Journal of Cellular Biochemistry 23:211–222 (1983)

Binding of Cytosolic Proteins to the Erythrocyte Membrane

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Certain enzymes and other proteins, traditionally thought to be exclusively cytosolic, have very often been found to be associated with isolated plasma membranes. These findings raise fundamental questions about concepts of membrane structure and the methods used to isolate membranes. It is conceivable that the interaction of cytosolic proteins is central to the overall structure and function of the cell in vivo. However, it is necessary to consider the specificity of the various interactions and evidence for their occurrence under conditions which can exist within the cell.

The interaction of cytoplasmic proteins with the erythrocyte membrane has enjoyed renewed interest [1]. Although earlier work considered the problem of the interaction of hemoglobin with the membrane [2–17], more recent studies have shown that certain glycolytic enzymes also bind to specific sites [7,18–22] and that a class of peripherally associated, so-called cytoskeletal, proteins exist [23–28]. The interaction of hemoglobin and the glycolytic enzymes has been shown to be largely electrostatic in nature and the discussion of the physiologic significance of these interactions is accelerating.

One might at first disregard the electrostatic association of hemoglobin and the glycolytic enzymes as simply an isolation artifact. However, closer examination has shown a surprising degree of specificity in binding even under the nonphysiologic conditions necessary to study binding stoichiometry. The site of high affinity binding has been shown to be band 3 protein, the integral membrane protein involved in Cl^-/HCO^{3-} exchange [1]. In this paper, I shall attempt to critically summarize the later

Abbreviations used: G3PD, glyceraldehyde-3P dehydrogenase; EPR, electron paramagnetic resonance; PFK, phosphofructokinase; DIDS, 4,4 -bis (isothiocyano)-2,2 -stilbene disulfonate; DPG, 2,3-diphosphoglycerate; PMB, p-chloromercuribenzoate; NEM, N-ethylmaleimide; NAD^+, nicotinamide adenine dinucleotide (oxidized); ATP, adenosine triphosphate.

Received August 8, 1983; accepted September 7, 1983.

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studies in this field with the hope of placing the interactions in perspective. I shall not discuss the interaction of the cytoskeletal proteins (spectrin, actin, band 4.1, and ankyrin, etc) except as they directly relate to the discussion of cytosolic protein binding. Reviews on the cytoskeletal proteins are plentiful and may be consulted for further details [23–28].

SITES OF MEMBRANE BINDING AND ALTERATIONS IN FUNCTION OF MEMBRANE-BOUND CYTOSOLIC PROTEINS

It does not seem surprising that proteins could adsorb to the red cell membrane at low ionic strength and/or acidic pH owing to the highly charged nature of the membrane and the high concentrations prevailing at the surface. Despite this expectation, a specific association with the cytoplasmic pole of band 3 protein has been demonstrated for several glycolytic enzymes and hemoglobin. A specific association was first demonstrated for G3PD [29]. No other binding sites seem to exist under the conditions used. The binding stoichiometry is one G3PD tetramer per band 3 monomer. Furthermore, the isolated peptide containing the NH_2-terminal 23 residues of band 3 displaces G3PD from the membrane and also inhibits the enzyme [30]. However, the ability of this fragment to displace G3PD was 30-fold lower than intact band 3 in solution, while the ability of the same fragment to inhibit the enzyme was virtually identical. Perhaps enzymatic inhibition requires only the last 23 residues, while the total binding site encompasses a greater portion of the cytoplasmic fragment of band 3 protein.

Beth et al [31] have recently performed saturation transfer EPR studies using rabbit muscle G3PD labeled with a maleimide spin label. They showed considerable immobilization of the enzyme upon binding. However, comparison of the mobility of the enzyme-band 3 complex with the mobility of the integral portion of band 3 [32] showed greater mobility of the cytoplasmic portion of band 3 than the integral portion. The authors also showed that G3PD undergoes a rearrangement of the ionic residues in the area of the active site consequent to binding. This could certainly reflect the insertion of the acidic NH_2-terminal residues of the cytoplasmic fragment of band 3 into the active site of the enzyme, as suggested by Steck's studies [30].

