Targeted Cross-Linking of a Molten Globule Form of Acetylcholinesterase by the Virucidal Agent Hypericin†

Lev Weiner,*,‡ Esther Roth,§ Yehuda Mazur,|| and Israel Silman§

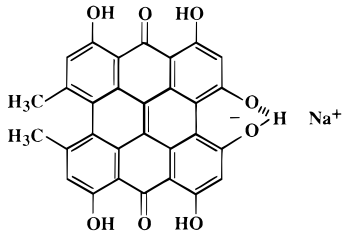
Departments of Chemical Services, Neurobiology, and Organic Chemistry, The Weizmann Institute of Science, 76100 Rehovot, Israel

Received May 18, 1999; Revised Manuscript Received July 9, 1999

ABSTRACT: The natural product hypericin is a photosensitive polycyclic aromatic dione compound, which has been widely investigated because of its virucidal and antitumor properties. Although it has been suggested that singlet oxygen or a radical species might be responsible for its biological action, its mechanism of action remains unknown. Due to its amphiphilic characteristics, we considered the possibility that it might interact preferentially with partially unfolded proteins which exhibit exposed hydrophobic surfaces. We here demonstrate that hypericin binds to a molten globule species generated from *Torpedo* acetylcholinesterase, but not to the corresponding native enzyme. Irradiation with visible light, under aerobic conditions, causes chemical cross-linking of the catalytic subunits, to dimers and heavier species, under conditions where no cross-linking is observed for the native enzyme. Both anaerobiosis and sodium azide greatly reduce the extent of cross-linking, suggesting that singlet oxygen is responsible for the phenomenon. This agrees with our observation, using spin traps, that mainly singlet oxygen is produced by the complex of hypericin with the molten globule of acetylcholinesterase. Cross-linking is enhanced in the presence of liposomes to which the molten globule of acetylcholinesterase is quantitatively adsorbed. This may be due to high local concentrations of both hypericin and the protein resulting in close proximity, and hence in a high yield of cross-linking. Molten globule species are believed to be intermediates in both protein folding and translocation through biological membranes. Thus, hypericin may serve as a valuable tool for trapping such intermediates. This might also explain its therapeutic effectiveness toward virus-infected or tumor cells.

The natural compound hypericin (Hy;[1] Chart 1) is a photosensitive polycyclic aromatic dione compound, which has been widely investigated because of its strong virucidal properties (*1*, *2*). Various photophysical processes have been put forward to explain its photosensitizing characteristics; the fact that its action is strongly enhanced by oxygen suggests that either singlet oxygen (*3*, *4*), superoxide radical (*5*), or a Hy radical (*5*, *6*) could be responsible for its biological action. It has been shown that Hy is effective only against enveloped viruses (*7*); this suggests that, as might be expected from its hydrophobic character, it is sequestered in the plasma membrane where it exerts its effect (*4*). It has further been demonstrated that Hy produces photodynamically induced cross-linking of membrane proteins of vesicular stomatitis and other viruses (*8*, *9*), which led to the suggestion that the loss of infectivity may result from inactivation of fusion (*8*). Nevertheless, the molecular basis for the virucidal action of Hy is still the subject of controversy.

Chart 1



The folding and assembly of proteins and multiprotein complexes is currently the topic of intensive fundamental research (*10*). It is now generally accepted that nascent polypeptide chains, immediately after synthesis on the ribosome, collapse to a compact structure known as a "molten globule" (MG) (*11*, *12*), which is then converted to the unique native structure, with or without the help of molecular chaperones (*13*). The MG possesses substantial secondary structure, but is devoid of the tertiary structure of the native state, and exhibits hydrophobic surfaces as visualized by binding of amphiphilic probes, like 1-anilino-8-naphthalene sulfonate (ANS) (*14*, *15*). Due to their exposed hydrophobic surfaces, proteins in the MG state can interact with lipid bilayers (*16*−*19*), and it has been proposed that proteins can be translocated through plasma membranes in this state (*20*). When Hy's amphiphilic character was taken into account

† Supported by a grant from the Israel Science Foundation. I.S. is Bernstein-Mason Professor of Neurochemistry.

