Photoresponsive Polypeptides. Photochromic and Conformational Behavior of Spiropyran-Containing Poly(L-glutamate)s under Acid Conditions

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ABSTRACT: High molecular weight poly(L-glutamic acid) was chemically modified with a spiropyran reagent to give polypeptides containing 35 and 85 mol % spiropyran units in the side chains. To investigate the photochromic and conformational behavior in acid conditions, the polypeptides were dissolved in hexafluoro-2-propanol (HFP) and a small amount of trifluoroacetic acid was added (TFA, $c = 5 \times 10^{-4}$ g/mL). In the absence of acid, the same polypeptides were found to respond to light, giving reversible coil/α-helix transitions. In the presence of TFA, by contrast, photoisomerization of the chromophores in the side chains did not result in any conformational variation of the macromolecular main chains, and CD spectra showed that the macromolecules adopted a random coil structure, both in the dark and after light exposure. However, when appropriate amounts of cosolvents such as methanol (MeOH) or trifluoroethanol (TFE) were added to the HFP/TFA solutions, the system again responded to light, giving reversible coil/α-helix transitions of the macromolecular structure. The extent of the photoinduced conformational changes depended on solvent composition and the photoresponse could be modulated by the combined action of light and chemical environment. For HFP/MeOH solvent mixture, it is possible to trigger the photostimulated coil/α-helix transition in a narrow window of solvent composition, so that the system is characterized by a gated photoresponse. The molecular processes responsible for the observed photoinduced conformational changes are discussed on the basis of absorption and fluorescence results. These results also allow us to set up a detailed picture of the molecular mechanisms responsible for the described chemo-modulated photosensory system.

Introduction

The salient, common features of biological photoreceptors such as rhodopsins, phytochromes and the photoactive-yellow-protein are as follows: (a) they contain a low-molecular-weight photochromic moiety incorporated into a macromolecular matrix, such as a membrane or a cytoplasmatic protein; (b) on irradiation, the photochromic moiety undergoes reversible stereochemical rearrangements between two or more isomeric forms, the direction being determined by the wavelength of the incident light; (c) the primary photochemical reaction induces a conformational change in the macromolecular matrix, the "photosignaling state", which yields, directly or indirectly, the physiological photoresponse.[1−5]

Like biological systems, synthetic polymers containing photochromic moieties have been found to be able to act as efficient photosensors.[6−9] In particular, remarkable photoinduced conformational changes have been observed for poly(L-glutamic acid) bearing spiropyran units in the side chains in hexafluoro-2-propanol (HFP) solutions. In the dark, the photochromic side chains are present as dipolar merocyanine ("open") form and the macromolecules adopt a random coil conformation. Exposure to light, and the consequent photoisomerization to the spiro ("closed") form, induces the transition to the α-helix structure.[10]

The isomerization reaction of the photochromic side chains is the very first event of the photoresponse, but the actual driving force responsible for the conformational change has been recently shown to be the intermolecular interaction between the photochromic side chains.[8] In the dark, the dipolar merocyanine side chains undergo aggregation phenomena with formation of dimeric species which oblige the macromolecule to adopt a disordered structure. Following irradiation, merocyanine units photoisomerize to apolar spiropyran species, which only exist in the monomeric form, and the macromolecules adopt the helical structure.[8]

Previous results have shown that HFP solutions of spiropyran-modified poly(L-glutamate)s in the presence of trifluoroacetic acid (TFA) do not exhibit any light-induced conformational variation, the macromolecules being random coil when the samples are kept in the dark as well as when they are exposed to light. However, when appropriate amounts of methanol (MeOH) are added to the HFP/TFA solutions, the polypeptide can respond to light again giving random coil → α-helix transitions.[10]

The "structural sensitivity" of the system with respect to light and solvent composition might be exploited in designing synthetic photosensors/photoswitches whose characteristics and performances might be modulated by the combined action of light and of its chemical environment. To further characterize this synthetic photoresponsive system, we have performed a spectroscopic study on poly(L-glutamate)s having various contents of spiropyran units in the side chains and on a low molecular weight spiropyran compound, dissolved in different solvent mixtures, such as HFP/MeOH and

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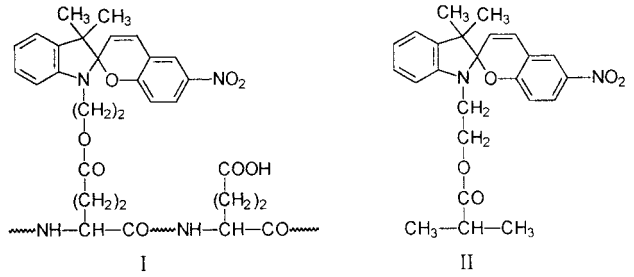

Figure 1. Chemical structure of poly(L-glutamic acid) containing spiropyran units in the side chains (I) and of a low molecular weight model compound (II).

