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QUANTITATIVE CHARACTERIZATION OF THE INTERACTIONS OF ALDOLASE AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE WITH ERYTHROCYTE MEMBRANES

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Results of studies on the interactions of aldolase and glyceraldehyde-3-phosphate dehydrogenase with erythrocyte ghosts have been reinterpreted by making allowance for possible multivalency of the enzymes in regard to their interactions with matrix sites. It is shown that the curvilinearity of the experimental Scatchard plots may be attributed fully to the formation of enzyme-membrane complexes in which tetravalent enzyme may form crosslinks between several membrane sites. This interpretation of the results is preferable to earlier analyses based on heterogeneity of membrane sites in that (a) it takes into account the tetrameric nature of aldolase and glyceraldehyde-3-phosphate dehydrogenase, and (b) it is consistent with experimental demonstrations that band 3 protein is the sole site for enzyme interaction with the erythrocyte matrix. The dependence on ionic strength of the intrinsic association constant for either interaction is such that the binding of neither aldolase nor glyceraldehyde-3-phosphate dehydrogenase could be detected at ionic strengths in excess of 0.08 *I*. This finding is discussed in relation to the claims and counterclaims concerning the physiological significance of these interactions between glycolytic enzymes and erythrocyte membranes.

Introduction

A recurrent feature of binding studies on the interactions between glycolytic enzymes and erythrocyte ghosts has been the curvilinearity of Scatchard [1] plots obtained for both aldolase [2] and glyceraldehyde-3-phosphate dehydrogenase [3,4]. Although this curvilinearity has been considered to reflect either heterogeneity or negative cooperativity of erythrocyte membrane sites, the results have invariably been analyzed in terms of the former concept. However, such existence of two classes of binding sites on the erythrocyte membrane is rendered extremely unlikely by demonstrations that both enzymes bind to band 3 protein [2,5–9], and the fact that the total number of binding sites for either enzyme [1–4] corresponds to the band 3 content of the erythrocytes [10]. An alternative explanation of the observed curvilinearity of the Scatchard plots resides in the potential multivalency of the enzymes, both of which are tetrameric and hence possibly tetravalent with respect to their interactions with the erythrocyte membrane.

Provided that the interactions of an f-valent solute, A, with matrix sites, X, are governed by a single intrinsic [11] association constant, k_{AX}, the concentration of A in the solution phase, m_A, is related to its total concentration, $\overline{m}_A$, by the ex-

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pression [12]

$$k_{AX} = \frac{\left[1-(m_A/\bar{m}_A)^{1/f}\right]}{(m_A/\bar{m}_A)^{1/f}\left\{\bar{m}_X - f\bar{m}_A\left[1-(m_A/\bar{m}_A)^{1/f}\right]\right\}} \quad (1)$$

where $\bar{m}_X$ is the total concentration of matrix sites. Since this expression has previously been shown to provide adequate descriptions, with $f = 4$, of the binding of aldolase to phosphocellulose [12] and muscle myofibrils [13], it was considered of interest to determine the extent to which the observed curvilinearity of the Scatchard plots might be attributable to multivalency of the enzymes. To this end Eqn. 1 has been applied to published results on the interactions of aldolase [2,7] and glyceraldehyde-3-phosphate dehydrogenase [3,4] with erythrocyte ghosts; and also to the results of experiments performed in the present investigation to characterize the effect of ionic strength on the two interactions.

