BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **237,** 277–282 (1997)
ARTICLE NO. RC977131

Effect of Heat-Induced Structural Perturbation of Secondary and Tertiary Structures on the Chaperone Activity of α-Crystallin

Jiahn-Shing Lee,* Takanori Satoh,† Hiroshi Shinoda,† Tatsuya Samejima,† Shih-Hsiung Wu,‡ and Shyh-Horng Chiou‡,§,[1]

**Department of Ophthalmology, Chang-Gung Memorial Hospital, §Laboratory of Crystallin Research, Institute of Biochemical Sciences, National Taiwan University, and ‡Institute of Biological Chemistry, P.O. Box 23-106, Academia, Taipei, Taiwan; and †Department of Chemistry, College of Science and Engineering, Aoyama Gakuin University, Tokyo, Japan*

Received July 2, 1997

α-Crystallin, a major protein of the lens, is known to have chaperone activity to protect other proteins against thermal aggregation. Heat-induced structural change of α-crystallin was previously shown to increase its chaperone activity. In this report, we studied the thermal reversibility of α-crystallin and the effect of change in secondary structure on its chaperone function *in vitro*. The heat-induced conformational changes in the aromatic region of near-UV CD spectra showed only a small degree of reversibility. The structural transitions from 50 to 70°C were largely reversible if the incubation time was short. However, the protective ability to inhibit thermal aggregation of alcohol dehydrogenase by α-crystallin was essentially similar at 48 and 70°C. Under long-term heating at high temperatures, there was a time-dependent irreversibility of structural change in α-crystallin as revealed by CD spectroscopy. Such denatured α-crystallin by long-term heating can still preserve its ability to prevent UV-induced aggregation of γ-crystallin at room temperature, indicating relatively little effect of heat-induced changes in secondary structure on the chaperone activity of α-crystallin.

α-Crystallin of the ocular lens is a multimeric protein composed of two polypeptides, αA and αB subunits, that have considerable sequence homology and are held noncovalently to form aggregates ranging from 700 to more than 1000 kDa (1). This protein is believed to play an important role in the maintenance of lens transparency (2). α-Crystallin shows structural and functional similarities to small heat-shock proteins (3); similarly its expression can also be induced by thermal (4) and osmotic stress (5). *In vitro* studies of α-crystallin also indicate chaperone-like activity of this lens protein in preventing the aggregation of other proteins (6,7). However, the detailed molecular mechanism for its chaperone-like action remains unknown.

Due to its large size and no crystallographic data available, the detailed conformation of α-crystallin has not been established and various molecular packing models (8-12) for the association of α-crystallin subunits *in vivo* remain a matter of controversy. In the absence of high-resolution structural data, one way in which information could be obtained is through probing the environments of various amino acids or the location of hydrophobic areas (13). The other way is using circular dichroism (CD), Fourier-transform infrared (FT-IR) spectroscopy and/or differential scanning calorimetry (DSC) in conjunction, because these techniques are highly complementary (14). By these methods, it was shown that α-crystallin undergoes an irreversible conformational transition with a marked increase in surface hydrophobicity from 38 to 60°C (15) together with a loss of its native secondary structure between 50 and 70°C (16).

In our previous report (17) using CD spectroscopy, we have also found both secondary and tertiary structural changes in α-crystallin under thermal perturbation. Incubation of α-crystallin at 50°C for 3 h did not increase its molecular size, whereas 3 h-incubation at 60°C changed its Stokes radius from 85Å to 95Å. A kinetic-energy barrier may exist in α-crystallin that prevents its denaturation and aggregation at temperatures lower than 50°C. Under high temperatures, the thermal transition of α-crystallin was identified as a conformational change in tertiary structure (partial un-

[1] Corresponding address: S.-H. Chiou, Institute of Biological Chemistry, Academia Sinica, P.O. Box 23-106, Taipei, Taiwan. Fax: (886)-2-3635038. E-mail: shchiou@gate.sinica.edu.tw.

0006-291X/97 $25.00

folding) and quaternary structure of high-molecular-weight (HMW) aggregates, along with a loss of β-sheet backbone secondary structure (18). The heat-induced structural change in the secondary structure of α-crystallin, unlike most of other crystallins, is shown to be largely reversible (19).

