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Hydration-dependent conformational states of hemoglobin

Equilibrium and kinetic behavior

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The equilibrium and kinetics of methemoglobin conversion to hemichrome induced by dehydration were investigated by visible absorption spectroscopy. Below about 0.20 g water per g hemoglobin only hemichrome was present in the sample; above this value, an increasing proportion of methemoglobin appeared with the increase in hydration. The transition between the two derivatives showed a time-dependent biphasic behavior and was observed to be reversible. The rates obtained for the transition of methemoglobin to hemichrome were 0.31 and 1.93 min^{-1} and for hemichrome to methemoglobin 0.05 and 0.47 min^{-1}. We suggest that hemichrome is a reversible conformational state of hemoglobin and that the two rates observed for the transition between the two derivatives reflect the α- and β-chains of hemoglobin.

1. Introduction

Water is a key element in biological systems. The nature of the biological role played by water is unclear, however, it is certainly essential to establish the structural and dynamical aspects of the macromolecules.

The interactions of a protein with water are crucial in stabilizing its native structure, although dehydration does not necessarily imply that unfolding occurs. Based mostly on studies using lysozyme [1–3], it is believed that a dehydrated protein is in a latent functional state, i.e., biological function can be restored if the protein is rehydrated above a certain critical hydration value.

Several types of experimental results have appeared in the literature concerning the dehydration of proteins. Some data indicate the occurrence of minor conformational changes in the protein (ref. 3 and references therein), while none were observed in ref. 2. These conformational changes, when they occur, are reversible upon rehydration. It is now accepted that the lack of activity of a dried protein sample is correlated (at least in part) with the lack of structural flexibility, which is recovered when the protein is rehydrated above a critical threshold value. Proteins are currently considered as dynamical systems whose structures fluctuate around an average value determined by X-ray diffraction, proceeding through several different conformational states [4,5]. The fluctuations are 'frozen' at low temperatures, where it is possible to follow the kinetics of different conformational substates. Structural flexibility is also affected by dehydration, however, very few experiments have been focussed on correlating hydration with conformational states. The present work is aimed in this direction.

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We have investigated the equilibrium and kinetics of the conformational transitions induced by dehydration of human hemoglobin (Hb) in a film using visible absorption spectroscopy. Previous studies on hemoglobin dehydration have shown that the dried protein forms a derivative that is optically and magnetically distinct from that observed in solution [6,7]. This state is known as hemichrome or hemochrome depending on the heme iron being in the ferric or ferrous state, respectively. It was proposed that in this derivative the iron is coordinated to the proximal and distal histidines [8–10]; thus, the term bis-His has also been used to designate this derivative. The bis-His state has been observed in solution in several investigations [11–17]. However, it is unclear as to whether this conformational state is a stable Hb structure or a step in the process of protein denaturation. In this study, we have determined the critical hydration values for Hb, its conformational states and the dependence of structural flexibility on the degree of hydration.

2. Materials and methods

Hb in the oxy form was prepared from human blood according to a standard procedure [18]. The Met derivative was prepared by addition of potassium ferricyanide to HbO_2 at neutral pH and in very small excess over the heme (10%). The ferrocyanide formed was removed by gel filtration [19].

In order to follow the formation of the film, 15–20 μl of a 5–8 mM MetHb solution was spread on a glass slide and located in a closed sample holder in the presence of a desiccant (P_2O_5 and saturated salt solutions were used [20]). The sample holder was introduced into a Cary 17 spectrophotometer keeping the glass slide in the horizontal position. Several spectra were recorded as a function of time until no further spectral changes were observed. To rehydrate the film, the desiccant was substituted by distilled water.

Measurements were also made on samples equilibrated for 24 h in environments with different relative humidities (R.H.). Hydration and dehydration were also followed at a fixed wavelength.