* To whom correspondence should be addressed. Fax: +972-8-934412. E-mail: cilev@weizmann.weizmann.ac.il.

‡ Department of Chemical Services.

§ Department of Neurobiology.

|| Department of Organic Chemistry.

[1] Abbreviations: Hy, hypericin; MG, molten globule; ANS, 1-anilino-8-naphthalenesulfonate; AChE, acetylcholinesterase; GdnHCl, guanidine hydrochloride; DMPC, dimyristoylphosphatidylcholine; DMPO, 5,5′-dimethyl-1-pyrroline *N*-oxide; TEMP, 2,2,6,6-tetramethyl-4-piperidone; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi991147+ CCC: $18.00 © 1999 American Chemical Society
Published on Web 08/12/1999

(see Chart 1), it occurred to us that Hy might interact preferentially with either soluble or membrane-bound proteins in the MG state. This could explain both its selective action on enveloped viruses and the suggestion that it may inhibit fusion by viruses. Indeed, virus assembly is currently a topic of intensive research (*21*), and recent studies have suggested that partially folded, molten globule intermediates may play an important role in assembly of capsid viruses (*22*, *23*).

To test the above hypothesis that Hy might exert its virucidal action by preferentially interacting with partially folded intermediates, we utilized a well-characterized MG species produced from *Torpedo* acetylcholinesterase (AChE) by exposure to a low concentration of guanidine hydrochloride (GdnHCl) (*24*, *25*). In the following, we show that Hy, under illumination in the presence of oxygen, produces cross-linking of both membrane-bound and soluble MG species, under conditions in which the native enzyme is completely resistant.

MATERIALS AND METHODS

Materials. AChE was the G_2 dimer purified from electric organ tissue of *Torpedo californica* by affinity chromatography after solubilization with phosphatidylinositol-specific phospholipase C (*26*). Hy, in the form of its sodium salt, Hy-Na (see Chart 1), was prepared as described previously (*27*), and employed throughout this study. Dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). GdnHCl (Ultrapure) was from Schwartz/Mann Biotech (Cleveland, OH). 5,5′-Dimethyl-1-pyrroline *N*-oxide (DMPO) and 2,2,6,6-tetramethyl-4-piperidone (TEMP) were from Sigma (St. Louis, MO). DMPO was repurified before use as described previously (*28*). Low-molecular weight markers were from Bio-Rad (Hercules, CA), and cross-linked phosphorylase *b*, obtained from Sigma, provided the high-molecular weight markers.

Sample Preparation. A MG species of AChE, MG−AChE, was produced as described previously by exposure to 1.5 M GdnHCl (*24*, *25*). Small unilamellar phospholipid vesicles were prepared by sonication of DMPC (*17*, *18*). Protein concentrations were determined as described previously (*24*).

Acrylamide Gel Electrophoresis under Denaturing Conditions. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out as described previously (*24*).

Sucrose Gradient Centrifugation. Analytical sucrose gradient centrifugation was performed on linear 5 to 20% sucrose gradients as described previously (*25*).

Photosensitization. Photosensitization of Hy, both for measuring photoinduced ESR spectra (see below) and in cross-linking experiments, was performed using an HMBO 200 W superpressure mercury lamp as a light source. A glass filter (2 cm thick) containing 1% aqueous $CuSO_4$ was used. The visible light power employed was ca. 20 mW cm^{-2}. In experiments conducted under anaerobic conditions, samples were deoxygenated by bubbling nitrogen through the samples for 40 min.

Spectroscopic Measurements. Fluorescence measurements were performed in a Shimadzu RF-540 spectrofluorometer at 23 °C. Excitation was at 590 nm. ESR spectra were recorded in a Bruker ER 200 D-SRC spectrometer in a flat quartz cell (200 μL). Photoinduced ESR spectra were recorded as described previously (*5*). Quasielastic light scattering was performed at 22 °C as described previously (*25*).

