Stabilization of photosystem II reaction centers: influence of bile salt detergents and low pH

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Abstract Rapid deterioration of samples is a major obstacle in research on the isolated reaction center of photosystem II. Its stability was tested systematically using a wide range of detergents, varying pH and temperature. Stability and activity did not depend on ionic properties of detergents or on critical micellar concentration. However, both were significantly increased by bile salt detergents in the dark as well as in the light. Low pH (5.5) and low temperature further improved stability. The results suggest that in particular the zwitterionic bile salt detergent, CHAPS, in pH 5.5 buffers is a very useful detergent and even superior to dodecylmaltoside for work with photosystem II reaction centers.

Key words: Photosynthesis; Photosystem II; Reaction center; D1-D2-cytochrome b_{559} complex; Detergent; Stability

1. Introduction

The reaction center of photosystem II (PS II-RC) is believed to be similar to that of the purple bacteria, the structure of which is known from X-ray crystallography [1–5]. Similarities include homologies among the two main proteins (D1 and D2 vs. L and M, respectively), and the presence of two de-metallated tetrapyrroles, viz. pheophytin (Phe) and bacteriopheophytin (BPhe), respectively. However, there are significant differences in type and number of the cofactors (in bacterial RC 4 BChl/2 BPhe, in PS II-RC 6 Chl *a*/2 Phe *a*). Another difference has profound consequences for practical work: in contrast to bacterial RC, PS II-RC is highly labile and can be handled and investigated only under dim light at low temperature (on ice), and for rather limited times. Currently, there is no explanation for this instability at the molecular level. A contributing factor may be the lack of the plastoquinone cofactors and of the H-subunit, which both lead to decreased stability in bacterial RC. There is also evidence, from experiments conducted at different pHs, that hydrogen bonds play a major role in the stabilization of the complex [6].

Pigment exchange has been used in bacterial RC as a valuable tool to study their functions. When trying to adapt this technique to PS II-RC, their lability was a major obstacle. In bacterial RC pigment exchange is usually achieved by incubating the RC with a surplus of chemically modified pigment at nearly denaturing temperatures (approx. 41–43°C [7,8], but see [9]): these temperatures are unsuitable for work with PS II-RC. Some attempts to stabilize PS II-RC have been reviewed by Seibert [10] and include: detergent exchange [11], precipitation with polyethylene glycol [12] or the use of an oxygen-scrubbing system [12]. The most common way to stabilize PS II-RC depends on using an appropriate detergent. The positive effect of dodecylmaltoside (DM) is widely recognized in the preparation of PS II-RC; at the end of the isolation procedure it replaces the Triton X-100 (TX100) used for the solubilization of PS II-enriched thylakoids. However, to our knowledge no systematic study has yet been published on the influence of different types of detergents on RC stability.

We present such a comparative study, which surveys the most important detergent classes and includes the pH and temperature dependence of the stability of PS II-RC. Conditions are defined which stabilize this complex considerably more than those commonly used.

2. Materials and methods

PS II-RC were isolated from pea leaves using essentially the method of Braun et al. [13] and of Barber et al. [14] but with some modifications: for thylakoid solubilization, the Chl *a* concentration was adjusted to 1.7 mg/ml; PS II-enriched thylakoids were solubilized with 4.4% TX100; before applying to the DEAE column to isolate PS II-RC, NaCl was added (final concentration 30 mM); RC were eluted from the column with a gradient of 30–250 mM NaCl; a second, small DEAE column was used for RC concentration and detergent exchange; 400 mM NaCl and 0.03% DM was used to elute purified RC from the second DEAE column.

This protocol yields PS II-RC in Tris buffer (50 mM, pH 7.2) containing 0.03% DM and 400 mM NaCl. Aliquots of the preparations were frozen in liquid nitrogen and stored at −80°C.

