Biochimica et Biophysica Acta, 983 (1989) 199–204
Elsevier

BBAMEM 74498

Association of hemin with protein 4.1 as compared to spectrin and actin

Irit Solar and Nurith Shaklai

Sackler Institute of Molecular Medicine, Sackler Faculty of Medicine, Tel-Aviv University, Ramat Aviv (Israel)

(Received 21 October 1988)
(Revised manuscript received 8 February 1989)

Key words: Protein 4.1; Spectrin; Actin; Hemin; Fluorescence; Circular dichroism

The interaction of hemin with protein 4.1 isolated from red cell membrane cytoskeleton has been studied. Spectrophotometric titration has shown one strong binding site and additional lower affinity sites for hemin. From fluorescence quenching data an association binding constant of $1.3 \cdot 10^7$ M^{-1} has been calculated for the primary site. The conformation of cytoskeletal proteins after hemin binding was followed by the use of far UV circular dichroism and compared to that of the serum hemin trap, albumin. The secondary structure of albumin was unchanged in the presence of high hemin concentrations. Both spectrin and actin lost their conformation upon hemin binding in a ligand-concentration and time-dependent manner. Unlike spectrin and actin, the secondary structure of protein 4.1 was unaffected by hemin binding to the primary site, but, at higher hemin concentrations, some reduction in the ellipticity of protein 4.1 appeared. The findings of this study suggest that protein 4.1 may serve as the cytoskeletal temporary sink for small amounts of membrane-intercalated hemin similarly to the function of albumin in the serum. However, an increased release of hemin under pathological conditions may cause hemin association with the cytoskeletal proteins and as a result the cell membrane is expected to be distorted.

Introduction

Hemin is a highly active molecule participating in many vital processes. However, being so active, free hemin is also likely to be involved in undesired reactions and therefore should not reside in a free form. Its transportation to the right locations, for metabolic as well as catabolic purposes, requires binding to mediators. Indeed the main protein in the serum, albumin, has been known to serve as a temporary sink for free hemin which is finally catabolized in the liver [1,2].

Of all cells, the erythrocytes has the highest density of hemin where it is bound tightly to globin. Normal red cells contain negligible amounts of globin 'free' hemin. However, under various pathological conditions, an increased level of 'free' hemin has been found [3,4]. Despite the ability of the cell cytosol to temporarily inhibit transfer of free hemin by binding it to agents such as glutathione, free hemin, being a hydrophobic molecule, will eventually intercalate in the red cell membrane [5]. The slowly phospholipid-accumulated hemin can normally be released by passive diffusion through the membrane to be trapped by serum proteins, such as albumin [6,7]. In order to prevent undesired interactions of protein components with the transfering hemin, it should be directed, analogously to the serum system, to unaffected temporary sinks. Such hemin traps are likely to be situated in the membrane/cytosol interface, which is the first to be encountered by globin-free hemin. The natural candidates are the membrane cytoskeleton proteins which partition the membrane lipid core and the cytosol.

The red cell cytoskeleton plays a key role in regulating the stability and deformability of the membrane. It is composed of three major proteins spectrin, actin and protein 4.1, which form a two-dimensional network. Spectrin and actin have already been shown to bind hemin [8,9] but the possible association of protein 4.1 is less clear. Some clues to the ability of 4.1 to bind hemin can be found in previous literature demonstrating an increase in electrophoretic mobility of protein 4.1 in the

Abbreviations: CD, circular dichroism; DTT, 1,4-dithiothreitol; SDS, sodium dodecyl sulfate; UV, ultraviolet.

Correspondence: N. Shaklai, Sackler Institute of Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel.

presence of hemin [10]. The current work has examined the ability of protein 4.1 to associate with hemin and has screened the ability of the cytoskeletal proteins to serve as temporary sinks for the hemin transporting through the membrane.

