*Biochimica et Biophysica Acta*, 689 (1982) 203–209
Elsevier Biomedical Press



BBA 71263

# INTERACTION OF HEMOGLOBIN WITH THE RED BLOOD CELL MEMBRANE

## A SATURATION TRANSFER ELECTRON PARAMAGNETIC RESONANCE STUDY *

ROBERT CASSOLY

*Institut de Biologie Physico-chimique, 13, rue Pierre et Marie Curie, 75005 Paris (France)*

(Received February 11th, 1982)

*Key words: Saturation transfer ESR; Hemoglobin; ESR; Erythrocyte membrane*

**Human hemoglobin has been labeled on cysteine 93($\beta$) with the maleimide spin label, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl and reassociated with erythrocyte membrane previously stripped of hemoglobin and glyceraldehyde-3-phosphate dehydrogenase. The affinity of hemoglobin for the membrane is not affected by the presence of the label. Saturation transfer electron paramagnetic resonance measurements show that the diffusion rotational movements of hemoglobin are considerably slowed down when it is bound to the erythrocyte membrane. The correlation time of rotation, $\tau_c$, is found to be $8 \cdot 10^{-6}$ s as compared with $2 \cdot 10^{-8}$ s when the hemoglobin molecule is in solution. The same values are obtained whether the protein is associated with its high- or low-affinity binding sites. They depend on the viscosity of the solution. The high-affinity sites are presumably located on the segment of the band 3 protein which extends into the cytoplasm and which links through ankyrin, the spectrin-actin cytoskeleton to the membrane. When band 3 is cross-linked into a dimer after reaction with the copper-*ortho*-phenanthroline chelate, the correlation time of rotation of spin-labelled hemoglobin is unchanged. It is also independent of the presence of the spectrin-actin network and ankyrin. These results show that the movements of hemoglobin bound by ionic linkage to different part (protein or phospholipid) of the cytoplasmic surface of the membrane are similarly highly restricted by some potential or energetic barrier. They give also evidence for independent movements and flexibility in the assembly of the macromolecules which link the spectrin-actin cytoskeleton to the erythrocyte membrane.**

## Introduction

The interactions of hemoglobin with the red blood cell membrane depend strongly on the pH and ionic strength. They are essentially ionic. Quantitative studies performed in vitro with different techniques have shown the existence of two classes of binding site [1,2]. The high-affinity type ($10^6$ sites per cell) is presumably located on the cytoplasmic part of the transmembrane band 3 protein * [4], in a region which is also concerned with the binding of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase [1,2,5]. The low-affinity sites which are more abundant ($4 \cdot 10^6$ sites per cell) are believed to represent glycophorin and some of the polar head-groups of

* This work has been dedicated to Professor A. Rossi-Fanelli at the International Symposium on 'Relationships between Structure and Function in Biological Systems' held in his honour (Rome, September 28–30, 1981).

Abbreviations: ST-EPR, saturation transfer electron paramagnetic resonance; MSL-Hb, maleimide spin-labelled hemoglobin.

* Nomenclature of human erythrocyte proteins is according to Steck [3].

0005-2736/82/0000–0000/$02.75 

the phospholipids present on the cytoplasmic surface of the membrane [6].

The association of cytoplasmic proteins with the red blood cell membrane has recently been reviewed and their physiological importance discussed [7]. In the present work we provide a new experimental approach for the study of the interactions between hemoglobin and the erythrocyte membrane with the purpose of gaining further information on the different binding sites. We have performed saturation transfer electron paramagnetic resonance (ST-EPR) experiments on membrane-bound maleimide spin-labelled hemoglobin (MSL-Hb) and have determined from the shape of the spectra the rotational correlation time of the labelled protein. Red blood cell membranes of which the band 3 protein has been cross-linked as a dimer have also been studied. In order to evaluate if the ankyrin-spectrin-actin cytoskeleton could restrict the movements of hemoglobin, experiments have also been made with membranes previously depleted of these proteins.

