FEBS Letters 345 (1994) 154–158

FEBS 14040

Thermal stability of lipid-depleted purple membranes at neutral and low pH values

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Received 17 March 1994; revised version received 8 April 1994

Abstract

Differential scanning calorimetry was used to compare the thermal behavior of native and delipidated purple membrane fragments at pH values corresponding to purple, blue and acid-purple forms. At neutral pH, delipidation results in a 2.5- to 3-times increase in the cooperativity of the denaturational transition, accompanied by a minor increase in its temperature. At pH values below 5 the delipidated membranes exhibit considerably higher thermal stability than the native membranes. The reversible predenaturational transition observed in the native state is not detectable upon delipidation. There is no strict correlation between color changes upon acidification and deionization of either native or delipidated purple membranes and their thermal stability.

Key words: Purple membrane; Bacteriorhodopsin; Differential scanning calorimetry; Delipidation

1. Introduction

The molecules of the integral protein, bacteriorhodopsin (bR), are packed in clusters of three. These clusters form a two-dimensional hexagonal lattice with p3 symmetry of the purple membrane (PM) from *Halobacterium halobium* [1,2]. In the PM, neutral lipids constitute 7–9% of the lipids; the remaining are acidic lipids which consist exclusively of phytanyl analogues of phospholipids (mainly phosphatidylglycerophosphate) and glycolipids (mainly a sulfated triglycosylglycerol diether) [3,4]. A rather low ratio of 10–12 lipid molecules per 1 bR molecule is characteristic for native PM. The glycolipids are present only on the outer side of the membrane [5]. The exact topology of the other lipids in PM is not yet well specified. The purple membrane is associated with about 4–5 mol of Ca^{2+} and/or Mg^{2+} per mole bR in deionized water, bulk pH ~5.5 [6].

The purple color (absorption maximum at 568 nm) of the membranes at neutral pH values is due to the retinal chromophore covalently bound to a lysine residue (Lys-216) via a protonated Schiff base [7]. A number of factors, including pH, ion and lipid environment, can modulate the light-absorbing properties of the retinal chromophore. Acid titration to pH values between 1 and 3, as well as removal of the cations bound to PM, induce a reversible transition to the blue form (absorption maximum at 605 nm) [6,9,10]. Further acidification to pH values below 1 turns the blue form to an acid-purple form, indistinguishable by its absorption from the native purple form [10]. Removal of 75% of the lipids from the native PM shifts the pK of the purple-to-blue transition from values between 3.0 and 4.0 to 1.4 [12,13]. Also, the purple-to-blue transition is no longer cation dependent in delipidated PM [12]. Perturbations such as changes in pH, ion and lipid environment, have been established to influence the main function of bR, namely the light-driven transfer of protons. This function is strongly suppressed in delipidated PM [14] and fully inhibited in the blue species (both acid-induced and deionized) [6,9] and also in the acid-purple species [15].

The thermal behavior of bR in native PM is characterized by two prominent endothermic events, a reversible 'pretransition' at ca. 80°C, attributed to a structural change in the crystal lattice of the membrane, followed by an irreversible transition at ca. 100°C, associated with partial thermal unfolding of the membrane protein [16–23]. In the present study the thermal behavior of partially delipidated PM as a function of pH and bound cations removal has been compared to that of native PM. It has been established that delipidation strongly increases the cooperativity of the bR denaturation. The color changes upon acidification of native and delipidated PM do not correlate precisely with their thermal stability.

2. Materials and methods

PM from *Halobacterium halobium* were isolated according to the procedure described by Oesterhelt and Stoeckenius [24]. Protein concentration was determined spectrophotometrically using an extinction coefficient of $\varepsilon_{568} = 63{,}000\ M^{-1} \cdot cm^{-1}$ [7].

PM were delipidated by treatment with the zwiterionic bile salt 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) (Sigma) according to the procedure described by Szundi and Stoeckenius [12]. PM (10 mg) were incubated overnight with CHAPS (20 mM) in 5 ml 5 mM sodium acetate buffer (pH 5.4). The delipidation procedure was repeated three times. The delipidated samples were washed

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SSDI 0014-5793(94)00426-V

4–5 times with distilled water by centrifugation. Deionization of PM on a cation-exchange column (Dowex AG-50) was performed as described by Kimura et al. [10]. The pH of the suspension was adjusted with HCl or NaOH and measured with a Radelkis pH meter. Absorption spectra were recorded at room temperature using a Specord UV VIS spectrometer (Karl Zeiss, Jena).