Materials and Methods

The resins for column chromatography were diethylaminoethyl cellulose from Whatman and Sepharose 4B from Pharmacia. Reagents for SDS-polyacrylamide electrophoresis were obtained from Bio-Rad and other reagents were purchased from Sigma. All reagents were of analytical grade.

Preparation of proteins

Spectrin. Red cell membranes (ghosts) were prepared according to previously described procedures [11]. Spectrin tetramers were extracted [12] and isolated by gel filtration chromatography on Sepharose 4B column in 10 mM Tris/100 mM NaCl/0.1 mM EDTA/0.5 mM DTT at pH 7.6 [13].

Protein 4.1. Protein 4.1 was purified from the membranes of fresh erythrocytes using previous procedures [14,15].

Actin. This protein was prepared from rabbit skeletal muscle according to established procedures [16].

Protein concentrations. Concentrations were determined by using an absorption coefficient ϵ_{280} of 0.8 $(mg/ml)^{-1} \cdot cm^{-1}$ for protein 4.1 and 1.0 $(mg/ml)^{-1} \cdot cm^{-1}$ for spectrin [17]. $\epsilon_{290} = 0.63$ $(mg/ml)^{-1} \cdot cm^{-1}$ was used for actin [18].

Hemin preparation

Hemin was freshly prepared as stock solution by dissolving it in 5 mM NaOH and centrifuging for 10 min at 40000 × *g*. Hemin concentration was determined spectrophotometrically using an absorption coefficient ϵ_{385} of $58.4 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ in NaOH.

For ligand binding experiments all proteins were dissolved in buffer A containing 20 mM phosphate/0.5 mM EGTA/20 mM NaCl at pH 7.3 and used with several days of preparation.

SDS-polyacrylamide gel electrophoresis

The purity of the protein was tested using gel electrophoresis in the presence of SDS according to Fairbanks [19].

Instruments

Spectrophotometric measurements were performed on a Cary 219 spectrophotometer. Fluorescence measurements were carried out using a Hitachi-Perkin-Elmer MPF-44B spectrofluorometer. For circular-dichroism (CD) experiments a Jasco spectropolarimeter model J-500C was used.

Results

Binding of hemin to protein 4.1

In Fig. 1 the Soret band in the presence of protein 4.1 as well as other proteins is compared to that of free hemin in aqueous solution. Spectra were recorded at low hemin concentrations, at which it is mostly monomeric [20]. It has been illustrated that addition of protein 4.1 induced changes in the spectral characteristics of hemin, namely, an increase in the absorption coefficient and a small shift of the maximal absorption to the red. These observations are indicative of the ability of protein 4.1 to interact with hemin. To avoid free-hemin contribution in the protein solutions, the difference spectrum of hemin in the presence of protein 4.1 and albumin is compared in the inset of Fig. 1. It is seen that the difference spectrum of both proteins is similar unlike the cases of the other cytoskeletal proteins, spectrin [8] and actin [9].

For binding analysis, protein 4.1 was titrated with increasing hemin concentrations. Aqueous hemin in the concentration range used in this study exists as a mixture of the monomeric and dimeric forms with different

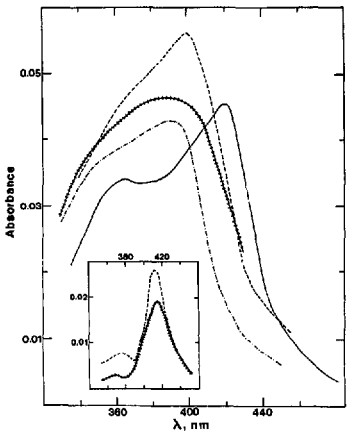

Fig. 1. Spectral characteristics of free and protein-bound hemin. Hemin (1 μM) was dissolved in buffer A and the solutions kept at room temperature. ·–·–·, hemin only; ———, in the presence of 4.5 μM protein 4.1; - - - - -, in the presence of 5.0 μM human serum albumin; ———, in the presence of 4 μM crude spectrin (EDTA extract). Inset: difference spectrum of free and protein-bound hemin. - - - - -, in presence and absence of human serum albumin (5 μM); ———, in the presence and absence of protein 4.1 (4.5 μM).