To test the influence of detergents, PS II-RC were diluted to a Chl *a* concentration of approx. 4 μg/ml (A_{676} of approx. 0.5/cm) with Tris buffer (50 mM, pH 7.2) containing the respective detergent at its critical micellar concentration (CMC). RC were placed into the cold cuvette within the photometer. Then the cold buffer was added, the solution was mixed well with the pipette tip and the measurement was started. CMC data were obtained from information sheets provided by the suppliers; in case of differing values the highest was used. Temperature dependence of stability was tested in Tris buffer (50 mM, pH 7.2), pH dependence in Tris buffer (50 mM, pH 7.0), MES buffer (50 mM, pH 5.5) and Tricine buffer (50 mM, pH 8.5), each containing 0.03% DM. Measurements were performed as described above. If not otherwise stated, samples were placed into cuvette holders (thermostatted at 4°C) in a Perkin Elmer Lambda 2 spectrophotometer and spectra were recorded automatically at intervals of 30 min. For the screening, we used the empirical formula of Braun [6] to estimate the changes in the relative activities of the primary

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Abbreviations: %P680, spectroscopic screening parameter for the relative activity of the primary donor P680, according to [6]; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; Chl *a*, chlorophyll *a*; CMC, critical micellar concentration; CTAB, cetyltrimethylammoniumbromide; DM, dodecyl-β-D-maltoside; LDAO, lauryldimethylamine oxide; OG, n-octyl-β-D-glucopyranoside; Phe *a*, pheophytin *a*; PS II-RC, photosystem II reaction center = D1-D2-cyt b_{559} complex; RC, reaction center; SDS, sodium dodecyl sulfate; TX100, Triton X-100

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donor, P680 (expressed as %P680). It is calculated from the absorption values at 679 and 672 nm (A_{679} and A_{672}) (see Section 3).

Absorption spectra were recorded on a Lambda 2 spectrophotometer (Perkin-Elmer). RC purity was assayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel containing 6 M urea, and stained with Coomassie blue [14]. Photobleaching experiments were done with a TIDAS diode array spectrometer (J&M, Aalen, Germany) at room temperature. White actinic light was supplied by a cold light lamp (Schott KL1500 electronic). The light intensity at the cuvette surface was approximately 4700 μmol m^{-2} s^{-1}.

3. Results and discussion

3.1. Influence of detergent

Stability of membrane proteins, in particular of PS II-RC, depends strongly on the detergent(s) used for solubilization and storage. Because it is not yet possible to predict the best detergent for a given membrane protein, empirical testing is required as shown in Fig. 1, where the percentage of the activity of the primary donor (%P680) was plotted against incubation time. The %P680 values were determined using the following equation:

$$\%\mathrm{P680} = (2.38 \cdot A_{679}/A_{672} - 1.64) \cdot 100 \quad (1)$$

This formula was developed by Braun [6], whose data were confirmed independently by the transient fluorescence measurements of Booth et al. [15]. It was chosen here as an operational screening criterion to estimate changes in RC activity from spectral parameters.

From the results shown in Fig. 1, two parameters were derived. The first is the kinetics of the decrease of activity, the second is the apparent maximal activity immediately after addition of the detergent, expressed as %P680 at $t = 0$. High initial %P680 does not necessarily imply slow deactivation and vice versa.

The losses of apparent activity (%P680) were fitted to a single exponential where possible. Comparison of the time constants of decay of activity and of percentage of remaining RC activity (Table 1) does not necessarily suggest the use of the same detergent: this is probably due to the $\%\mathrm{P680}_\infty$ value in Eq. 2, which was used for fitting the data:

$$\%\mathrm{P680} = \%\mathrm{P680}_\infty + a_1 \cdot e^{-t/\tau_1} \quad (2)$$

where $\%\mathrm{P680}_\infty$ is a constant offset, a_1 the amplitude and τ_1 the time constant of the exponential. The biochemical significance of $\%\mathrm{P680}_\infty$ which greatly influences the appearance of the decay curves is unclear; nonetheless, irrespective of which of the parameters is used, the main conclusions of this paper remain the same.

