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Claudio Nicolini [a,*], Victor Erokhin [b], Francesco Antolini [a], Paolo Catasti [a] and Paolo Facci [a]

[a] *Institute of Biophysics, University of Genova, Via Giotto 2, Genova Sestri Ponente, 16153 Genova (Italy) and* [b] *Technobiochip, Marciana, Livorno (Italy)*

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Key words: Langmuir-Blodgett film; Reaction center; CD; Thermal stability; Protein secondary structure

The temperature dependence of the secondary structure of photosynthetic reaction centres from *Rhodobacter sphaeroides* in solution and in Langmuir-Blodgett film was studied by circular dichroism. It was shown that the secondary structure of the protein was not affected in Langmuir-Blodgett films by heating up to 200°C, while in solution it was completely lost at 55°C. Molecular order rather than decreased hydration degree was held responsible.

Introduction

Secondary structure stabilization is necessary, albeit insufficient, for functional stabilization of proteins. This important goal is usually approached from the molecular dynamics and protein engineering point of view and requires much effort without guaranteed success. In the present work, we have tried another approach, namely exploiting the properties arising from arranging the proteins in closely packed two-dimensional arrays by means of the Langmuir-Blodgett (LB) technique. This is a promising technique for achieving molecular and biomolecular ordered structures and systems [1], because complicated molecular systems can be constructed in which individual molecules can be considered as functional units [2]. The technique has been successfully applied in the formation of lipid [2] and protein films [3]. In particular, the LB technique has been widely applied in the formation of films of the photosynthetic reaction centre (RC) [4–6]: a large membrane protein (three subunits) [7,8] which, in bacterial photosynthesis, provides light-induced transmembrane electron transport with 100% quantum efficiency [9]. This protein is interesting both from a fundamental and technological point of view, constituting an important sample for photo-electrical energy-converting systems and monoelectron transfer chains.

In the present work, we tried to determine by circular dichroism (CD) whether, and in which ways, the secondary structure of the protein (RC) is affected in LB film by heating up to 200°C and to compare these results with those obtained in solution.

Materials and Methods

Preparation of the RC solution

RC proteins from *Rhodobacter sphaeroides* were purified according to a standard procedure [10] in Moscow State University. The proteins were suspended in 3 mM phosphate buffer (pH 7.2) and 0.05% LDAO. RC concentration in solution was calculated by measuring the absorbance (Jasco 7800 UV/VIS spectrophotometer) at 802 nm (molar extinction coefficient = 288 $cm^{-1}\,mM^{-1}$; [11]), and was estimated to be 0.41 mg/ml. The molecular mass of the RC was assumed to be 93.5 kDa [12], disregarding the molecular mass of pigments, because the value was used for calculating molar ellipticity, which is affected only by chiral structure.

Formation and deposition of LB films

LB films were formed and studied in an LB trough (MM-MDT): the trough size is 240 × 100 mm and its volume is 300 ml; there are two barriers which compress the monolayer from two sides, resulting in improvement of the homogeneity of the monolayer in the central part where the deposition and the measurements of the surface pressure take place. This instru-

* Corresponding author. Fax: +39 10 6513106.

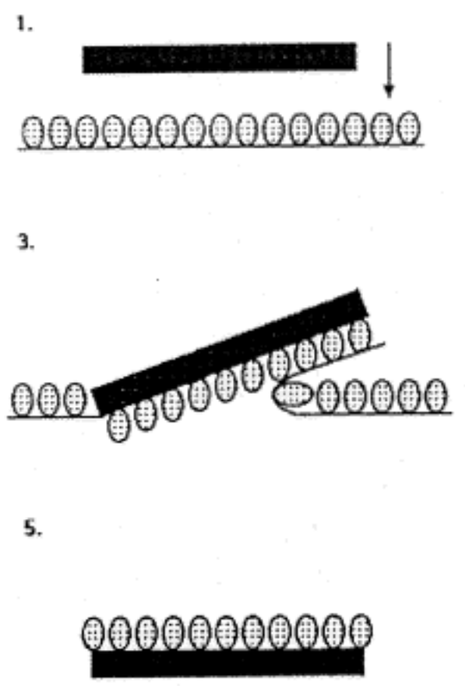

Fig. 1. Scheme of the horizontal lift (Langmuir-Schaefer) method of RC deposition.

ment is equipped with a Wilhelmy balance with a surface pressure sensitivity of 0.05 mN/m [13].