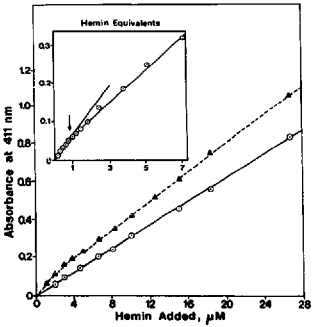

Fig. 2. Spectrophotometric titration of protein 4.1 with hemin. Hemin in buffer A was titrated by successive additions from a stock solution and the absorbance at 411 nm, the isosbestic point of hemin monomer and dimer [21] was measured at room temperature (approx. 22°C). ⊙, without protein; △, in the presence of 4.5 μM protein. Inset. Difference titration at 411 nm. The reference cuvette contained increasing concentrations of hemin in a buffer solution, while in the reaction sample hemin was added to a buffered protein solution. Protein 4.1 at a concentration of 4.5 μM was kept in buffer A and at room temperature. The arrow demonstrates the point of intersection of the two straight lines.

extinction coefficients. Therefore, titration of protein 4.1 with hemin was carried out at 411 nm, which is the isosbestic wavelength of the two forms [21]. In this manner, the differences in absorption reflect protein-bound hemin only and are free of those differences resulting from unbalanced monomer and dimer concentrations of the uncomplexed hemin. The blank titration of the protein with hemin is demonstrated in Fig. 2. It can be seen that the slope of absorption at 411 nm vs. hemin concentration is higher in the protein-containing solution throughout the entire titration concentration range, indicating binding of hemin to the protein. The difference in titration demonstrated in the inset to Fig. 2 indicates that the titration is composed of two linear portions which intersect at a hemin concentration smaller than one equivalent. We assumed from these findings that only one high-affinity site exists for hemin but lower-affinity sites exist as well.

To calculate the binding affinity of the primary site without expression of the lower affinities, fluorescence techniques were used. The maximum of emission from protein 4.1 excited at 280 nm was at 340 nm, emission typical for tryptophan-containing proteins. Addition of hemin to protein 4.1 resulted in quenching of the fluorescence intensity due to radiationless energy transfer from the excited tryptophan donors to the protein-bound

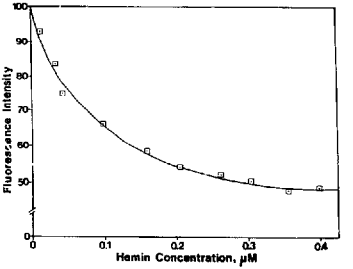

Fig. 3. Binding of hemin to 4.1 as demonstrated by quenching of the intrinsic protein fluorescence. Fluorescence was monitored at 348 nm and the protein solution was excited at 280 nm. Protein 4.1 was kept in buffer A at a concentration of 0.025 μM and thermostated at 25°C. Experimental data representing an average of three different experiments are demonstrated. The full line represents a theoretical curve based on one binding site per protein molecule and a binding affinity of $K_d = 1.3 \cdot 10^7\ M^{-1}$.

hemin acceptors [22]. The fluorescence quenching curve of protein 4.1 in the presence of increasing hemin concentrations is shown in Fig. 3.