3. Results

3.1. Sorption and desorption of water in MetHb

Fig. 1 shows the optical absorption spectra of MetHb (pH 6.8) in the range 450–700 nm. The hemoglobin was initially in solution and its dehydration in an environment of 11% R.H. was followed as a function of time. The spectra drawn as continuous lines (fig. 1a–c) show absorption bands at 500 and 630 nm, typical of the MetHb derivative in solution [21]. Analysis of these spectra indicates a decrease in the absolute spectral intensity, while the relative intensities of the bands and the shape of the spectra are invariant; this effect is probably due to the migration of Hb to the border of the film. After this time period, different spectra (drawn as broken lines; fig. 1d–g) were recorded. A decrease in intensity of the bands at 500 and 630 nm and a simultaneous increase at about

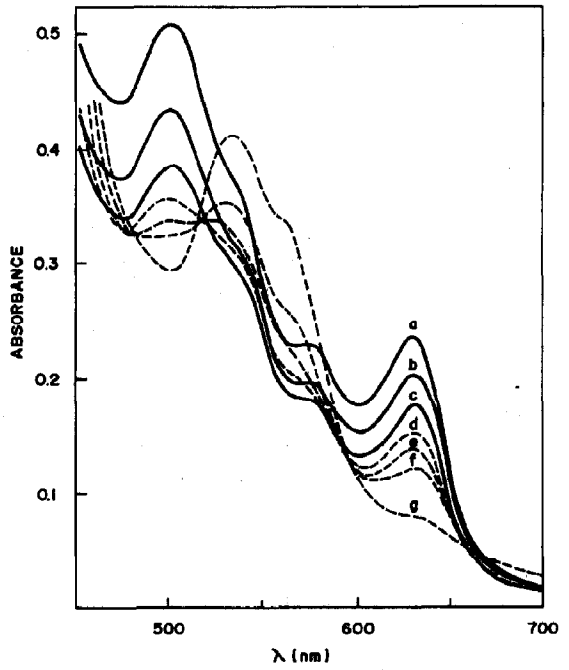

Fig. 1. Dehydration process: absorption spectra obtained during dehydration of a MetHb solution in an 11% R.H. environment. Spectra were taken (a) 14, (b) 30, (c) 39, (d) 45, (e) 48, (f) 51, (g) 60, 83 and 122 min after the beginning of the process. Scan time, 2 min.

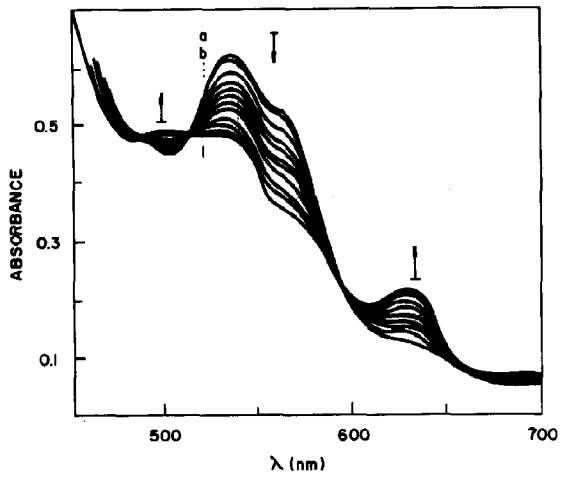

Fig. 2. Rehydration process: absorption spectra obtained during the rehydration of a Hb film initially at 75% R.H. and transferred to a 100% R.H. environment. Spectra were taken (a) 0, (b) 3, (c) 6, (d) 9, (e) 12, (f) 15, (g) 18, (h) 23, (i) 31, (j) 39, (k) 53, (l) 72 and 115 min after the beginning of the process. Arrows indicate directions of the changes with time. Scan time, 2 min.

535 nm were observed. After about 1 h this spectral transition was completed and no further changes were observed. On protein dehydration reaching completion, the spectrum shows an absorption band at 536 nm and a shoulder near 565 nm, characteristic of the hemichrome observed in hemoproteins [6].

The reversibility of the MetHb to hemichrome transition was verified by rehydration of a film equilibrated at 75% R.H. The film was transferred to a 100% R.H. environment and the spectra recorded as a function of time, as shown in fig. 2. The characteristic hemichrome bands decrease in intensity as a function of time, while the bands at 500 and 630 nm increase in intensity, indicating the tendency of the hemichrome to undergo reconversion to MetHb. Even after reaching equilibrium at 100% R.H., a mixture of hemichrome and MetHb is observed; complete conversion was achieved only when water was dropped onto the film.

Another characteristic of the spectra shown in fig. 2 is the presence of four isosbestic points at 485, 519, 595 and 660 nm, suggesting that only the two derivatives, hemichrome and MetHb, are present in the film. Isosbestic points very close to these were also observed in the MetHb to hemichrome transition induced by sodium salicylate in solution [22].

