these figures, the average number of FITC molecules bound per GAPDH tetramer is found to be 2.3–6.0.

The enzymatic activities of unlabelled and FITC-labelled GAPDH were compared by photometrically measuring the rate of NAD^+ reduction [46,47].

Standard reagents were purchased from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), or Sigma (Deisenhofen, Germany) and were of p.a. quality (if available).

2.2. Analytical ultracentrifugation

Sedimentation equilibrium and sedimentation velocity experiments used a Beckman Optima XL-A analytical ultracentrifuge, in connection with an An-50 Ti or An-60 Ti rotor and 12 mm Epon 6-channel or double sector centerpieces. Rotor speed was between 6000 and 8000 rpm in the sedimentation equilibrium and 40 000 rpm in the sedimentation velocity runs. Rotor temperature was 4°C. Sample volume was 135 μl (sedimentation equilibrium runs) or 400 μl (sedimentation velocity runs). The absorbance versus radius data, $A(r)$ or $A(r,t)$, were collected at 497 nm for samples containing FITC–GAPDH and at 280 nm for unlabelled GAPDH and for controls on the state of the band 3 samples. Protein concentrations in the ultracentrifuge cell were independently adjusted to 80–360 μg/ml for band 3, 90–510 μg/ml for GAPDH and 80–620 μg/ml for aldolase. If not stated otherwise, the buffer used was 10 mM sodium phosphate (pH 7.5), 0.2% (w/w) $C_{12}E_9$. The experiments and evaluations followed the strategies for unravelling heterologous protein–protein associations reviewed in [37] and applied by us several-fold [15–17]. The evaluations used the following values for the effective molar masses $M_{eff} = M(1-\bar{v}\rho_o)$ of the proteins studied: $M_{eff} = 32\,200$ for the band 3 protomer/detergent complex [17], 37 700 for tetrameric GAPDH (based on $M = 35\,700$ g/mol for the protomer [48], a partial specific volume $\bar{v}$ of 0.730 mg/ml [43], and a (mean) solvent density ρ_o of 1.008 g/ml), and 41 500 for tetrameric aldolase (based on $M = 39\,300$ g/mol [49] and $\bar{v} = 0.730$ ml/g (from [50] after correcting for temperature [51])). Since the absorbance increase during the runs resulting from the oxidation of dithiothreitol cannot be controlled, the reference cell contained water only. Baseline determination was performed as described earlier [16].

3. Results

3.1. The state of association of FITC-labelled and unlabelled GAPDH is identical

In sedimentation equilibrium experiments, the $A(r)$ profiles for both the unlabelled and the FITC-labelled GAPDH could be fitted by a single exponential, indicating homogeneity of the particle mass. The resulting effective molar masses, $M_{eff} = M(1-\bar{v}\rho_o)$, were $38\,100 \pm 500$ for both the unlabelled and the dye-labelled protein and thus agreed, within the experimental error, with the figure calculated for the

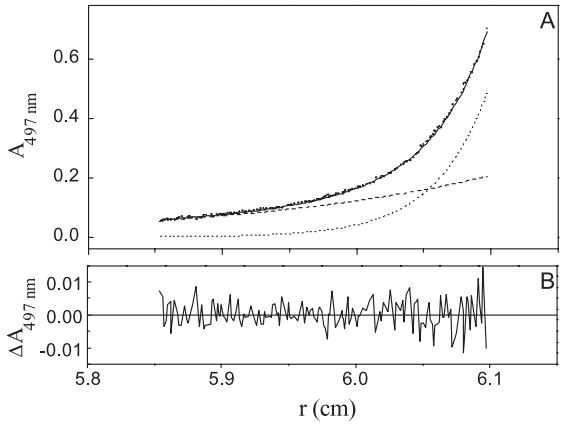

Fig. 1. (A) Sedimentation equilibrium analysis on mixtures of FITC–GAPDH and band 3 at low GAPDH/band 3 ratio: experimental absorbance values $A(r)$ at 497 nm, curve fitted to them assuming the presence of free enzyme and a complex of one GAPDH tetramer and four band 3 protomers (straight line), and calculated contributions to $A(r)$ of the free enzyme (dashed line) and of the complex (dotted line). (B) Residuals of the fit. Protein concentrations: 190 μg/ml (GAPDH) and 310 μg/ml (band 3). Buffer: 10 mM sodium phosphate (pH 7.5), 0.2% $C_{12}E_9$. Rotor speed: 8000 rpm.