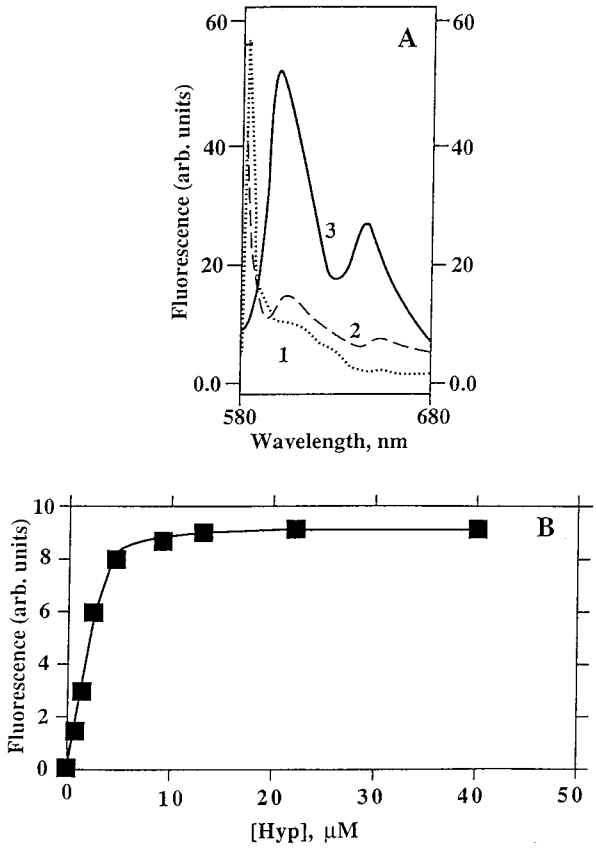


FIGURE 1: Interaction of Hy with MG−AChE. (A) Influence of AChE on the fluorescence emission spectrum of Hy: (1) Hy (10 μM) in 10 mM Tris-HCl (pH 7.3), (2) Hy in the presence of native AChE (15 μM), and (3) Hy in the presence of MG−AChE (15 μM). (B) Fluorescence titration of MG−AChE with Hy. The protein concentration was 15 μM. The solid line represents the experimental data fitted to a saturation curve.

RESULTS

Figure 1A shows the fluorescence emission spectrum of Hy alone, and in the presence of either native *Torpedo* AChE or a MG species, MG−AChE, obtained by treatment with 1.5 M GdnHCl, which was then removed by gel filtration (*25*). It can be seen that Hy alone exhibits very little fluorescence, due to the fact that it aggregates in aqueous solution (*29*). In the presence of an excess of the native enzyme, only a slight increase in fluorescence is noted. In the presence of a similar concentration of MG−AChE, however, Hy fluorescence is greatly enhanced, and the emission spectrum that is obtained resembles that seen when Hy is dissolved in polar organic solvents, where it is known to exist as a monomeric species (*29*, *30*). Figure 1B shows the concentration dependence of the emission of Hy at 600 nm, at a constant MG−AChE concentration. A saturation curve is obtained, with a K_{app} of 4.8×10^6 M^{-1}. It should be borne in mind that this is not a true dissociation constant, both due to the fact that Hy is aggregated in aqueous solution (*29*) and because it is not known whether it is bound at independent sites with equal affinity.