The water used for the subphase was purified by a Milli-Q system to a resistivity of 18 MΩ cm.

Films were transferred onto quartz substrates by the Langmuir-Schaefer method (horizontal lift) [14] at 35 mN/m surface pressure. The deposition procedure is illustrated in Fig. 1. As the RC is a membrane protein with two hydrophilic regions in opposite positions it is rational to suppose that, being spread on the water surface, the molecules form a monolayer in which one hydrophilic region is in the water and the opposite one is in the air [5]. After monolayer formation, a hydrophilic substrate horizontally touches the film. The best deposition is observed using substrates just treated with weak oxygen-containing plasma (very bad deposition takes place when hydrophobic substrates are used) [3]. Molecules attached to the substrate are closely packed and interact with their neighbours by hydrophobic bonding. Touching the film with the substrate results in a slight immersion into subphase of the part of RC monolayer under the substrate and in a consequent separation of this area from the initial layer. During the upward motion, some water is also transferred onto the substrate and some RC molecules, not connected with the substrate, are also transferred and can face the upper meniscus of water droplets on the substrate. To obtain a good deposited monolayer it is therefore necessary to remove the excess water and protein molecules not attached directly to the substrate surface (they are not regularly distributed and due to the drying process they will be adsorbed irregularly on the substrate). We use a nitrogen flow which removes the water droplets without allowing them to dry out on the substrate. This technique was shown to provide a good quality of RC film deposit [5]. The quality of the deposited films was checked by viewing the homogeneity of the interference colours on a silicon substrate after deposition of the RC multilayer film and by ellipsometry. The mass deposited was measured by a nanogravimetric technique using quartz resonators [13] and was found to be reliable in successive layer depositions.

Initially, hydrophobic regions of RC are surrounded by the detergent molecules. Once on the air/water interface, the detergent molecules leave the proteins and transfer to the water surface. Thus, just after spreading, a mixed monolayer of RC and detergent is formed.

