EDITORIAL REVIEW

Human Erythrocyte Membrane Enzymes: Current Status and Clinical Correlates

By Stanley L. Schrier

THE PAST TWENTY YEARS have seen a remarkable growth in information concerning the metabolism of the human erythrocyte. It became apparent that, depending on the method of preparation, some of the enzymes of the human erythrocyte can be recovered partially or totally in an operationally defined membrane fraction. Therefore, there now exists a substantial body of information relating to the membrane-associated enzymes of the human erythrocyte. Attempts have been made to classify these enzymes, to indicate their orientation, and to propose specific physiologic functions for them. As a subject of interest for the hematologist, there is good evidence that acquired and genetically determined disorders of the human erythrocyte may involve disordered erythrocyte membrane function. In more general terms, the human erythrocyte membrane has been a favorite subject for students of plasma membrane biology because of its ready availability as a plasma membrane preparation free of other intracellular membranes.

PROPERTIES OF MEMBRANE-ASSOCIATED ENZYMES

The general biologic importance of membrane-bound enzymes has recently been discussed in a thoughtful review by Coleman, who argues that membrane enzymes impose a geography on the workings of the cell. The membrane produces a compartmentation of enzymes and substrates and provides a framework on which enzymes can exist in multienzyme sequences. Therefore, there is a potential microenvironment in the membrane consisting of other enzymes, substrates, and charged molecules with binding sites for inhibitors or stimulators of function. The existence of an enzyme on a membrane provides for a vectorial orientation of catalysis, such that substrates and cofactors can be moved in specific directions across the membrane resulting in their movement in or out of the cell. Finally, since almost all plasma membranes have a substantial lipid component, the unique properties of the lipid, interacting with the membrane enzyme proteins, may alter the catalytic properties of those enzymes.

There have been recent reviews covering erythrocyte membrane function in disease, the protein structure of the erythrocyte membrane, and the localization of red cell membrane constituents with emphasis on the lipid content of membranes. Hanahan has recently reviewed erythrocyte membrane enzyme
activity, emphasizing the variability of activity as a consequence of the species and the age of the subject, and the method of preparation of the membrane to be studied.

The aim of this review is to present a catalog of the membrane-associated enzymes of the mature human erythrocyte, to propose a functional classification, and to summarize relevant clinical data. Studies on other species and studies on the enzyme activities of reticulocyte membranes have not been included. The enzymes of lipid metabolism and lipid renewal have also been omitted since an inspection of the subject indicates that study of plasma membrane lipids and the enzymes involved in lipid metabolism constitutes a separate field. There are two recent comprehensive reviews on erythrocyte lipids and their renewal.5,7

CRITERIA FOR MEMBRANE ASSOCIATION

There is real difficulty in defining exactly what constitutes an erythrocyte membrane enzyme. Erythrocyte membrane proteins, including enzymes, have been classified as being integral or peripheral, intrinsic or extrinsic, or membrane associated.4 These distinctions are usually based on the kinds of treatments which are necessary to dissociate the component under study from the membrane.4 If the membrane component can be removed by relatively gentle treatments, it is thought to be peripheral, or extrinsic, or membrane associated. Conversely, if the treatment required is drastic and leads to destruction of membrane structure, then the protein component under study has been termed intrinsic or integral. Juliano has dealt with these problems in a recent review.4

The most common method of preparation recorded in the literature involves the use of hypotonic lysis, but as Hanahan6 points out, the osmotic stress may lead to an artifactual association of enzymes with the inner surface of the membrane. Therefore, while the white “hemoglobin-free” ghost has achieved a degree of standard acceptance, it is subject to variations in lipid content. There is irregularity of substrate penetration through this membrane, and enzyme binding may be artifically induced.