HFP/trifluoroethanol (TFE), in the presence of the strong protonating agent trifluoroacetic acid. The results obtained from absorption, fluorescence, and circular dichroism measurements provided information about the influence of the acid and the role of the solvent on photochromic and conformational behavior of spiropyran-containing polypeptides. Moreover, they allowed us to have a rather detailed picture of the mechanism of the combined action of light and solvent in modulating the folding/unfolding processes of the macromolecular structure.

Experimental Section

Chemicals and Sample Preparation. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) was of spectroscopic grade from Merck. When the commercial product was not completely transparent until 200 nm, it was purified by catalytic hydrogenation under 70 atm in the presence of 10% palladium on charcoal, at 80 °C, for 48 h. At the end of the reaction the catalyst was filtered off and the solvent was distilled over calcium oxide.

Poly(L-glutamic acid) was obtained by polymerization of γ-benzyl-L-glutamate *N*-carboxyanhydride, followed by removal of the γ-benzyl groups with anhydrous HBr. The polymer was dialyzed against water (pH 8) and then precipitated by dialysis against 0.01 N HCl. The lyophilized poly(L-glutamic acid) had intrinsic viscosity $[\eta] = 0.33$ dL g^{-1} (in water, pH 7.3, 25 °C) corresponding to an average molecular weight $M_V = 250\,000$.

Poly(L-glutamic acid) was reacted with the spiropyran reagent 1-(β-hydroxyethyl)-3′,3′-dimethyl-6-nitrospiro(indoline-2′,2-[2*H*-1]-benzopyran) in the presence of dicyclohexylcarbodiimide and 4-pyrrolidinopyridine, following the procedure already reported.[11] Two modified polymers, having the structure shown in Figure 1, were obtained. Their spiropyran content was evaluated by comparing the absorption spectra of the polymers, in the colorless spiro form, with that of the model compound II (see Figure 1), corresponding to the isobutanoyl ester derivative of the spiropyran reagent (spiro form: $\lambda_{max} = 355$ nm; $\epsilon_{max} = 11\,200$ M^{-1} cm^{-1}, in HFP). The two polymers used in our experiments contained 35 and 85 mol % spiropyran units, respectively.

Solutions of the photochromic compounds were prepared under a red safe light and stored in the dark. For the preparation of samples containing protonated photochromic units, trifluoroacetic acid (TFA) was added to HFP solutions ([TFA] = 5×10^{-4} g/mL). The resulting solutions contained about 100 equiv of acid with respect to the amino acidic residues. The concentration of the acid was in excess with respect to the molar content of photochromic groups but absolutely negligible with respect to the concentration needed to cause denaturation of the polypeptide conformation.

Irradiations of the samples were carried out by means of a 75 W Xe OSRAM lamp, coupled to suitable BALZERS interference filters. Irradiation times of about 1 min were enough to produce the complete bleaching of the solutions. The back reaction was obtained by keeping the sample in the dark at 25 °C for ≅ 20 h. No photoisomerization was detected during recording of absorption, fluorescence and circular dichroism spectra.