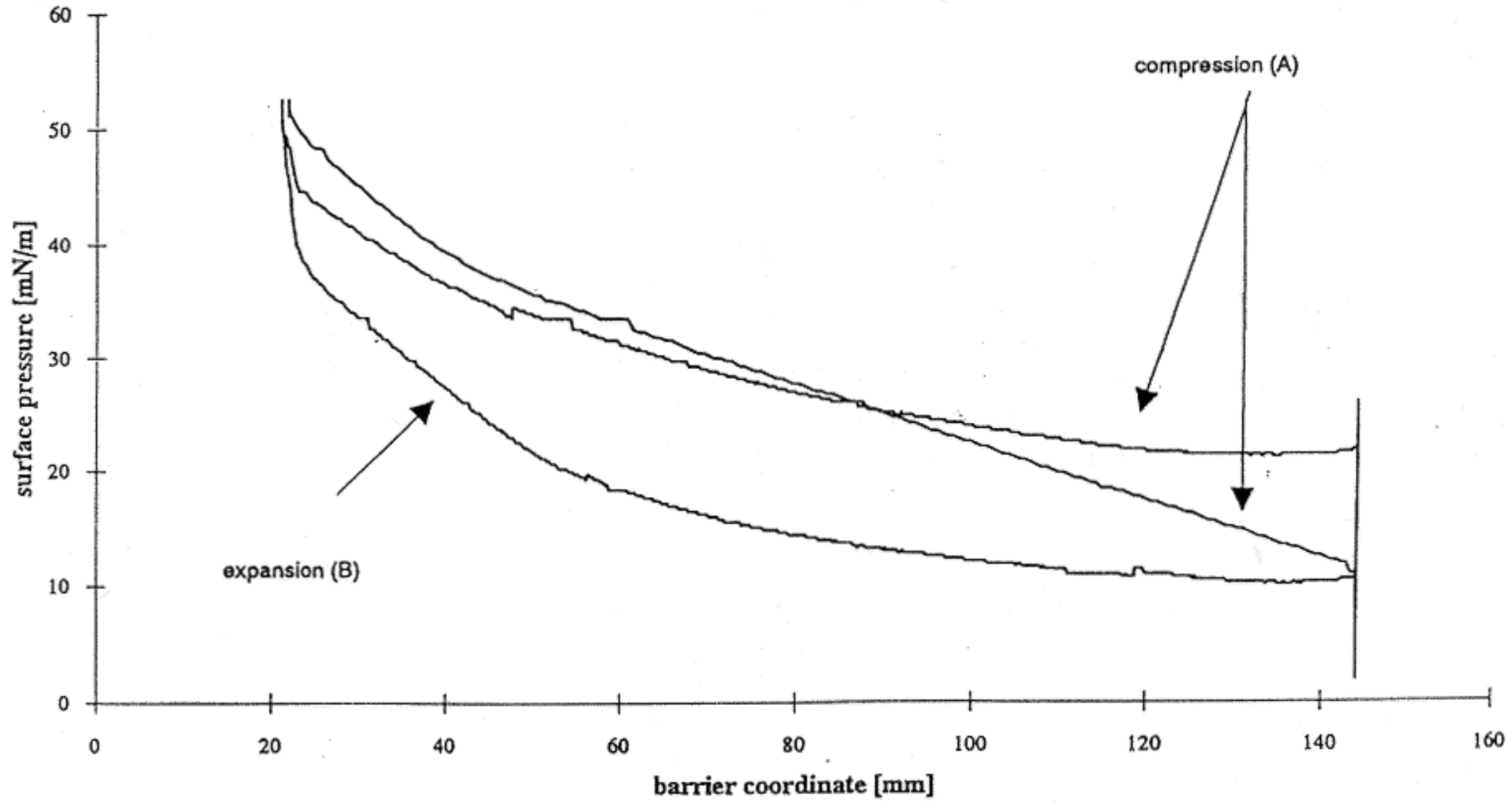

Fig. 2. π-A isotherm of RC monolayer. The picture shows two compression-expansion cycles.

The presence of detergent molecules resulted in irreproducibility of π-A isotherms during compression and expansion for the first two cycles. This behaviour is illustrated in Fig. 2. Initially the increase in the surface pressure is due not only to the presence of RC but also to detergent molecules. After compression to quite high pressures, the surface concentration of the detergent is decreased due to the formation of micro-collapses (the hydrocarbon chains of the detergent are too short to form stable monolayers at high surface pressure [15]) which can be included in the subphase volume in the shape of micelles [3]. Performing two compression-expansion cycles makes the isotherm reproducible; this behaviour is in good agreement with the literature [16], indicating improvement in the order and homogeneity of the monolayer. It is possible to assume that, after such action, it is mainly RC molecules that participate in monolayer formation.

The samples for CD measurements containing 18 monomolecular RC layers were deposited onto standard spectropolarimeter quartz cuvettes.

CD measurements

Measurements of the solution were carried out at different temperatures, by directly regulating the sample temperature inside the spectropolarimeter. To ensure that no renaturation took place, experiments were performed both at the denaturation temperature and after cooling the sample down to room temperature (data not shown).

The film samples were analyzed at various temperatures by heating them according to the following procedure: each sample was heated for 10 min at the desired temperature in an oven. After waiting for 10 min in order to come back to room temperature, samples were analyzed.

In all cases, a Jasco J-710 spectropolarimeter was used in the range 185–260 nm.

The calculation of molar ellipticity in the film was performed normalizing the absorbance of the films to the solution counterpart according to the following formulae:

$$K(\lambda) = \frac{A_{film}(\lambda) - [A_{film}(260) - A_{sol}(260)]}{A_{sol}(\lambda)}$$

$$= \frac{C_{film}(\lambda) \cdot 1_{film} \cdot \epsilon_{film}(\lambda)}{C_{sol}(\lambda) \cdot 1_{sol} \cdot \epsilon_{sol}(\lambda)} \quad (1)$$

$$K(\lambda) \cong \frac{C_{film}(\lambda) \cdot l_{film}}{C_{sol}(\lambda) \cdot l_{sol}} \quad (2)$$

$$K(\lambda) \cdot C_{sol}(\lambda) \cdot l_{sol} = C_{film}(\lambda) \cdot l_{film} \quad (3)$$

$$(\Omega) = \frac{\Theta_{obs} \cdot M_w}{10^4 \cdot n \cdot C_{film}(\lambda)} \cdot l_{film} \quad (4)$$

Where (Ω) is molar ellipticity (expressed in deg cm^2 dmol^{-1}), Ω_{obs} is the ellipticity observed by spectopolarimeter and n is the number of amino acids in the protein (850 in RC).