GAPDH tetramer (see above). Our data thus disagree with those of Ovadi et al. [52,53] reporting self-association of tetrameric FITC–GAPDH but agree with others on the unlabelled enzyme [43,54,55]. It follows that the FITC-labelling applied by us does not disturb the enzyme's native quaternary structure. In agreement with [53], measurements of the rate of NAD^+ reduction by unlabelled and labelled GAPDH showed that FITC-labelling also does not significantly impair the enzyme's activity.

3.2. The sole or predominant binding site for FITC–GAPDH is the band 3 tetramer

In 10 mM sodium phosphate (pH 7.5), 0.2% $C_{12}E_9$, $A(r)$ profiles from sedimentation equilibrium runs on mixtures of band 3 and the dye-labelled enzyme showed the presence of particles larger than the band 3 tetramer. This indicates the formation of heterologous complexes. When $A(r)$ was measured at 497 nm, the nature of the complexes could be unravelled, the simplest results being obtained at relatively low abundance of GAPDH:

1. At band 3/enzyme weight ratios above 1.5, all curves could be perfectly fitted by only two terms: a term for the free enzyme and a second one for a complex characterized by $M_{ceff} = 166\,500$ g/mol, i.e. the effective molar mass of a complex composed of four band 3 molecules and one GAPDH tetramer. They could not be fitted by using the M_{ceff} of complexes containing one or two band 3 protomers (Fig. 1). This finding strongly suggests that the band 3 tetramer represents the sole or at least the predominant binding site for GAPDH. The conclusion was supported by consecutive fits in which M_{ceff} was varied: The opti-

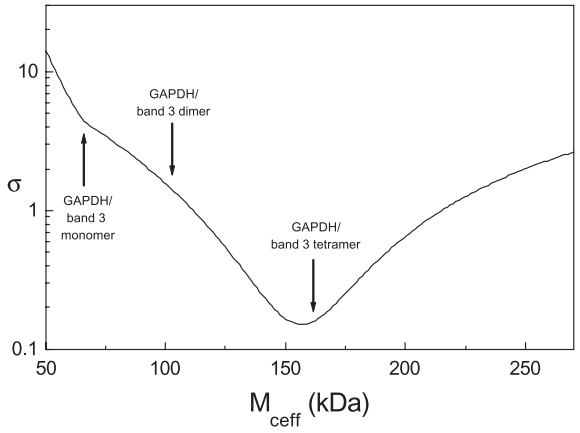

Fig. 2. An extension of the analysis of Fig. 1: dependency of the sum of the squared residuals of the fit, σ, on the effective molar mass of the assumed complex, M_{ceff}. The arrows indicate the calculated effective molar masses of complexes made up of one GAPDH tetramer and a band 3 monomer, dimer, and tetramer, respectively.

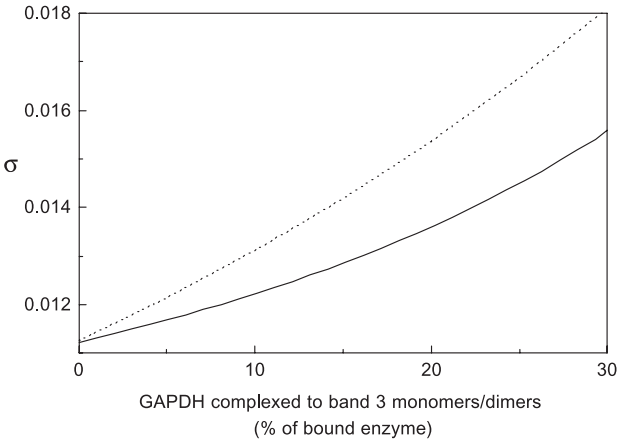

Fig. 3. Allowing for additional complexes: effect on σ of the consideration, in the fits to the data of Fig. 1, of fixed amounts of complexes consisting of a GAPDH tetramer and either a band 3 monomer (dashed line) or dimer (straight line).

mum M_{ceff} found was 161 000 g/mol and thus close to the figure applied above (Fig. 2).

2. The lack of significant contributions of complexes containing, apart from one GAPDH tetramer, a band 3 monomer or dimer was indicated by fits using, besides the two terms applied above, a term representing a fixed amount of the respective smaller complex [15–17,37]: the quality of the fits progressively deteriorated with increasing amount of the added complex, as demonstrated by the increase in the sum of the squared residuals (Fig. 3). Thus, at maximum a few percent of the complexed enzyme may be bound to band 3 monomers or dimers.