Measurements were also made for samples equilibrated in environments of differing relative humidity at room temperature. For these spectra, the ratio R of the absorbance at 630 nm to that at 595 nm (one of the isosbestic points) was calculated, giving the MetHb fraction in the film. In the absence of MetHb $R = 0.4$, as obtained for a film equilibrated in P_2O_5, while $R = 1.3$ corresponds to 100% MetHb in the film, as determined for the spectrum of MetHb in solution. A plot of R as a function of relative humidity is presented in fig. 3. Below about 75% R.H. the protein is totally in the hemichrome form and, as the R.H. increases the fraction of MetHb increases; however, at 100% R.H., the protein does not revert completely to the MetHb form. The midpoint of the transition (corresponding to a 50:50% mixture of the two derivatives) is observed at about 93% R.H. The R.H. values can be converted to water content (h) in g water per g protein using the Hb hydration isotherms determined by Brausse et al. [23]. From the 20 °C isotherm, 75% R.H. corresponds to $h = 0.20$,

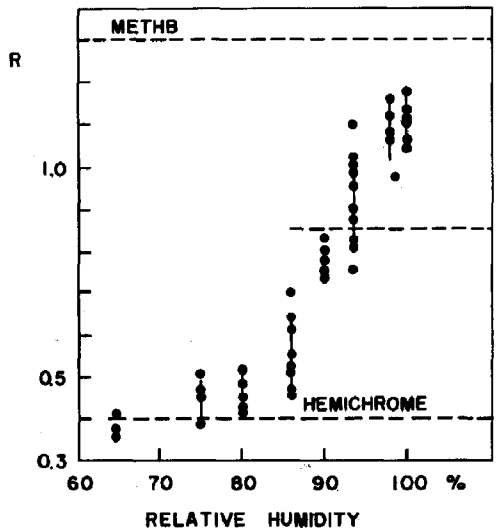

Fig. 3. Hemichrome-MetHb transition: ratio R of the absorbance at 630 nm to that at 595 nm as a function of hydration (R.H.). Results from several experiments are plotted and the bars denote the errors in the mean values.

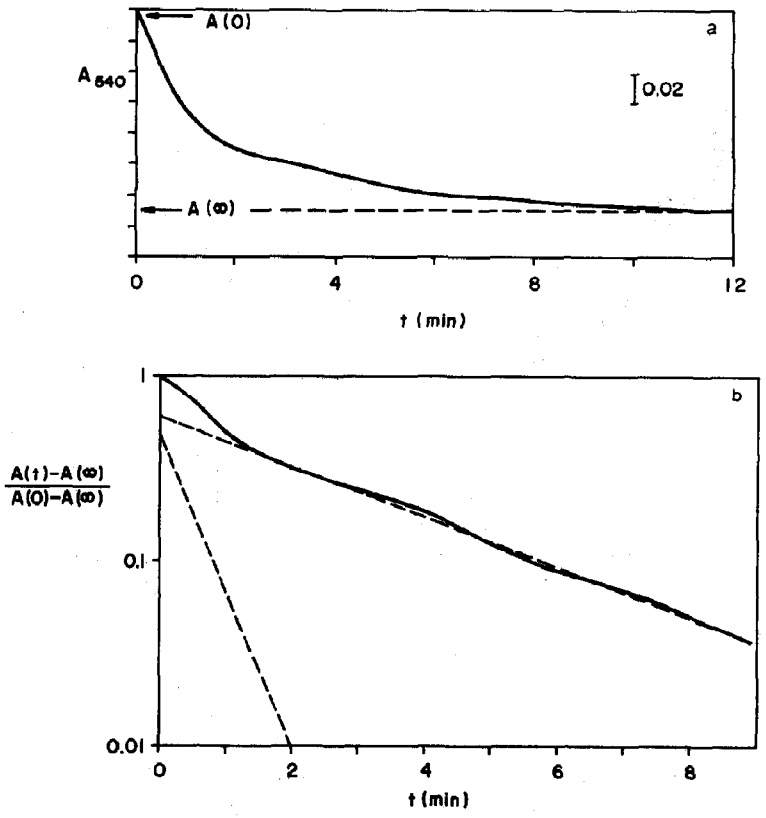

Fig. 4. Dehydration process: change in absorbance at 540 nm for a MetHb film as a function of dehydration time on (a) linear and (b) logarithmic scales. The lines in panel b were fitted to the experimental points and correspond to rates of 0.31 and 1.93 min^{-1}.