Calorimetric measurements were performed using high-sensitivity DASM-1M and DASM-4 differential adiabatic scanning microcalorimeters [25]. Sample concentrations between 1 and 5 mg/ml were used. Runs were routinely made in the temperature range 20–100 or 20–120°C with heating rates of 0.5 or 1°C/min. No influence of the heating rate on the thermodynamic parameters was observed. Particular runs were started at 0°C, however, no thermal events were registered in the range 0–20°C. The thermograms were corrected for the instrumental baseline recorded after each scan. The temperature at the maximum of the excess heat capacity curve was indicated as the transition temperature T_m. The calorimetric enthalpy of the transition ΔH_{cal} was determined as the area under the excess heat capacity curve. The van't Hoff enthalpy, ΔH_{vH}, was calculated from the calorimetric data by using the relationship [26]:

$$\Delta H_{vH} = 4RT_m^2 C_p^{max}/\Delta H_{cal}$$

where C_p^{max} is the maximum excess heat capacity.

3. Results

3.1. Thermal behavior of lipid-depleted PM at neutral pH

DSC scans for native and delipidated PM at neutral pH are presented in Fig. 1. No thermal transitions were observed upon immediate repetition of these heating scans. The thermodynamic parameters (peak temperature, half-width and enthalpy) of the transitions, as determined from the thermograms, are given in Table 1. The parameters of both pretransition and denaturational transition of native PM agree well with the previously published data [16,17,19–21]. The denaturational transitions for native and delipidated PM samples are characterized by similar values of T_m (96.2°C and 97.5°C, respectively) and practically identical values of ΔH_{cal} (96.6 and 96.3 kcal/mol, respectively). A pronounced difference was observed in their cooperativity, however. The denaturational transition of delipidated PM is characterized by a 5–6 times smaller half-width than that of native PM (Figs. 1 and 2, Table 1). As a consequence of the transition narrowing, the ΔH_{vH} values strongly increase upon PM delipidation (Table 1). This results in an increase by several times of the ratio $\Delta H_{vH}/\Delta H_{cal}$, the so called 'cooperative unit'.

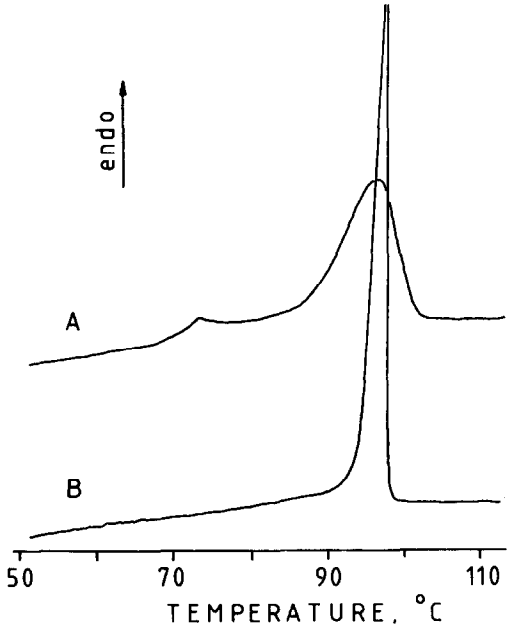


Fig. 1. DSC scans of native (A) and delipidated (B) PM at pH 6.4; bR concentration 5.55 mg/ml; heating rate 1°C/min.

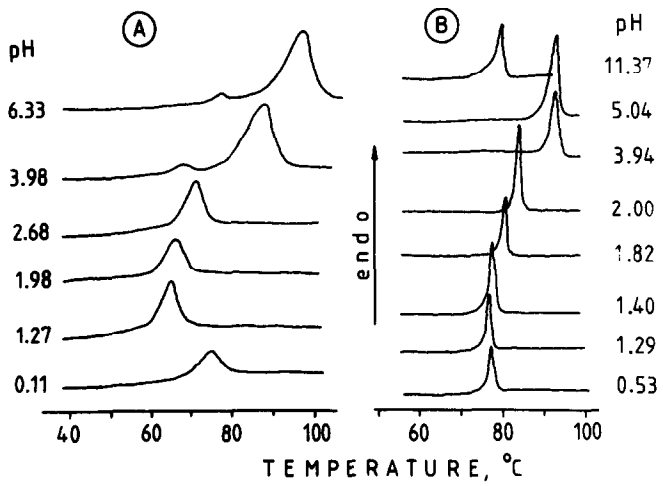


Fig. 2. DSC scans of native (A) and delipidated (B) PM at different pH values.