Aldolase, like G3PD, also seems to bind to the same portion of band 3 protein [33,34]. The issue of site identity has been raised based on studies with intact membranes [35]. In the latter studies, G3PD was able to inhibit the binding of aldolase while the reverse was not true. This finding contrasts with relatively simpler studies using isolated fragments of band 3 [36]. The 23K-dalton fragment was an effective inhibitor of both enzymes, and this inhibition was comparable to the effect of membrane binding on enzymatic activity in both cases. However, the fragments do not appear to be as potent in displacing the enzymes from the membranes, as was already noted for G3PD. The relative affinity of the fragments for G3PD was considerably *less* than for aldolase. Once again, if the 23K-dalton piece of band 3 is an effective inhibitor but does not encompass the entire binding site on the 43K-dalton cytoplasmic portion of band 3, enzymatic inhibition studies could be identical but affinities or even the site of binding on the larger fragment could be different. Nevertheless, it is difficult to envisage how these relatively large enzymes could interact with the fragments in solution, but *not* show mutually exclusive binding in membrane studies. Detailed studies using the entire 43K-dalton cytoplasmic fragment,

measuring individual binding constants under the same conditions and performing classical competitive binding studies where specific inhibition constants are extracted and compared with the binding constants, should clarify these points.

PFK is another enzyme which is thought to bind to band 3 [37,38]. The binding characteristics of this enzyme are similar to those of G3PD and aldolase at pH 7. Both aldolase and G3PD dissociate PFK from the membrane under appropriate conditions, suggesting competition for the same site [38]. In the earlier work on PFK binding to membranes, it was reported that hemoglobin did not compete with PFK for the same site, since addition of hemoglobin did not cause a significant amount of bound PFK to be released. This result would seem to contradict other studies discussed below which show that hemoglobin does bind to band 3. The explanation for this apparent discrepancy lies in the fact that the PFK-displacement studies with hemoglobin were performed at pH 7 and at hemoglobin concentrations fully 25 times lower than exist within the red cell under physiologic conditions. It seems likely, based on the studies mentioned below, that hemoglobin and PFK will show competitive binding for band 3 when conditions are properly chosen. As we will see shortly, a recurring problem in work with hemoglobin binding is the failure to consider or use physiologic hemoglobin concentrations when studies are performed at physiological intracellular pH and ionic strength.

Besides the glycolytic enzymes, there is evidence that both red cell catalase [39] and ankyrin [40,41] bind to band 3. Ankyrin is the protein which binds to spectrin and serves to attach the cytoskeletal "meshwork" to the membrane via *some* of the band 3 molecules [26]. This protein binds very tightly to band 3 at physiologic pH and ionic strength. Ankyrin binding is totally unaffected by the presence of G3PD under the conditions used [41]. The usual conditions for G3PD binding are low ionic strength (5 mM phosphate) and pH 8. The ankyrin-G3PD competition experiments were performed at near physiologic salt concentrations and pH 7.5 [41]. This result means that G3PD's relative binding constant to band 3 at physiologic conditions is much weaker than ankyrin's. Furthermore, this difference in affinity does *not* mean that both G3PD and ankyrin couldn't be simultaneously membrane bound. There are far more copies of band 3 than copies of ankyrin molecules [26]. Therefore, several band 3 sites could exist for glycolytic enzymes and hemoglobin binding if the conditions are favorable. What seems clear, however, is that neither the glycolytic enzymes tested so far nor hemoglobin have binding constants, under physiologic conditions, which could displace ankyrin.

Unlike most of the cytoplasmic proteins discussed above, the binding capacity of erythrocyte membranes for hemoglobin at low pH (pH 6) in 5 mM phosphate, is about five times greater than the number of band 3 monomers present in the membrane [42]. At least two classes of sites exist which have widely different affinities [42,43]. The high-affinity class constitutes about 1×10^6 sites and was shown to be sensitive to selective protolysis of band 3 at the cytoplasmic surface [42], to the addition of G3PD [42,44], and to DIDS binding to band 3 [42,45]. This latter transmembrane effect of stilbine disulfonate binding to band 3 was first shown in the work with hemoglobin binding [42,45] and has since been demonstrated in several other types of studies [46–48].