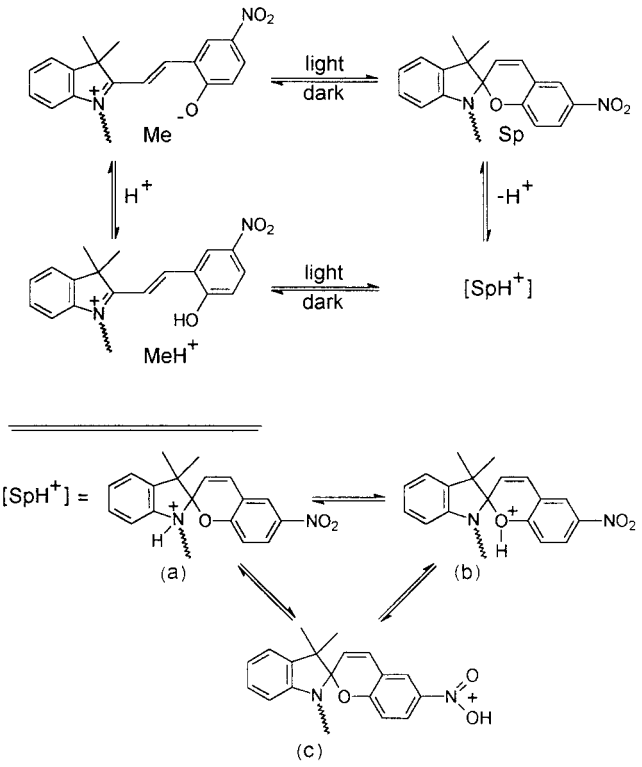

Figure 2. Photochromic reactions of spiropyran compounds in HFP solution in the absence and in the presence of acid.

Spectroscopic Measurements. Absorption spectra were recorded by means of a JASCO 7850 spectrophotometer.

Fluorescence emission spectra were recorded by means of a Perkin-Elmer LS 50B spectrofluorometer. For all fluorescence measurements, sample concentration values were low enough to avoid artifacts due to inner filter and self-absorption. All fluorescence measurements were performed at excitation wavelengths in the range 290−500 nm.

Circular dichroism (CD) spectra were recorded by means of a JASCO J-500A spectropolarimeter. CD intensities are expressed in terms of molar ellipticity values, [Θ] (deg·cm^2·dmol^{-1}), based on the mean residue molecular weight. The fraction of helical polypeptide (*f*) was estimated using the equation

$$f = ([\Theta]_{obs} - [\Theta]_{coil})\cdot([\Theta]_{helix} - [\Theta]_{coil})^{-1} \times 100$$

where $[\Theta]_{obs}$ is the measured value, and $[\Theta]_{helix}$ and $[\Theta]_{coil}$ are the ellipticities for the fully helical and the fully coiled conformations at 222 nm.[12]

Results and Discussion

Photochromic Behavior under Acid Conditions. Poly(L-glutamate)s having the structure shown in Figure 1, containing respectively 35 and 85 mol % spiropyran units in the side chains, were dissolved in HFP, and the solutions had a small amount of TFA added ($c = 5 \times 10^{-4}$ g/mL).

The photochromic behavior of spiropyran compounds in acid conditions is reported in the literature,[13−17] but the photochemical reactions involved have not been described in detail. On the basis of the data available in the literature, the gross photochromic cycle illustrated in Figure 2 can be proposed. The acid protonates the phenoxy group of the merocyanine Me to give the open O-protonated species MeH$^+$. This last is converted by light into the protonated closed spiro species SpH$^+$; since the proton-accepting ability of the open form is much higher than that of the closed spiro

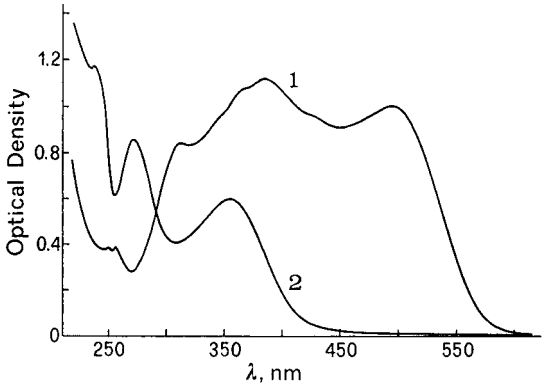

Figure 3. Poly(L-glutamic acid) containing 35 mol % spiropyran units: absorption spectra in HFP for the dark adapted (1) and the irradiated sample (2).

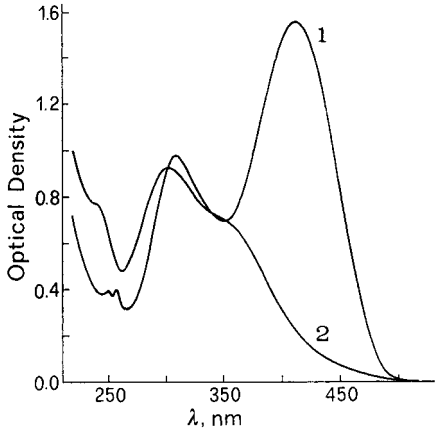

Figure 4. Poly(L-glutamic acid) containing 35 mol % spiropyran units: absorption spectra in HFP in the presence of TFA ($c = 5 \times 10^{-4}$ g/mL) for the dark adapted (1) and the irradiated sample (2).

form, deprotonation of SpH^+ can occur, giving again the neutral spiro structure Sp.