93% R.H. to $h \simeq 0.35$ and 100% R.H. to $h > 0.50$. Our results indicate, therefore, that below about 0.20 g water per g protein, only hemichrome is present in the film and above this value hemichrome can be converted to MetHb. Equal proportions of the two derivatives are observed for a degree of hydration of about 0.35 g water per g protein and hemichrome is present even at values above about 0.50 g water per g protein.

3.2. Kinetics of the MetHb-hemichrome transition

The transition from one derivative to the other was investigated following the changes in absorbance at a chosen wavelength. The hydration and dehydration processes were initiated by transferring the humid film (100% R.H.) to a dry environment (P_2O_5), or a dried film (in P_2O_5) to a humid environment (100% R.H.).

Figs. 4a and 5a show the change in absorbance at 540 nm as a function of time for the dehydration and hydration processes, respectively. It is observed that 50% of the transition occurs during the first minute for dehydration, while about 10 min are necessary for 50% of the transition in the time course of hydration. Figs. 4b and 5b show plots vs. time of the ratio $[A(t) - A(\infty)]/[A(0) - A(\infty)]$ obtained from figs. 4a and 5a, respectively. A sum of two exponentials was fitted to these data and the transition rates obtained are given in the legends to figs. 4 and 5.

These results are typical of two independent conversion processes, reflecting the existence of either two distinct Hb populations or an inter-

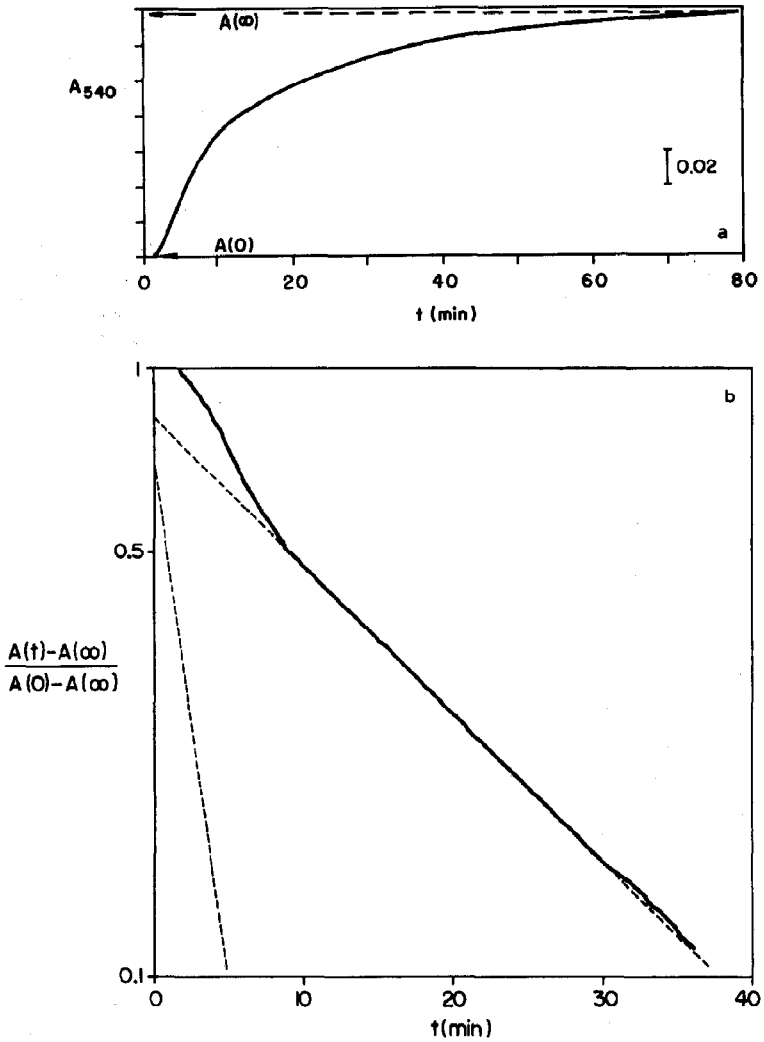

Fig. 5. Rehydration process: change in absorbance at 540 nm for a Hb film as a function of rehydration time on (a) linear and (b) logarithmic scales. The lines in panel b were fitted to the experimental points and correspond to rates of 0.05 and 0.47 min^{-1}.

mediate state in the MetHb-hemichrome transition. The latter possibility, however, is inconsistent with the sorption/desorption experiments that indicate only two states and no intermediate.