## Materials and Methods

*Preparation of red blood cell membrane and hemoglobin*

Freshly drawn human blood was washed six times in isotonic NaCl solution. Unsealed ghosts were prepared from the packed cells by the method of Dodge et al. [8] with the slight modification that hemolysis was conducted in the presence of 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. The hemolysate was concentrated under vacuum and hemoglobin used without further purification after dialysis against 5 mM phosphate buffer, pH 6. The ghosts were depleted of glyceraldehyde-3-phosphate dehydrogenase according to Kant and Steck [9]. They were subsequently stripped of the spectrin-actin cytoskeleton by extraction with water 30 min at 35°C and of ankyrin by the method of Tyler et al. [10]. The transmembrane protein band 3 was cross-linked into a dimer by reaction with copper-*ortho*-phenanthroline according to Steck [11]. The amount of membrane proteins was assayed by the method of Lowry et al. [12]. The quality of the preparations was controlled each time by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

*Spin-labelling of hemoglobin and its association with red blood cell membrane*

Hemoglobin concentration (per heme) was measured spectrophotometrically as the oxygenated derivative using the values $A^{mM}_{542nm} = 14.61$ and $A^{mM}_{578nm} = 15.34$. Spin labelling was performed in 5 mM phosphate buffer, pH 6, at 2°C, by incubating the protein 12 h with a 5-fold molar excess of 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl dissolved in minute amounts of dimethyl sulfoxide. Unreacted maleimides were eliminated by an exhaustive dialysis against 5 mM phosphate buffer, pH 6. In order to compare the binding characteristics of MSL-Hb with those of native hemoglobin, the light-scattering method of Salhany et al. [2] was used.

Glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts at a protein concentration of 1 mg/ml were incubated with the desired amounts of MSL-Hb for 30 min in 5 mM phosphate buffer, pH 6, at 20°C. The membranes were subsequently pelleted 5 min at $40000 \times g$ and washed twice with the same buffer. The number of ghosts was estimated according to Lepke et al. [13] using the value $5.13 \cdot 10^{-10}$ mg protein/ghost. In order to study the high-affinity binding sites, $1 \cdot 10^6$ molecules of MSL-Hb (tetramer) were added per ghost. It is unlikely that the high-affinity sites alone are titrated. In the present experimental conditions one can calculate that about one-fifth of the hemoglobin molecules are also bound to the low-affinity site, assuming a 40-fold difference in their affinities [14]. The same remark pertains also when one wants to study the weakly bound population of hemoglobin molecules, i.e., one-fifth of the hemoglobin settles on the high-affinity sites. In this last case, however, one can use glyceraldehyde-3-phosphate dehydrogenase-unstripped ghosts to circumvent this difficulty. As will be shown later in the Results section, the impossibility of titrating the high-affinity site exclusively is without consequence in the interpretation of the experimental data. When the ankyrin-spectrin-actin-depleted membranes were studied, a 2-fold excess of MSL-Hb was added per mg membrane protein to take into account the fact that band 3 protein is in this case the main protein present.

*Recording of EPR spectra*

Conventional and ST-EPR spectra were recorded with a Varian E-109 spectrometer interfaced with a microcomputer Digital Minc II used for data acquisition and signal averaging. The samples were studied at 20°C in 50 $\mu$l quartz cells set in a quartz Dewar for temperature control. First-harmonic in-phase spectra were recorded at 1 G modulation (100 kHz) and second-harmonic out-of-phase spectra at 5 G modulation (50 kHz). The ST-EPR spectra were analyzed according to Thomas et al. [15] by comparison with reference spectra obtained with MSL-Hb in solution. The microwave strength received by the sample was calibrated with peroxylamine disulfonate [15].

## Results

All experiments reported in this work have been performed in 5 mM phosphate buffer, pH 6, which favors strong association of hemoglobin with the red blood cell membrane [1,2]. Spin-labelling of hemoglobin on the residue cysteine 93($\beta$) does not affect significantly its capacity for binding as demonstrated by light-scattering titration experiments performed at different protein concentrations (curves not shown). The conventional EPR spectrum of MSL-Hb in solution shows that most of the label is immobilized (Fig. 1a). The corresponding ST-EPR spectrum shown in Fig. 1b is similar to the reference spectra given by Thomas et al. [15] and corresponds to a correlation time, $\tau_c$, of about $2 \cdot 10^{-8}$ s. When MSL-Hb is bound to the red blood cell membrane the shape in the ST-EPR spectrum is modified and shows a large decrease in the rotational mobilities of MSL-Hb ($\tau_c = 8 \cdot 10^{-6}$ s). The interaction of MSL-Hb with the high- and low-affinity binding sites have been studied by adding different amounts of hemoglobin to the ghosts. As has been shown in Materials and Methods, it is not possible to avoid in the first case the presence of about 20% of hemoglobin linked on the other type of the sites under study. This contamination has been proved to be of no importance to the results, since one obtains exactly the same ST-EPR spectra for the two populations of sites (Fig. 1). The only difference lies in the precision of the measurements. When one studies MSL-Hb strongly bound to the membrane, the amounts