It appears that different photochemical intermediates are involved in acidic and nonacidic conditions; moreover, many questions remain to be answered, such as the precise structural nature of the protonated spiro species SpH^+.

In HFP in the absence of acid, the dark-adapted 35 mol % polypeptide shows broad and intense absorption bands with maxima at about 500 and 390 nm (Figure 3, line 1). This spectrum has been previously discussed[8] and can be assigned to the presence of the zwitterionic merocyanine species Me. Irradiation at 500 nm completely cancels absorption in the visible region, and the colorless solution exhibits absorption bands at 350, 270, and 240 nm (Figure 3, line 2), typical of the closed spiro structure Sp.

Addition of TFA to the HFP solution produces drastic variations of the absorption spectra (Figure 4). In the dark-adapted sample in the presence of acid, in fact, the band at 500 nm is canceled and the resulting spectrum is characterized by an intense absorption band at 410 nm and a weaker band at 310 nm (Figure 4, line 1): such a spectrum corresponds to that reported in the literature for the cationic species MeH^+.[15] Formation of the O-protonated merocyanine species MeH^+ (Figure 2) is also in agreement with resonance Raman studies carried out on spiropyrans in alcoholic solutions acidified with HCl.[16]

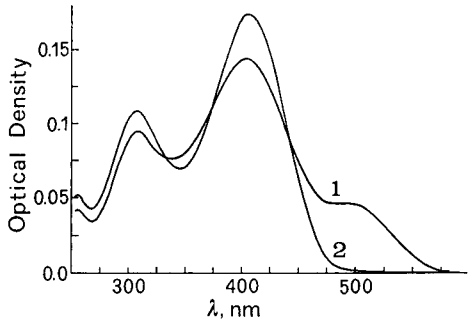

Figure 5. Absorption spectra of the model spiropyran compound II (dark adapted sample) in HFP without acid (1) and in the presence of TFA ($c = 5 \times 10^{-4}$ g/mL) (2).

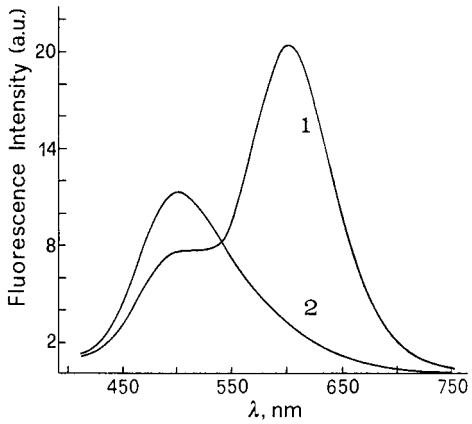

Figure 6. Fluorescence spectra of the model spiropyran compound II (dark adapted sample) in HFP without acid (1) and in the presence of TFA ($c = 5 \times 10^{-4}$ g/mL) (2).

For the low molecular weight spiropyran compound II not bound to a macromolecular matrix, the spectrum in HFP without acid shows absorption bands at 500, 410, and 310 nm (Figure 5, line 1), indicating the simultaneous presence of the zwitterionic merocyanine species Me and the protonated merocyanine species MeH^+. Addition of TFA produces the disappearance of the band at 500 nm and a consequent enhancement of the bands at 310 and 410 nm (Figure 5, line 2), thus confirming that the latter bands must be assigned to the O-protonated merocyanine form MeH^+. In agreement with this, while the emission spectrum of the spiropyran II in HFP without TFA shows two bands at 500 and 600 nm (Figure 6, line 1), the emission spectrum in the presence of acid presents only an enhanced band at 500 nm (Figure 6, line 2).

The presence of both the zwitterionic (Me) and protonated species (MeH^+) in pure HFP is likely to be due to the protonating action of the solvent itself, as observed to occur for spiropyrans in trifluoroethanol solution.[16] The protonated species MeH^+ does not seem to be present in pure HFP when the dye is bound to polypeptide chains, probably because the macromolecular matrix favors formation of zwitterionic merocyanine dimers.[8]

When acidified HFP solutions of the spiropyran-containing polypeptides and of the low molecular weight model compound II are irradiated at 445 nm, the intense absorption band at 410 nm is completely canceled and the spectrum of the bleached solution shows poorly resolved bands at about 350, 300, and 240 nm (Figure 4, line 2). This spectrum should be assigned to a protonated ring-closed spiro form SpH^+.