Materials and Methods

Preparation and characterization of erythrocyte ghosts. Unsealed human erythrocyte ghosts were prepared from outdated blood samples, kindly provided by the Red Cross Blood Transfusion Service in Brisbane, essentially in accordance with the method of Steck and Kant [14]. Endogenous aldolase and glyceraldehyde-3-phosphate dehydrogenase were then removed by washing the ghosts twice with 20 volumes of phosphate-buffered saline (5 mM sodium phosphate/150 mM sodium chloride (pH 8.0)) [15]. These enzyme-depleted ghosts were then placed in the required medium by three further washes with the appropriate buffer. The protein content of the resultant erythrocyte membrane preparation was determined by a modification [16] of the procedure of Lowry et al. [17], standardized on the basis of colour yields obtained with defined concentrations (10–100 μg/ml) of bovine serum albumin. The erythrocyte ghosts were also assayed for permeability and sidedness by means of the marker enzymes acetylcholinesterase, glyceraldehyde-3-phosphate dehydrogenase and NADH-dependent cytochrome *c* oxidoreductase [14]. Inclusion of Triton X-100 to render permeable any resealed ghosts had no significant effect on the activity of acetylcholinesterase, an enzyme located on the outer membrane surface, or of glyceraldehyde-3-phosphate dehydrogenase, an enzyme associated with the cytoplasmic face of erythrocyte membranes, irrespective of whether the experiments were conducted in 5 mM phosphate (pH 8.0) (conditions under which erythrocyte ghosts are unsealed), or in phosphate-buffered saline (pH 8.0). Although substitution of the latter for 5 mM phosphate (pH 8.0) as the medium for suspension of erythrocyte ghosts caused an 84% decrease in the measured activity of NADH-dependent cytochrome *c* oxidoreductase, a similar decrease (81%) in the spontaneous rate of the reaction was also observed, signifying that the decreased enzymic activity of the erythrocyte ghost preparation in phosphate-buffered saline was an effect of ionic strength on the reaction rather than an indication of partial inaccessibility of enzyme due to its location on the inner membrane surface of resealed ghosts. On the basis of experiments with all three marker enzymes it was therefore concluded that even in phosphate-buffered saline the erythrocyte ghosts exhibited sufficient permeability to allow access of added glyceraldehyde-3-phosphate dehydrogenase and aldolase to the cytoplasmic face of the membranes.

Preparation of enzyme solutions. Ammonium sulphate was first removed from commercial preparations of glyceraldehyde-3-phosphate dehydrogenase (Boehringer) and aldolase (Sigma), both rabbit muscle enzymes, by dialysis for 24 h at 4°C against the appropriate buffer (three times 500 ml), after which the enzyme concentrations were determined spectrophotometrically at 280 nm on the basis of values of 10.0 and 9.1 for the respective extinction coefficients ($A^{1\%}_{1\,cm}$) of glyceraldehyde-3-phosphate dehydrogenase [18] and aldolase [19].

Binding studies. In studies of the interaction of aldolase with erythrocyte ghosts at low ionic strength (0.014 *I*), ghosts (0.24 mg protein/ml) and aldolase (0.035–1.84 mg/ml) in 5 mM phosphate (pH 7.8), were incubated at 4°C for 2 h in Microfuge tubes, after which the ghosts were pelleted by centrifuging at 15000 × *g* for 30 min at the same temperature. The supernatants were then assayed for aldolase by the method of Jagannathan et al. [20]. Studies of the effect of ionic

strength on the interaction entailed the same experimental protocol except that the concentration of erythrocyte ghosts was increased to 0.37 mg protein/ml, and that a single enzyme concentration (35 μg/ml) was used. In a parallel series of experiments with glyceraldehyde-3-phosphate dehydrogenase, the erythrocyte ghost concentration was 0.32 mg protein/ml and the enzyme concentration 21 μg/ml. In this case the supernatants were assayed by observing the formation of NADH in reaction mixtures containing glyceraldehyde-3-phosphate (1.8 mM) and NAD^+ (1.0 mM) in 5 mM phosphate buffer (pH 7.8) made 1 mM with respect to dithiothreitol, sodium arsenate (13 mM) being included in the assay mixtures to render the reaction irreversible [21]. Throughout these binding studies control experiments were performed to test for any elution of endogenous enzymic activity, and also for dependence of enzymic activity upon ionic strength.