In the erythrocyte membrane, the band 3/GAPDH association is strongly dependent on pH and ionic strength I [21,22,27]. We have studied whether the influence of both parameters is mimicked in our system and whether the qualitative results described for pH 7.5 and 10 mM phosphate are valid also under other experimental conditions. We found that at pH 7.0 and 8.0 the results were qualitatively the same as at pH 7.5: the band 3 tetramer was the sole or by far the most predominant GAPDH binding site. However, the relative amount of band 3-bound GAPDH distinctly decreased with increasing pH, as in the native system [21,22,27]. Analogously, increasing the phosphate concentration, at pH 7.5, up to 100 mM led to a gradual decrease in the percentage of the bound GAPDH, with virtually no detectable complex at the highest phosphate concentration. Complex stoichiometry, however, was the same as at 10 mM phosphate.

As in our previous studies on the binding of ankyrin and of aldolase to band 3 [13,16], there is an alternative way of fitting the experimental $A(r)$ data (though with slightly inferior quality of the fits). In this alternative model it is assumed that the stoichiometry of the complex is, instead of 4:1 (band 3 protomers/GAPDH tetramers), 2:2. Two of the criteria used previously to discriminate between the different stoichiometries [13,16] were applied also to the present data: (1) at very high band 3/GAPDH ratio, the 2:2 complex should be accompanied by a 2:1 complex; this complex could, however, not be detected. (2) Since there is only a single binding site

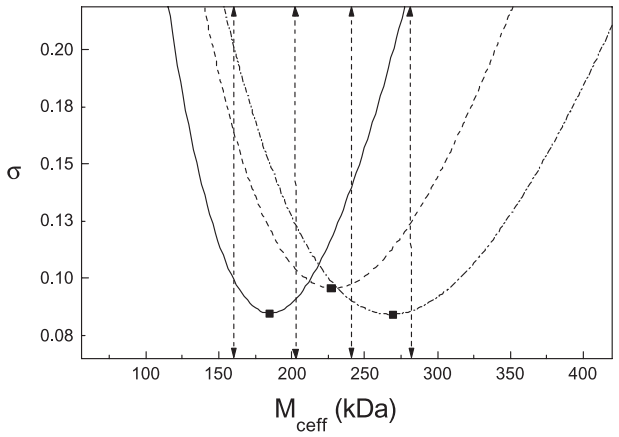

Fig. 4. Increase in M_{ceff} at higher abundancy of GAPDH: dependency of σ on the assumed M_{ceff}, in two-component fits to data collected at different GAPDH/band 3 weight ratios. The vertical lines indicate the calculated M_{ceff} values of complexes of the band 3 tetramer and one to four GAPDH tetramers. Protein concentrations: 180 μg/ml GAPDH, 330 μg/ml band 3 (straight line); 210 μg/ml GAPDH, 240 μg/ml band 3 (dashed line); 520 μg/ml GAPDH, 180 μg/ml band 3 (dashed/dotted line). Rotor speed was 8000 rpm.

for the GAPDH tetramer per band 3 protomer [1,2,20,21], the 2:2 complex should be the largest one occurring (with a possible exception at high band 3 concentrations where the formation of 4:4 complexes via further association of band 3 could be favored). This was found not to be the case (see below).

In sedimentation velocity experiments, the sample did not show separate boundaries which could be correlated to the free enzyme ($s_{20,w} = 7.05$) and the complex. Instead, an ill-defined broad boundary with an approximate average $s_{20,w}$ value of 10–14 S was observed. As was already described for other heterologous complexes of band 3 [13,15–17], this indicates that the band 3/GAPDH complex is unstable on the time scale of the experiment. However, it is more stable than at least the complexes involving aldolase and band 4.1, as indicated by its larger s value.

3.3. The band 3 tetramer can bind up to four tetramers of FITC–GAPDH

Using band 3/GAPDH mixtures of higher enzyme content than above, the quality of the fits to the $A(r)$ data analogous to that of Fig. 1 deteriorated with

increasing enzyme content, in particular at r values near the cell bottom. Calculations of the type shown in Fig. 2 indicated that higher complex masses were required to fit the data. The maximum values for M_{ceff} obtained were close to that for a complex of the band 3 tetramer and four GAPDH tetramers. Intermediate values were found for intermediate GAPDH concentrations (Fig. 4). Qualitatively similar results were obtained at higher phosphate concentrations ($\leq$80 mM), the average complex mass being increasingly shifted, however, towards that of the 4:1 complex.