With the existence of two classes of sites, it is necessary to show that binding to band 3 is specific, since it has often been supposed that any protein would adsorb to a membrane under these conditions. There are several experiments which demonstrate

that high-affinity hemoglobin binding is specific for band 3, even under the nonphysiologic conditions used. In the clearest experiments, it was shown that the isolated cytoplasmic fragment of band 3 binds to hemoglobin in 5 mM phosphate pH 6 [42,49], but that myoglobin does not bind under the same conditions [50,51]. Myoglobin will bind to the low affinity sites on the membrane with about the same affinity as hemoglobin, but there is no detectable high-affinity binding to band 3 [51]. This seems to speak strongly against nonspecific binding to band 3.

Fung [52] has obtained evidence for low-affinity hemoglobin binding at physiologic pH but low ionic strength using EPR spectroscopy. She chose to label the membrane −SH groups with a maleimide spin label and study spectroscopic changes upon addition of hemoglobin. Unfortunately, the difficulty in interpreting this work is in deciding where the spin labels are located. This was also a problem in a second study using sickle cell hemoglobin, where tighter binding to the membrane was shown [53]. This latter result reproduced the finding of Shaklai et al [54], who showed tighter binding for sickle hemoglobin.

Cassoly [55] took a different and more interpretable approach by specifically labeling hemoglobin at the beta -93 sulfhydryl group and studying binding. Identical results were obtained when binding curves for labeled hemoglobin were compared using either the ESR method or the light-scattering method of Salhany et al [42]. The saturation transfer ESR measurements showed that the diffusional rotational movements of hemoglobin were slowed considerably, as was the case for G3PD [31]. Rotational correlation times of 8×10^{-6} sec were measured as compared with 2×10^{-8} sec in solution. However, the degree of rotational immobility was not different for high- vs low-affinity binding sites. As we shall see shortly, the low-affinity binding sites are composed of phospholipid and glycophorin, while the high-affinity site consists of the cytoplasmic portion of band 3.

The identification of low-affinity hemoglobin binding sites was accomplished by Rauenbuehler et al [56], who used selective proteolytic and lipolytic enzyme digestion to measure the release of hemoglobin bound stoichiometrically to sites on inside-out vesicles. They confirmed that the band 3-bound hemoglobin constituted about 25% of the total and was sensitive to chymotrypsin. Lipolysis with various phospholipase C preparations demonstrated that about 38% of the hemoglobin was bound to phospholipid head groups on the inner surface. About 30% to 40% of the bound hemoglobin was not released by either of these enzymes. The only other integral component of the membrane in sufficient quantity to be able to account for this rather large fraction was glycophorin [57]. Rauenbuehler et al [56] used rabbit inside-out vesicles which lack glycophorin [58,59] to show that this proteolytic and lipolytic insensitive component involved hemoglobin binding to the cytoplasmic aspect of glycophorin. Glycophorin reconstitution into phosphatidyl choline vesicles confirmed that hemoglobin can bind to the highly charged [60] cytoplasmic aspect of glycophorin under the conditions used. Recent preliminary experiments in our laboratory have suggested that the affinity of hemoglobin for glycophorin in solution is low even at low pH and ionic strength. The interaction of these two molecules seems unlikely under physiological conditions.

Subsequent work on the interaction of hemoglobin with the cytoplasmic fragment of band 3 has confirmed the earlier work and significantly extended the understanding of the interaction. Cassoly [49,61] studied the interaction of hemoglobin with the isolated cytoplasmic fragment of band 3 using rate zonal centrifugation