4. Discussion

Our results on sorption/desorption are indicative of a reversible MetHb-hemichrome transition. A reversible process was also observed for the deoxyHb-hemochrome transition, deoxyHb retaining its oxygen-binding ability after rehydration [24]. Thus, the dehydration-induced conformational state cannot be considered as a denatured derivative of Hb, but, rather, represents a thermodynamically accessible conformation of Hb. Our results suggest, therefore, that Hb dehydration induces a reversible conformational change at the distal side of the heme group that facilitates binding of the distal His to the heme iron. This view is in accordance with Raman scattering stud-

HbCO indicating that dehydration effects on the Hb conformation are localized main at the distal side of the heme group [25]. Preliminary results with horse and sperm whale myoglobins (Mb) also suggest a bis-His conformation at low degrees of hydration [24].

The picture that is emerging is that dehydration induces a structural state in which the imidazole-N of His E7 is sterically accessible for binding to the iron ion, competing with the ligand for the sixth coordination position. Data from photoacoustic spectroscopy agree with this interpretation, since they show that the conversion to the bis-His conformation induced by lyophilization is of smaller extent for ligands with higher affinity for Hb in solution [26].

The critical hydration values obtained from fig. 3 indicate that the Hb structure is sensitive to perturbations in the water at high hydration levels such as 0.50 g water per g protein. This value is much greater than those obtained using different techniques for determining the 'water of hydration' for this protein, namely, in the range 0.32–0.42 g water per g Hb [27,28]. We believe that such procedures underestimate the real degree of hydration, since they are based on the freezing of water. DSC data show that during a slow freezing process, the hydration water can be transformed partially into crystalline ice [29], therefore, part of the water of hydration is also considered as being in the 'free' state. This also explains the formation of bis-His observed during the slow freezing of Met and deoxy solutions of Hb and Mb [17,30,31].

Several lines of evidence from spectroscopy such as optical absorption [24,32], infrared absorption [33], Raman scattering [25] and EPR [34] suggest that at high hydration levels the hemoproteins exhibit two conformations, one of which is the solution conformer and the other the conformer observed in the dehydrated state. These results, therefore, are in agreement with our observations at high water contents, and corroborate the idea that at these hydration levels the conformational dynamics of the protein begin to occur on a slower scale, allowing the detection of conformers that are not observed in solution.

Concerning the low hydration limit, $h = 0.20$ corresponds approximately to the amount of water necessary to saturate the charged and polar groups in the protein [27]. The hemichrome-MetHb transition therefore occurs only when the electrostatic effects are balanced, an essential condition for attaining the solution conformation of the protein.

The dehydration and hydration kinetics shown in figs. 4 and 5 demonstrate biphasic behavior which may be indicative of the existence of either an intermediate in the MetHb-hemichrome transition or two structurally distinct Hb populations. The presence of isosbestic points in our previous spectra suggests that only two chromophores are involved in the transition. Therefore, there are probably two distinct MetHb populations that undergo conversion to hemichrome at different rates. Preliminary results indicate monophasic kinetics for the Met to hemichrome transition in Mb [24]. It is reasonable to suggest, therefore, that the two populations in Hb originate from its α- and β-chains, although the possibility of some other heterogeneity in the protein cannot be excluded. Consequently, in such a case, the two forms of kinetics indicate different rates of transition from the α- and β-chains of MetHb to the hemichrome derivative.

Studies with isolated ferric α- and β-chains indicate a rate of spontaneous conversion to hemichrome that is 20-times faster for α- than for β-chains [22]. This value is of the same order as those observed in our studies for the two forms of kinetics. Analysis of the MetHb heme pocket structure indicates that Val-11 is closer to the water coordinated to the iron in the β-chains as compared to the α-chains [35], which may explain the ease with which the β-chains undergo reduction compared to the α-chains. Taking all of the results together, it is highly tempting to relate the two observed populations to the existence of two distinct chains of Hb, although conclusive evidence remains to be obtained.