$A_{film}(\lambda)$ and $A_{sol}(\lambda)$ are the absorbance values obtained from absorption spectra. The absorbance ratio $K(\lambda)$ between films, measured at different temperatures, and in solution at room temperature, was calculated from the values of $A_{film}(\lambda)$ and $A_{sol}(\lambda)$ over the range 185 to 260 nm. Scattering due to RC film was minimized by subtracting an offset at 260 nm (offset =

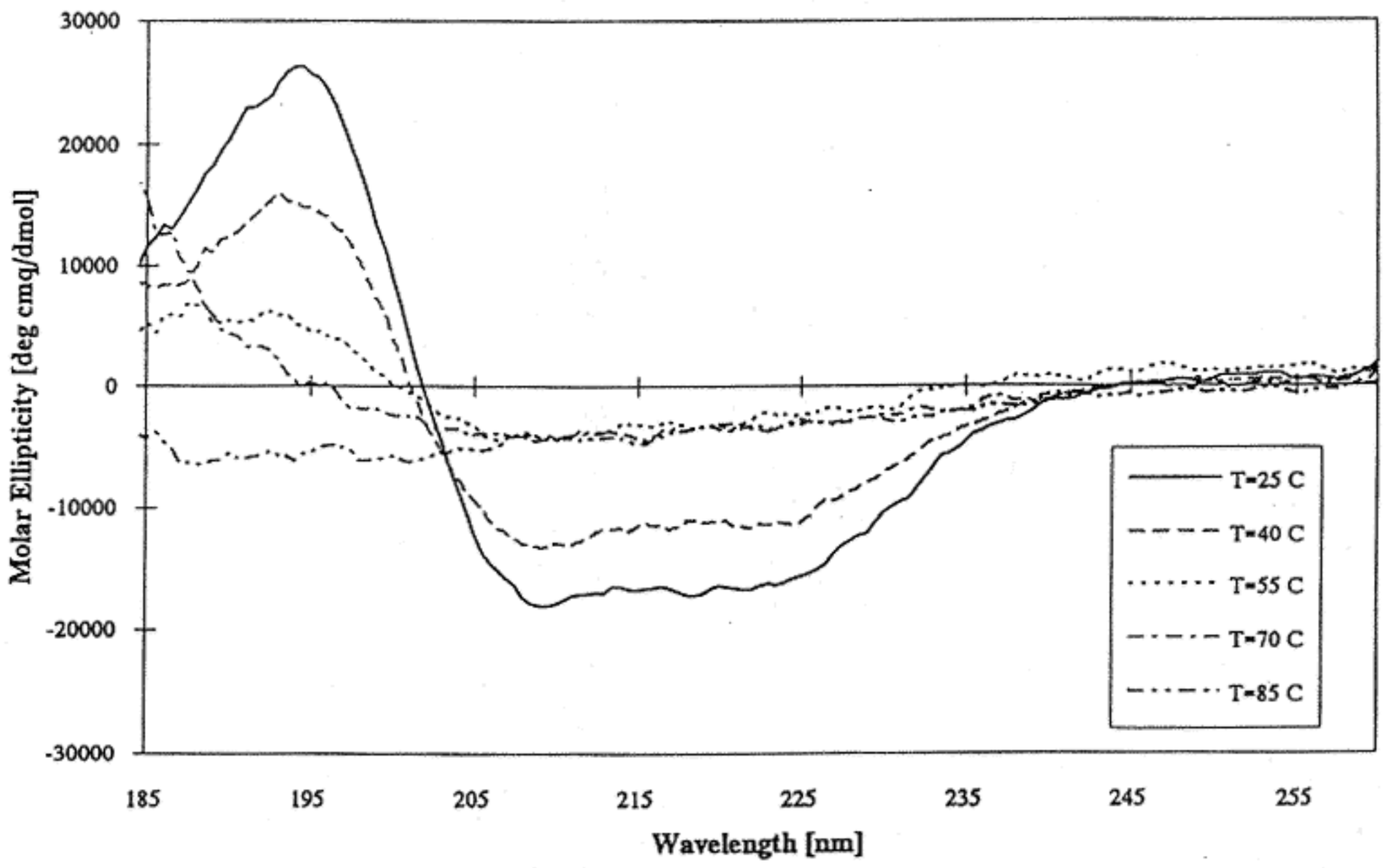

Fig. 3. CD spectra of RC in a solution at various temperatures. These spectra indicate that the secondary structure is practically lost at 55°C.

TABLE I

RC secondary structure at room temperature in solution and in LB film

Percentages of α-helix, β-sheet, β-turn and random coil determined from X-ray measurements were taken from Ref. 17. The coincidence of the data suggests that secondary structure in ordered layers is preserved.