On the other hand, it was demonstrated that the ability of α-crystallin to protect irradiation-induced aggregation of γ-crystallin is strongly temperature dependent (20). The short-term preheating of α-crystallin showed that the preheated crystallin seemed to have better ability than the native one to prevent dithiothreitol (DTT)-induced aggregation of insulin B chain (16,18), and to inhibit UV-induced aggregation of γ-crystallin (21) at room temperature. These results suggest that the conformational transitions induced by heating are probably involved in modulating the chaperone activity of α-crystallin. However which structural features of α-crystallin responsible for the chaperonin function of this crystallin is still not clear. In this report we have further studied the heat-induced denaturation and aggregation of α-crystallin by CD spectroscopy to examine the possible effect of heat-induced changes in secondary and tertiary structures on the chaperone activity of α-crystallin.

MATERIALS AND METHODS

Isolation of lens crystallins. Lens crystallins were isolated by gel-filtration chromatography as described previously (17). In brief, decapsulated lenses from freshly slaughtered steers (1-2 years old) were thoroughly homogenized in an elution buffer containing 0.05 M ammonium bicarbonate, 5 mM EDTA, 0.01% β-mercaptoethanol, and 0.02% sodium azide, pH 7.5. After centrifugation at 27,000 $\times$ g for 30 min, the supernatant (adjusted to A_{280nm} = 100) was applied to a column of TSK HW-55 (S) (2.5 $\times$ 110 cm) and eluted at 25 ml/h. Five well-resolved peaks were obtained and identified as HMα-, α-, βH-, βL- and γ-crystallins based on SDS-PAGE. The pooled α-crystallin solution was further dialyzed at 0.1 M sodium phosphate pH 7.5 overnight for CD measurements.

Circular dichroism. The CD spectra of α-crystallins were measured with a Jasco J-600 automatic recording dichrograph with an Adventec LMC-13D temperature-control accessory part. In the thermal perturbation experiments, the average heating rate is 50-60°C / h and after reaching the set temperature, the cooling rate to room temperature is 35-45°C /h. Protein concentrations were 0.3 mg/ml with a 0.1 cm light path for far-UV CD measurements, and 1.5 mg/ml with a 1.0 cm light path for near-UV CD measurements. In both far- and near-UV region, [θ] is the mean residue ellipticity in degrees.cm^2/decimole based on the mean residue weight of 114 from the known sequence of αA_2 crystallin chain (17,22).

Assay for chaperone activity of α-crystallin under UV-irradiation. Photoaggregation of γ-crystallin (0.5 mg/ml) was monitored at 25°C, in the buffer of TSK gel-filtration column used for crystallin isolation, in the absence or presence of native or preheated α-crystallins. The sample solutions were irradiated at 295 nm with the excitation bandpass of 20 nm for a fixed time period in a Jasco-FP777 spectrofluorometer, and after irradiation the excitation and emission of the monochrometer were set at 600 nm with a bandpass of 1.5 nm each to measure the relative scattering as described in the previous report (20). The arbitrary unit of relative scattering was plotted as a function of irradiation or incubation time. The preheated α-crystallins were incubated at 82°C for 3 h and cooled down to room temperature overnight.

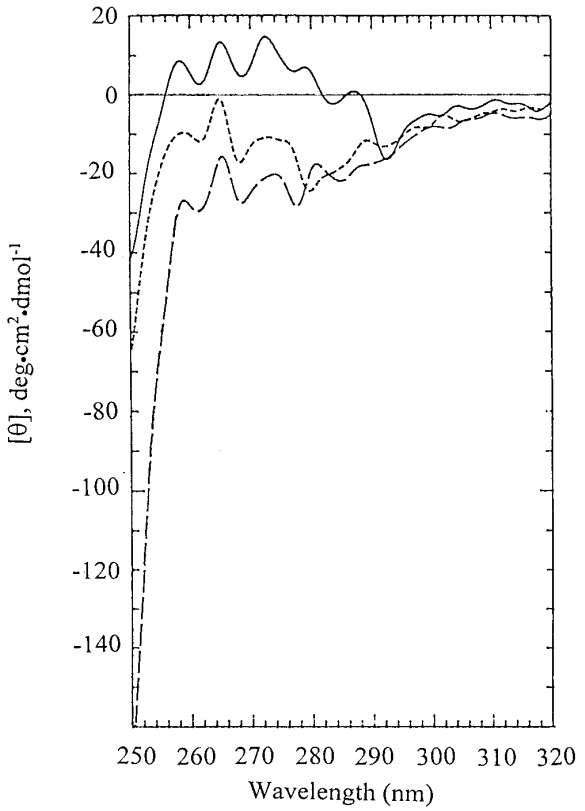

FIG. 1. Near-UV CD spectra of α-crystallin at 25°C (solid line), 82°C (dash line) and 82°C sample cooled to 25°C (dot line).