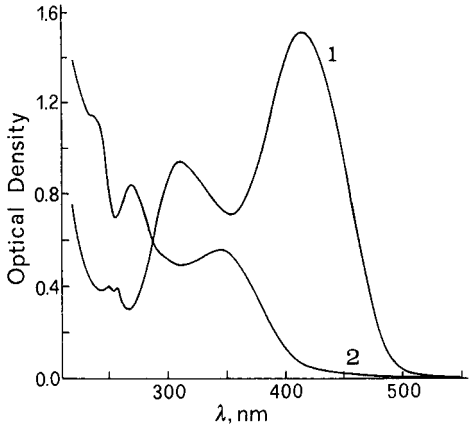

Figure 7. Poly(L-glutamic acid) containing 35 mol % spiropyran units: absorption spectra in HFP/TFE = 30/70 in the presence of TFA ($c = 5 \times 10^{-4}$ g/mL): (1) dark-adapted sample; (2) irradiated sample.

The structure of the species SpH^+ is controversial. Bertelson[18] reports that the N-protonated spiro structure SpH^+(a) (Figure 2) is rapidly formed when a nitrospiropyran is treated with HCl in alcohol at −78 °C. This primary species is then thermally converted to the neutral structure Sp. Conversely, no evidence for N-protonation of the ring-closed structure was obtained by NMR measurements.[19] Bercovici et al.[14] have measured the absorption spectrum of a species obtained by treating a nitrobenzospiropyran with a large excess of acid. The spectrum is characterized by two bands at about 300 and 350 nm and is very similar to that obtained by us irradiating the sample in acidified HFP (Figure 4, line 2). They proposed that such a spectrum should be assigned to a protonated species, but not necessarily the N-protonated spiro structure SpH^+(a): N, O, or even nitro group protonation [SpH^+(a), SpH^+-(b), SpH^+(c) (see Figure 2)] might be involved. Indeed, if the N-protonated structure SpH^+(a) were formed, it would be expected to absorb at shorter wavelengths with respect to the neutral species Sp.

Addition of cosolvents, such as methanol up to 40% or trifluoroethanol up to 70%, does not produce any variation of the spectra for the solutions kept in the dark (Figure 7, line 1) (photochromic groups present as O-protonated species MeH^+). For the irradiated samples, by contrast, the above cosolvents produce significant variations of the spectra which lead to disappearance of the 310 nm band and the simultaneous appearance of a band at 270 nm (Figure 7, line 2). So the resulting spectrum shows the same bands (350, 270, and 240 nm) observed for the neutral spiro structure Sp obtained by irradiation in the absence of acid (Figure 3, line 2). This suggests that the protonated spiro species SpH^+ formed by irradiating the acidified HFP solution, upon addition of 40% methanol or 70% trifluoroethanol, releases a proton and gives the neutral spiro structure Sp. This may be due to the decreased polarity of the medium which inhibits the dissociation of trifluoroacetic acid. Actually it was reported that the influence of acid on photochromism of spiropyrans is linked to the degree of dissociation of the acid in the solvent and no protonation occurs when acid is added to spiropyrans dissolved in nonpolar solvents.[20]

Photomodulation of Polypeptide Conformation: Effect of Solvent. Figure 8 reports the CD spectra of poly(L-glutamic acid) containing 35 mol % spiropyran units, in the presence of TFA at various

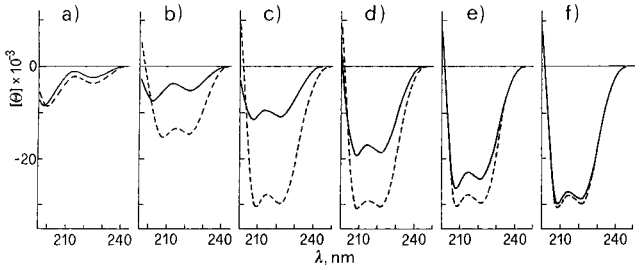

Figure 8. CD spectra of poly(L-glutamic acid) containing 35 mol % spiropyran units in various HFP/TFA/MeOH solvent mixtures. TFA = 5×10^{-4} g/mL; MeOH % (v/v): (a) 0%; (b) 5%; (c) 10%; (d) 15%; (e) 20%; (f) 30%. Key: (—) dark-adapted samples; (- - -) irradiated samples.