The predenaturational transition, clearly expressed in the thermograms of native PM, was not detectable after delipidation (Fig. 1).

3.2. Thermal stability of native and delipidated PM at low pH

Representative thermograms from native and delipidated PM samples at different pH values are shown in Fig. 2. The pH-dependencies of the thermal unfolding temperature and enthalpy are given in Fig. 3B,C and the numerical values are summarized in Table 1. Fig. 3A shows the pH-dependence of the absorption maximum of native and delipidated PM samples. The decrease in pH from 6 to 1.7 lowers the temperature of denaturation of bR in native PM by more than 30°C with a pK_a value between 3.0 and 3.5 (Fig. 3B). Further decreases in pH to values below 1 increase the temperature of the DSC endotherm, but not to that of the native purple state (Fig. 3B). The calorimetric enthalpy of the denaturational transition in native PM has lower values at acidic than at neutral pH (Fig. 3C). The temperature of the pretransition also decreases upon lowering of the pH to 4 (Fig. 3B; Table 1). At pH's below 3, pretransition is not observable on the thermograms of native PM (Fig. 2; Table 1). The color change from purple to blue takes place at

pH's between 4 and 3, and from blue to acid-purple at pH < 1.5 (Fig. 3A). While the characteristic pK_a value of the purple-to-blue color change (Fig. 3A; cf. $pK_a = 3.2$ reported by Oesterhelt and Stoeckenius [7]) is similar to that of the transition temperature drop, the latter occurs across a considerably wider pH range.

Higher thermal stability is characteristic for bR in delipidated PM – its denaturation takes place at higher temperature than in native PM in the whole pH range studied (Fig. 3B). The pH-dependence of the temperature of denaturation in delipidated PM follows a different pattern compared to native PM: first, the downward course of the T_m vs. pH curve starts at a considerably lower pH value (ca. 3) and has a pK_a value of 2.0–2.2, i.e. bR in delipidated PMs exhibits higher stability to acidification-induced denaturation; second, delipidation moderates the overall acidification-induced decrease in T_m from >30°C to ca. 20°C (Fig. 3B). The enthalpy of bR denaturation in delipidated PM is not considerably influenced by the acidification in the pH range from 6 to 2.2 but sharply decreases twice at pH's between 2.2 and 2.0 (Fig. 3C). The half-width of the denaturational transition of delipidated PM is considerably lower than that of native PM in the whole pH range studied and seems not to be systematically influenced by the change in pH, while that of native PM decreases twice in the pH range 3–1.2 and slightly increases upon further decreases in pH (Table 1). The color changes in delipidated PM follow the pattern described by Szundi and Stoeckenius [12,13]. The pK_a value of the purple-to-blue transition becomes ca. 1.5–1.6 (Fig. 3A).

It has been reported that increasing the pH above neutral has a similar effect on the thermal behavior of native PM as its lowering in the acid range, namely it provokes a decrease in the temperature and enthalpy of the denaturational transition [17]. Our control measurement shows that the same holds for delipidated PM: the thermal behavior of delipidated PM at pH 11.8 is practically identical to that at pH 1.6 (Fig. 2B, Table 1).