Assay for chaperone activity of α-crystallin under thermal heating. Thermoaggregation of alcohol dehydrogenase (0.2 mg/ml) was monitored at 48 or 70°C respectively for 30 min in the absence or presence of various amounts of native α-crystallins (0.05-0.8 mg/ml). The relative scattering was monitored at 360 nm to evaluate thermal aggregation. The turbidity change was expressed by the difference in absorbance between pre- and post-incubation. The turbidity change of thermal aggregation of alcohol dehydrogenase in the absence of α-crystallin was used as control (100%). The percentage of turbidity change was plotted against the ratio of α-crystallin to alcohol dehydrogenase under 48 and 70°C respectively.

RESULTS

The CD spectra of α-crystallin in the near-UV region (Fig. 1) changed minimally in the range of 290 to 320 nm, and gradually shifted from essentially positive to totally negative ellipticity below 290 nm upon heating the protein solution to 82°C, indicating a loss of fine structure in some aromatic region of this protein at high temperatures. However, after cooling back to room temperature, most of the near-UV CD spectra could not be reversed. This result is probably related to previous reports (16,18,20) which showed an irreversible temperature-induced quaternary structural change as de-

tected by hydrophobic probe ANS (1-anilino-8-naphthalene sulfonate) after short-term incubation.

The far-UV CD spectra (Fig. 2A) of native α-crystallin at room temperature showed a minimum at around 217 nm, which is indicative of the predominate presence of a high proportion of β-sheet conformation in this protein (17). As the temperature increased to above 80°C, there was a drastic shift of CD minimum at 217 nm. The minimum changed to approximately 200-205 nm at temperatures higher than 85°C (207.2 nm at 82°C), indicating a more disordered structure formed at high temperatures (16,18). However after cooling back to room temperature, the shape of far-UV CD spectra is similar to that of the native one, *i.e.* the CD minimum moves back to 217 nm, indicative of a partial recovery of secondary structure.

In Fig. 2B, we shows the change in ellipticity at 205 nm with respect to temperature graphically. The major transition of secondary structural change is found clearly between 50 and 70°C. This result is in agreement with the previous report (16). When the temperature returned to room temperature again, the change in ellipticity at 205 nm in contrast to that at 217 nm showed a high degree of reversibility. CD ellipticity at 217 nm showed an increase in band intensity after cooling when compared with the native and unheated sample, which is different from the result of Maiti et al. (19), which showed more or less reversible by heating/cooling.

Because the major secondary structural change of α-crystallin takes place from 50 to 70°C, we have further examined its chaperone activity to prevent thermal aggregation of alcohol dehydrogenase at 48 and 70°C, two temperatures cover this transition region. At 48°C, the backbone conformation of α-crystallin did not change while a massive change of secondary structure was noted at 70°C. Nevertheless it is of interest to find that the protective ability of α-crystallin for this enzyme is very similar at these two different temperatures (Fig. 3).

In order to confirm the irreversibility of the heat-induced secondary structural change in α-crystallin, we have performed an additional experiment in which aliquots of α-crystallin (0.3 mg/ml) were preheated at different high temperatures for various time intervals and allowed to equilibrate back to room temperature overnight before far-UV CD measurements. As a result, we found a time-dependent secondary structural change of preheated α-crystallins. For simplicity, in Fig. 4, we show only the result of α-crystallins preheating at 82°C/15 min and 82°C/3 h as compared with the native one. For α-crystallin preheating at 82°C/3 h, the far-UV CD spectra of α-crystallin showed a large irreversible negative shift at both 217 and 205 nm.