HFP/MeOH solvent compositions. In the absence of methanol (Figure 8a), the polypeptide is essentially in a random-coil structure and photoisomerization of the chromophores in the side chains does not result in any conformational variation of the macromolecular main chains.

When MeOH concentration is higher than 30% (Figure 8f), the polypeptide shows the α-helix CD spectrum, characterized by the two typical minima at 208 and 222 nm. Also in these conditions, the CD spectrum indicates that no conformational variation of the polypeptide occurs upon irradiation or dark-adaptation. Conversely, at MeOH concentrations between 5 and 30% (Figure 8b−e), exposure of the sample to light or dark conditions produces reversible variations of the CD spectra, indicative of reversible variations of the helix content, the extent of the photoresponse depending on solvent composition. CD bands in the near-UV and visible region, corresponding to the electronic transitions of the photochromic side chains are also present, but they are very small both for the dark and the irradiated samples and are not reported in Figure 8.

The extent of the photoresponse, measured as photoinduced variation of the α-helix structure, cannot be exactly estimated on the basis of the CD spectra. In fact, for several polypeptides all having α-helical conformation, the intensities of the CD bands were found to show significant differences when the CD spectra were measured in HFP. On the basis of the literature[21] values reported for 100% α-helix in HFP ($[\Theta]_{222} = -30\,000$ to $-40\,000$), the intensities of the CD bands observed for the 35% spiropyran-modified polypeptide in HFP/TFA/MeOH should correspond to a maximum helix content of about 75−100%, while the maximum photoinduced variation of helical structure, observed in HFP/MeOH = 90/10, can be estimated at about 65%.

The polypeptide containing 85 mol % spiropyran units show an analogous behavior and an even more pronounced conformational photoresponse (Figure 9). When methanol concentration is below 5% (Figure 9a), both the dark-adapted and the irradiated samples show the typical CD pattern of disordered polypeptides. In HFP/MeOH = 90/10 (Figure 9b), the sample kept in the dark is a random coil, whereas the sample exposed to light displays the standard CD spectrum of the α-helix. The intensity of the 208 and 222 nm CD bands indicates that under these conditions light causes the full conversion from random coil to 100% α-helix. On increasing methanol concentration, also the dark-adapted sample becomes partially helical, and finally when methanol concentration is higher than 40%, both the dark and the irradiated samples are fully helical (Figures 9c and 9d).

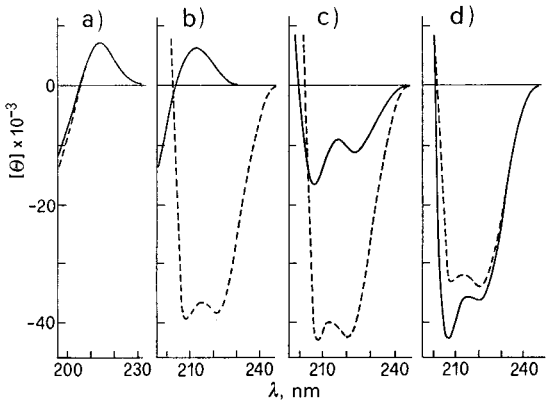

Figure 9. CD spectra of poly(L-glutamic acid) containing 85 mol % spiropyran units in various HFP/TFA/MeOH solvent mixtures. TFA = 5×10^{-4} g/mL; MeOH % (v/v): (a) 0−5%; (b) 10%; (c) 20%; (d) 40%. Key: (—) dark-adapted samples; (- - -) irradiated samples.

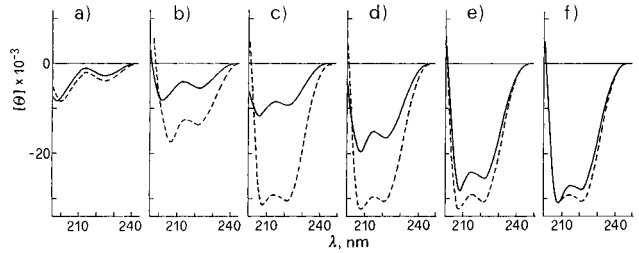

Figure 10. CD spectra of poly(L-glutamic acid) containing 35 mol % spiropyran units in various HFP/TFA/TFE solvent mixtures. TFA = 5×10^{-4} g/mL; TFE % (v/v): (a) 0%; (b) 15%; (c) 30%; (d) 50%; (e) 75%; (f) 90%. Key: (—) dark-adapted samples; (- - -) irradiated samples.