The fluorescence quenching data were translated into a fraction of ligand saturation assuming $\Delta F/\Delta F_\infty = \nu$, where ΔF represents the fluorescence intensity quenched

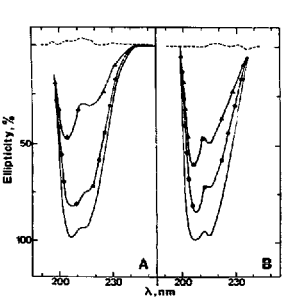

Fig. 4. CD spectra of spectrin (A) and actin (B) in the presence of hemin. Proteins were kept in solution A at pH 7.3 and at 23°C. The cell path length was 0.2 cm; protein concentrations were 0.034 mg/ml for spectrin and 0.15 mg/ml for actin. The ellipticity of the proteins in the absence of hemin was taken as 100%. Molar ellipticities (in the absence of hemin): for spectrin $29.1 \cdot 10^{-3}$ deg·cm^2·dmol^{-1}; for actin: $6.6 \cdot 10^{-3}$ deg·cm^2·dmol^{-1}. Note the difference in molar ellipticity of the proteins. ------, baseline of buffer solution only; ———, protein only; ●———●, 100 min incubation with 1 μM hemin; ▲———▲, 40 min incubation with 100 μM hemin.

at various hemin concentrations and ΔF_∞ is the amount of fluorescence quenched at infinite hemin concentration. ΔF_∞ was calculated from the intercept of the double-reciprocal plot [23] of fluorescence quenched vs. hemin concentration (data not shown). Considering one site of high affinity, the fluorescence quenching data were found to fit best an association affinity of $K_a = 1.3 \cdot 10^7\ M^{-1}$. The total hemin concentration was related to free-hemin monomer concentration considering the monomer–dimer equilibrium constant of $10^6\ M^{-1}$ [24]. The fluorescence quenching, ΔF, was calculated from the protein total concentration and the fraction of bound protein, ν, where the simple equation of a single binding site relates to the free hemin monomer, c, by: $K_a c = \nu/(1-\nu)$. The theoretical curve is shown in Fig. 3 and the measured data are shown on the same scale for comparison.

The effect of bound hemin on the conformation of cytoskeletal proteins

As it appeared that all three cytoskeletal proteins can associate with hemin, it was important to find out next whether binding of hemin has any effect on the structure of the proteins discussed. CD spectra in the far UV are convenient to follow changes in the protein structure [25]. Protein solutions were incubated at room temperature in the absence or presence of hemin and the far-UV CD spectra of the samples were followed. In Fig. 4 a reduction in the typical far-UV CD bands of spectrin and actin in the presence of hemin is demonstrated.

At a concentration of 1 μM hemin, a reduction in ellipticity could be observed after less than 2 h. At hemin concentrations of 100 μm after 40 min, about 50% of ellipticity was already lost for actin and in the case of spectrin ellipticity was reduced to 70% of the hemin-lacking sample incubated under the same conditions. It thus appeared that both proteins lost their ellipticity in a time- and hemin-concentration-dependent manner. Moreover, spectrin is shown to be more susceptible to bound hemin than actin. Whether a loss of ellipticity is a general feature of hemin-binding proteins was not clear at this point. We thus measured the far CD spectrum of albumin, a thoroughly studied hemin-binding protein, in the presence of hemin as well. The results, as demonstrated in Fig. 5, show that the ellipticity of albumin in the far UV was completely retained in the presence of hemin, even at concentrations as high as 100 μM under the same conditions studied for spectrin and actin. Since the present study has shown that protein 4.1 binds hemin but with different characteristics than those of spectrin and actin, the ellipticity of the above protein was followed as well. Protein 4.1 was incubated at room temperature under the same conditions which showed reduction in ellipticity for the other cytoskeletal proteins spectrin and actin.

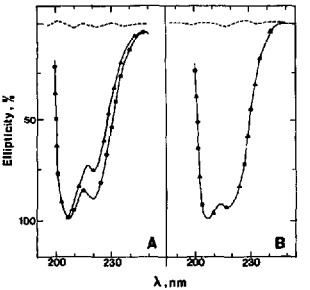

Fig. 5. CD spectra of protein 4.1 (A) and human serum albumin (B) in the presence of hemin. Proteins were kept in solution A at pH 7.3 and at 23°C. The cell path length was 0.2 cm and protein concentrations were 0.05 mg/ml for protein 4.1 and 0.02 mg/ml for albumin. The ellipticity of the proteins in the absence of hemin was taken as 100%. Molar ellipticities (in the absence of hemin): for protein 4.1, $9.9 \cdot 10^{-3}$ deg·cm^2·dmol^{-1}; for albumin, $49.5 \cdot 10^{-3}$ deg·cm^2·dmol^{-1}. Note that despite similarities in mol. wt. protein 4.1 has much lower ellipticity compared to albumin. ------, baseline of buffer solution only: ———, protein only: ●———●, 120 min incubation with 1 μM hemin; ▲———▲, 70 min incubation with 100 μM hemin.