To further assess the effect of heat-induced secondary structural changes on the chaperone activity of α-crystallin, we also compare the protective ability between the native and 82°C/3 h preheated α-crystallin for the inhibition of UV-induced aggregation of γ-crystallin at room temperature. As shown in Fig. 5, photo-aggregation of γ-crystallin leads to aggregation and this process can be suppressed by α-crystallin. When

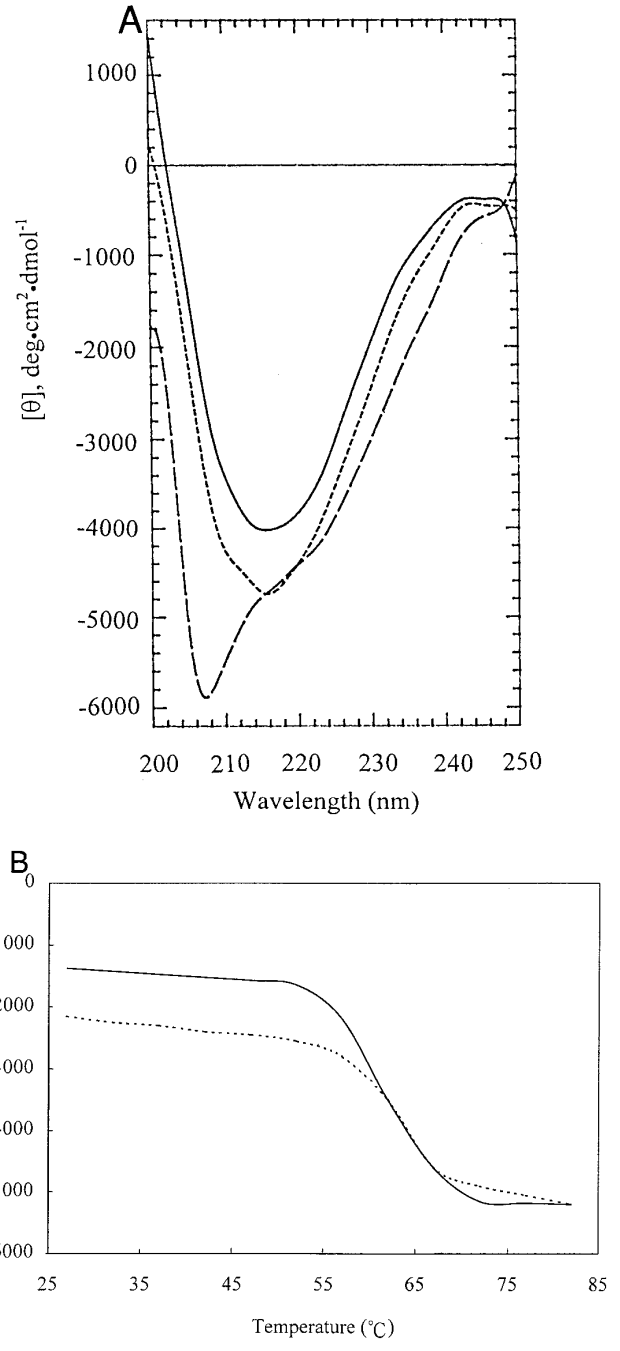

FIG. 2. (A) Far-UV CD spectra of α-crystallin at 25°C (solid line), 82°C (dash line) and 82°C sample cooled to 25°C (dot line). (B) Change in ellipticity at 205 nm with respect to temperature. Ellipticities were monitored during the heating cycle (solid line) at a heating rate of 50-60°C/h, and after reaching 82°C for about 10 min, the cooling cycle (dot line) at a cooling rate of 35-45°C/h.

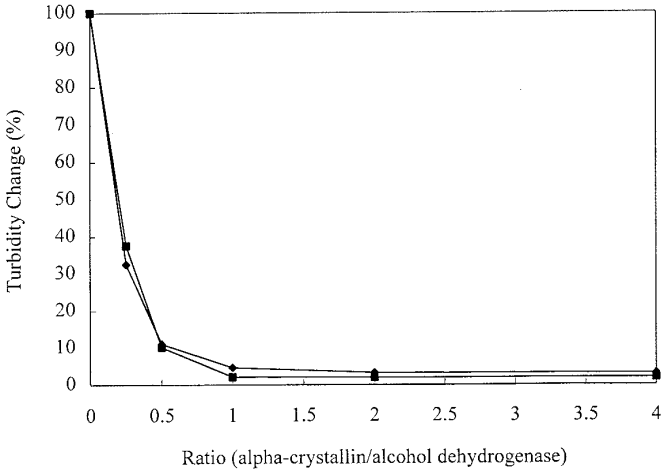

FIG. 3. Thermal aggregation of alcohol dehydrogenase (0.2 mg/ml) at 48(– ◆ –) and 70°C(– ■ –), in the absence and presence of various amounts of α-crystallins (0.05-0.8 mg/ml). The turbidity change was expressed by the difference in absorbance (at 360 nm) between pre- and post-incubation. The turbidity change of thermal aggregation of alcohol dehydrogenase in the absence of α-crystallin was used as control (100%).