Results and discussion

The binding of aldolase to erythrocyte membranes in 5 mM phosphate (pH 7.0)

In order to determine the extent to which the curvilinearity of Scatchard plots for the binding of aldolase to erythrocyte membranes might be attributable to enzyme multivalency, advantage has been taken of the extensive set of binding data reported in Fig. 2 of Strapazon and Steck [2] for the interaction of rabbit muscle aldolase with human erythrocyte ghosts in 5 mM phosphate (pH 7.0). Those results, displayed (●) in Fig. 1, refer to experiments with $7 \cdot 10^8$ erythrocyte ghosts in a total volume of 1.1 ml. On the basis of the reported existence of $1.43 \cdot 10^6$ binding sites per ghost [2], a value of 1.51 μM is calculated for $\bar{m}_X$, the total concentration of matrix sites. Application of Eqn. 1 to the results using this calculated value of $\bar{m}_X$, a value of 4 for f, and values of m_A and $\bar{m}_A$ derived from the experimental points, yielded a series of estimates of k_{AX}, the mean being $3.5 \cdot 10^6$ M^{-1}. The relative constancy of these calculated values was taken to signify conformity of the system to the multivalent model with the selected value of 4 for f, which is in keeping with the tetrameric nature of aldolase and also the tetravalency of the enzyme in its interaction with phosphocellulose [12] and muscle myofibrils [13]. The theoretical Scatchard plot for the system with $\bar{m}_X = 1.51$ μM, $f = 4$ and $k_{AX} = 3.5 \cdot 10^6$ M^{-1} is shown as the solid line in Fig. 1, which clearly provides a plausible description of the results. Moreover, from the molecular viewpoint, this description of the binding of aldolase to erythrocyte membranes in terms of a single type of interaction between enzyme and a single class of membrane site is fully consistent with the proposition that aldolase forms a 1:1 complex with band 3 protein, the sole matrix site for the binding of the enzyme to erythrocyte membranes [2]; and is thus preferable to the original analysis of the results [2] in terms of membrane site heterogeneity, a concept in direct conflict with such a proposition.

Although tetravalency of aldolase in its interaction with band 3 protein undoubtedly provides an

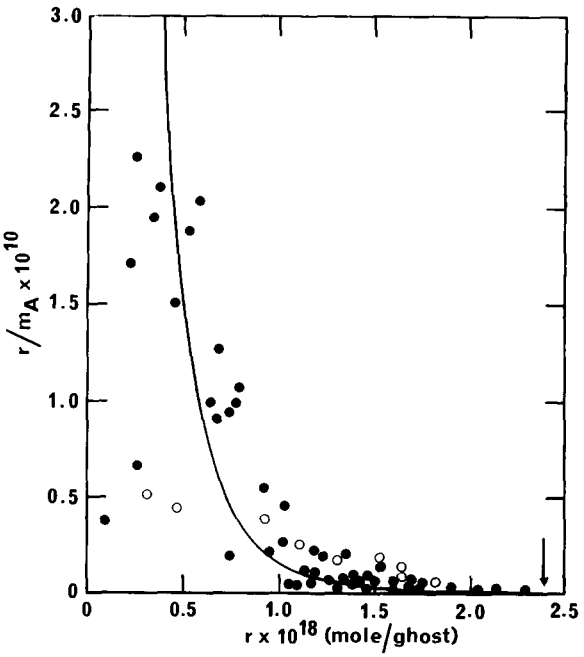

Fig. 1. Scatchard plot of binding data for the interaction of aldolase with human erythrocyte ghosts in 5 mM phosphate (pH 7.0): ●, rabbit muscle enzyme (taken from Fig. 2 of Strapazon and Steck [2]); ○, human erythrocyte enzyme (taken from Fig. 3 of Strapazon and Steck [7]). The curve is the theoretical relationship predicted by Eqn. 1 and an intrinsic binding constant (k_{AX}) of $3.5 \cdot 10^6$ M^{-1} for the interaction of tetravalent enzyme with a single class of sites on erythrocyte ghosts: the arrow denotes the abscissa intercept based on the band 3 protein content of erythrocyte ghosts.

adequate explanation of the curvilinear Scatchard plot obtained with rabbit muscle enzyme, the reported linearity of the corresponding plot for human erythrocyte aldolase [7], also a tetramer, is seemingly at variance with such interpretation. However, the results of that investigation [7] were far less extensive (○ in Fig. 1), and it is debatable whether any distinction should be made between results for aldolase from the two sources.