in a sucrose density gradient, by quenching of the fragment fluorescence by hemoglobin, and by flash photolysis of HbCO bound in a stoichiometric complex with the fragment. He found a stoichiometry of one hemoglobin tetramer per band 3 monomer. Furthermore, both fluorescence quenching and flash photolysis kinetic results showed that the liganded hemoglobin dimer is stabilized *on the fragment* when liganded hemoglobin is added. This finding is in excellent agreement with the kinetic results of Salhany and Shaklai [62], who added HbCO stoichiometrically to band 3 on the membrane. The fraction of fast CO-binding hemoglobin (ie, dimer) present as a function of band 3 fragment added was shown to fit a model where both dimers and tetramers can bind. Two dimer binding sites on the fragment and an isomerization to form tetramers had to be postulated to fit the data. One hemoglobin dimer binding site alone did not fit the results. Furthermore, the effective tetramer dissociation constant had to be about two orders of magnitude *smaller* than the dimer dissociation constant to give a good fit. The functional properties of the tetramer on the fragment were also altered. In summary then, we get a picture of the existence of two hemoglobin alpha-beta dimer binding sites on one band 3 monomer fragment which, when filled, isomerizes to form a complex containing an equilibrium mixture of bound dimers and tetramers (for liganded hemoglobin), with the equilibrium biased toward the liganded hemoglobin dimer. Deoxygenation should shift the equilibrium to all bound tetramer because of the much greater tendency of deoxyhemoglobin to associate. Furthermore, deoxyhemoglobin should have a higher affinity for the fragment. There is conflicting evidence concerning the relative affinity of oxy- and deoxyhemoglobin (Hb) for the fragment. One group finds a higher affinity [63], while the other finds a lower affinity [64]. Work with low concentrations of deoxyhemoglobin is difficult, and these difficulties may account for the discrepancies.

With regard to the suggestion from the Cassoly model that two dimer binding sites exist, it is interesting to note that the recent amino acid sequence of the cytoplasmic fragment of band 3 shows the presence of a repeat in the NH_2-terminal sequence [65]. It is conceivable, but yet to be demonstrated, that each repeated segment constitutes one alpha-beta dimer binding site, so accommodating one hemoglobin tetramer.

Recently, Arnone et al [66] have published an abstract on the effect of a *synthetic* 11-residue, acidic NH_2-terminal peptide of band 3. They report that this peptide *reduces* the oxygen affinity of hemoglobin and, indeed, is more effective at this than DPG. X-ray results showed that the peptide binding site extends deep into deoxy Hb in the cleft between the beta chains along the dyad axis of symmetry, the DPG binding site. The effect of this synthetic peptide seems somewhat different than the effect of the larger 40,000-dalton cytoplasmic fragment which, at least under the conditions used in several laboratories, stabilizes the high affinity form of liganded hemoglobin [61,62]. Any attempt to rationalize all of these results must consider three major issues: First, one study uses a small synthetic peptide of band 3, while the others use the entire cytoplasmic fragment of band 3. As we mentioned above for the glycolytic enzymes, there is some reason to be concerned about whether the smaller peptide constitutes the entire hemoglobin or enzyme binding site on band 3. Clearly, a direct comparison of the peptide binding constant to hemoglobin vs that of the 40,000-dalton fragment is needed to clarify this situation. Will the entire natural fragment also lower the oxygen affinity? Second, comparisons must be made under the same conditions of pH, ionic strength, and relative protein stoichiometries. Arnone et al [66] observed

their effects at physiologic salt and pH 7.2 and at near stoichiometric levels of hemoglobin and peptide. Although other functional (usually kinetic) studies were made at stoichiometric fragment to hemoglobin concentrations [49,61,62], the studies had to be performed at pH 6 in 5 mM phosphate and at low hemoglobin concentrations for technical reasons related to an inability in using high hemoglobin concentrations in the stopped flow apparatus. These differences will affect both band 3 structure [67] and the stability of the liganded hemoglobin tetramer [68]. Finally, owing to the highly changed nature of the 11-residue peptide, one might ask whether the lowered oxygen affinity is simply an effect any polyanion should have on hemoglobin's oxygen affinity [69]. In this regard, it is significant that Cassoly [49] has found that the CO binding rate to deoxyhemoglobin bound to the fragment is slower, implying a lower "oxygen" affinity of the tetramer within the complex (see below). This result was observed in both flash-photolysis experiments on CO hemoglobin and in forwardflow CO binding experiments to dexoyhemoglobin bound to the fragment.

The functional properties of the hemoglobin-band 3 fragment complex were studied further by Cassoly and Salhany [70] by measuring the O_2-CO replacement reaction. This is a measure of the initial rate of oxygen release from liganded hemoglobin dimers and tetramers. A similar series of experiments was conducted by Salhany and Shaklai [62] for oxyhemoglobin bound to band 3 on intact, isolated membranes. In both situations, strongly biphasic oxygen release kinetics were observed. The changes were associated with a spectral change in the beta chain of oxyhemoglobin upon binding band 3 in solution. Based on the flash-photolysis results [49], the fast component in oxygen release was assigned to O_2 release from the bound dimer while the *relatively* slower component was assigned to oxygen release from the bound tetramer.