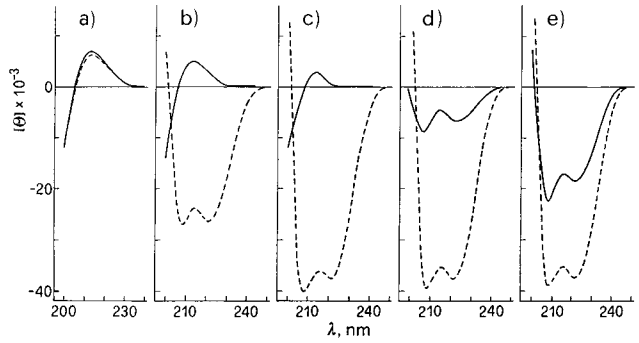

Figure 11. CD spectra of poly(L-glutamic acid) containing 85 mol % spiropyran units in various HFP/TFA/TFE solvent mixtures. TFA = 5×10^{-4} g/mL; TFE % (v/v): (a) 0−10%; (b) 30%; (c) 50%; (d) 70%; (e) 90%. Key: (—) dark-adapted samples; (- - -) irradiated samples.

When TFE is used as a cosolvent (Figures 10 and 11), the polypeptides exhibit CD spectra similar to those displayed in the presence of methanol (Figures 8 and 9), even though there are significant differences. In HFP/MeOH, in fact, photoinduced variations of the CD spectra were found to occur below 30−40% methanol concentration. In HFP/TFE, by contrast, exposure to light induces variations of the CD spectra, indicating the occurrence of photostimulated variations of the helical structure, at all solvent compositions up to 90% TFE concentration. Moreover for the polypeptide containing 85 mol % photochromic units in HFP/MeOH, both the dark-adapted and the irradiated samples were found to be fully helical at a relatively low methanol concentration (40%) (Figure 9d). In HFP/TFE by contrast, the dark sample does not achieve the full helical

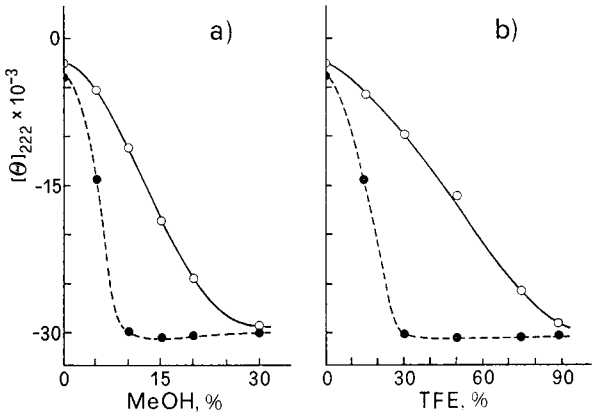

Figure 12. Poly(L-glutamic acid) containing 35 mol % spiropyran units. Variation of ellipticity at 222 nm as a function of solvent composition, for the dark-adapted (—) and the irradiated samples (- - -): (a) in HFP/TFA/MeOH; (b) in HFP/TFA/TFE. TFA = 5×10^{-4} g/mL.

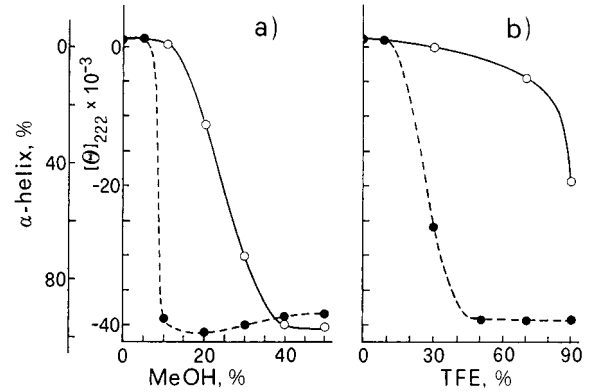

Figure 13. Poly(L-glutamic acid) containing 85 mol % spiropyran units. Coil/α-helix transitions induced by solvent composition, for the dark-adapted (—) and the irradiated samples (- - -): (a) in HFP/TFA/MeOH; (b) in HFP/TFA/TFE. TFA = 5×10^{-4} g/mL.

structure even at TFE concentrations up to 90% (Figure 11e). For the same polymer in the HFP/MeOH solvent mixture, the maximum photoinduced conformational variation, from random coil to 100% α-helix, was observed at 10% methanol concentration (Figure 9b), while in HFP/TFE it occurs at relatively higher concentrations of the cosolvent (TFE = 50%) (Figure 11c).