The binding of glyceraldehyde-3-phosphate dehydrogenase to erythrocyte membranes in 5 mM phosphate (pH 8.0)

Results reported in Fig. 1 of Beth et al. [4] for the binding of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase to human erythrocyte membranes are re-presented in Scatchard format in Fig. 2, and refer to experiments with $3.3 \cdot 10^9$ ghosts in a total volume of 1 ml. On the basis that there is competition between aldolase and glyceraldehyde-3-phosphate dehydrogenase for membrane sites [22] and that a 1 : 1 stoichiometry has been established for the enzyme-band 3 protein interaction [5], $\bar{m}_X$ has been based on the existence of $1.43 \cdot 10^6$ binding sites per ghost, the value used above for aldolase. In that regard we note that an estimate of $1.46 \cdot 10^6$ binding sites per ghost has been obtained from experimental studies of the erythrocyte interaction with glyceraldehyde-3-phosphate dehydrogenase [3]. Analysis of the results from Fig. 2 in terms of a tetravalent solute ($f = 4$ in Eqn. 1) yielded a value of $1.0 \cdot 10^6$ M^{-1} for k_{AX}, and a theoretical binding curve (solid line in Fig. 2) in excellent agreement with experimental findings. For this system too, therefore, the observed curvilinearity of the Scatchard plot may be explained fully by invoking the concept of cross-linking of matrix sites due to enzyme multivalency, rather than that of site heterogeneity or of negative cooperativity of membrane sites.

Initially, an attempt was made to analyze the results of Fig. 2 in terms of a bivalent solute ($f = 2$) in view of (a) the presence of two tightly-bound NAD^+ molecules per glyceraldehyde-3-phosphate dehydrogenase tetramer [23,24], and (b) the possible involvement of the coenzyme-binding sites in the enzyme-membrane interaction [15,25]. However, the curvilinearity predicted for such a system was insufficient to provide an acceptable

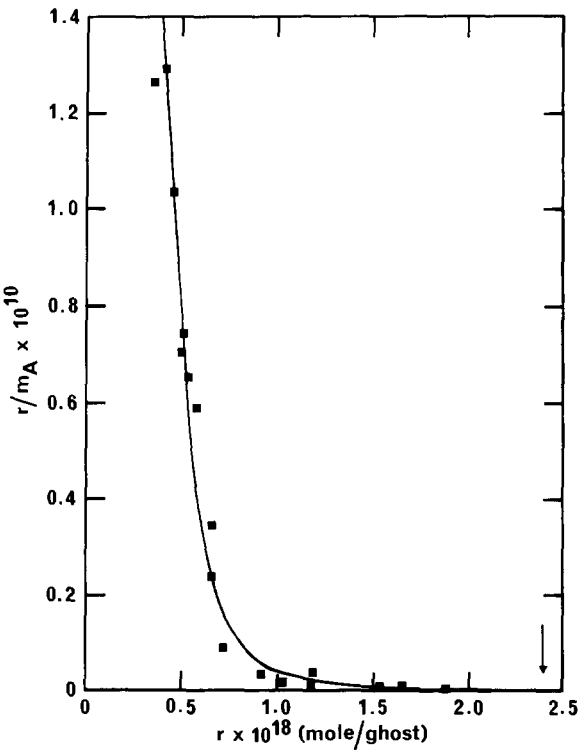

Fig. 2. Scatchard plot of binding data reported in Fig. 1 of Beth et al. [4] for the interaction of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase with human erythrocyte ghosts in 5 mM phosphate (pH 8.0). The curve is the theoretical relationship predicted by Eqn. 1 and an intrinsic binding constant of $1.0 \cdot 10^6$ M^{-1} for the interaction of tetravalent enzyme with a single class of sites on erythrocyte ghosts: the arrow denotes the abscissa intercept based on the band 3 protein content of erythrocyte ghosts.