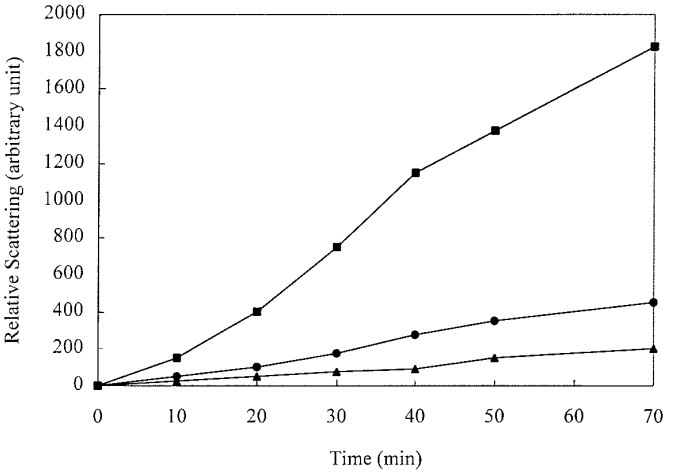

FIG. 5. Photoaggregation of γ-crystallin irradiated at 295 nm in the absence (– ■ –) or presence of native (– ● –) or preheated (– ▲ –) α-crystallins. The concentration of γ-crystallin used in the experiment was 0.5 mg/ml and that of α-crystallin was 0.21 mg/ml. The preheated α-crystallin was incubated at 82°C for 3 h and then cooled down to room temperature overnight before the experiment.

compared with the native one, the 82°C/3 h heat-denatured α-crystallin, of which the secondary structure has shown a great degree of irreversible change (as in Fig. 4), can still preserve its chaperone activity. This result is similar to that of short-term preheating experiment in our previous report (21), attesting to the existence of some other factor(s) besides secondary structural changes to account for the chaperone activity of α-crystallin under these *in vitro* experimental conditions.

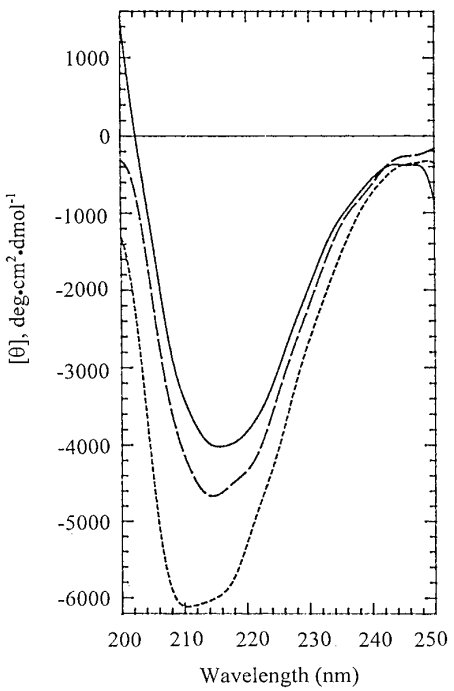

FIG. 4. Far-UV CD spectra of α-crystallin, measured at 25°C for native state (solid line), preincubation at 82°C for 15 min and cooling down to room temperature overnight (dash line), preincubation at 82°C for 3 h and cooling down to room temperature overnight (dot line).

DISCUSSION

There is a considerable interest in the functional role of α-crystallin, especially after it was shown to have *in vitro* chaperone activity (6). However, the mechanism of this chaperone action of α-crystallin is still not very clear. From some thermodynamic studies (15-19), it was found that this protein is not really "thermostable", and some heat-induced structural transitions may be involved in modulating its chaperone activity (16,18,20,21). Thus the conformation of α-crystallin induced by thermal perturbation is of import to elucidate its chaperone mechanism. The information obtained from heat-induced conformational change of α-crystallin itself is also important to provide some insights regarding the interactions between α-crystallin and its substrates underlying these chaperone reactions during thermal denaturation (23).