When the kinetics and the optical spectral changes were studied as a function of band 3 fragment concentration, it was observed that the appearance of the spectral change had a maximum as fragment concentration increased. It was suggested that this occurs as a consequence of the formation of higher aggregates of band 3-Hb complexes in solution (ie, that hemoglobin dimers can "cross-link" separate band 3 dimers to each other to form higher polymers). It is known that the cytoplasmic fragment of band 3 exists as a homodimer in solution [71,72]. There is increasing evidence that at least at concentrations prevailing within the membrane, band 3 can form tetramers and possibly higher oligomers [73–75]. Oxyhemoglobin dimers, then, may facilitate this association of band 3 dimers into tetramers. However, direct proof for this association has yet to be demonstrated.

Very recently, Salhany and Cassoly [unpublished results] have extended their studies to investigate the kinetics of PMB binding to the –SH groups on the cytoplasmic fragment of band 3 in the absence and presence of hemoglobin whose beta-93 –SH group was blocked with NEM. Two classes of –SH groups were identified and both were shown to be significantly affected by hemoglobin binding. These results suggest that hemoglobin binding to band 3 induces widespread conformational changes in the cytoplasmic fragment.

The functional properties of G3PD, aldolase, and PFK, like hemoglobin, are all reversibly altered when bound to the cytoplasmic fragment of band 3. It has been shown that band 3 acts as an inhibitor of G3PD and is competitive with NAD^+ and phosphate [30]. The percent of G3PD bound to ghosts was exactly comparable to the

percent inhibition of enzymatic activity. Furthermore, Triton X-100-solubilized band 3 also caused full enzyme inhibition; however, the shape of the inhibition curve was sigmoidal. The 23,000-dalton, S-cyanylation-cleaved fragment of the cytoplasmic, NH_2-terminal portion of band 3, purified in water soluble form, also completely inhibited enzyme activity.

Similar studies with aldolase were also performed by Steck's group [36]. Analysis of that data suggested that simple competitive inhibition was not operative. Furthermore, inhibition was never complete. These effects occurred despite an apparent competition between band 3 and enzyme substrate. It was suggested that binding a single band 3 peptide reduces the affinity of the remaining protomers for band 3, independent of their catalytic activity, implying a high degree of negative cooperativity among aldolase subunits.

Initial studies with PFK showed that membrane binding, presumably to band 3 [37], caused a loss of sensitivity to the allosteric effectors of the enzyme (eg, ATP and DPG) without loss of catalytic activity per se [38]. More recently, Jenkins and Steck [76] have shown that both inside-out vesicles and the 23K-dalton NH_2-terminal portion of band 3 not only causes the immediate alteration of allosteric properties, but they also promote gradual loss of catalytic activity. Inactivation was shown to be reversible. The mechanism of inactivation was suggested to be caused by the band 3-induced dissociation of PFK (either stabilization of dimers or destabilization of tetramers) by analogy with other studies on the functional properties of PFK [77]. This effect of band 3 on the state of association of PFK seems similar to the effect on oxyhemoglobin [62,49].

Finally, catalase has been shown to bind to the membrane (presumably also to band 3) under conditions of acidic pH and low ionic strength [39]. However, unlike the glycolytic enzymes and hemoglobin, the enzymatic activity of catalase was not altered. It was suggested that fully functional, membrane-bound catalase may provide protection to the cell membrane against peroxidative damage. This, of course, assumes that binding occurs under physiologic conditions.

BINDING OF CYTOSOLIC PROTEINS TO THE MEMBRANE UNDER PHYSIOLOGIC CONDITIONS AND IN INTACT CELLS

The research with isolated membranes has focused on the definition of the binding sites and the binding characteristics of cytosolic proteins. An alternate goal of this work is to establish whether binding occurs within intact cells under physiologic conditions. This goal can very often be elusive, both with respect to apparent positive or negative results.