3.3. *Thermal behavior of native and delipidated PM after deionization*

DSC thermograms of deionized native and delipidated PM are shown in Fig. 4. The thermodynamic parameters of the unfolding transition in these thermograms are shown in Table 1. In agreement with previous DSC studies [19,20], removal of bound cations (Ca^{2+}, Mg^{2+}) from native PM results in a considerable reduction of both temperature and enthalpy of denaturation (Fig. 4A). Pretransition is not observable on the thermograms of deionized native PM. It is possible that it either disappears or merges with the main transition. The thermody-

Table 1
Thermodynamic parameters of the phase transitions in purple membranes

pH	Pretransition		Denaturational transition			
	T_m (°C)	ΔH_{cal} (kcal/mol)	T_m (°C)	$\Delta T_{1/2}$ (°C)	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)
Native purple membranes						
0.11	–	–	74.2	6.0	13.4	221.5
0.60	–	–	66.6	5.6	11.8	174.6
1.27	–	–	68.0	5.8	13.0	176.4
1.68	–	–	65.3	5.8	20.8	152.2
1.98	–	–	67.5	5.2	12.0	196.2
2.68	–	–	71.3	5.0	21.8	174.4
2.90	–	–	77.0	10.0	24.7	176.8
3.98	67.0	2.7	87.0	8.8	46.3	135.5
5.00	75.0	5.0	95.8	7.8	72.0	152.2
6.00	74.5	6.0	96.2	7.6	96.6	154.0
6.33	76.0	5.0	96.0	7.8	96.0	153.6
Delipidated purple membranes						
0.53	–	–	78.8	1.6	42.0	337.9
1.07	–	–	76.2	3.2	32.0	272.0
1.29	–	–	77.5	2.2	31.0	357.4
1.40	–	–	79.0	1.7	37.5	466.5
1.82	–	–	83.0	2.0	33.0	405.6
2.00	–	–	87.0	2.0	30.8	484.0
2.18	–	–	88.2	2.2	66.1	379.6
3.94	–	–	97.0	2.0	70.0	438.0
5.04	–	–	97.2	1.8	80.0	487.3
5.85	–	–	97.5	1.4	96.3	405.0
11.84	–	–	81.5	2.6	42.3	434.0
Deionized purple membranes						
4.60	–	–	69.8	7.0	34.0	105.4
Deionized delipidated purple membranes						
4.80	–	–	94.0	4.2	97.5	146.2

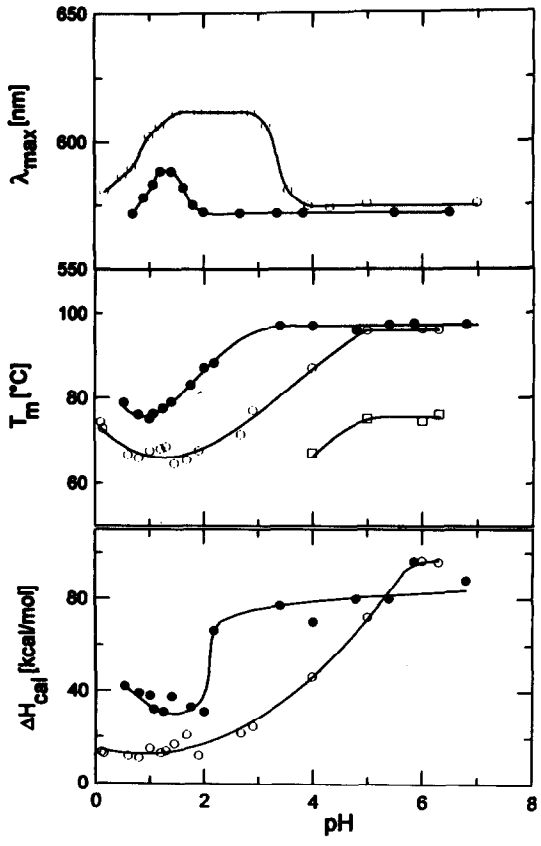


Fig. 3. pH dependencies of the absorption maximum (A), T_m (B), and ΔH_{cal} (C) of native PM (O, denaturational transition; □, pretransition) and delipidated PM (●).

namic parameters of the denaturational transition in delipidated PM appear to be practically cation independent (Fig. 4B, Table 1).

4. Discussion

4.1. Higher cooperativity of bR thermal unfolding in delipidated PM

From the present DSC data it is evident that, at neutral pH, bR in delipidated PM denatures at approximately the same temperature as in native PM and that the enthalpy of the protein unfolding is also not affected by lipid removal (Table 1). The major change in the thermal behavior of bR in delipidated membranes is observed in the shape of the denaturational transition (Fig. 1). The cooperative unit for this transition is increased between 2.5 and 3 times. Since the denaturating structural units in native PM have been supposed to be dimers and trimers [21,28], it follows from our data that cooperative units of 4–6 protein molecules should be assumed for the denaturation of delipidated PM.