The increase in M_{ceff} with increasing enzyme concentration was observed both at the lowest and the highest band 3 concentrations applied. Thus, it is not the consequence of an association, via band 3, of 2:2 complexes (band 3 protomers/GAPDH tetramers) but of consecutive binding of up to four GAPDH tetramers to band 3 tetramers. These findings are analogous to those reported with the ligand proteins hemoglobin, aldolase and band 4.1 [15–17] but differ from those found for the band 3/ankyrin complex where the band 3 tetramer binds only one ankyrin molecule ([19] and Doelle, F., Mayer, G. and Schubert, D., manuscript in preparation).

The ease by which the 4:1 complex, the 4:4 complex (band 3 protomers/GAPDH tetramers), or complexes of intermediate size could be found under a variety of conditions argues against a pronounced positive or negative cooperativity of GAPDH binding. The same holds for aldolase binding to band 3 [16].

3.4. Unlabelled and FITC-labelled GAPDH compete for binding to the band 3 tetramer

Using the same experimental conditions as above, part of the FITC–GAPDH was substituted by unlabelled enzyme. The effective complex mass M_{ceff}, as determined from $A(r)$ data collected at 497 nm, remained unchanged, as well as the relative contribution of the complex to the total absorbance. On the other hand, addition of unlabelled GAPDH at a fixed concentration of labelled enzyme and band 3 reduced the percentage of dye label associated with the complex (in addition, at low GAPDH/band 3 ratios it led to an increase of M_{ceff}). For example, in samples containing 140 μg band 3 and 190 μg FITC–GAPDH per ml, the presence of 240 μg/ml or 470 μg/ml unlabelled GAPDH led to a reduction of the relative amount of complex-bound FITC–GAPDH from 38% to 21% and 3%, respectively. This shows that the native and the modified enzyme compete for the same binding sites. Due to relatively large limits of error in the determination of the relative amount of complex-bound dye, it remains unclear whether or not the affinities of the two enzyme preparations towards band 3 may differ. It is, however, apparent that, at least qualitatively, the binding behavior of the FITC–GAPDH towards band 3 is representative for the native enzyme.

3.5. Aldolase and FITC–GAPDH compete for binding to the band 3 tetramer

Published data on competition between the glycolytic enzymes for binding to band 3 are controversial, in particular with respect to the combination aldolase/GAPDH (see, e.g., [30,35,36] versus [24]). The technique applied in the present paper allows an easy and reliable access to solving this problem, by studying mixtures which contain, besides band 3 and FITC–GAPDH at fixed concentrations, varying concentrations of aldolase. Under all conditions tested: at pH values of 7.0 and 8.0, ionic strengths between 5 and 50 mM, band 3 concentrations around 150 or 360 μg/ml, and GAPDH concentrations of 110 or 490 μg/ml, the relative amount of complex-bound dye in the mixtures strongly decreased with increasing aldolase/GAPDH ratio. Results obtained at both low and high molar ratio of FITC–GAPDH and band 3 (the latter granting, in the absence of aldolase, nearly complete occupancy of the GAPDH binding sites on band 3) are shown in Fig. 5. In the latter experiments, the complex-bound dye is found at virtually unchanged effective average complex mass M_{ceff} (data not shown). Both data sets demonstrate displacement of the labelled enzyme by the aldolase and thus competition for binding to band 3. In addition, the latter data show that binding of aldolase to band 3 at sites independent of the GAPDH binding sites, or to band 3-bound GAPDH (and vice versa), does not occur. Consistent with this conclusion we could not detect complex formation between FITC–GAPDH and aldolase in the absence of band 3, in agreement with [34,35] but in contra-

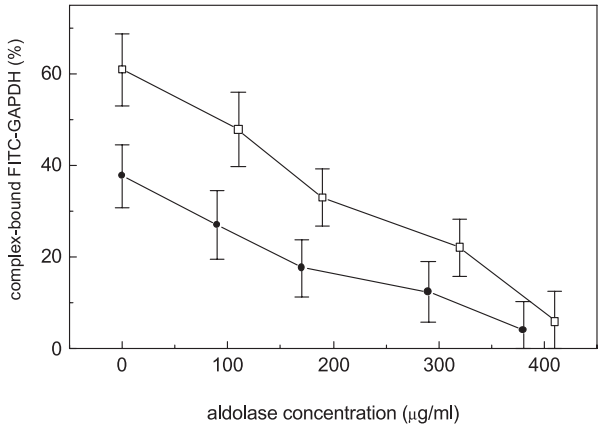

Fig. 5. Competition between GAPDH and aldolase for binding to band 3: dependency of the percentage of complex-bound FITC–GAPDH on the concentration of added aldolase. The concentration of GAPDH was 110 μg/ml (□) and 490 μg/ml (●), that of band 3 was 360 μg/ml (□) and 170 μg/ml (●), respectively. Buffer: 10 mM sodium phosphate (pH 7.5), 0.2% $C_{12}E_9$. Rotor speed: 8000 rpm. The error bars include the contributions due to the uncertainty of the bottom position of the cell.

diction to [52,53]. In the experiments at low GAPDH/band 3 ratios, higher aldolase/GAPDH ratios were required for displacing FITC–GAPDH from the complexes. In addition, an increase in M_{ceff} was observed (see below).