Techniques and/or conditions	α helix (%)	β sheet (%)	coil (%) *
X-ray	51	15.6	33.4
CD in solution Room Temp.	54	11	35
CD in film (18 Layers) Room Temp. (+)	46 ± 5	14 ± 1	40 ± 5

(+) S.D. reflects the error in the estimation of the K factor (see Eqns. 1 and 2).
(*) This value is comprehensive of coil and turn.

$[A_{film}(260) - A_{sol}(260)]$, outside the absorption band of the peptide bonds (see Eqn. 1). Once the scattering in the range 190–230 nm is removed, the only absorbing chromophore is the peptide bond. Therefore, $K(\lambda)$ does not depend upon the extinction coefficient, and using Eqn. 2 for the different curves, we found an average value of $K_{max} = 2.2 \pm 0.2$ for the 18-layer film. From K_{max} it is possible to calculate the product $C_{film}(\lambda)l_{film}$ (see Eqn. 3) and to convert the CD spectra into molar ellipticity for secondary structure prediction purposes as indicated in Eqn. 4.

Results and Discussion

Initially, the temperature stability of RC secondary structure in solution was studied. Circular dichroism spectra of RC in solution at different temperatures are presented in the Fig. 3. The measurements were performed at 25, 40, 55, 70 and 85°C. From these data the percentage of α-helix, β-sheet, β-turn and random coil of RC was calculated. Results obtained at 25°C (Table I) are in good agreement with the data calculated from X-ray crystallography [17]. Increasing the temperature up to 70°C, a decrease in α-helix content and a corresponding increase in random coil and β-turn content (Table II), up to the point of complete denaturation, took place. The data also suggested an increase of β-sheet with temperature in the range from 25°C to 55°C.

TABLE II

Temperature dependence of RC secondary structure in solution

Percentage of α-helix, β-sheet and random coil calculated from CD spectra in solution of RC at different temperatures.

Temp. (c)	α helix (%)	β sheet (%)	coil (%) *
25	54	11	35
40	36	16	47
55	7	49	43
70	10	1	89

This value is comprehensive of coil and β-turn.

The thermal denaturation was shown to be irreversible: renaturation did not take place, since the CD spectra of the protein solution measured at 85°C–90°C and then after return to room temperature were identical (data not shown).

The spectra of RC LB films shown in Fig. 4 suggest that the solid LB organization of RC produces just slight modifications in their secondary structure. This fact may be due to the interactions of each RC protein