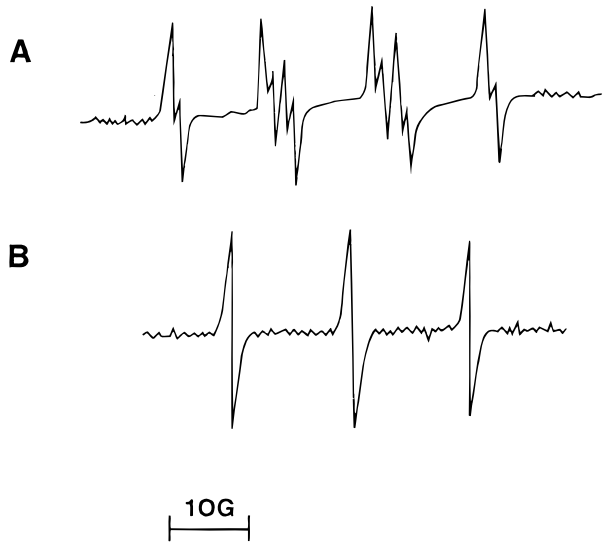


FIGURE 2: Detection of reactive oxygen species formed during aerobic illumination of Hy. Hy (11 μM) in 10 mM Tris-HCl (pH 7.3) was irradiated in the absence (A) and presence (B) of 15 μM MG−AChE. The concentrations of DMPO (A) and TEMP (B) were 0.1 and 0.2 M, respectively. EPR spectra were measured 5 min after irradiation had started. The EPR conditions were as follows: (A) microwave power of 20 mW, modulation amplitude of 0.8 G, and receiver gain of 2×10^5 and (B) microwave power of 20 mW, modulation amplitude of 1 G, and receiver gain of 3.2×10^5.

It has been clearly established that Hy in the monomeric form, e.g., either in an organic solvent (*30*) or bound to micelles (*31*), generates singlet oxygen upon irradiation with visible light, whereas when aggregated, e.g., in aqueous solution, the main form of active oxygen that is generated is superoxide (*5*). Figure 2A utilizes the spin trap technique, with DMPO, to confirm that aggregated Hy, in aqueous buffer, where it lacks fluorescence (see Figure 1A), generates an ESR spectrum which is characteristic of the spin adduct of DMPO with superoxide (*5*). In contrast, as is apparent from Figure 2B, using TEMP as a spin trap (*32*), Hy bound to MG−AChE, conditions under which it is monomeric (Figure 1A), is an effective generator of singlet oxygen.

Figure 3 displays the patterns obtained upon SDS−PAGE, under reducing conditions, of native *Torpedo* AChE and of MG−AChE, which had been irradiated in the presence of Hy. Under reducing conditions, both native AChE and MG−AChE preparations (lanes 1 and 2, respectively) exhibit a single ca. 65 kDa polypeptide band, generated by reduction of the intersubunit disulfide bond (*33*). Upon irradiation in the presence of Hy, the native enzyme exhibits a pattern (lane 3) similar to that of the control, with only traces of higher-molecular weight species, whereas MG−AChE (lane 4) displays very little monomer, with the concomitant appearance of heavier species corresponding to dimers, larger oligomers, and aggregated forms migrating close to the origin. Upon similar irradiation in the presence of Hy, but under anaerobic conditions, much more monomer remained (lane 5). Irradiation of either the native or MG species in the absence of Hy did not produce any higher-molecular weight species, nor did incubation of MG−AChE with Hy in the absence of irradiation (not shown).

As already mentioned, Hy, due to its amphiphilic characteristics, is mainly sequestered in membranous compartments when added to cell cultures (*4*). When added to

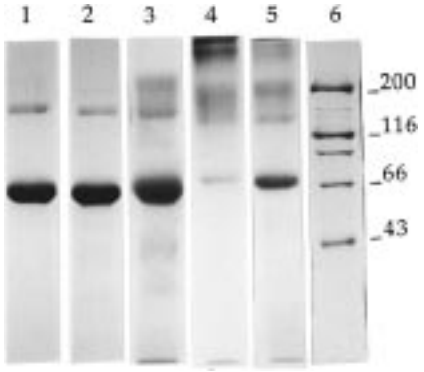


FIGURE 3: Cross-linking of MG−AChE by Hy as demonstrated by SDS−PAGE: (1) native AChE, (2) MG−AChE, (3) native AChE and Hy with irradiation for 1 h, (4) MG−AChE and Hy with irradiation for 1 h, (5) MG−AChE and Hy with irradiation for 1 h under anaerobic conditions, and (6) molecular weight markers. Concentrations of AChE and Hy-Na were 17 and 12 μM, respectively, in 10 mM Tris-HCl (pH 7.3). SDS−PAGE was performed under reducing conditions, on 5 to 15% gradient polyacrylamide gels, after reduction of the samples with 10 mM DTT (see Materials and Methods).