3.6. Aldolase and FITC–GAPDH can bind simultaneously to the band 3 tetramer

3.6.1. The problem

Given the numerous and conflicting reports on multienzyme complexes bound to the erythrocyte membrane (reviewed, e.g., in [1,3]) it appeared worthwhile to determine whether, in the experiments of Section 3.5, aldolase completely displaced GAPDH from part of the band 3 tetramers but did not bind to others, or whether mixed band 3/GAPDH/aldolase complexes were formed. Since, due to the similarity of the molar masses of the two enzymes, the complex composition cannot be deduced from M_c, it had first to be clarified whether and how sedimentation equilibrium analysis could solve the problem.

In case that mixed band 3/GAPDH/aldolase complexes do not exist, binding of one aldolase tetramer to the band 3 tetramer will have to displace all GAPDH tetramers already bound. Thus, a dye-labelled complex would disappear from the solution. On the other hand, the released GAPDH could bind to other (free) band 3 tetramers or to preexisting band 3/GAPDH complexes. Due to the 'trapping' of part of the band 3 tetramers in GAPDH-free band 3/aldolase complexes, this effect could, in addition, profit from the decrease in the amount of band 3 available for binding GAPDH. Thus, an increase in the measured M_c following the addition of aldolase does not necessarily indicate the formation of ternary band 3/GAPDH/aldolase complexes but could also accompany the formation of GAPDH-free band 3/aldolase complexes, in a physically totally different way. We have performed model calculations to find out whether the dependency of M_c on the concentrations of the added aldolase could, nevertheless, yield information on the types of complexes formed. The calculations were done for low GAPDH/band 3 ratios only, equivalent to a large proportion of free band 3 tetramers and 4:1 (band 3 protomers/GAPDH tetramers) as the predominant complex stoichiometry (at high ratios, the question raised certainly cannot be answered). Essentially, the method of calculation applied represents textbook physical biochemistry on binding phenomena governed by the law of mass action (see, e.g., [56]), which was adapted to serve the requirements of the present study. A detailed description has been given elsewhere [57]. In this paper, we will only describe the crucial results.

3.6.2. Results of model calculations

Model I: simultaneous binding of GAPDH and aldolase.

To simplify the calculations, two assumptions were made:

1. The association constants governing the degree of occupancy of the individual GAPDH and aldolase binding sites, respectively, on the band 3 tetramer are identical and independent (equivalent to a lack of cooperativity of binding; this is supported by the findings described in Section 3.3 and [16]). The figures chosen were 2×10^6 M^{-1} (GAPDH) [27,58] and 10^6 M^{-1} (aldolase) [27,59,60].

2. All band 3 molecules are in the form of tetramers (other percentages could be used equally well).

The calculations showed that the added aldolase was first bound virtually exclusively either to uncomplexed band 3 tetramers or to 4:1 complexes (band 3 protomers/GAPDH tetramers) of band 3 and GAPDH. Under these conditions, displacement of GAPDH from band 3 tetramers is a very rare event: only one out of approximately 25 aldolase tetramers bound to band 3 displaces a GAPDH tetramer. As a consequence, M_c shows a marked increase with increasing aldolase concentration. The error bars (mainly determined by the shape of the parabola in $\sigma(M_c)$ plots similar to Figs. 2 and 3 [61,62]) are small enough not to obscure this effect. At higher aldolase levels, increasing displacement of GAPDH from its binding site leads to a deterioration of the signal/noise ratio of the $A(r)$ data. As a consequence it also leads to broader $\sigma(M_c)$ curves and thus to larger uncertainties in M_c. This is shown by Fig. 6 (curve a), where M_c is plotted versus the 'aldolase binding function' r_A, which represents the fraction of aldolase binding sites on the band 3 tetramer that are occupied.

Model II: separate band 3/GAPDH and band 3/aldolase complexes.