While deactivation of the RC occurs in all detergents tested, the kinetics were very different, and this effect was reprodu-

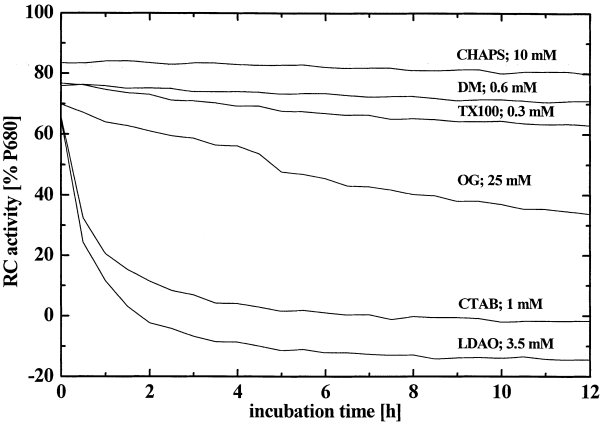

Fig. 1. Deactivation of PS II-RC with selected detergents (at 4°C). RC, solubilized by DM, were diluted into Tris buffer containing the respective detergent at its CMC. Activity was determined according to Braun [6]. For details see text.

Table 1
Activity decrease of PS II-RC after addition of selected detergents to a preparation in DM

Detergent	Concentration (mM)	Activity ($t = 0$ h) (%P680)	Activity ($t = 12$ h) (%P680)	Remaining activity after 12 h (%)	τ_1 (h)	τ_2 (h)
Deoxycholate	8	86	83	97	172	
CHAPS	10	84	80	95	211	
Digitonin	0.7	78	73	94	81.8	
Dodecylmaltoside	0.6	76	71	93	33.5	
Dodecylglucoside	0.2	81	74	92	7.65	
Cholate	15	86	79	91	11.6	
Tween 20	0.06	79	69	88	7.82	
Sucrose monocaprate	2.5	76	66	87	22.5	
Triton X-100	0.3	77	63	82	6.31	
Brij 58	0.95	71	50	70	0.74 (6)	11.2 (94)
Octylthioglucoside	9	78	54	69	5.07	
NP 40	0.3	71	49	68	8.1	
Mega 10	7	78	47	60	6.26	
Triton BG10	(0.2%)	67	40	60	0.35 (36)	8.29 (64)
Brij 35	0.1	76	43	57	11.6	
Decylglucoside	3	71	40	56	6.17	
Triton X-114	0.35	78	43	55	7.58	
Octylglucoside	25	70	34	48	10.9	
Deriphat 160	2.7	79	2	3	4.00	
SB 12	4	72	−1	0	0.51 (46)	4.33 (54)
LDAO	3.5	65	−14	0	0.42 (60)	2.15 (40)
CTAB	1	66	−2	0	0.38	
SDS	10	54	−8	0	0.37	

Detergents are listed in order of decreasing percentage of remaining activity after 12 h incubation. τ_1 and τ_2 are the time constants obtained by fitting experimental curves like those shown in Fig. 1 with mono- (if possible) or bi-exponential functions. Numbers in parentheses give the relative proportion of the respective amplitudes, a_1 and a_2.

cible in different RC samples. It is noteworthy, when comparing RC stability in the different detergents, that the method used relies on dilution and, therefore, a constant concentration of DM (0.03 mM/0.0015%) is always present, in addition to the test detergent. Therefore, results reflect not only the influence of the detergent added, but also the ability of the test detergent to either co-act with and/or to remove DM from PS II-RC. This was necessary for practical reasons: complete detergent exchange requires such long periods that a comparison of detergent effects becomes impossible, especially when deactivation occurs in less than 1 h. Since comparison of the stabilizing effects of various detergents was the main goal of this work, incomplete detergent exchange was unavoidable.

Further, the detergent concentrations had to be at their respective CMC values which vary with environmental conditions; for example, CMC values of ionic and zwitterionic detergents vary considerably with NaCl concentration [16] which is required for the elution of PS II-RC from the purification column. This variability in the ratio of test detergent to RC and DM could not be avoided. Nonetheless, considerable differences were found among the detergents tested, and our results are useful for the selection of appropriate detergents for use with PS II-RC.

The apparent maximal activity immediately after addition of the detergent, expressed as %P680 at $t = 0$, was less than 90% with all detergents used. This is somewhat lower than the 95–100% values obtained by Braun [6] and Booth et al. [15]. The RC used in this study meet the usual purity criteria, so the slightly lower activities probably do not arise from RC preparation procedures but rather from small photometric errors which greatly influence %P680 values. Some calculated values of %P680 were less than zero and are obviously insignificant: only %P680 values above 10% were regarded as significant [6]. The maximum %P680 value is a characteristic of the detergent used and, presumably, reflects specific effects of the detergents on the spectral properties (see below), and a changed equilibrium between active and inactive P680 which is arrived at during the setup of the experiment (approx. 1 min).