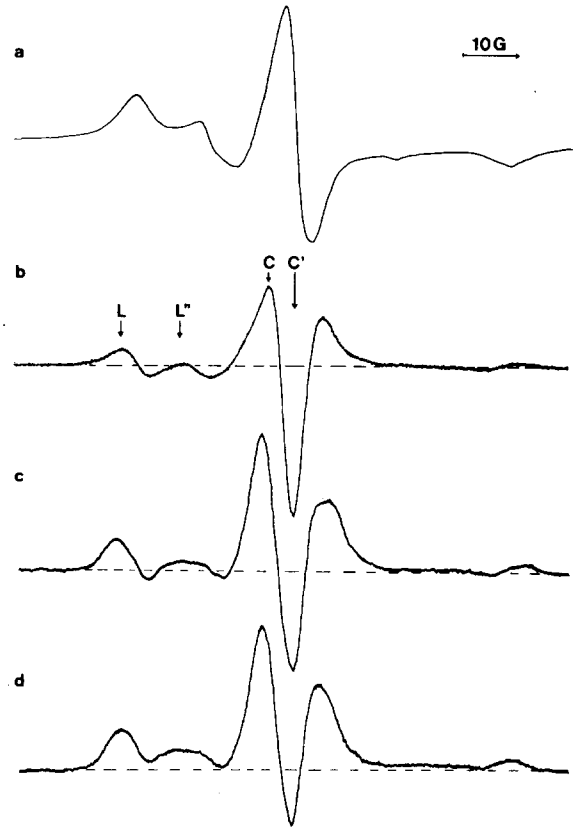


Fig. 1. Conventional (a) and ST-EPR spectra (b, c, d) of MSL-Hb in solution (a, b) and bound to the erythrocyte membrane (c, d). In experiments c and d, saturating amounts of MSL-Hb ($5 \cdot 10^6$ molecules) were added per ghost. Identical but more noisy spectra have been obtained when hemoglobin was associated mainly with the high affinity binding sites ($1 \cdot 10^6$ molecules per cell). (a, b, c) 5 mM phosphate buffer, pH 6; (d) 5 mM phosphate buffer (pH 6)/50% glycerol. Other experimental conditions are given in the text.

of spin label in the sample are small. In these conditions there is a large imprecision in the ratio of peak intensities measured at high magnetic field as compared with the values obtained for the ratios $L''/L$ and $C'/C$.

Table I gives the rotation correlation times, $\tau_c$, of MSL-Hb measured under different conditions. They are not significantly different when the spin-labelled protein is bound to glyceraldehyde-3-phosphate dehydrogenase-depleted ghosts or spectrin-actin-ankyrin-stripped membranes, or when band 3 protein has been cross-linked into a dimer with Cu-*o*-phenanthroline. However they do depend on the viscosity of the solution.

TABLE I

ROTATIONAL CORRELATION TIME $\tau_c$ OF MSL-Hb, MSL-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND MSL-ANKYRIN BOUND TO THE ERYTHROCYTE MEMBRANE

For MSL-Hb the values are determined from the ratios of peak intensities, $L''/L$, measured on the ST-EPR spectra of Fig. 1.

| | $L''/L$ | $\tau_c$ (s) |
|---|---|---|
| MSL-Hb in solution | 0.08 | $2 \cdot 10^{-8}$ |
| $1 \cdot 10^6$ MSL-Hb molecules per: | | |
| ghost | 0.37 | $8 \cdot 10^{-6}$ |
| ghost stripped of spectrin-actin-ankyrin | 0.37 | $8 \cdot 10^{-6}$ |
| ghost with cross-linked band 3 protein | 0.38 | $9 \cdot 10^{-6}$ |
| $5 \cdot 10^6$ MSL-Hb molecules per: | | |
| ghost | 0.38 | $9 \cdot 10^{-6}$ |
| ghost in presence of 50% glycerol | 0.50 | $2 \cdot 10^{-5}$ |
| ghost in 0.05 M phosphate buffer, pH 7.5 | 0.10 | $2 \cdot 10^{-8}$ |
| Membrane-bound MSL -glyceraldehyde-3-phosphate dehydrogenase [a] | | $2 \cdot 10^{-5}$ |
| Membrane-bound MSL-ankyrin [b] | | $5 \cdot 10^{-5}$ |

[a] Data from Beth et al. [18];
[b] Cassoly, R. and Lemaigre-Dubreuil, Y., unpublished data.