The red cell contains far more hemoglobin molecules than band 3 sites. Only about 1% of the total pool of hemoglobin tetramers would be needed to saturate all of the band 3 sites. There are several experiments in the literature where a very small amount of hemoglobin was added to membranes under physiologic conditions of pH and ionic strength and no binding observed and conclusions reached that hemoglobin does not bind [eg, 78]. These experiments do not seem very meaningful, since physiologic concentrations of hemoglobin were not used for technical reasons. If the binding constant of hemoglobin for band 3 were 1 to 2 mM under physiologic conditions, there would be significant binding owing to the fact that red cell concen-

tration of hemoglobin approaches 5 mM tetramers. The experimental question which needs to be answered is the value of the binding constant to band 3 under physiologic conditions. Unfortunately, this is a relatively difficult number to obtain by most optical spectroscopic methods owing to the strong absorptivity of hemoglobin over a wide range of the UV-visible spectrum. As mentioned above, Fung [52] has used EPR spectroscopy to measure binding at physiologic pH but *not* physiologic ionic strength. However, Arnone et al [66] showed an effect of a peptide of the cytoplasmic fragment of band 3 on the oxygen affinity of hemoglobin under physiological conditions. Using a different approach [79], Eisinger et al [80] attempted to measure the quenching of fluorescent probes on band 3 and in the lipid bilayer of intact cells as a function of cell pH under otherwise physiological conditions. They could show increased fluorescent quenching of membrane probes with decreased intracellular pH between pH 7 and 7.2. Intracellular pH values as low as 7 have been reported in intact cells using 31-P NMR [81,82]. Despite the clear demonstration of increased quenching, there are limitations in interpretation which depend on the model used to calculate the distance between the quencher (Hb) and the probes. These matters were discussed in the original paper [80] and have been discussed more recently by Eisinger and Flores [83]. Direct evidence for hemoglobin binding to band 3 under physiologic conditions has been obtained by J. Belsky, working in Cantley's laboratory at the Biological Laboratories at Harvard University. The purified, 43,000-dalton cytoplasmic fragment of band 3 was labeled with a fluorescent maleimide and showed quenching by hemoglobin in 159 mM KCL, pH 6.8, indicating binding [84]. Aldolase, G3PD, and the unlabeled fragment of band 3 reversed the quenching. Clearly, conclusions that hemoglobin does not bind at physiologic pH and salt must also consider physiologic hemoglobin concentrations. When this is accomplished and sensitive techniques are used, binding is observed.

One potentially important aspect of the hemoglobin binding problem has to do with the interaction of sickle cell hemoglobin with the membrane. The mutational change involves replacement of beta-6 glutamic acid by valine, which results in greater positive charge and surface hydrophobicity. Human sickle cell hemoglobin forms polymers upon deoxygeneration. This process is accelerated by the presence of "nuclei" of deoxyhemoglobin polymers [85]. Interaction of two membrane-bound deoxyhemoglobins, one on each monomer of the band 3 dimer, for example, could serve to form a nucleus and thereby accelerate polymerization. Although there is evidence for increased interaction of sickle cell hemoglobin with the membrane [53,54,78,86], Goldberg et al [87] were unable to demonstrate an acceleration in the rate of polymerization of deoxy HbS upon adding membranes, after correcting for expected excluded volume effects.

Another consequence of an increase in membrane-bound hemoglobin could be an effect on membrane shape and rigidity. Although this would not directly affect the rate of HbS polymerization, alterations in membrane rigidity or shape could affect the speed with which the cell traverses the capillary bed. Longer capillary dwell-times could lead to more hemoglobin deoxygenation and consequently a greater degree of sickling. There is evidence that cytosolic proteins can affect the shape of the human erythrocyte under certain conditions. Cordes and Salhany [51] showed that G3PD and hemoglobin, but *not* myoglobin, could inhibit the endocytotic process associated with ion depletion [88], suggesting that cytosolic protein binding can affect cell shape directly, at least under the conditions used. Wiedenmann and Elbaum [78]

have observed and reported some nonspecific effects of adding either hemoglobin or albumin under conditions where there was no binding. It is difficult to relate these experiments to other studies where specific binding of both G3PD and hemoglobin was first established. Although a direct effect of membrane binding on the rate of HbS polymerization can probably be ruled-out, a role for HbS binding in influencing membrane shape and flexibility might be worth further consideration.