In this work representatives of all important detergent classes, including ionic, non-ionic and zwitterionic detergents were used. The effects of the different detergents are compared to TX100 and DM. The former was substantial in the first preparations and is still used for dissociation of PS II-enriched thylakoid particles. Since its destabilizing effect was soon recognized, it is replaced by DM in the later steps of the RC preparations. While the destabilizing effect of TX100 is well documented [11,13], it is only moderately so if compared with some other detergents. In particular, SDS, CTAB, SB12 and LDAO lead to almost complete inactivation within less than 2 h. Because of the significant deactivating effect of LDAO it is remarkable that this detergent is so widely used in studies on bacterial RC [17]. Deriphat 160, used for non-denaturing gels of antenna systems and of PS II-RC [6], also shows strong destabilizing behavior. The denaturing effect of sucrose monocaprate, already used in PS II-RC work [18], is similar to that of TX100 and to the members of the Mega, Tween and Brij series which were tested here. Remarkably, the kinetics of deactivation measured in DM are not as different from those in TX100 as anticipated. It has been generally accepted that DM is much less detrimental for PS II-RC, and relatively stable RC could be isolated omitting the use of TX100 altogether, by using DM in combination with the chaotrope $LiClO_4$ [19–21]. This result is in part due to the concentration of TX100 employed here (0.02%), which is much lower than that used in most isolation protocols (0.2%): with TX100 concentrations of 0.1% and 0.2%, the activity of P680 remaining after 12 h incubation is only 54% and 32%, respectively, which clearly shows that the detrimental effect of TX100 is concentration-dependent.

Another unexpected result was the relatively rapid deactivation of PS II-RC in the presence of the non-ionic detergent, β-octylglucoside (OG), which is usually regarded as a mild detergent: a 35 mM solution was used for the isolation of PS II-RC by sucrose density gradient centrifugation [22]. Dodecylglucoside is much better and stabilizes PS II-RC almost as much as DM.

It is clearly shown that PS II-RC is highly stable with the bile salt detergents cholate, deoxycholate, CHAPS and digitonin (Fig. 2): the stabilizing effect of digitonin, reported previously [23], is even exceeded by most of the other members of this group. The positive effect of deoxycholate for bacterial RC has been previously noted [24]: the ESR signals of RC in deoxycholate buffers were most similar to those occurring in the native membrane (chromatophores). All bile salt detergents tested showed strong stabilizing properties, suggesting that this effect is due to the specific features of the bile salt structure rather than to ionic properties. Bile salt detergents form micelles with a low aggregation number, and their polycyclic hydrophobic moiety is very rigid. Possibly, the insertion of the rigid and relatively large polycyclic detergent system between proteins is energetically unfavorable and, therefore, does not cause dissociation. Womack et al. [25] suggested that the rigid structure of bile salt detergents prevents them adapting to the surface of proteins to cause dissociation or denaturation; however, detergents which contain a long aliphatic chain and only a small hydrophilic group (e.g. LDAO, CTAB or SDS) consistently show a pronounced destabilization. On the other hand, a long hydrophobic tail in the carbohydrate group of detergents leads to increased stabilizing properties, as can be seen when comparing the measurements in octyl-, decyl- and dodecylglucoside. A bigger or longer hydrophilic moiety also seems to increase stability, but this effect is less pronounced (see e.g. dodecylmaltoside vs. dode-

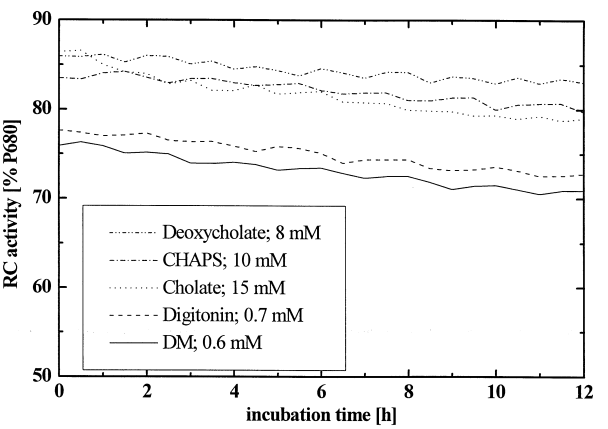

Fig. 2. Deactivation of PS II-RC with bile salt detergents (at 4°C). The *y*-scale has been expanded, relative to Fig. 1, to note small changes.