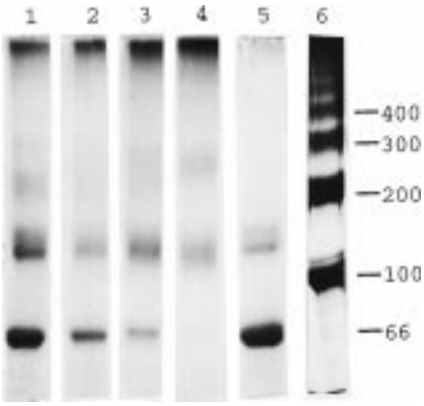


FIGURE 4: Enhancement of cross-linking of MG−AChE by Hy in the presence of liposomes: (1) MG−AChE and Hy with irradiation for 20 min, (2) MG−AChE and Hy with irradiation for 1 h, (3) MG−AChE, DMPC liposomes, and Hy with irradiation for 20 min, (4) MG−AChE, liposomes, and Hy with irradiation for 1 h, (5) native AChE, liposomes, and Hy with irradiation for 1 h, and (6) high-molecular weight markers. Reaction conditions were as described in the legend of Figure 3, except that reaction mixtures corresponding to lanes 3−5 contained 5.5 mg/mL DMPC liposomes. Other conditions were as described in the legend of Figure 3, except that the gel gradient was from 3.5 to 10%.

liposomes, in which it exists in monomeric form, it can generate both singlet oxygen and oxygen radicals (*32*). We have already demonstrated a strong association of MG species of AChE with liposomes (*17*), and decided, therefore, to investigate the effect of Hy on the membrane-bound species. Figure 4 shows the SDS−PAGE results, under reducing conditions, of MG−AChE which had been irradiated in the presence of Hy in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of liposomes. In the absence of liposomes, after 20 min, the bulk of the protein still migrated as monomer, whereas after 1 h, only a minor fraction was still monomeric. In the presence of the liposomes, after irradiation for 20 min only a small amount of monomer was already visible, and after 1 h, the monomer was barely detectable. Upon irradiation of native AChE with Hy in the presence of liposomes, only traces of heavier bands could be observed (lane 5).

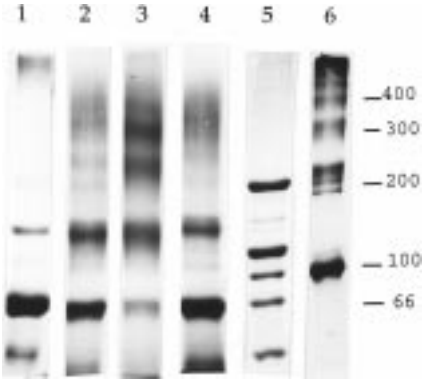


FIGURE 5: Resolution of oligomeric products of cross-linking of MG−AChE by Hy by SDS−PAGE under reducing conditions: (1) MG−AChE, (2) MG−AChE and Hy with irradiation for 30 min, (3) MG−AChE, Hy, and DMPC liposomes with irradiation for 30 min, (4) MG−AChE, Hy, DMPC liposomes, and 1 mM sodium azide with irradiation for 30 min, (5) low-molecular weight markers, and (6) high-molecular weight markers. The DMPC concentration was 5.0 mg/mL. Other conditions were as described in the legend of Figure 3, except that the gel gradient was from 3.5 to 7.5%.

Use of 3.5 to 7.5% acrylamide gels permitted resolution of the cross-linked polypeptide species obtained by irradiation in the presence of Hy. Figure 5 (lane 2) clearly shows the appearance of dimers, trimers, and tetramers, as well as discrete heavier species. When gels were run under the same conditions for samples in which irradiation was carried out in the presence of liposomes (lane 3), similar patterns were observed, but the species corresponding to trimer was notably absent. In the presence of sodium azide, a known quencher of singlet oxygen, the extent of cross-linking was greatly reduced (compare lanes 4 and 3).