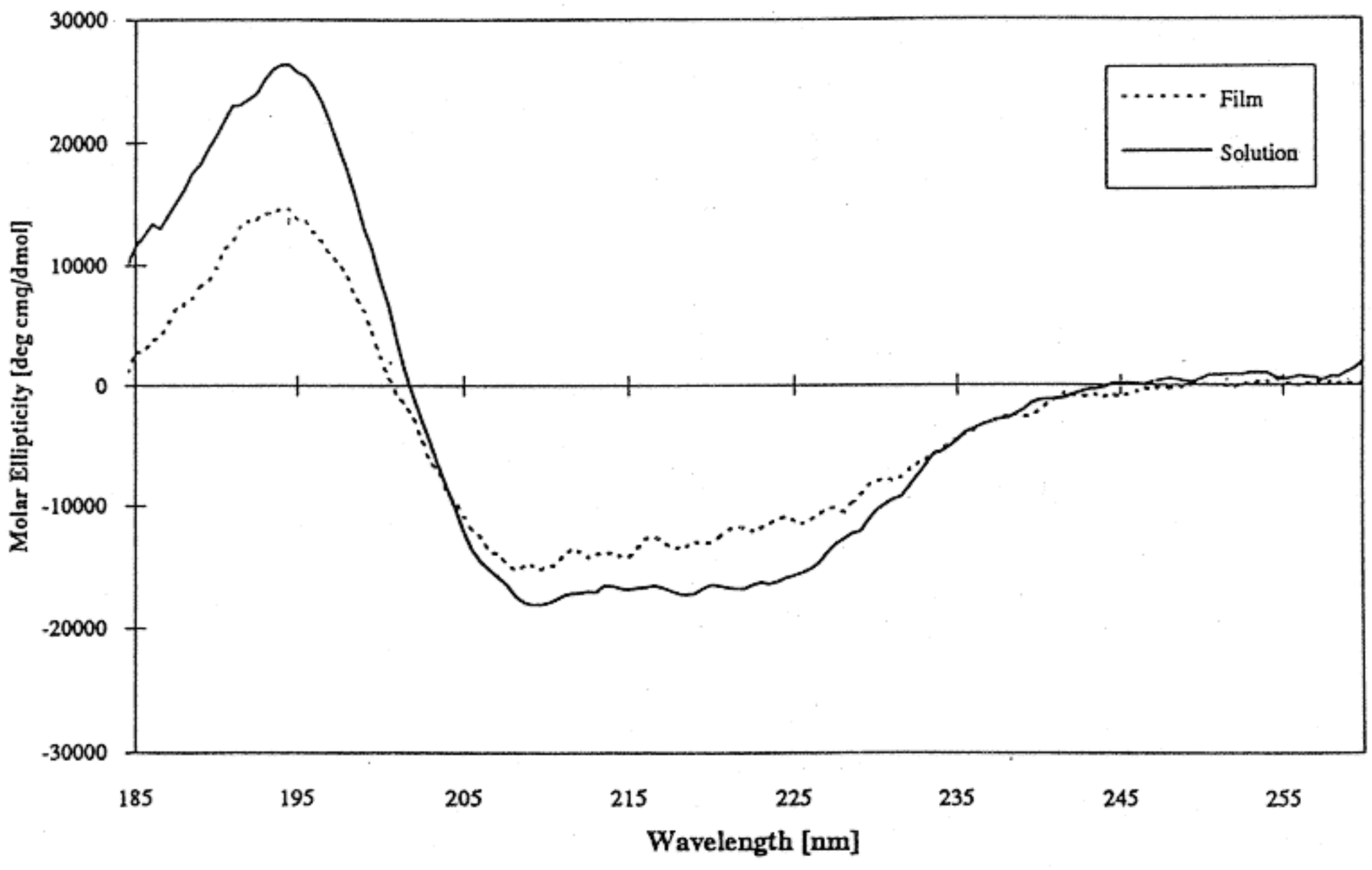

Fig. 4. Comparison between CD spectra of RC in solution and in LB film.