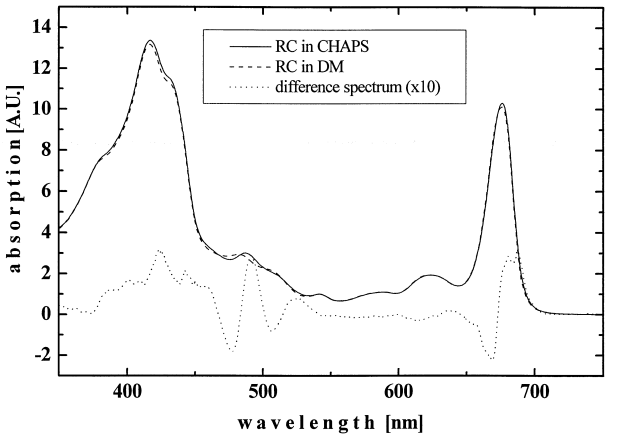

Fig. 3. Absorption spectra of PS II-RC in Tris buffer (50 mM, pH 7.2) containing 0.03% DM or 0.62% CHAPS and the difference spectrum (10-fold expanded). Spectra were normalized at the Phe *a* Q_x band.

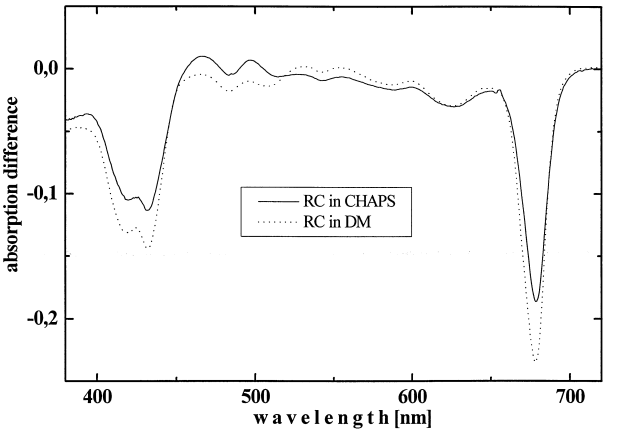

Fig. 5. Difference spectra of PS II-RC in CHAPS and DM before and after 4 min illumination. Chl *a* concentration was approx. 3.5 μg/ml (A_{676} = 0.40/cm).

cylglucoside or Triton X-100 vs. Triton X-114). Introduction of a sulfur atom, as in octylthioglucoside, also leads to increased stabilization.

With the bile salt detergents the time course of deactivation is very slow but, more importantly, the RC activity at the beginning of the experiment is also considerably higher than in all other detergents. Since such changes can be brought about already by a red shift of the Q_y band of as little as 0.5 nm (Fig. 3), this may in part relate to a spectral rather than a functional detergent effect. Fig. 4 indicates, however, that increased activity in the detergent we focused on, viz. CHAPS, is not an artefact of the formula used; it corresponds to higher activity measured by light-induced accumulation of Phe a^-. The higher activity of PS II-RC in CHAPS solution is also reflected by a lower fluorescence quantum yield (approximately 60% of that in DM) and by a smaller rate of photobleaching with white light (approximately 80% of that in DM; see Fig. 5).

The comparison of the detergents tested shows that there is no consistent effect of detergent charge on PS II-RC stability. Both anionic SDS and cationic CTAB are strongly destabilizing, but anionic deoxycholate was the most stabilizing of all detergents tested. Similarly CHAPS and SB12 are both zwitterionic, but show very different behavior. CMC size does not influence PS II-RC stability; for example, both CHAPS and SDS have a CMC of about 10 mM, but have very different effects on PS II-RC. It has been suggested that detergents with high CMC values are best suited for solubilization [26], and that those with low CMC should be useful for storage [27]. The latter suggestion is not true for PS II-RC, where both CHAPS and cholate, despite their high CMC, show very good properties for storage (Fig. 6). All assertions related to CMC values should be taken with caution [28], but our stated exceptions to published suggestions are clearly beyond any inaccuracy of the CMC values and their changes with environmental conditions.