At hemin concentrations up to at least 10 μM no changes in the protein far-UV CD spectrum characteristics could be observed. Only at 100 μM could a minor reduction in the proteins' ellipticity, which was unchanged with time, be noticed.

Discussion

The current study suggests that protein 4.1, like the cytoskeletal proteins spectrin and actin, can associate with hemin. The spectral characteristics of protein-bound hemin reflect the protein moiety composing the hemin binding site. The typical absorption of spectrin-bound hemin shown here as well as in previous literature and that of hemin-bound actin, resemble each other [8,9]. In both cases the Soret band is characteristic of hemin-iron chelation with nitrogenous amino-acid side groups [8,9,26]. The Soret band of hemin bound to protein 4.1 is different and is more typical of monomeric hemin in an hydrophobic environment [21,27]. From the spectra of protein 4.1 and albumin (Fig. 1, inset), we infer that hemin binds to both proteins in similar protein regions and the small red shift of hemin bound to protein 4.1 resembles the hemin-protein complex in the case of the protein ligandin [21]. Thus, it seems that protein 4.1 belongs to a group of proteins such as ligandin, protein A and albumin, where binding of hemin is governed by hydrophobic interactions of the

porphyrin ring [21,27]. However, involvement of iron chelation to amino-acid side chains has not been ruled out in the case of protein 4.1.

Differences in the characteristics of hemin binding sites of spectrin and actin and protein 4.1 are expressed in the affinities of the above proteins for hemin as well. In the case of actin, earlier studies have demonstrated a high-affinity site of $K_a = 5.3 \cdot 10^6\ M^{-1}$ from fluorescence quenching data and additional secondary lower-affinity sites have been suggested as well [9].

For spectrin, an average binding constant of $K_a = 5 \cdot 10^5\ M^{-1}$ has been reported for all available (approx. 15 per dimer) hemin binding sites [8]. In the current study we show that protein 4.1 exhibits a strong binding site for hemin with an association constant of $K_a = 1.3 \cdot 10^7\ M^{-1}$. Thus, hemin affinity to the primary site in protein 4.1 is higher than that of the other cytoskeletal proteins. Once reaching the cytoskeleton, it is reasonable to suggest that residual free hemin will first saturate the highest-affinity sites. Therefore, by affinity criteria, protein 4.1 is a natural candidate to serve as the temporary sink for small quantities of free cytosolic hemin passing through the membrane cytoskeleton.

It is expected that hemin binding to a protein sink will not induce changes in its secondary structure for the following reasons. (1) Conformational changes which require energy are unlikely to be invested in a passive transport. Moreover, in the case of a membrane-associated protein, further conformational changes may be induced in attached components. (2) Reversibility of the process is a necessary condition for any repetitive process. This renders conformational changes, which tend to be irreversible, unlikely.

Being concerned with the possible role of the cytoskeletal proteins as temporary hemin sinks, their secondary structure before and after hemin binding was followed. Albumin was chosen for comparative reasons, since it has already been shown to serve as the temporary hemin sink in the serum prior to hemin transportation via hemopexin for catabolism in the liver [1,2]. Our study demonstrates (Fig. 5) that the secondary structure of albumin is maintained for at least few hours in the presence of hemin concentrations as high as 100 μM. In this manner, albumin withstands the aforementioned expectations of a physiological hemin sink.