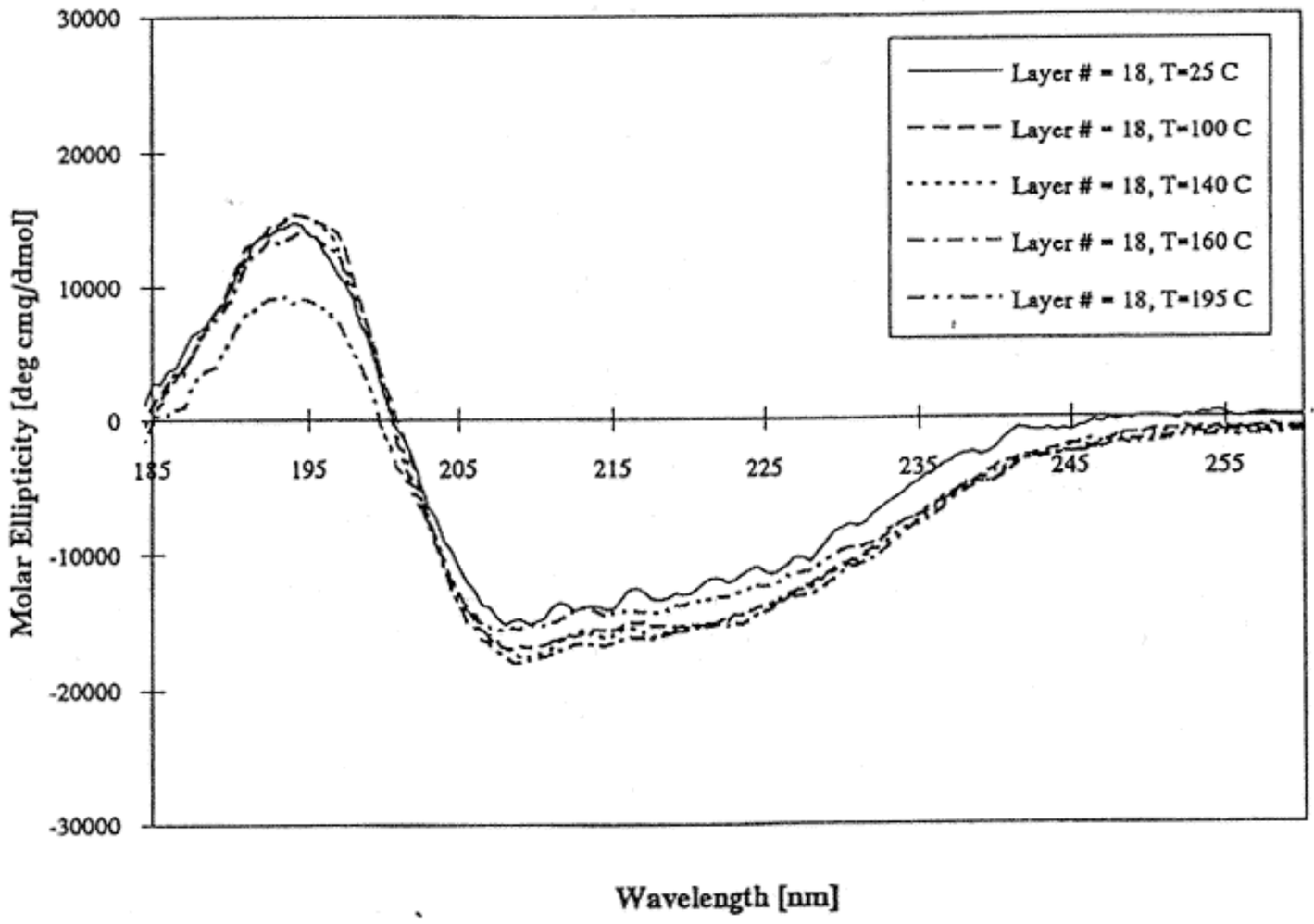

Fig. 5. CD spectra of RC in LB films at various temperatures. Heating of the sample (18 layers) was carried out using a Jouan (Apparecchi scientifici) heater. Each sample was placed into the heater for 10 min after the desired temperature was reached. The measurements were performed thereafter at room temperature.

with the surrounding ones and/or to the fact that in an LB film the RC proteins assume a preferential orientation, which is absent in the case of solution (random orientation). The calculations of α-helix, β-sheet and random coil percentage reported in Table I confirm that, at room temperature, the secondary structure of RC is the same in crystal, in solution and in LB film. Moreover, they also suggest that in the film the secondary structure of RC is preserved and the difference from solution can be explained as previously indicated.

CD spectra of RC in LB films (18 layers) at different temperatures are presented in Fig. 5. The resulting percentages of the secondary structure, calculated by a modified Hennessey-Johnson procedure [18], are shown in Table III. These data confirm the preservation of