Acknowledgements

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References

1 P.H. Yang and J.A. Rupley, Biochemistry 18 (1979) 2654.
2 J.A. Rupley, E. Gratton and G. Careri, Trends Biochem. Sci. 8 (1983) 18.
3 J.L. Finney and P.L. Poole, Comments Mol. Cell. Biophys. 2 (1984) 129.
4 R.H. Austin, K. Beeson, L. Eisenstein, H. Frauenfelder, I. C. Gunsalus and V.P. Marshall, Phys. Rev. Lett. 32 (1974) 403.
5 A. Ansari, J. Beredzen, D. Braunstein, B.R. Cowen, H. Frauenfelder, M.K. Hong, I.E.T. Iben, J.B. Johnson, P. Ormos, T.B. Sauke, R. Scholl, A. Schulte, P.J. Steinbach, J. Vittitow and R.D. Young, Biophys. Chem. 26 (1987) 337.
6 D. Keilin and E.F. Hartree, Nature 170 (1952) 161.
7 G.C. Papaefthymiou, B.H. Huynh, C.S. Yen, J.L. Groves and C.S. Wu, J. Chem. Phys. 62 (1975) 2995.
8 S. Maricic, G. Pifat and V. Pravidic, Ber. Bunsenges. Phys. Chem. 68 (1964) 787.
9 M.F. Perutz, E.J. Heidner, J.E. Ladner, J.G. Beetlestone, C. Ho and E.F. Slade, Biochemistry 13 (1974) 2187.
10 G.B. Ogunmola, A. Zipp, F. Chen and W. Kauzmann, Proc. Natl. Acad. Sci. U.S.A. 74 (1977) 1.
11 W.S. Caughey, W.Y. Fujimoto, A.J. Bearden and T.H. Moss, Biochemistry 5 (1966) 1255.
12 A. Zipp and W. Kauzmann, Biochemistry 12 (1972) 4217.
13 D.L. Drabkin and J.H. Austin, J. Biol. Chem. 112 (1935) 89.
14 E.A. Rachmilewitz, Sem. Hematol. 11 (1974) 441.
15 D.J. Bucher and W.D. Brown, Biochemistry 12 (1971) 4239.
16 T. Iuzuka and M. Kotani, Biochim. Biophys. Acta 194 (1969) 351.
17 H. Eicher, W. Bode and F. Parak, J. Chem. Phys. 64 (1976) 1446.
18 A. Riggs, Methods Enzymol. 76 (1981) 5.
19 M.W. Neal and J.R. Florini, Anal. Biochem. 55 (1981) 328.
20 M.J. Schneider and A.S. Schneider, J. Membrane Biol. 9 (1972) 127.
21 E. Antonini and M. Brunori, Hemoglobin and myoglobin in their reactions with ligands (North-Holland, Amsterdam, 1971).
22 E.A. Rachmilewitz, J. Peisach and W.E. Blumberg, J. Biol. Chem. 246 (1971) 3356.
23 G. Brausse, A. Mayer, T. Nedetzka, P. Schlecht and H. Vogel, J. Phys. Chem. 72 (1968) 3098.
24 M.F. Colombo, Ph.D. Thesis, Instituto de Física e Química de S. Carlos, Universidade de S. Paulo, Brazil (1988).
25 E.W. Findsen, P. Simons and M.R. Ondrias, Biochemistry 25 (1986) 7912.
26 G.M. Alter, J. Biol. Chem. 258 (1983) 14960.
27 I.D. Kuntz, Jr and W. Kauzmann, Adv. Protein Chem. 28 (1974) 239.
28 A.J. Hopfinger, Intermolecular interactions and biomolecular organizations (Wiley, New York, 1977).
29 W. Doster, A. Bachleitner, R. Dunau, M. Hiebl and E. Luscher, Biophys. J. 50 (1986) 213.
30 K. Gray and E.F. Slade, Biochem. Biophys. Res. Commun. 49 (1972) 1019.
31 A. Levy and J.M. Rifkind, Biochemistry 24 (1985) 6050.
32 S. Bohm and L.V. Abaturov, FEBS Lett. 77 (1977) 21.
33 W.E. Brown III, J.W. Sutcliffe and P.D. Pulsinelli, Biochemistry 22 (1983) 2914.
34 R. Sanches, Biochim. Biophys. Acta 955 (1988) 310.
35 J.T. Edsall and H.A. McKenzie, Adv. Biophys. 16 (1983) 53.