The values of $\tau_c$ are independent of the dilution of the membranes in the buffer. When an excess of a concentrated solution of MSL-Hb is added to MSL-Hb-saturated ghosts the ST-EPR spectrum shows a decrease in the value of $L''/L$, indicating qualitatively that the rotational diffusion of unbound MSL-Hb is not influenced by the presence of the highly packed membranes. Furthermore, when the pH and ionic strength of a suspension of membranes saturated with MSL-Hb are increased by the addition of a small amount of a concentrated buffer which promotes the dissociation of hemoglobin, the ST-EPR spectrum becomes similar to that of MSL-Hb in solution (Table I).

## Discussion

ST-EPR spectroscopy [15] allows one to discriminate between rotational correlation times of macromolecules in the range $10^{-7}$–$10^{-3}$ s. It is thus a powerful tool for exploring the movements of proteins included in supramolecular structures. Among a large variety of biological systems it has been applied to the study of several erythrocyte membrane proteins *: spectrin [16,17], glyceraldehyde-3-phosphate dehydrogenase [18], ankyrin and band 4.1 (Cassoly, R. and Lemaigre-Dubreuil, Y., unpublished data). The rotational diffusion movements of spectrin measured by this method are independent either of the state of polymerization of the molecule or of its binding to ankyrin on the red blood cell membrane [17]. These results have emphasized the flexible character of spectrin. On the other hand, the movements of the globular hemoglobin molecule in solution reflect more the rotation of the molecule as a whole [15]. In the present work, one has considered the rotational dynamics of MSL-Hb when it is associated with the high- and low-affinity binding sites of the erythrocyte membrane. It is most important to know in this case if the movements of MSL-Hb are not overestimated by the contribution of a large dissociation rate constant of the membrane-bound hemoglobin implying a fast turnover of the molecules. This possibility can be excluded in the present cases: as shown by Shaklai and Sharma [14] the dissociation rate constants measured for oxyhemoglobin are very low (0.07 and 0.4 $s^{-1}$) for the high- and low-affinity binding sites.

It is known that maleimide spin-labelled hemoglobins show generally a decrease in oxygen-binding cooperativity [19], as is often the case when the cysteine 93($\beta$) residue has been modified. The perturbations brought by the presence of the spin label on the structure of hemoglobin have been studied in detail by X-ray difference Fourier techniques [19]. They are located in the environment of the heme groups and in the region of the $\alpha_1\beta_2$ interface, but do not concern the surface of the protein, which is most probably involved in binding to band 3 protein. Furthermore, spin-labelled hemoglobin does not show any trace of oxidation of the heme, even after long storage in the cold room. We have shown further for the purpose of this work that its binding to the erythrocyte membrane is similar to that of unlabelled hemoglobin.

The rotational correlation time, $\tau_c$, of MSL-Hb strongly bound to the membrane is about $8 \cdot 10^{-6}$ s (Table I). It is almost one order of magnitude

* Nomenclature of human erythrocyte proteins according to Steck [3].

smaller when it is calculated in the central part of the spectrum ($C'/C$). This difference reflects anisotropic rotation of the labelled protein and one should consider only those values of $\tau_c$ measured from the ratio $L''/L$ as relevant to the motion [20]. A value for $\tau_c$ of $8 \cdot 10^{-6}$ s as compared with $2 \cdot 10^{-8}$ s for MSL-Hb in solution indicates that the rotational movements of MSL-Hb have been considerably slowed down when it is associated with its high-affinity binding sites.

This decrease is directly correlated with the binding of hemoglobin to the membrane. It cannot be ascribed to the presence of the thick suspension of the packed membranes, as shown by the independent movements of unbound MSL-Hb when it is added to hemoglobin-saturated ghosts or when the membranes are incubated at higher pH and ionic strength.

It has been suggested for a long time that in the erythrocyte membrane the high-affinity binding site for hemoglobin is located on the segment of band 3 protein which extends into the cytoplasm. This attribution is strongly supported by the recent finding from rate zonal centrifugation and fluorescence quenching measurements that hemoglobin and the purified fragment of band 3 strongly interact [4]. Band 3 protein is also the receptor for several glycolytic enzymes, including glyceraldehyde-3-phosphate dehydrogenase (140 kDa) and for ankyrin (210 kDa) which links the spectrin-actin cytoskeleton [5,21]. It is worth taking into account the relative size of all these interacting proteins and to notice that the cytoplasmic part of band 3 protein involved in these associations is only 41 kDa [21,22].