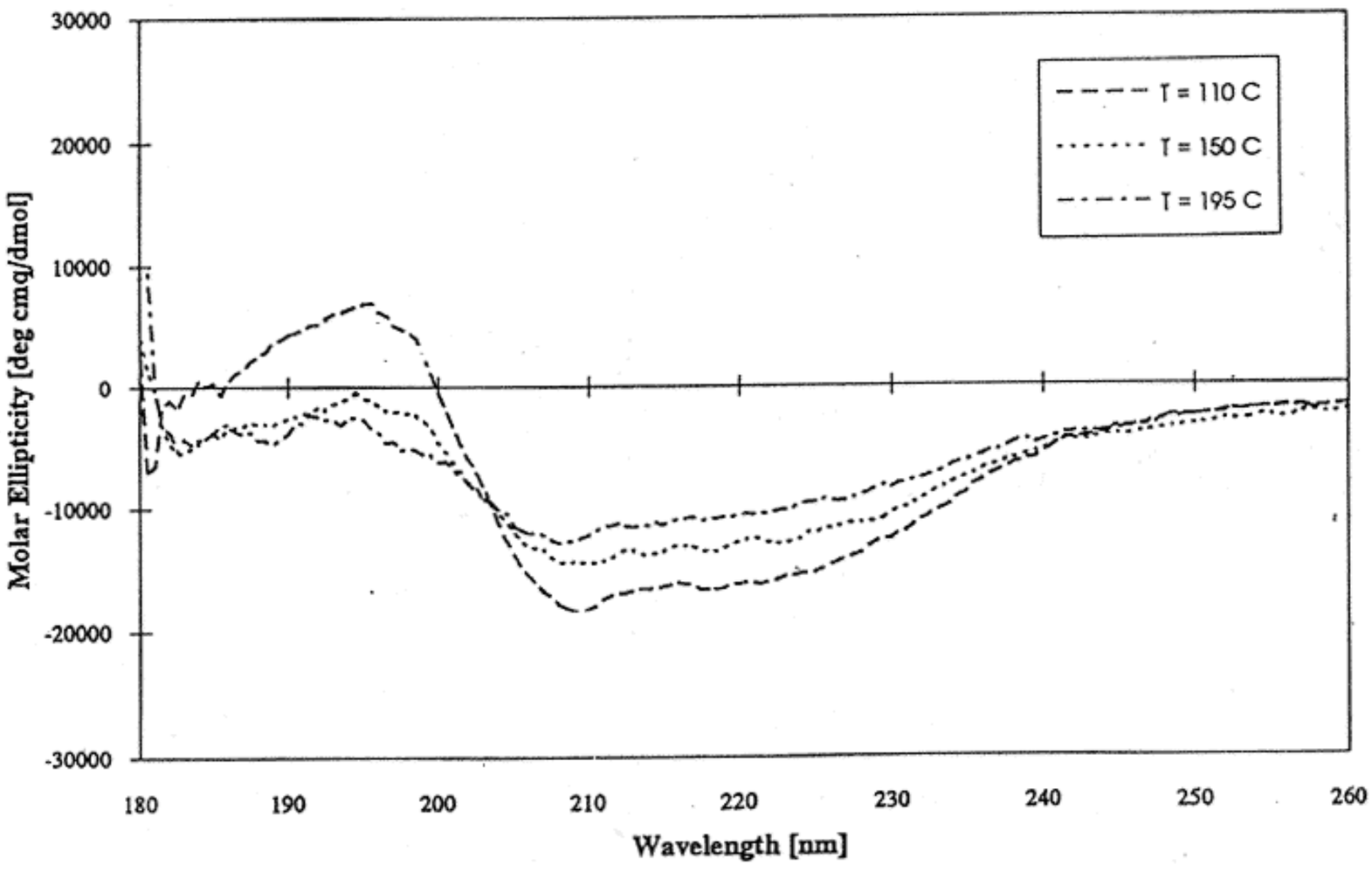

Fig. 6. CD spectra of RC film formed by spreading the protein solution over a quartz slide and by drying it for 4 h.

TABLE III

Temperature dependence of RC secondary structures in LB film

S.D. reflects the error in the estimation of the K factor.

T[C] (Layer#)	α-helix (%)	β-sheet (%)	β-turn (%)	coil (%) *
25 (18 Layers)	46 ± 5	14 ± 1	15 ± 2	25 ± 3
100 (18 Layers)	54 ± 5	12 ± 1	12 ± 1	23 ± 3
140 (18 Layers)	55 ± 5	12 ± 1	12 ± 1	22 ± 2
165 (18 Layers)	57 ± 5	10 ± 1	11 ± 2	22 ± 2
195 (18 Layers)	49 ± 5	12 ± 1	13 ± 1	25 ± 2

the secondary structure up to 160°C. At 195°C we can see only a slight decrease in the α-helix content, indicating that the unfolding process begins to take place.

Conversely, partial denaturation was observed already at 150°C in air-dried films, formed by spreading the protein solution onto a quartz slide and letting them dry out (Fig. 6). This test was performed in order to give an insight into the role of order with respect to dehydration in stabilization of secondary structure. The result shows that order plays a more important role than dehydration. Nevertheless, in this case we cannot completely neglect the possibility of partial order induced by the slow drying process. Even if the effect of the detergent on the protein secondary structure is not completely clear, it seems that the larger amount of detergent in the dried film with respect to the LB film should not play a very important role in structure stability, as most of the detergent molecules are attached to the hydrophobic area. This bound detergent is the 'natural' environment of RC in solution [10] and does not act to destroy protein structure.

It would thus appear that the stability of the RC secondary structure is strongly affected by the conditions under which the proteins are organized. In particular, the insertion of RC proteins into an LB film confers unique stability to their secondary structure, as a result of both specific intermolecular interactions and decreased hydration. This is evident also from the significant enhancement of α-helix stability in the air-dried samples (up to 110°C).

Conclusion

To summarize, LB appears to be an efficient and easy technique for stabilizing protein secondary structure, which is necessary but certainly not sufficient for the preservation of function.

The main stabilizing factor of secondary structure was found to be the order induced by LB film formation. Dehydration, being of course a stabilizing factor, too, in this case does not play a primary role, as was shown by the experiment on the dried sample. This is in agreement with other experiments on antibodies (a detailed description will be published elsewhere) where heating lyophilized IgG molecules resulted in their denaturation, contrary to what happened in the case of the LB film, where protein secondary structure was preserved together with antigen recognition properties.

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