When the intensity of the CD band at 222 nm, which can be also considered a parameter of the α-helix content, is plotted as a function of methanol or trifluoroethanol concentration, one observes that addition of cosolvent to HFP solutions induces coil−helix transitions (Figures 12 and 13). However the amount of methanol or trifluoroethanol needed to induce the conformational transition is different for the dark-adapted samples and the irradiated ones, so two separate curves are observed. Irradiation at solvent compositions in the range between the two curves gives rise to reversible folding−unfolding of the macromolecular chains.

Conclusions

The mechanism of the photoresponse can be well rationalized on the basis of the spectroscopic measurements and the chemical reactions illustrated in Figure 2.

Absorption and fluorescence spectra, in fact, confirm that in dark conditions, spiropyran compounds dissolved

in HFP acidified upon addition of TFA are present as protonated merocyanine species MeH^+. Exposure to light converts the species MeH^+ into the ring-closed spiro form SpH^+. In the presence of TFA, therefore, the photochromic side chains of the macromolecules are present as cationic species in the dark as well as in light conditions. In both cases the repulsive electrostatic interactions among the charged side chains force the macromolecules to adopt an extended random coil structure, and no photoinduced conformational change is observed as a result of photoisomerization.

When appropriate amounts of the cosolvents MeOH or TFE are added to the HFP/TFA solutions, the protonated species MeH^+ present in the dark-adapted solutions are not altered, but the equilibrium between protonated and unprotonated spiro units present in the irradiated solutions is shifted toward the neutral form, thus removing the repulsive electrostatic interactions among side chains. In these conditions irradiation induces formation of α-helix as it does in HFP without acid.[8,10] MeOH is more efficient than TFE in causing deprotonation of the ring-closed spiro form, so the coil/α-helix transition occurs at lower alcohol concentrations for HFP/TFA/MeOH than for HFP/TFA/TFE solvent mixtures (Figures 12 and 13).

Formation of α-helix even in the dark-adapted samples (photochromic units present in the charged form MeH^+) at high MeOH or TFE concentration may be due to the same effect observed for poly(α-amino acid)s with charged side chains, such as poly(sodium L-glutamate)[22] and poly(L-lysine hydrochloride),[23] which are random coils in a very polar solvent such water, but become helical upon addition of methanol. Such an effect seems to be due to the ability of MeOH to favor "contact ion pairs" between polymer charges and counterions, thus providing a shielding effect among the charged side chains and stabilizing the α-helical structure.[22,23]

In conclusion, the role played by MeOH or TFE cosolvents seems to be different for the irradiated and the dark-adapted solutions. In the former case, the cosolvent should shift the equilibrium between SpH^+ and Sp toward the neutral species. In the latter case, the cosolvent should provide a shielding effect. This may be the reason for the different slope of the coil/α-helix transition curves observed in irradiated and dark-adapted solutions (Figures 12 and 13).

The system described can be considered a synthetic molecular photosensory system in which the primary photochemical event occurring in the photochromic units attached to a polypeptide macromolecular matrix is amplified and transduced by the coil/helix variation of the macromolecules. The extent of the photoinduced structural changes depends on solvent composition, so that the photoresponse is actually modulated by the chemical environment.

More specifically in HFP/MeOH solvent mixture, the methanol concentrations suitable to obtain photostimulated conformational changes are included in the limited range between 10 and 30% for the polypeptide containing 35 mol % photochromic units (Figure 12a), and 10−40% for the polypeptide containing 85 mol % photochromic units (Figure 13a). We can say that this system is characterized by a so-called *gated photoresponse*,[24,25] in the sense that the photoisomerization of the photochromic side chains is able to trigger the coil/α-helix transition of the macromolecular main chains only in a narrow "window" of solvent composition.

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