description of the experimental results, the close conformity of which to the theoretical curve constructed with $f = 4$ seemingly, therefore, precludes the requirement of a vacant coenzyme-binding site for the interaction of glyceraldehyde-3-phosphate dehydrogenase with a membrane site. At first sight this conclusion appears to be in conflict with the observation that membrane-bound enzyme is eluted by pyridine nucleotides [15,25,26], but closer examination of those effects indicates nonconformity of the system with the simplest form of ligand-facilitated elution. Whereas low concentrations of NADH cause complete dissociation of the enzyme-membrane complex, NAD^+ is of low efficacy as an eluent [25,26], despite its comparable

affinity for glyceraldehyde-3-phosphate dehydrogenase [27,28]. Furthermore, NAD^+ is found to protect against elution of the enzyme by NADH [25,26]. Since these findings signify that the elution of glyceraldehyde-3-phosphate dehydrogenase from erythrocyte membranes does not reflect direct competition between coenzyme and matrix sites for the enzyme, there is clearly no necessity for the valence of glyceraldehyde-3-phosphate dehydrogenase toward membrane sites to be dependent on the coenzyme content of the enzyme: the present results argue against any such correlation.

Results from other experimental studies with glyceraldehyde-3-phosphate dehydrogenase may now be considered in terms of enzyme tetravalency towards erythrocyte membrane sites. The curvilinear Scatchard plots obtained by McDaniel et al. [3] for glyceraldehyde-3-phosphate dehydrogenases from rabbit muscle and human erythrocytes are certainly qualitatively consistent with this concept, the results for the human enzyme yielding, for example, an intrinsic association constant of $4.5(\pm 1.6)\cdot 10^5\ M^{-1}$ for the interaction in 5 mM phosphate (pH 7.5). In fact the only result seemingly at variance with the concept of enzyme multivalency is a report of positively cooperative binding [5]. This may, however, have been artefactual in origin, since at very low enzyme concentrations the error in experimental points is large. Indeed, similar evidence for positive cooperativity in the interaction of aldolase with erythrocyte membranes is provided by the experimental points at low r in Fig. 1; but Strapazon and Steck [2] have argued strongly in favour of those results being a reflection of the high experimental uncertainty associated with determinations of r and r/m_A in this region of the Scatchard plot.

Dependence of the enzyme-membrane interactions on ionic strength

Having employed the extensive sets of results reported by Strapazon and Steck [2] and Beth et al. [4] to justify consideration of the interactions of aldolase and glyceraldehyde-3-phosphate dehydrogenase with erythrocyte membranes in terms of Eqn. 1, we have applied a similar analysis to results obtained in the present investigation at pH 7.8. Studies of the binding of rabbit muscle aldolase to human erythrocyte ghosts in 5 mM phosphate (pH 7.8) are summarized in Fig. 3a, where the value of $\bar{m}_X$ used for the estimation of r has been based on the weight concentration of membrane protein, the value of $6\cdot 10^{-13}$ g protein per ghost [29], and the existence of $1.43\cdot 10^6$ binding sites per ghost [2]. The theoretical curve shown in Fig. 3a refers to a system with $\bar{m}_X = 0.96\ \mu M$, $f = 4$ and $k_{AX} = 5.0\cdot 10^5\ M^{-1}$, the average experimental value. Clearly the concept of crosslinking of matrix sites due to enzyme tetravalency also provides a reasonable description of these results.

On the basis that the above results (Figs. 1, 2 and 3a) at low ionic strength (0.011–0.015 I) are described adequately by Eqn. 1, the effect of ionic strength on the magnitude of the intrinsic association constant has been screened by measuring m_A, the free enzyme concentration, in reaction mixtures containing fixed concentrations of enzyme and erythrocyte ghosts ($\bar{m}_A$, $\bar{m}_X$) in buffers (pH 7.8) with ionic strengths in the range 0.014–0.15 I. From the results (Fig. 3b) it is evident that for both enzymes k_{AX} exhibits a pronounced inverse dependence on ionic strength; and that k_{AX} is of unmeasurable magnitude at ionic strengths in excess of 0.08 I. Two additional points are noted. First, the value of $8.9\cdot 10^5\ M^{-1}$