The listing of membrane enzymes has been divided into two categories. Table 1 contains a catalog of enzymes whose activities are reported to be solely in the membrane and not in the membrane-free hemolysate or cytosol. The presumption is that these are indisputably membrane-associated enzymes. The enzymes listed in Table 2 have been reported to be membrane associated, although a varying fraction of the enzyme activity is also found in the cytosol. There is considerable disagreement about the likelihood of real in vivo membrane association of the enzymes listed in Table 2. It has been argued that the method of hypotonic lysis used produces an artifactual association of cytosol enzymes with the membrane.6

In the latter category, glyceraldehyde-3-phosphate-dehydrogenase (GAPD) is the best studied example, having first been reported in 1963 as a membrane-associated enzyme.6 Relying almost exclusively on hypotonic hemolysis, membranes contain from 7% to 60% of the total erythrocyte content of GAPD, which in its monomeric form appears as band 6 on acrylamide gel electrophoresis of sodium dodecyl sulfate (SDS)-solubilized membranes. Membrane GAPD is identical to cytosol GAPD and membrane association does not alter its catalytic properties.11 GAPD has been localized to the inner face of the
Table 1. Enzymes Whose Activity Is Confined to the Membrane

<table>
<thead>
<tr>
<th>Enzymes of nucleotide metabolism (ATPases considered separately)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2',3' cAMP cyclic nucleotide 3'-phosphohydrolase</td>
<td>60</td>
</tr>
<tr>
<td>Adenyl cyclase</td>
<td>61</td>
</tr>
<tr>
<td>UTPase *</td>
<td>41</td>
</tr>
<tr>
<td>5' AMP phosphatase *</td>
<td>42</td>
</tr>
</tbody>
</table>

II. Enzymes of carbohydrate metabolism

- Neuraminidase | 62 |
- CMP-N-acetylneuraminic acid: glycoprotein sialyltransferase | 17 |
- N-acetyl-β-glucosaminidase * | 43 |
- α-D-glucosidase * | 43 |
- β-D-glucosidase * | 43 |
- α-D-galactosidase * | 43 |
- β-D-galactosidase * | 43 |
- α-L-fucosidase * | 43 |
- β-L-fucosidase * | 43 |
- β-D-xylanidase * | 43 |
- α-D-mannosidase * | 43 |
- N-acetyl-β-D-galactosaminidase * | 43 |
- β-D-Glucuronidase | 43 |
- N-acetyl-galactosaminyl transferase | 18 |

III. Phosphatases

- Vitamin B₆ phosphate phosphatase | 63 |
- p-nitrophenyl phosphatase (not K⁺ dependent, for K⁺ dependent nitrophenyl phosphatase see ATPases) | 64 |
- Mg²⁺ dependent phosphoprotein phosphatase | 36 |

IV. Proteinases

- 3 proteinases pH optima 7.4, 7.4, 3.2 | 37 |
- 2 proteinases pH optima 3.4, 7.4 | 38, 39 |

V. ATPases

- ATPases not linked to fibrous membrane proteins
  - Mg²⁺ ATPase | 65 |
  - Na⁺, K⁺-ATPase, ouabain inhibited | 65 |
  - (K⁺ dependent p-nitrophenyl phosphatase, ouabain inhibited) | 64 |
  - Ca²⁺, Mg²⁺ ATPase | 66 |
- Possibly two affinities for Ca²⁺, the low-affinity enzyme may be the Ca²⁺ transporting enzyme | 24, 26 |
- Monovalent cation stimulated, not related to active Ca²⁺ extrusion | 26, 27 |
- ATPases associated with fibrillar membrane proteins (spectrin)
  - Ca²⁺ ATPase inhibited by Mg²⁺ (approximately 5% as active as the Ca²⁺, Mg²⁺ ATPase) | 29, 31 |
  - Mg²⁺ ATPase, low activity, stimulated by actin, thus resembling actomyosin | 31 |

VI. Protein kinases

- cAMP independent, stimulated by mono and divalent cations to phosphorylate band 2, spectrin | 34 |
- cAMP stimulated, Ca²⁺ inhibited, monovalent cation inhibited | 32-34 |

VII. Miscellaneous

- NAD⁺ glycohydrolase (DPNase) * | 44 |
- NADP⁺ glycohydrolase (TPNase) * | 44 |
- NADH: acceptor oxidoreductase | 49 |
- Acetylcholinesterase * | 19 |

* Externally oriented.
† See Mg²⁺ dependent phosphoprotein phosphatase.
Table 2. Enzymes Whose Activity Is Found in Both the Cytosol and the Membrane