In this model, binding of one aldolase tetramer to a band 3/GAPDH complex will displace all bound GAPDH from its binding sites. As previously stated, the displaced GAPDH may form complexes with free band 3 tetramer, or bind to preexisting band 3/GAPDH complexes. At the same time, the concentration of band 3 tetramers available for GAPDH binding will decrease, which will favor the formation of complexes containing more than one GAPDH tetramer. According to the calculations, the overall effect is a preferential disappearance of the 4:1 complexes of band 3 and GAPDH, as compared to the larger complexes, which will lead to an albeit small increase in the observed M_c. Due to the strong reduction of all dye-labelled complexes, the experimental uncertainty in the determination of M_c will, however, drastically increase. This will completely obscure the M_c increase (Fig. 6, curve b). Thus, despite the slightly positive slope of the M_c versus aldolase supply curve, discrimination between the two models should be easy.

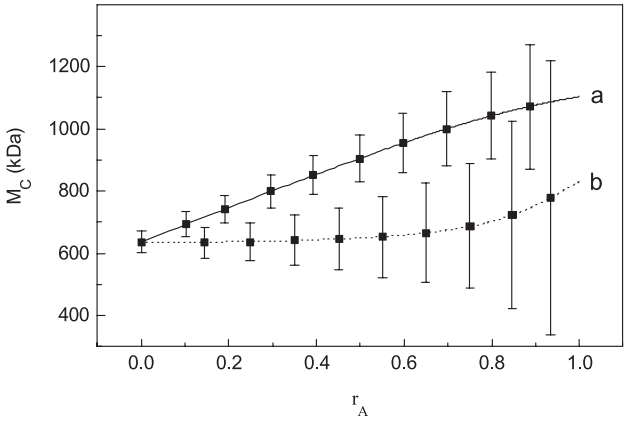

Fig. 6. Calculated dependency of the molar mass M_c of FITC-labelled complexes, in ternary systems band 3/FITC–GAPDH/aldolase, on the fraction r_A of aldolase binding sites on the band 3 tetramer which are occupied. (a) Simultaneous binding of both enzymes; (b) separate band 3/GAPDH and band 3/aldolase complexes. The error bars are based on the uncertainty of the M_c determination from plots $\sigma(M_c)$, assuming typical noise levels of the $A(r)$ data.

The above results were obtained using a number of simplifying assumptions. It was to be clarified therefore whether they are valid in more general cases. This was accomplished in part by simulations comparable to those described above, in part by qualitative considerations. Concerning the association constant for binding of GAPDH to band 3, the calculations show, in agreement with intuition, that the average molar mass and relative abundance of band 3/GAPDH complex present at a certain enzyme concentration would be changed. Thus, in order to establish the desired starting mixture of free band 3 tetramers and 4:1 complexes of band 3 and GAPDH (corresponding, in Fig. 6, to $M_c(r_A = 0)$), a different GAPDH concentration would have to be chosen. Concerning the assumptions on the concentration of band 3 tetramers and the association constants for aldolase we note that the abscissa of Fig. 6 refers to the average state of occupancy of the aldolase binding sites on the band 3 tetramers (including the aldolase-free fraction), r_A. Thus, an incorrect figure for one of the parameters specified would lead to a shift in the abscissa position considered but not to changes in the shape of the curve. The relationship between the experimental parameter accessible, total aldolase concentration, and r_A will in any case be non-linear; consequently a comparison of experimental M_c data collected at different aldolase concentrations with the curves of Fig. 6 will necessarily consider qualitative aspects only. With respect to the assumptions made on the lack of cooperativity in

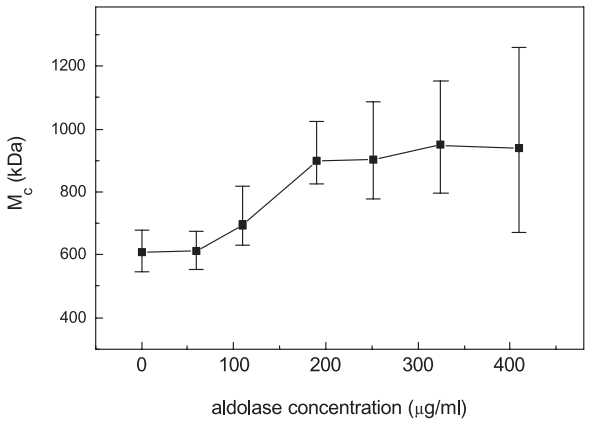

Fig. 7. Dependency of the molar mass M_c of FITC-labelled complexes, in ternary band 3/FITC–GAPDH/aldolase systems, on aldolase concentration: experimental data. The concentration of band 3 was 360 μg/ml, that of FITC–GAPDH 110 μg/ml. For other data see Fig. 5.

the binding of each of the enzymes to band 3 it should be noted that a pronounced positive or negative cooperativity is ruled out by the results of Section 3.3 and [16]. In addition, if band 3/GAPDH complexes, in model I, showed an increased tendency for binding aldolase (as compared to free band 3 tetramers), or if formation of a band 3/aldolase complex favored GAPDH binding, curve a would have shown a higher slope than in Fig. 6; in the opposite cases the situation and thus the resulting curve would become more similar to model II. This means that, in any case, an experimentally significant and marked increase in M_c with increasing aldolase concentration would be compatible with model I only.