DISCUSSION

The main finding of this study is the demonstration that the photosensitive drug, Hy, cross-links a partially unfolded, MG, state of a protein, whereas it has no detectable effect on the fully folded native species. It should be noted that the MG−AChE preparation, which is derived from the native dimer, is long-lived (several hours) under the experimental conditions, during which period heavier species cannot be detected by either sucrose gradient centrifugation or quasielastic light scattering (*25*).

The ability of Hy to cross-link preferentially the MG species of AChE, under conditions in which the native species is not affected, can be ascribed to the fact that Hy is absorbed to the hydrophobic surfaces of the MG as a monomeric species, as evidenced by its fluorescence emission spectrum in the presence of MG−AChE. As already mentioned, it is the monomeric species of Hy which generates singlet oxygen preferentially upon irradiation, and it is this species which is believed to be responsible for the cross-linking of various proteins (*34*). In various model polypeptides and in proteins, it was shown that certain amino acids, namely, histidine, lysine, and tyrosine, are attacked preferentially by $^1O_2$, with subsequent formation of chemical cross-links (*35*, *36*). Our data suggest that, in the case of MG−AChE, cross-linking occurs both between the two subunits within the dimer and between dimers (Figure 3). In the three-dimensional structure of *Tc*AChE (*37*), there is a four-helix bundle at the subunit interface. Within this bundle, pairs of candidates for cross-linking include K436Nϵ in the two subunits (distance of 13.0 Å), the imidazoles of H362 in one subunit and H536 in the other (distance between centers of rings, 9.0 Å), K530Nϵ in one subunit and K357Nϵ in the other (12.2 Å), and H513Nδ in one subunit and K386Nϵ in the other (14.6 Å). Although these distances appear to be large, it has been shown that MG states of a number of proteins are characterized by an absence of fixed side chain packing (*11*, *12*). In particular, NMR data support considerable flexibility of polypeptide chains in MG-like states (*38*, *39*). Furthermore, our own data, concerning both disulfide reshuffling (*24*) and interaction of an amphipathic polypeptide sequence with liposomes (*17*), argue for considerable conformational flexibility in the partially unfolded *Tc*AChE dimer. As for production of cross-linked oligomers (Figures 3−5), examination of the three-dimensional structure of *Tc*AChE reveals several candidates with exposed side chains, in particular, H26, K107, Y137, K192, H264, K270, K346, Y472, H486, and K501. Obviously, the conformational flexibility in the MG state just referenced might make other side chains, buried in the native crystal structure, that are available for cross-links between subunits.

The mean free pathway of $^1O_2$ in biological systems is 100−500 Å (*40*, *41*). Thus, in a dilute aqueous solution of a protein to which it was not absorbed, viz. a native species, Hy would be expected to be ineffective both due to the dilution factor and due to the fact that it is aggregated (*5*). In the case of the liposome-bound MG−AChE, our data clearly show enhanced cross-linking (Figures 4 and 5). Since our published data show that MG−AChE is completely bound to the liposome (*17*, *18*), and Hy itself is sequestered in the membrane, the high local concentrations of both protein and cross-linker should result in close proximity, and hence in a high yield of cross-linking. However, it should be kept in mind that Hadjur et al. (*32*) have presented evidence that Hy in the lipid phase may photoreact by both type I and type II mechanisms; this might be an alternative explanation for the enhanced cross-linking of MG−AChE bound to liposomes. It should be noted, however, that in a system generating $^{\bullet}$OH radicals, we observed peptide bond cleavage, rather than cross-linking of *Tc*AChE (*42*); furthermore, it has been reported that the quantum yield for singlet oxygen production by hypericin bound to DMPC liposomes is quite high, 0.43 (*43*). Both types of data support a type II mechanism in our system. The necessity that Hy be very close to its target for the $^1O_2$ which it generates to be effective is similar to the requirement that adriamycin be complexed to DNA so that the oxygen radicals generated by this drug will serve as an effective "chemical" nuclease (*44*).