<table>
<thead>
<tr>
<th>Enzymes of nucleotide metabolism</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP deaminase</td>
<td>58, 67</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>46, 68</td>
</tr>
<tr>
<td>UDPase*</td>
<td>41</td>
</tr>
<tr>
<td>Hypoxanthine phosphoribosyl transferase*</td>
<td>40</td>
</tr>
<tr>
<td>Guanine phosphoribosyl transferase*</td>
<td>40</td>
</tr>
<tr>
<td>Adenine phosphoribosyl transferase*</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phosphatases</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>43</td>
</tr>
<tr>
<td>2,3 DPG phosphatase (may be found only in the membrane)</td>
<td>69</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Enzymes of glucose metabolism</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>8, 20, 46, 68, 70</td>
</tr>
<tr>
<td>Aldolase</td>
<td>8, 20, 68, 70</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>8, 46, 70</td>
</tr>
<tr>
<td>Lactic dehydrogenase</td>
<td>71</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>20</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>20</td>
</tr>
<tr>
<td>Transketolase</td>
<td>8</td>
</tr>
<tr>
<td>Phosphoriboseisomerase</td>
<td>8</td>
</tr>
<tr>
<td>Purine nucleoside phosphorylase</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes of glutathione metabolism (weakly bound)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione (GSH) peroxidase</td>
<td>70</td>
</tr>
<tr>
<td>Glutathione (GSSG) reductase</td>
<td>20, 70, 72</td>
</tr>
</tbody>
</table>

*Externally oriented.

As reported by several laboratories, the active sites of membrane-associated enzymes may be oriented to face either outward into the external medium or inward, facing either the membrane core or the cytosol. The active sites of membrane-associated enzymes may be oriented to face either outward into the external medium or inward, facing either the membrane core or the cytosol. In determining whether or not an erythrocyte membrane is externally oriented, a substrate is added to a suspension of carefully washed erythrocytes. Changes in the substrates of the incubating media in the absence of hemolysis are taken to indicate the action of externally oriented enzymes.
erythrocyte membrane enzymes. When there are serum enzymes with similar activities, it can be difficult to determine whether or not the erythrocyte membrane has taken up the enzyme from the suspending plasma. Conversely, the plasma enzyme may be a dissociated erythrocyte membrane enzyme. The classical example of an externally oriented enzyme is acetylcholinesterase. Examples of enzymes bound to the inner membrane surface and oriented toward the cytosol are GAPD and NADH oxidoreductase. An enzyme may be bound to the inner membrane face, yet appear to have a portion of its active site associated with the lipid components of the membrane. A preferred example is phosphoglycerate kinase. Externally oriented enzymes are indicated by an asterisk in Tables 1 and 2. The orientation of the other membrane enzymes is either not known or is thought to be at the inner membrane surface facing the cytosol.

CONSIDERATION OF SPECIFIC MEMBRANE ENZYMES

Adenosinetriphosphatases (ATPases)

The ATPase enzymes are confined to the membrane and have been the subject of considerable investigation. There are at least three catalytic activities which can be physically separated. The Mg$^{2+}$-ATPase requires Mg$^{2+}$ and ATP for activation and has no known physiologic function. The ouabain-inhibitable Na$^+$-, K$^+$-ATPase accounts for the active transport of sodium and potassium in the human erythrocyte. Perhaps the K$^+$-dependent, ouabain-inhibitable, P-nitrophenylphosphatase reflects the second K$^+$-dependent dephosphorylation step of Na$^+$, K$^+$-ATPase action. The Ca$^{2+}$, Mg$^{2+}$-ATPase is linked to the pump which actively extrudes calcium against a 50-fold concentration gradient. There may be more than one Ca$^{2+}$, Mg$^{2+}$-ATPase. It has been affirmed and refuted that the Ca$^{2+}$, Mg$^{2+}$-ATPase has two distinct affinity patterns for Ca$^{2+}$. In one report, the low-affinity Ca$^{2+}$, Mg$^{2+}$-ATPase appears to be the Ca$^{2+}$ pump-related enzyme. A monovalent cation-stimulated Ca$^{2+}$, Mg$^{2+}$-ATPase has also been described and is thought not to be related to the active calcium extrusion process. Casual readers in the field of red cell membrane enzymology may not be aware that the Ca$^{2+}$, Mg$^{2+}$-ATPase, as calculated either per milligram of membrane protein or per number of ghosts studied, is between three and eight times as active as the Na$^+$, K$^+$-ATPase under certain defined experimental conditions.