3.6.3. Simultaneous binding: experimental evidence

The experimentally accessible equivalent to Fig. 6 is a graph M_c versus total aldolase concentration, as stated above. Data from three different series of experiments, each of them using the same set of stock solutions, are shown in Fig. 7 (part of the data are from the same experiments as those in Fig. 5). The error bars were derived from the respective $\sigma(M_c)$ plots (analogous to Figs. 2 and 3) and correspond to a 5% increase in σ; their asymmetry results from that of $\sigma(M_c)$. It is obvious from the figure that the experimentally determined (average) M_c increases with increasing aldolase supply and that, at least for three of the data points, the increase in M_c is statistically significant. According to the arguments described above, this is evidence for the occurrence of mixed band 3/GAPDH/aldolase complexes.

3.7. Stable band 3 dimers represent GAPDH binding sites

The 'state a' band 3 samples applied in the experiments described above and containing monomers, dimers, and tetramers of the protein can easily be converted largely or even completely into stable dimers. This is achieved by incubation (due to the action of highly reactive degradation products of the polyether detergent [6,7,16,17] ('state b')), or alternatively by crosslinking via S–S bridges [42] ('state c'). In previous studies our group has shown that both types of stable dimers can bind hemoglobin [15], aldolase [16], band 4.1 [17], and ankyrin ([13] and Doelle, F., Mayer, G. and Schubert, D., manuscript in preparation) at least as effectively as the 'state a' tetramers, in contrast to the 'state a' dimers. We have now performed analogous binding studies with FITC–GAPDH as the ligand protein. A typical result, using 'type b' band 3 dimers, is shown in Fig. 8. It is apparent from the figure that the $A(r)$ data can be perfectly fitted assuming that the only dye-labelled particles contributing to $A(r)$ are free GAPDH and 2:1 complexes (band 3 protomers/

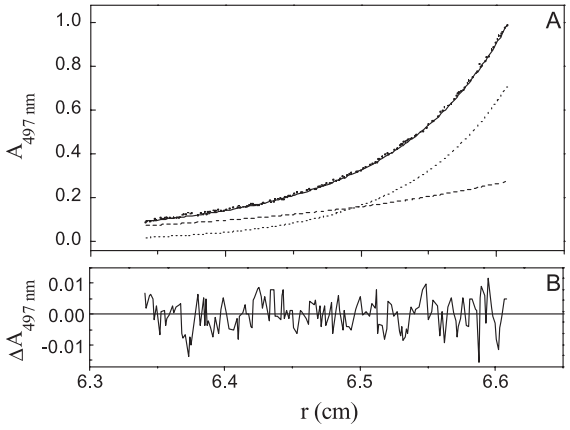

Fig. 8. Binding of GAPDH to stabilized band 3 dimers: (A) experimental $A(r)$ data, fit to the data assuming that the only dye-labelled particles present are the free enzyme and a complex of one GAPDH tetramer and one band 3 dimer (straight line), and calculated contributions of free GAPDH (dashed line) and of complex (dotted line). (B) Residuals of the fit. Protein concentrations: 160 μg/ml (GAPDH) and 330 μg/ml (band 3). Rotor speed: 7000 rpm.

GAPDH tetramers). At higher abundancy of the ligand, (average) stoichiometries up to 2:2 were observed. Analogous results were obtained using 'type c' band 3 dimers (data not shown). Thus, stabilizing the band 3 dimer confers to it the ability to bind GAPDH. These results again demonstrate that detergent-solubilized band 3 protein can occur in different conformations, coupled with differences in oligomeric structure and binding properties [6,7,16–18]. As emphasized earlier [6,7,18], we consider 'state a' as the native one.