As mentioned in the introductory section, it is widely accepted that the MG intermediate is involved in protein folding (*11*, *12*), and may also be involved in translocation of proteins across membranes (*20*). Thus, Hy may serve as a valuable tool for trapping nascent polypeptides at early stages of their folding, as well as partially unfolded species occurring during the course of translocation or generated by stress responses. It may also permit the trapping of transient intramolecular complexes. As already mentioned, our MG−AChE preparation is long-lived, and significant amounts of aggregates appear only after many hours. Yet, oligomeric species appear rapidly upon irradiation in the presence of Hy. Finally, we would like to draw attention to the important

implications of the phenomenon observed in this study. First, Hy may provide a tool for studying folding, oligomerization, and assembly of proteins both in solution and at the membrane surface, as well as partially unfolded species that exist during the course of translocation or generated by stress responses (*13*). Second, our data may offer an explanation for the therapeutic effect of Hy and related photosensitive compounds. Since nascent proteins are believed to reach the fully folded native conformation via a MG intermediate (*11*, *12*), they should be very vulnerable to Hy, particularly if it is kept in mind that, in vivo, folding occurs at the surface of the endoplasmic reticulum (*13*). Protein synthesis in cells which are undergoing rapid division (e.g., cancer cells or virus-infected cells) should be particularly susceptible. Indeed, as already mentioned, partially unfolded states were observed in assembly of capsid viruses (*22*, *23*), and treatment of a virus infection with an inhibitor of protein folding and traffic was recently reported (*45*).

ACKNOWLEDGMENT

We thank Charles Millard for help in examination of the three-dimensional structure of *Tc*AChE.