Previous studies have shown that the native oligomeric structure of spectrin and actin is destroyed in the presence of hemin. However, it required an incubation time of several hours at hemin concentrations as high as 100–200 μM [9,28,29]. On the other hand, the stability of red cell cytoskeletons, which requires the physiological oligomeric state of the cytoskeletal proteins [30,31], was shown in an earlier study to be reduced at hemin concentrations as low as 1 μM [10]. These phenomena are expected to be the outcome of the same process but there is a large difference in the hemin concentration range reported for the two cases. The observations of the current study shed light on the apparent discrepancy. We have shown that hemin added to spectrin, as well as actin, induced reduction of the CD bands in the far-UV, namely in the peptide-bond region (Fig. 4). This observation has been interpreted as the failure of both proteins to maintain conformational stability after hemin binding. The loss of protein structure is a time- and hemin-concentration-dependent phenomenon. The slow reduction of the secondary structure demonstrated in this study at low hemin concentrations of 1 μM is typical of irreversible conformational changes, namely, denaturation. Thus, it seems that the above described loss of protein conformation which has already occurred at low hemin concentrations, is the first process and therefore the crucial event in the cytoskeleton's impairment. From the binding affinity of spectrin and hemin [8], it is calculated that at 1 μM ligand concentration only about 15% of the spectrin should be hemin-bound. Since the data demonstrated a reduction in ellipticity of the order of 30%, it seems that the hemin-bound molecules undergo total loss of structure, namely denaturation. Unlike spectrin and actin, the third cytoskeletal protein 4.1 is resistant to conformation changes upon hemin binding as demonstrated by ellipticity of the helical segments (Fig. 5). It takes saturation of lower-affinity sites to cause a change in the secondary structure of the helical portion of protein 4.1. It seems that the resistance of protein 4.1 and albumin to hemin-induced loss of secondary structure may be a more general phenomenon for proteins exhibiting preexisting sites for hemin. In general, such proteins will have higher affinity for hemin and will be less vulnerable to hemin-induced unfolding.

The physiological significance of the cytoskeletal protein lattice in determining the shape and mechanical properties of the erythrocyte is well-documented. Spectrin–actin interactions predominate in forming the filamentous meshwork of the cytoskeleton, and protein 4.1 has a key role in regulating the interactions between spectrin and actin [33]. The current study adds another dimension to the possible functions of protein 4.1, namely, protection of the vulnerable proteins in the cytoskeleton. These oligomeric proteins, especially the elongated spectrin, escape hemin-induced loss of their native conformation by the binding of hemin to protein 4.1. Moreover, the use of protein 4.1 as a hemin sink may be extended to other hydrophobic molecules passing through the membrane in the process of their clearance. In general, it seems that the physiological concentrations of free hemin in the membrane are low enough to enable protein 4.1 to function as a useful hemin trap. However, under pathological conditions, an increased level of free hemin may linger in the cytoskeleton area thereby losing its option to be drained.

Protein 4.1 will no longer be sufficient as the trap for hemin accumulating in the cytoskeleton. Finally, as shown in this study, the hemin associated with the other cytoskeletal proteins will cause loss of their function, resulting in cell distortion.

Acknowledgements

N.S. is deeply grateful to Dr. W.B. Gratzer for many helpful discussions. This study was initiated during a visit of N.S. to Dr. Gratzers' laboratory in the Biophysics unit, King's College, London. This visit was supported by a short term grant from the EMBO foundation. We thank Mr. Dan Amar from the organic chemistry department of the Weizman Research Institute for allowing the use of the Jasco spectropolarimeter and for his extremely helpful assistance and advice. Mrs. Helene Cohen is acknowledged for her editorial assistance. This study was partially supported by the Binational foundation Grant No. 850096.