Our results suggest that bile salts are by far the best detergents for use with PS II-RC and even distinctly better than the commonly used DM. Because the stability is also increased at

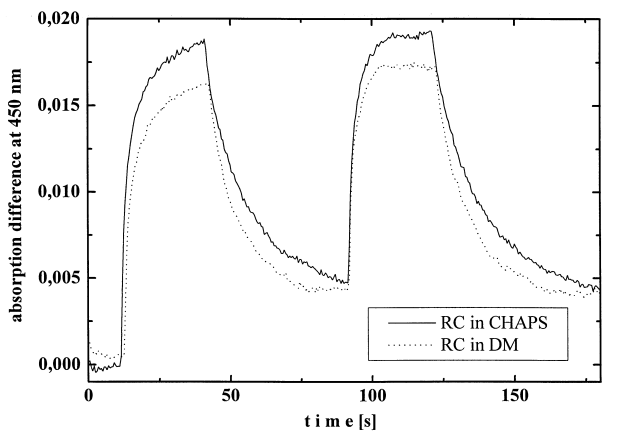

Fig. 4. Photochemical activity (light-induced accumulation of Phe a^-) of PS II-RC in Tris buffer (50 mM, pH 7.2) containing 0.03% DM or 0.62% CHAPS. Both samples were from the same batch. Chl *a* concentration was approx. 3.7 μg/ml (A_{676} = 0.43/cm).

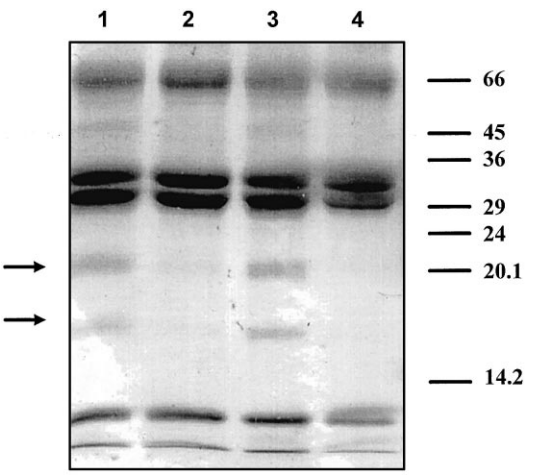

Fig. 6. SDS gel of PS II-RC that were stored for 10 months at −80°C in Tris buffer (Chl *a* concentration approx. 61 μg/ml) containing 10 mM CHAPS (lane 2 and 4) or 0.6 mM DM (lane 1 and 3); samples (all derived from the same batch) where thawed, incubated on ice for 1 h and then frozen slowly at −20°C (lanes 1 and 2) or shock-frozen in liquid nitrogen (lanes 3 and 4); no cryoprotectant was added. RC were thawed again, mixed with sample buffer and put on the gel.

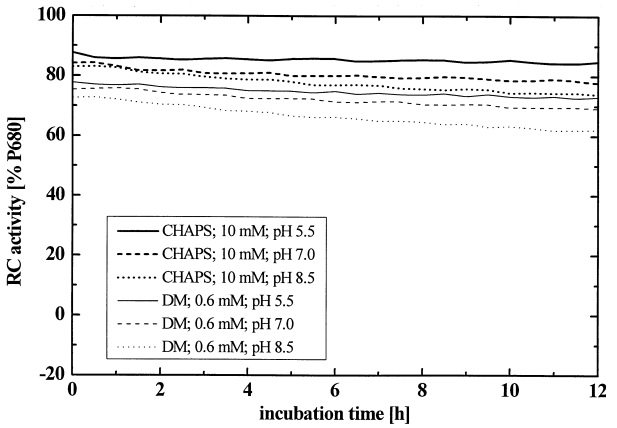

Fig. 7. pH dependence of PS II-RC activity (in CHAPS and DM at 4°C).

low pH values (see below, Fig. 7), we favor the use of CHAPS, since deoxycholate and cholate tend to precipitate at pH < 7.2 and < 6, respectively. CHAPS also proved superior to DM in experiments with PS II-RC under high hydrostatic pressure [29].