REFERENCES

1. Lavie, G., Valentine, F., Levin, B., Mazur, Y., Gallo, G., Lavie, D., Weiner, D., and Meruelo, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5963−5967.
2. Kraus, G. A., Zhang, W., Fehr, M. J., Petrich, J. W., Wannemuehler, Y., and Carpenter, S. (1996) *Chem. Rev. 96*, 523−535.
3. Duran, N., and Song, P.-S. (1986) *Photochem. Photobiol. 43*, 677−680.
4. Thomas, C., MacGill, R. C., Miller, G. C., and Pardini, R. (1992) *Photochem. Photobiol. 55*, 47−53.
5. Weiner, L., and Mazur, Y. (1992) *J. Chem. Soc., Perkin Trans. 2*, 1439−1442.
6. Fehr, M. J., Carpenter, S. L., and Petrich, J. W. (1994) *Bioorg. Med. Chem. Lett. 4*, 1339−1344.
7. Tang, J. M., Colacino, J., Larsen, S. H., and Spitzer, W. (1990) *Antiviral Res. 13*, 313−325.
8. Lenard, J., Rabson, A., and Vanderoef, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 158−162.
9. Lavie, G., Mazur, Y., Lavie, D., and Meruelo, D. (1995) *Med. Res. Rev. 15*, 111−119.
10. Creighton, T. E., Ed. (1992) *Protein Folding*, W. H. Freeman & Co., New York.
11. Ptitsyn, O. B. (1995) *Adv. Protein Chem. 47*, 83−229.
12. Fink, A. (1995) *Annu. Rev. Biophys. Biomol. Struct. 24*, 495−522.
13. Smith, D. F., Whitesell, L., and Katsanis, E. (1998) *Pharmacol. Rev. 50*, 493−514.
14. Goto, Y., and Fink, A. L. (1989) *Biochemistry 28*, 945−952.
15. Dolginova, E. A., Roth, E., Silman, I., and Weiner, L. (1992) *Biochemistry 31*, 12248−12254.
16. van der Goot, F. G., Gonzalez-Mañas, J. M., Lakey, J. H., and Pattus, F. (1991) *Nature 354*, 408−410.
17. Shin, I., Silman, I., and Weiner, L. (1996) *Protein Sci. 5*, 42−51.
18. Shin, I., Kreimer, D. I., Silman, I., and Weiner, L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2848−2852.
19. Montich, G. G., and Marsh, D. (1995) *Biochemistry 34*, 13139−13145.
20. Bychkova, V. E., Pain, R. H., and Ptitsyn, O. B. (1988) *FEBS Lett. 238*, 231−234.
21. Liljas, L. (1999) *Curr. Opin. Struct. Biol. 9*, 129−134.
22. Tuma, R., and Thomas, G. J., Jr. (1997) *Biophys. Chem. 68*, 17−31.
23. Kirkitadze, M. D., Barlow, P. N., Price, N. C., Kelly, S. M., Boutell, C. J., Rixon, F. J., and McLelland, D. A. (1998) *J. Virol. 72*, 10066−10072.
24. Eichler, J., Kreimer, D. I., Varon, L., Silman, I., and Weiner, L. (1994) *J. Biol. Chem. 269*, 30093−30096.
25. Kreimer, D. I., Shin, I., Shnyrov, V. L., Villar, E., Silman, I., and Weiner, L. (1996) *Protein Sci. 5*, 1852−1864.
26. Sussman, J. L., Harel, M., Frolow, F., Varon, L., Toker, L., Futerman, A. H., and Silman, I. (1988) *J. Mol. Biol. 203*, 821−823.
27. Mazur, Y., Bock, H., and Lavie, D. (1992) Canadian Patent Appl. 202993; *Chem. Abstr. 116*, 63432.
28. Buettner, G. R., and Oberley, L. W. (1978) *Biochem. Biophys. Res. Commun. 83*, 69−74.
29. Gai, F., Fehr, M. J., and Petrich, J. W. (1994) *J. Phys. Chem. 98*, 5784−5795.
30. Darmanyan, A. P., Burel, L., Eloy, D., and Jardon, P. (1994) *J. Chim. Phys. 91*, 1774−1785.
31. Racinet, H., Jardon, P., and Gautron, R. (1988) *J. Chim. Phys. 85*, 971−977.
32. Hadjur, C., Jeunet, A., and Jardon, P. (1994) *J. Photochem. Photobiol., B 26*, 67−74.
33. Silman, I., and Futerman, A. H. (1987) *Eur. J. Biochem. 170*, 11−22.
34. Straight, R. C., and Spikes, J. D. (1985) in *Singlet O_2, Vol. IV, Polymers and Biomolecules* (Frimer, A. A., Ed.) pp 91−144, CRC Press, Boca Raton, FL.
35. Balasubramanian, D., Du, X., and Zigler, J. S., Jr. (1990) *Photochem. Photobiol. 52*, 761−768.
36. Shen, H.-R., Spikes, J. D., Kopeckova, P., and Kopecek, J. (1996) *J. Photochem. Photobiol., B 35*, 213−219.
37. Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991) *Science 253*, 872−879.
38. Alexandrescu, A. T., Evans, P. A., Pitkeathly, M., Baum, J., and Dobson, C. M. (1993) *Biochemistry 32*, 1707−1718.
39. Dyson, H. J., and Wright, P. E. (1998) *Nat. Struct. Biol. 5*, 499−503.
40. Moan, J. (1990) *J. Photochem. Photobiol., B 6*, 343−347.
41. Kanofsky, J. R., Baker, A., and Sima, P. D. (1995) in *The Oxygen Paradox* (Davies, K. J., and Ursini, F., Eds.) pp 539−549, CLEUP University Press, Padova, Italy.
42. Weiner, L., Kreimer, D., Roth, E., and Silman, I. (1994) *Biochem. Biophys. Res. Commun. 198*, 915−922.
43. Ehrenberg, B., Anderson, J. L., and Foote, C. S. (1998) *Photochem. Photobiol. 68*, 135−140.
44. Feinstein, E., Canaani, E., and Weiner, L. (1993) *Biochemistry 32*, 13156−13161.
45. Block, T. M., Lu, X., Mehta, A. S., Blumberg, B. S., Tennant, B., Ebling, M., Korba, B., Lansky, D. M., Jacob, G. S., and Dwek, R. A. (1998) *Nat. Med. 4*, 610−614.

BI991147+