Although it is stated that delipidation does not alter considerably the crystal lattice in PM [29], the pretransition, attributed to restructuring of the crystal lattice [16], is not detectable in delipidated PM. The lack of pretransition in delipidated PM could signify participation of the boundary lipid layer in the predenaturational transition. Since delipidation reduces the lipid: bR ratio and consequently the number of lipid–protein interactions, the pretransition appears to be largely controlled by these interactions. It is suggested also that during the pretransition a diminution in the protein intermolecular cooperativity takes place, i.e. the long-range interactions between the trimers are lost [17]. Thus, the disappearance of the pretransition from the thermograms may indicate that a tight intermolecular organization of protein persists up to ca. 100°C in delipidated PM, i.e. until the thermal unfolding takes place. This version satisfactorily rationalises the pronounced increase in the cooperativity of bR thermal unfolding in lipid-depleted PM.

4.2. Effect of pH and deionization on the thermal stability of native and delipidated PM

The thermodynamic characteristics of both pretransition and thermal denaturation of bR in native PM are strongly dependent on pH. The main transition takes place at lower temperatures and pretransition is not observable at pH <3, as in deionized-blue species. It has been demonstrated that pH and deionization also considerably modulate the light-absorbing properties of bR [6,9,10]. It has been suggested that the color of PM is primarily regulated by a negative charge near the protonated Schiff base, presumably provided by the unprotonated Asp-85 residue [8,11,27]. Its protonation (pK_a ~3.2) results in the formation of the blue membrane. An alternative explanation for the effect of pH infers a conformational change in the protein that indirectly regulates the color of PM, possibly by moving the counter ion away from the Schiff base [12,13]. Although a gross correlation between the thermal behavior and color change upon a decrease in pH exists, a considerable difference still remains. While the purple-to-blue color change takes place in the pH range 3.8–3, the decrease

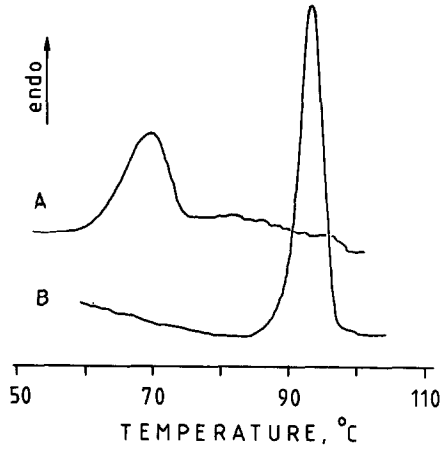


Fig. 4. DSC scans of deionized PM (A), and deionized and delipidated PM (B).

in the denaturation temperature and enthalpy occur over a much wider range of pH values, $1.5 < pH < 5$ (Fig. 3). This indicates that the bR destabilization upon a pH decrease cannot solely be accounted for by the Asp-85 protonation.

In delipidated PM the decrease in pH also destabilizes bR against thermal unfolding but this destabilization is considerably moderated by the lipid removal. Our results demonstrate that a decrease in the thermal stability of delipidated PM starts at lower pH values compared to native PM (Fig. 3B), i.e. delipidation stabilizes bR against pH-induced denaturation. In delipidated PM the decrease in pH also induces a color change from purple to blue. It is shifted to a lower pH and takes place in the range $1.4 < pH < 2$ (Fig. 3). Again, transition temperature decreases over a wider pH range ($1 < pH < 3$), reflecting larger-scale structural changes than the restructuring responsible for the color change. Interestingly, the enthalpy drop upon pH lowering is rather precipitous in the case of delipidated PM and does not coincide with pH with the color change (cf. $pK_a \sim 1.5$ for the purple-to-blue transition and $pK_a \sim 2.1$ for the enthalpy drop (Fig. 3)).