3.2. Influence of pH

The stability of proteins is highly dependent on pH since hydrogen bonds, which are essential for the secondary structure of most proteins, are very sensitive to pH change of the environment and concomitant protonations or deprotonations. At pH 5.5 PS II-RC activity decreased only slightly over a 12 h period, but activity decreased more rapidly at higher pH (Fig. 7). In this case, there is also a correlation with the %P680 value at the start of the experiment, which is highest at low pH. Obviously, there is again an increase in the %P680 value, which occurs in the period between mixing of RC and buffer and commencing measurements (approx. 1 min).

Changes in PS II-RC caused by pH were investigated by Braun [6]. Using CD and FT-IR spectroscopy, a significantly higher percentage of α-helical structure was demonstrated at low pH. Based on computer modeling, Braun suggested the presence of two hydrogen bonds in the interior of the complex between D1 and D2 with pK_a values of 7.8 and 5.8. Our measurements clearly support this observation and show that the pH effect is almost the same for the two detergents DM and CHAPS.

For the above reasons we suggest the use of pH 5.5 buffers, which is still higher than the isoelectric point of PS II-RC, viz. 4.9 [30]: this assures maximal stability of PS II-RC, but still allows the use of anion exchange materials for purification.

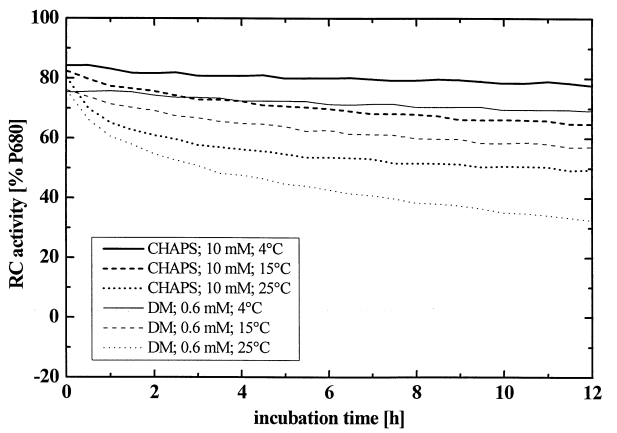

Fig. 8. Temperature-induced deactivation of PS II-RC (in CHAPS and DM at pH 7.2).

3.3. Stabilization with bile salts at higher temperatures

Pigment exchange experiments with bacterial RC depend on the use of higher temperatures (41–43°C). Therefore, knowledge of the dependence of stability of PS II-RC on temperature is essential. Whereas the complex is fairly stable in the presence of an appropriate detergent at 4°C, the activity drops rapidly at higher temperatures, in both DM and CHAPS solutions (Fig. 8). However, stability in CHAPS is again higher than in DM, especially at 25°C. At higher temperature, most deactivation occurs in the first 2 h, which is the period required for pigment exchange in bacterial RC. The processes occurring during deactivation at higher temperature are still poorly understood, but Braun [6] suggested that increased temperature increases the distance between the D1 and D2 proteins, changing the geometry of P680, and eventually leading to the observed changes in spectrum and activity. Loss of secondary structure in the D1 and D2 proteins is expected to occur only at much higher temperatures of 52°C and 51°C, respectively [31].

3.4. Concluding remarks

The results show that there is no detergent which allows PS II-RC storage without decrease in activity: the kinetics of the decrease vary considerably. Even in RC preparations containing the most stabilizing detergents, i.e. bile salts, there is a slow but steady decrease in activity. They also show that stability of PS II-RC can be significantly enhanced by the use of both CHAPS as detergent and buffers of about pH 5.5. While the stabilities of these preparations are still far from those known for purple bacterial RC, their improvement should be especially relevant to all methods relying on long storage or incubation periods (e.g. protein crystallography) or which use elevated temperature (e.g. pigment exchange).

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