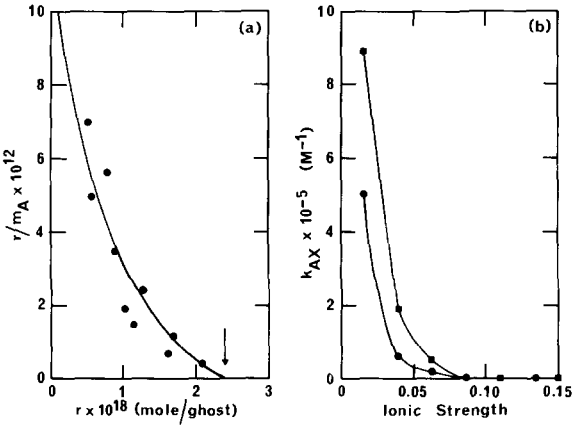

Fig. 3. Interactions of rabbit muscle aldolase (●) and glyceraldehyde-3-phosphate dehydrogenase (■) with human erythrocyte ghosts at pH 7.8. (a) Scatchard plot of results for aldolase in 5 mM phosphate (0.014 I), and the theoretical curve predicted by Eqn. 1 for a system with $k_{AX} = 5.0\cdot 10^5\ M^{-1}$. (b) Effect of ionic strength on the magnitude of the intrinsic binding constant (k_{AX}).

for the intrinsic association constant describing the interaction of glyceraldehyde-3-phosphate dehydrogenase with erythrocytes in 5 mM phosphate (pH 7.8) is in good agreement with the k_{AX} of $1.0 \cdot 10^6$ M^{-1} calculated from Fig. 2 for the same interaction under similar conditions (5 mM phosphate, pH 8.0). Secondly, in agreement with earlier findings [22,30], glyceraldehyde-3-phosphate dehydrogenase is bound more strongly than aldolase.

Concluding remarks

This investigation has provided a quantitative reappraisal of binding studies on the interactions of aldolase and glyceraldehyde-3-phosphate dehydrogenase with human erythrocyte membranes. It has shown that the results may be attributed fully to the formation of enzyme-membrane complexes in which the tetravalent enzyme molecules may be crosslinked to several matrix sites, there being no necessity to invoke the concepts of site heterogeneity or negative cooperativity. The dependence on ionic strength of the intrinsic association constants for both interactions is such that the binding of neither enzyme could be detected in experiments conducted at ionic strengths in excess of 0.08 I, a result that seemingly lends quantitative support to the claims [31–33] that the interactions of glycolytic enzymes with erythrocyte membranes are not physiologically significant phenomena. On the other hand, counterclaims for the existence of significant amounts of membrane-bound enzyme at physiological ionic strength [6,26,34,35] could also be accommodated by the present quantitative findings if the formation of enzyme-matrix complexes is favoured by covolume considerations; or, in other words, if the effective radius of the complex is smaller than the sum of those for the two separate reactants. Under those conditions the space-filling effects of the high concentration of other macromolecular components, notably haemoglobin, within the red blood cell could well lead to the required enhancement of the apparent equilibrium constant written in terms of concentrations rather than thermodynamic activities [36]. In this regard the recent detection of interactions between F-actin and glycolytic enzymes at physiological ionic strengths by means of partition studies with biphasic aqueous polymer solvent systems [37] attests to the pronounced displacements of equilibria that can be effected by thermodynamic nonideality emanating from the presence of high concentrations of inert macromolecular solutes. Further quantitative studies which take into account the likely effects of thermodynamic nonideality within the red blood cell are clearly required in order to resolve the dilemma concerning the physiological relevance of the interactions between erythrocyte membranes and glycolytic enzymes; and in this regard the present finding that such interactions may be considered in terms of a single intrinsic thermodynamic binding constant should provide a welcome simplification to an exceedingly complicated analytical problem.

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