Similar to the pH decrease, removal of bound cations (Ca^{2+}, Mg^{2+}) from native PM considerably reduces both the temperature and the enthalpy of the denaturation (Fig. 4A, Table 1). The effects of cation removal and pH decrease are also similar with respect to color changes – both induce a purple-to-blue transition. By contrast the transition temperature and enthalpy of delipidated PM are not particularly sensitive to cation removal (Table 1). The purple-to-blue color change in this case has also been reported to be cation independent [12]. This behavior has been interpreted as reflecting an indirect role of the cations in the purple-to-blue transition, i.e. that in native membranes cations act mainly via raising the low surface pH generated by the acidic groups of the lipids [12,13]. Such an hypothesis attributes a considerable role to membrane lipids in cation binding.

References

[1] Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28–32.
[2] Blaurock, A.E. (1975) J. Mol. Biol. 93, 139–158.
[3] Kushwaha, S.C., Kates, M. and Martin, W.G. (1975) Can. J. Biochem. 53, 284–292.
[4] Kates, M., Kushwaha, S.C. and Sprott, G.D. (1982) Methods Enzymol. 88, 98–105.
[5] Henderson, R., Jubb, J.S. and Whytock, S. (1978) J. Mol. Biol. 123, 259–274.
[6] Chang, C.-H., Chen, J.G., Govindjee, R. and Ebrey, T. (1985) Proc. Natl. Acad. Sci. USA 82, 396–400.
[7] Oesterhelt, D. and Stoeckenius, W. (1971) Nature New Biol. 223, 149–152.
[8] Soppa, J., Otomo, J., Straub, J., Tittor, S., Meessen, S. and Oesterhelt, D. (1989) J. Biol. Chem. 264, 13049–13056.
[9] Mowery, P.C., Lozier, R.H., Chae, Q., Tseng, Y.-W., Taylor, M. and Stoeckenius, W. (1979) Biochemistry 18, 4100–4107.
[10] Kimura, Y., Ikegami, A. and Stoeckenius, W. (1984) Photochem. Photobiol. 40, 641–646.
[11] Jonas, R., Koutalos, Y. and Ebrey, T.G. (1990) Photochem. Photobiol. 52, 1163–1177.
[12] Szundi, I. and Stoeckenius, W. (1987) Proc. Natl. Acad. Sci. USA 84, 3681–3684.
[13] Szundi, I. and Stoeckenius, W. (1988) Biophys. J. 54, 227–232.
[14] Liu, S.Y., Govindjee, R. and Ebrey, T.G. (1990) Biophys. J. 57, 951–963.
[15] Chronister, E.L., Corcoran, T.C., Song, L. and El-Sayed, M.A. (1986) Proc. Natl. Acad. Sci. USA 83, 8580–8584.
[16] Jackson, M.B. and Sturtevant, J.N. (1978) Biochemistry 17, 911–915.
[17] Brouillette, C.G., Muccio, D.P. and Finney, T.K. (1987) Biochemistry 26, 7431–7438.
[18] Brouillette, C.G., McMichens, R.B., Stern, L.J. and Khorana, H.G. (1989) Proteins: Struct. Funct. Genet. 5, 38–46.
[19] Cladera, J., Galisteo, M.L., Duäach, M., Mateo, P.L. and Padrâs, E. (1988) Biochim. Biophys. Acta 943, 148–156.
[20] Kresheck, G.C., Lin, C.T., Williamson, L.N., Mason, W.R., Jang, D.-J. and El-Sayed, M.A. (1990) J. Photochem. Photobiol. B 7, 289–302.
[21] Kahn, T.W., Sturtevant, J.M. and Engelman, D.M. (1992) Biochemistry 31, 8829–8839.
[22] Shnyrov, V.L. and Mateo, P.L. (1993) FEBS Lett. 324, 237–240.
[23] Galisteo, M. and Sanchez-Ruiz, J.M. (1993) Eur. J. Biophys. 22, 25–30.
[24] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678.
[25] Privalov, P.L., Plotnikov, V.V. and Filimonov, V.V. (1975) J. Chem. Thermodyn. 7, 41–47.
[26] Jackson, W.M. and Brandts, J.F. (1970) Biochemistry 9, 2294–2301.
[27] Metz, G., Siebert, F. and Engelhard, M. (1992) FEBS Lett. 303, 237–241.
[28] Hiraki, K., Hamanaka, T., Mitsui, T. and Kito, Y. (1981) Biochim. Biophys. Acta 647, 18.
[29] Glaeser, R.M., Jubb, J.S. and Henderson, R. (1985) Biophys. J. 48, 775–780.