Another aspect of the "hemoglobin"-membrane interaction worth mentioning concerns the interaction of denatured hemoglobin [89–91]. This type of interaction contrasts with those reported above for intact hemoglobin where, although the functional properties were altered upon binding, functionality was *always* uniformly restored upon dissociation from the band 3 complex [eg, 62]. Denatured hemoglobin can form after long interactions with phospholipid vesicles under extreme conditions [92,93]. An irreversible component in HbS binding to the membrane has been attributed to this kind of interaction [54]. Recently, Low and Waugh [94] have reported that denatured hemoglobin can bind tightly to the isolated cytoplasmic fragment of band 3. This may be relevant to the mechanism of Heinz body formation. However, it does not directly relate to the reversible binding of hemoglobin discussed above where *full* functionality can be restored by dissociation of hemoglobin from the band 3 complex.

Since raising the ionic strength or the addition of metabolites also elutes G3PD and the other glycolytic enzymes, one may also ask if these proteins are bound in the intact cell. Kliman and Steck [95] have addressed this question for G3PD binding using a rapid filtration method where saponin was used to eliminate the membrane barrier and dilution employed to initiate reequilibration of the bound enzyme. Using this approach, enzyme off-rates can be measured and, by extrapolation to zero time, the fraction of enzyme bound in the cell determined. Their results suggested that about two-thirds of the enzyme was bound when oxygenated cells were used.

Brindle et al [96] have recently used a sophisticated ^{1}H NMR method to measure the rates of glycolytic flux through the G3PD step in *intact* erythrocytes. The method involves using an ^{1}H NMR spin-echo experiment [97] to study the exchange of isotope between the C-2 position of lactate and the solvent. The exchange is catalyzed by a coupled system of four glycolytic enzymes: fructose bisphosphate aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase, and lactate dehydrogenase. The specific isotope-exchange equilibrium velocity of an individual enzyme can be measured in the intact erythrocyte if the active enzyme concentration is changed by specific irreversible inhibition. In order to test the effect of membrane binding of G3PD, the authors prepared membrane-bound G3PD at low ionic strength. Differences in velocities were noted which could be reversed by adding salt to elute the enzyme, so establishing the method by confirming the other results showing that the enzyme is inhibited in the bound state. When measurements were performed in intact cells at steady state conditions, there was no evidence that G3PD was limiting glycolytic flux. Indeed, somewhat higher equilibrium velocities were observed in situ, which is opposite to expectation if the enzyme were bound to the membrane to any significant degree.

Unfortunately, it is difficult to compare directly the rapid filtration method for determining binding with the NMR method just described. It is certainly true that the rapid filtration involves membrane disruption and that calculation of the amount of bound G3PD involves an extrapolation of data. The NMR method involves the use of

intact cells and so seems less disruptive. Perhaps further experiments attempting to directly compare these methods will resolve this important problem.

CONCLUDING REMARKS

In summary, we have seen that there is direct evidence that hemoglobin and certain glycolytic enzymes bind to band 3. Work to show the binding of hemoglobin under physiologic conditions of pH and ionic strength, at cellular concentrations of hemoglobin, is currently in progress and should be forthcoming in the literature. The presence of some binding is probably not too surprising owing to the binding constants reported under nonphysiologic conditions and to the relatively enormous concentration of hemoglobin within the red cell. Binding of the glycolytic enzymes in the intact cell seems somewhat more controversial at present. As the issue of binding under physiologic conditions becomes resolved, the questions for the future will certainly begin to focus on the consequence of cytosolic protein binding to cell function (ie, O_2 and CO_2 transport), cell metabolism, and cell flexibility.

ACKNOWLEDGMENTS

Work from the author's laboratory was supported by a grant from the Medical Research Service of the Veterans Administration. This review was written during the tenure of an Established Investigationship from the American Heart Association with funds contributed in part by the Nebraska Heart Association. I thank Karen Cordes, Peter Rauenbuehler, and Drs Jean D. Jenkins and Ted Steck for reading and commenting on this review. I thank Drs I. McCarra and L. Cantley for providing a preprint.

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