A low ionic strength extraction of red cell membrane yields fibrous membrane proteins, called spectrins, plus band 5, which is actin. Because of the possible similarity of these proteins with actomyosin, they have been studied with regard to their enzymatic capacities, and it appears that the fibrous proteins have a Ca$^{2+}$-ATPase which is inhibited by Mg$^{2+}$ and is only 5, as active as the major Ca$^{2+}$, Mg$^{2+}$ membrane ATPase. The fibrous proteins also have a low Mg$^{2+}$-ATPase activity which is stimulated by adding actin, thereby resembling actomyosin. These fibrillar ATPase activities are, however, very low and it may be that trace contamination of the low ionic strength extract produces the low activities seen. These studies have been initiated because an important theme in current studies on erythrocyte membrane biology is the search for mechanisms which can result in contraction or relaxation of the spectrin fila-
membranous network that may provide a framework underlying the cytoplasmic surface of the erythrocyte membrane.

**Protein Kinases**

There are at least two different classes of protein kinases which are exclusively associated with the erythrocyte membrane fraction. Since the protein kinases can phosphorylate membrane proteins in the presence of ATP, it is necessary to distinguish these enzyme systems from the classical ATPases. The distinction has been made on a solid chemical basis, since the classical ATPases phosphorylate membrane proteins through acyl bonds readily disrupted by hydroxylamine, whereas the protein kinases form phosphoserine and phosphothreonine bonds. Physiologic functions for the protein kinases have not yet been described. It is assumed from parallel studies in other tissues that under the impetus of a message, the protein kinase will phosphorylate proteins, which will then alter either their catalytic or structural function. The fact that one class of protein kinases is stimulated by cyclic 3',5'-adenosine monophosphate (cAMP) is consistent with this hypothesis. There is a Mg\(^{2+}\)-dependent phosphoprotein phosphatase which may function to restore the membrane protein to the dephosphorylated state.

**Proteases**

The membrane-associated proteolytic enzymes have no known physiologic function. Nevertheless, they are important for experimenters working with erythrocyte membrane systems because the proteases may account for the degradation of stored blood or membrane preparations.

**MISCELLANEOUS MEMBRANE ENZYMES**

There are several interesting points that deserve comment. The presence of hypoxanthine-, guanine-, and adenine-phosphoribosyl transferase enzymes in the membrane suggests that the membrane has or had the potential for utilizing this salvage pathway of nucleotide synthesis. While the ATPases are not externally oriented, UTPase (uridinetriphosphatase) is a marker of the external membrane face, while UMP phosphatase is found only in the red cell cytosol. Externally Oriented Membrane Enzymes

When the externally oriented enzymes are grouped, the glycosidases, acid phosphatase, and other hydrolytic enzymes are prominent. Some of these enzymes are those that appear in lysosomes, and this orientation suggest that the endocytic vacuole of the erythrocyte might have been the primitive phagolysosome. Alternatively, the concentration of lytic enzymes in the membrane may indicate that upon senescence and removal of the erythrocyte from the circulation there is an intrinsic self-destruction mechanism which, upon activation, aids the macrophages’ lytic systems.

**POSSIBLE BIOLOGIC ROLES OF MEMBRANE ENZYMES**

There has been speculation about the possible biologic roles of the other membrane-associated enzymes. The glycolytic enzymes, particularly the se-
quence of GAPD and phosphoglycerate kinase, could function to provide ATP\(^45\) at the cytosol surface of the plasma membrane but there is no proof that this occurs. Membrane adenylate kinase\(^46\) could function to rescue adenosine diphosphate (ADP) for reconversion to ATP. An idealized hypothesis would link biologic mediators like Ca\(^{2+}\) and cAMP with enzymes that by their action could modulate the deformability state of the membrane.\(^47\) The protein kinases are good candidates since they are stimulated by cAMP and are inhibited or stimulated by Ca\(^{2+}\). Conceivably, by their ability to phosphorylate specific membrane polypeptides, they could produce alterations of the physical state of the membrane. There is no proof that protein kinases play such a role. The erythrocyte must protect itself against attack by activated oxygen which, if uncontrolled, could lead to oxidative denaturation of the membrane.\(^48\) The defenses include the reduced pyridine nucleotide coenzymes, reduced glutathione and glutathione peroxidase and hemoglobin.\(^48\) Perhaps the cytosol-facing membrane enzyme NADH oxidoreductase serves as a last line of defense.\(^49\)

**MEMBRANE ENZYMES IN DISEASE STATES**

Abnormalities of erythrocyte membrane enzymes have been reported in some disease states. In some cases the recorded abnormalities appear to relate to the pathophysiology of the erythrocyte disorder as secondary alterations or epiphenomena. In other cases study of erythrocyte membrane enzymes serves as an effective surrogate for study of plasma membrane enzymes in tissues which are more difficult to obtain.