Although the movements of erythrocyte-bound MSL-Hb are two orders of magnitude slower than those measured for the protein in solution, they are yet very rapid as compared with those found by Cherry et al. [23] for a segment of band 3 protein which could be a part of the transmembrane spanning portion ($\tau_c \approx 10^{-3}$ s). The mobility of rotation of the 41 kDa cytoplasmic domain of band 3 protein in situ has not been determined until now and one can envisage that the values of $\tau_c$ found in the present work reflect the movements of the complex formed by the rigid association of MSL-Hb with the cytoplasmic segment of band 3 protein. This hypothesis which cannot be unambiguously demonstrated by the present results would indicate a high degree of flexibility of the cytoplasmic part of band 3 protein, in agreement with the freeze-fracture electron microscopy experiments of Weinstein et al. [24] which identify this portion of band 3 protein as a 'granulofibrillar' material. Furthermore, it has more recently been shown by hydrodynamic methods that this peptide purified from the erythrocyte membrane has a very elongated structure [25]. A value for $\tau_c$ of $8 \cdot 10^{-6}$ s for membrane-bound MSL-Hb as compared with $2 \cdot 10^{-8}$ s for the protein in solution indicates clearly that it is significantly hindered by being bound and that any hinge present must be highly restrictive. There must be some sort of potential either restricting the motion so that large enough amplitudes of reorientation of the saturation transfer are made unlikely or that there is an energetic barrier which must be crossed. In both cases one would expect to have diffusional behavior of the labelled protein with $\tau_c$ dependent on the viscosity of the medium and on the size of the proteins. As shown in Table I, this is indeed the case: $\tau_c$ increases with increasing the viscosity and glyceraldehyde-3-phosphate dehydrogenase and ankyrin, with 2- and 3-fold the molecular weight of hemoglobin (i.e., 140000 and 210000), give values for $\tau_c$ of $2 \cdot 10^{-5}$ s and $5 \cdot 10^{-5}$ s, respectively (Ref. 18 and Cassoly, R. and Lemaigre-Dubreuil, Y., unpublished data). This is reasonable, since $\tau_c$ is proportional to $R^3$ even in hindered systems.

The present results alone cannot, however, demonstrate whether the hinge limiting the rotation of band 3 protein-bound MSL-Hb is located at the level of their junction or further in the domain linking the transmembrane and the cytoplasmic portions of band 3 protein. In the first case the movements of MSL-Hb should be relatively independent of those of band 3 protein, in the second case they should reflect the movements of the part of band 3 protein which extends in the cytoplasm. Whatever the case, one can say, however, that the rotational correlation time of this functionally important segment cannot be smaller than $8 \cdot 10^{-6}$ s under the experimental conditions of this work.

It is not surprising that cross-linking band 3 protein has no effect on the rotational mobility of

membrane-bound MSL-Hb (Table I). This result was expected, since band 3 protein probably exists in the membrane as a noncovalent dimer [3,25].

MSL-Hb possesses rigourously the same rotational diffusion movements whether the membrane has been stripped of ankyrin and spectrin-actin, or not (Table I). Although the amounts of ankyrin per ghost are less than the number of copies of band 3 protein [21], changes in the rotational movements of MSL-Hb would have been detected by our method after stripping out of the membrane the peripheral proteins, if membrane-bound ankyrin and hemoglobin undergo concerted motions. This result cannot be correlated directly with the studies of Nigg and Cherry [26], who show by an independent method that ankyrin exerts some constraints on the rotational mobility of band 3 protein. It indicates however the existence of independent movements and flexibility in the assembly of some of the macromolecules which link the erythrocyte cytoskeleton to the membrane.