A high-resolution calorimetric study of α-crystallin (10) showed that it undergoes two thermal transitions, the first between 35 and 51°C and the second between 49 and 73°C, in which only partial reversibility (the second one) was observed. This result is similar to the observation that α-crystallin undergoes an irreversible conformational transition with a marked increase in surface hydrophobicity between 38 and 50°C (15), and a major loss of its native secondary structure occurring only at temperatures higher than 50°C (16).

The heat-induced tertiary structural change in α-crystallin as shown in our near-UV CD spectra was found to be largely irreversible, which is also reflected in the exposure of hydrophobic surface at high temperatures (15,21). Even at low temperature range (25-37°C) some subtle structural changes causing partial dissociation of the α-crystallin complex have been reported at low temperatures (24). At higher temperatures (>50°C), α-crystallin usually unfolded more quickly and resulted in the formation of HMW aggregates (17,18).

The previous report (16) indicated that the stability of α-crystallin is only comparable to, or even lower than that of other crystallins such as β- and γ-crystallins in terms of their transition midpoints of the heat-induced change in the secondary structure. This protein, unlike most other crystallins, showed a high degree of reversibility in its heat-induced secondary structural change (18). On the other hand, γ-crystallin became aggregated and precipitated after heating over its transition melting temperature. The reason for this difference is currently not clear. It is possible that such structural transition at high temperatures is not related with the dissociation of native α-crystallin complex which is supported by an NMR study (25). Another possibility is that the heat-induced backbone structural change is only a mild denaturation (18) other than a massive loss of secondary structure as observed by FTIR (16), which may make α-crystallin less stable due to the presence of deuterium oxide in the studied sample.

Whether the heat-induced secondary structural change is massive or not, the protective ability of α-crystallin to inhibit alcohol dehydrogenase was found to be similar at 48 and 70°C, which lie out of the transition region and may represent two different backbone structural conformations before and after melting transition. Such secondary structural change (massive loss or only 10% loss of β-sheet) (16,18) does not appear to affect its chaperone activity. However, whether there is some genuine difference in the heat-induced conformational change between α-crystallin alone or α-crystallin complex after binding its protective substrates, is now under intensive investigation in our laboratory.

In the present study, the thermodynamic properties of α-crystallin indeed show different degree of irreversibility in both its secondary and tertiary structures. The change in the tertiary structure is more pronounced and drastic. The finding that α-crystallin undergoes a time-dependent irreversible secondary structural change has also an important implication for studying its effect on the chaperone action of this protein. The 82°C/3 h preheated α-crystallin showed a slightly higher chaperone activity than the native one, which can be explained by its higher ANS fluorescence (21, data not shown) in spite of its being in a HMW aggregate state by heating (17,18). The results further suggest that there may be a relatively little correlation between heat-induced secondary structural change and the chaperone activity of α-crystallin.

It is generally believed that hydrophobic interactions play a major role in the chaperone action of α-crystallin and other heat-shock proteins (26). We and other researchers have recently shown that the increase in surface hydrophobicity of α-crystallin by short-term heating correlates with its increased chaperone activity (16,18,20,21). It is apparent that the heat-induced secondary structural changes cause little effect on the hydrophobic surface of α-crystallin as reflected by ANS binding. This may explain why there is little correlation between the secondary structural change and the chaperone activity of α-crystallin. In contrast to other crystallins, the high stability of α-crystallin under high temperatures and long-term incubation, is probably related with its unique characteristic of "primary amphiphilicity" (*i.e.* relatively hydrophobic N-terminal portion and a more polar C-terminal region) in its subunits (16).

α-Crystallin has recently been shown not to be lens-specific (3), and it is also not very thermostable in its structure as probed by several physical methods. However it is still in a sense "thermostable" by possessing chaperone-like function *in vitro*. This fact has probably some significance for its role in the intact lens even though there is virtually no turnover of crystallins during the lifetime of lens development. α-Crystallin could preserve or even enhance its chaperonin function despite some irreversible structural change by external stress.

ACKNOWLEDGMENTS

This work was supported by Academia Sinica and the National Science Council (NSC Grants 83-0418-B-001-020BA, 84-2311-B-001-050-BA, and 86-2311-B-002-031-B15), Taipei, Taiwan.

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