**Acetylcholinesterase in Disease States**

The abnormalities of the externally oriented enzyme acetylcholinesterase have been reviewed by Tanaka.\(^50\) Effective interpretation of acetylcholinesterase activity requires that the age of the erythrocyte population be carefully defined. In the following conditions, erythrocyte membrane acetylcholinesterase activity is decreased: paroxysmal nocturnal hemoglobinuria (PNH), autoimmune hemolytic anemia of the warm antibody (AHA) type, and ABO hemolytic disease of the newborn. Isolated examples of reduced acetylcholinesterase activity also occurred in myelofibrosis with myeloid metaplasia, refractory anemia, di Guglielmo syndrome, chronic uremia, microangiopathic hemolytic anemia, and spur cell anemia with hepatic cirrhosis. There is no single explanation for the findings. Selection of an abnormal clone of the erythroid cell line may be the explanation in PNH and the refractory anemias. Loss of membrane material or specifically of membrane containing the acetylcholinesterase may be the explanation for the decreased activity in AHA.

It would be of interest to determine if several of the other externally oriented membrane enzymes (Tables 1 and 2) are decreased in the same diseases where acetylcholinesterase is decreased. If it is found that the externally oriented membrane enzymes are affected while a marker of the inner membrane surface like NADH oxidoreductase is not, this finding would suggest that the alteration involves some specific perturbation of the externally oriented membrane proteins. One study that has dealt with this issue has shown that while acetylcholinesterase is significantly depressed in AHA, membrane ATPase is only minimally affected.\(^51\)
Membrane Enzymes in Hereditary Spherocytosis

Membrane enzymes have been studied extensively in hereditary spherocytosis (HS). The reported alterations are generally modest in degree and reproducible findings include elevated Na⁺, K⁺-ATPase and elevated Mg²⁺-ATPase. The reported decrease in the Ca²⁺ pump-associated Ca²⁺, Mg²⁺-ATPase has not been confirmed. The decreased membrane protein phosphorylation in HS erythrocytes after 60 min of incubation is not due to a defect in protein kinase activity. There are, however, fibrillar ATPases (Table 1), and in HS the fibrillar low Ca²⁺-ATPase is absent.

Other Diseases Associated With Abnormalities of Membrane Phosphorylation

While no clearcut physiologic role for membrane phosphorylation has been defined, abnormalities have been described in several disorders. Protein kinase activity appears to be decreased in sickle cell disease. Using the human erythrocyte as an easily obtained surrogate for muscle tissues, Appel and Roses have found decreased phosphorylation of band 3 in myotonic muscular dystrophy (dystrophy and myotonia), increased phosphorylation of bands 2 and 3 in Duchenne muscular dystrophy (dystrophy without myotonia), and normal phosphorylation in congenital myotonia (myotonia without dystrophy).

FUTURE EXPERIMENTAL WORK

Several lines of future experimental work can be suggested. If enzymes are to be designated to be membrane associated, then studies should be done with the membranes prepared by other means of hemolysis in addition to hypotonic lysis. Some generally accepted way of expressing activity would be advantageous. Perhaps an expression in terms of lipid phosphorus would be generally acceptable. Attempts to demonstrate microenvironment effects have been unsuccessful with GAPD, but membrane-associated AMP deaminase has a different substrate velocity curve than the soluble enzyme. With the use of suitable membrane probes it might be possible to demonstrate that membrane microenvironments do alter catalytic effects.

There is as yet no proof that there are linked enzyme sequences in erythrocyte membranes. However one report has suggested that the erythrocyte membrane may have a segmental composition such that there might be a “geography” within the membrane. Linked enzymes with modulating lipids and transporting proteins would constitute defined physical units within the membrane, and they might be sought for by using procedures that gently and selectively disrupt the membrane. The addition of bifunctional cross-linking agents or transglutaminases might be useful in this regard, keeping adjacent components together during the requisite disruptive and separative procedures. The field of erythrocyte membrane enzymology could prove particularly interesting at the moment because it could benefit from vastly improved techniques for characterizing erythrocyte membrane proteins.

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