References

1 Morgan, W.T., Liem, H.H., Sutor, R.P. and Muller Eberhard, U. (1976) Biochim. Biophys. Acta 444, 4744–4750.
2 Liem, H.H. and Muller Eberhard, U. (1972) Blood 40, 942.
3 Hebbel, R.P., Morgan, W.T., Eaton, J.W. and Hedlund, B.E. (1987) Blood 62A, 101.
4 Liu, S.C., Zhai, S. and J. Palek (1988) Blood 71, 1755–1758.
5 Shviro, Y. and Shaklai, N. (1987) Biochem. Pharmacol. 36, 3801–3807.
6 Cannon, J.B., Kuo, F.S., Pastrnack, R.F., Wong, N.M. and Muller-Eberhard, U. (1984) Biochemistry 23, 3715–3721.
7 Shaklai, N., Shviro, Y. Rabizadeh, E. and Kirschner-Zilber, I. (1985) Biochim. Biophys. Acta 821, 355–366.
8 Beaven, G.H. and Gratzer, W.B. (1978) Acta Haemat. 60, 321–328.
9 Avissar, N., Shaklai, M. and Shaklai, N. (1984) Biochim. Biophys. Acta 786, 179–187.
10 Liu, S.C., Zhai, S., Lawler, J. and Palek, J. (1985) J. Biol. Chem. 12234–12239.
11 Shaklai, N., Yguerabide, J. and Ranney, H.M. (1977) Biochemistry 16, 5585–5592.
12 Morrow, J.S. and Marchesi, V.T. (1981) J. Cell Biol. 88, 463–468.
13 Tyler, J.M., Hargreaves, W.R. and Branton, D. (1979) J. Biol. Chem. 254, 8620–8627.
14 Tyler, J.M., Reinhardt, B.N. and Branton, D. (1980) J. Biol. Chem. 255, 7034–7039.
15 Ungenwickell, E. and Gratzer, W. (1978) Eur. J. Biochem. 88, 379–385.
16 Kendrick-Jones, J., Lehman, W. and Szent Gyorgyi, A.G. (1970) J. Mol. Biol. 54, 313–326.
17 Becker, P.S., Cohen, C.M. and Lux, S.E. (1986) J. Biol. Chem. 261, 4620–4628.
18 Lehrer, S.S. and Kerwar, G. (1972) Biochemistry 11, 1211–1217.
19 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617.
20 Brown, S.B., Dean, T.C. and Jones, P. (1970) Biochem. J. 117, 733–739.
21 Tipping, E., Ketterer, B., Christodoulides, L. and Enderby, G. (1976) Biochem. J. 157, 461–467.
22 Forster, T. (1965) Mod. Quant. Chem. 3, 93–137.
23 Randall, C.S. and Zand, R. (1985) Biochemistry 24, 1998–2004.
24 Brown, S.B., Shillcock, M. and Jones, P. (1976) Biochem. J. 153, 279–285.
25 Ettinger, M.J. and Timasheff, S.N. (1971) Biochemistry 10, 824–830.
26 Avissar, N., Inbal, A., Rabisadeh, E., Shaklai, M. and Shaklai, N. (1984) Biochem. Int. 8, 113–120.
27 Beaven, G.H., Chen, S.H., D'Albis, A. and Gratzer, W.B. (1974) Eur. J. Biochem. 41, 539–546.
28 Nakano, M., Iwamaru, H. and Tobita, T. (1982) Biopolymers 21, 805–815.
29 Blauer, G. (1964) Biochim. Biophys. Acta 79, 547–562.
30 Mohandas, N., Chasis, J.A. and Shohet, S.B. (1983) Semin. Hematol. 20, 225–242.
31 Liu, S.C., Palek, J., Prchal, J. and Castleberry, R.P. (1981) J. Clin. Invest. 68, 597–605.
32 Shviro, Y., Zilber, I. and Shaklai, N. (1982) Biochim. Biophys. Acta 687, 63–70.
33 Ohanian, V., Wolfe, L.C., John, K.M., Pinder, J.C., Lux, S.E. and Gratzer, W.B. (1984) Biochemistry 23, 4416–4420.