4. Discussion

In the first part of this paper, we have characterized the binding of GAPDH to band 3 protein in detergent solution, with emphasis on the stoichiometry of the complexes formed. The results are completely analogous to those obtained earlier, applying the same methodology, on the binding to band 3 of another glycolytic enzyme, aldolase [16]: (1) the band 3 tetramer is the sole or at least the by far predominant GAPDH binding site, as compared to band 3 monomers or dimers. (2) The band 3 tetramer can bind up to four GAPDH tetramers. (3) Stabilized band 3 dimers can also effectively bind GAPDH. In view of the known similarities in the binding of the two enzymes to the band 3 protein [1,3,23,58,63], the results described are not surprising. On the other hand, it is also well-known that the GAPDH and the aldolase binding site on band 3 are not identical [1,3,24], so the separate study on the GAPDH/band 3 system presented in this paper does not seem to be needless. Above all, however, it represents the basis for the subsequent experiments on competition between GAPDH and aldolase for binding to band 3 and on the existence of mixed band 3/GAPDH/aldolase complexes. Two questions raised by our study have been extensively discussed in an earlier report [16]: (i) whether the results can be biologically relevant and (ii) why the band 3 tetramer is distinguished as a ligand binding site.

Each band 3 protomer possesses one binding site for both GAPDH and aldolase tetramers [1,3,20,30,58]. We have shown in this and in a previous paper [16] that these binding sites become functional only when the protomers are arranged in a tetrameric form (an exception is the case of stabilized band 3 dimers which we consider as artefacts). The relative locations of the two enzyme binding sites on the band 3 tetramer and the question of competition between the two enzymes for binding to band 3 are controversial to some extent. Most published data suggest at least a partial overlap of these sites. For example, well-defined fragments from the N-terminal part of band 3 were found to displace completely both enzymes from erythrocyte ghosts, although other fragments displaced aldolase only [23,63]. Analogously, the interactions of the two enzymes with ghosts were reported to be mutually inhibitory [30]. On the other hand, it has been shown that GAPDH has a marked detrimental effect on binding of aldolase but the reverse is not true. In addition, the aldolase site was found to be denatured under conditions which leave the GAPDH site nearly unperturbed [24]. Our data clearly contradict the claim that aldolase does not interfere with the binding of GAPDH to band 3 [24]; they are well compatible with the findings on mutual inhibition [30]. A major question still remaining open is whether the competition observed is really due to complete or at least partial overlap of the GAPDH and the aldolase binding site on band 3, or whether it is due to allosteric interactions between non-overlapping sites.

The primary aim of the present study was to search for mixed band 3/GAPDH/aldolase complexes. Such aggregates could constitute the nucleus of a glycolytic enzyme complex, as extensively discussed in the literature (e.g. [1,3,38–41]). We could demonstrate that this putative ternary aggregate in fact does exist, by this providing, for the first time, a structural basis for the 'glycolytic enzyme complex hypothesis' [1,3,38–41]. We would like to stress, however, that nevertheless we do not intend to advocate this hypothesis since, in our opinion, the arguments put forward against it are quite convincing [1]. Instead, we could imagine that, for the red cell, the mixed aggregates detected simply represent a means of storing or stabilizing the enzymes, as suggested by Low [1].

In conclusion, we would like to comment on a technical aspect concerning our studies on the ternary band 3/GAPDH/aldolase aggregates (Section 3.6). It is evident from a comparison of Figs. 6 and 7 that, with the technique applied, a discrimination

between the two models discussed touches the limits of the technique's potential. However, in our opinion there is only one alternative technique which might yield a clearer answer, namely fluorescence resonance energy transfer between band 3-bound donor-labelled GAPDH and acceptor-labelled aldolase (or vice versa). With this method, a simple qualitative criterion – the detectability of energy transfer – would suffice to prove the existence of the ternary aggregate (but would not allow any conclusion on its stoichiometry). However, the dimensions of the cytoplasmic part of the aggregate: four times the cytoplasmic domain of band 3 plus at least two different enzyme molecules, could be quite large (the length of the cytoplasmic domain of band 3 is thought to be approximately 250 Å [1]). The distance within a donor/acceptor pair on enzyme molecules bound to different band 3 protomers could therefore well exceed that range in which effective energy transfer can occur, up to approximately 100 Å [64]. A failure to detect energy transfer would therefore not necessarily mean that a ternary aggregate does not exist. On the other hand, a positive result could open the way towards more detailed studies on the distances between the bound ligand enzymes. These data could clarify whether another prerequisite for an active band 3-based glycolytic enzyme complex could be fulfilled, i.e. a strong interaction between the bound enzyme molecules by which the inactive enzymes in binary band 3/enzyme complexes [1,3,23,63] could possibly be converted into active ones [1].

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