The correlation times of rotation of MSL-Hb are identical whether the protein is associated with the high or with the low-affinity binding sites (Table I). The same hindrance restricting the movements of hemoglobin is present in both cases, although the two types of site are different. Erythrocyte ghosts can carry $4 \cdot 10^6$ hemoglobin molecules (tetramer) on the low-affinity sites. This represents an amount sufficient to cover completely the surface of the membrane with contiguous molecules. Glycophorin is in part responsible for this binding, but hemoglobin which carries a net positive charge at pH 6 is also engaged in ionic linkage with the charged phospholipidic head-groups present at the surface of the membrane [6]. From independent experiments performed with liposomes one can infer that phosphatidylserine could be a good partner [27]. Deuterium nuclear magnetic resonance and neutron diffraction measurements have given some insight into the orientation and the mobility of some phospholipidic head-groups [28], but very little is actually known about the ionic interactions between protein and lipids at a molecular level. Every hemoglobin molecule should be linked to several lipid molecules if one compares the approximate cross-section area of both (3000 $Å^2$ and 70 $Å^2$, respectively) [29]. The organization and the number of the phospholipid head-groups engaged in ionic interactions with hemoglobin appears specific if one considers that oxy- and deoxyhemoglobin, which present different quaternary structures, show an approx. 40-fold difference in their affinity for the erythrocyte membrane [14]. In this respect, the knowledge of the atomic coordinates of charged amino acid residues in hemoglobin and several of its mutants can be an appropriate way to study more precisely the ionic protein-lipid and protein-protein interactions described in the present work.

## Acknowledgements

I wish to thank Dr. Martin Karplus for useful discussions concerning the interpretation of the experiments and Ms. Alice Royer for efficient technical assistance. This work was supported by grants from the Centre National de la Recherche Scientifique (E.R. 157), the Délégation Générale à la Recherche Scientifique et Technique and the Institut National de la Santé et de la Recherche Médicale.

## References

1 Shaklai, N., Yguerabide, J. and Ranney, H.M. (1977) Biochemistry 16, 5593–5597
2 Salhany, J.M., Cordes, K.A. and Gaines, E.D. (1980) Biochemistry 19, 1447–1454
3 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
4 Cassoly, R. (1982) C.R. Acad. Sci. 294, 141–143
5 Yu, J. and Steck T.L. (1975) J. Biol. Chem. 250, 9176–9184
6 Rauenbuchler, P., Cordes, K.A. and Salhany, J.M. (1982) Biochemistry, in the press
7 Salhany, J.M. and Gaines, K.C. (1981) Trends Biochem. Sci. 6, 13
8 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
9 Kant, J.A. and Steck, T.L. (1973) J. Biol. Chem. 248, 8457–8464
10 Tyler, J.M., Hargreaves, W. and Branton, D. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5192–5196
11 Steck, T.L. (1972) J. Mol. Biol. 66, 295–305
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
13 Lepke, S., Fasold, H., Pring, M. and Passow, H. (1976) J. Membrane Biol. 29, 147–177
14 Shaklai, N. and Sharma, V.S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7147–7151
15 Thomas, D.D., Dalton, L.R. and Hyde, J.S. (1976) J. Chem. Phys. 65, 3006–3024
16 Fung, L.W.M., SooHoo, M.J. and Meena, W.A. (1979) FEBS Lett. 105, 379–383

17 Lemaigre-Dubreuil, Y., Henry, Y. and Cassoly, R. (1980) FEBS Lett. 113, 231–234
18 Beth, A.H., Balasubramanian, K., Wilder, R.T., Venkataramu, S.D., Robinson, B.H., Dalton, L.R., Pearson, D.E. and Park, J.H. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4955–4959
19 Moffat, J.K. (1971) J. Mol. Biol 55, 135–146
20 Robinson, B.H. and Dalton, L.R. (1980) J. Chem. Phys. 72, 1312–1324
21 Hargreaves, W.R., Giedd, K.N., Verkleij, A. and Branton, D. (1980) J. Biol. Chem. 255, 11965–11972
22 Steck, T.L., Ramas, B. and Strapazon, E. (1976) Biochemistry 15, 1154–1161
23 Cherry, R.J., Bürkli, A., Busslinger, M., Schneider, G. and Parish, G.R. (1976) Nature (London) 263, 389–393
24 Weinstein, R.S., Khodadad, J.K. and Steck, T.L. (1978) in The Red Cell (Brewer, G.J., ed.), pp. 413–417, Alan R. Liss, New York
25 Appell, K.C. and Low, P.S. (1981) J. Biol. Chem. 256, 11104–11111
26 Nigg, E.A. and Cherry, R.J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4702–4706
27 Calissano, P., Alema, S. and Rusca, G. (1972) Biochim. Biophys. Acta 255, 1009–1013
28 Seelig, J. and Seelig, A. (1980) Q. Rev. Biophys. 13, 19–61
29 Papahadjopoulos, D., Cowden, M. and Kimelberg, H. (1973) Biochim. Biophys. Acta 330, 8–26