Photochromic Polypeptides as Synthetic Models of Biological Photoreceptors: A Spectroscopic Study

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ABSTRACT L-Glutamic acid polypeptides containing photochromic nitrospiropyran bound to the side chains at various percentages ("local" concentration) have been synthesized and investigated as possible artificial models of biological photoreceptors. Absorption and fluorescence spectroscopy have been utilized to investigate the photophysical and photochemical properties of nitrospiropyrans, both inserted in the polypeptide chain and in solution as "free" dye. Conformational variations produced by dark storage and light exposure of the photochromic polypeptides have been studied by means of circular dichroism. Dark-kept "free" dyes in hexafluoro-2-propanol solution in the merocyanine form ("open" form) give rise to molecular aggregates, which have been characterized as merocyanine dimers. The equilibrium constant between the monomer and the dimer, K, and their molar extinction coefficients, ϵ, at several wavelengths have been determined. Fluorescence measurements on "free" and polypeptide-bound nitrospiropyrans suggest that the dimerization process between merocyanines is favored when the photochromic units are inserted in the polypeptide chain and that under these conditions an efficient energy transfer from the monomer (donor) to the dimer (acceptor) occurs. By varying "local" as well as total nitrospiropyran concentration, it has been shown that the dimeric species result from intermolecular interactions between photochromic groups inserted in the same polypeptide chain. The α-helix $\rightarrow$ random coil transition of the polypeptide structure after dark storage has eventually been shown to be the result of the dimerization process and not of the dark isomerization per se from the "closed" spiropyran form to the "open" merocyanine form of the dye.

INTRODUCTION

Artificial models of natural photoreceptors have been widely utilized with the aim of elucidating the molecular mechanisms of light-driven biological processes as well as of devising synthetic tools able to mimic the performance of biological light detectors and transducers, and suitable for technological applications.

Even a concise survey of the vast literature in the field is beyond the scope of this paper, but it is worth recalling that the study of model systems with photocoupling properties can provide physical/chemical data and concepts that contribute to understanding of the grounds of in vivo photosynthetic processes (Loach, 1997, and references therein), and at the same time promote the development of artificial systems for converting light energy into vectorial charge separation (see, e.g., Hong, 1995, and references therein; Steinberg-Yfrach et al., 1997).

Similarly, the study of model photosensing and phototransducing systems not only helps clarify the relationships between light-induced modifications in the photopigment and subsequent biophysical/biochemical signal transduction steps (Kinoshita, 1995, and references therein), but can also yield impressive advancements in natural pigment-based photonic devices for applications in holography, neural network optical computing, and optical memories (Birge et al., 1995, and references therein).

With the goal of understanding the basic mechanisms of perception and transduction of light signals in freely motile photoresponsive microorganisms (Lenci et al., 1991, and references therein), a series of experiments was started to set up reliable model systems. To simulate the structural and functional properties of pigment granules, the photoreceptor apparatus of the ciliated protozoa *Stentor coeruleus* and *Blepharisma japonicum*, hypericin-type chromophores, were embedded in liposomes at high local concentration and their photophysical properties studied (Lenci et al., 1995; Angelini et al., 1997). In these hypericin-type chromophores the transduction chain was suggested to be triggered by a charge transfer from the first excited singlet state (Wells et al., 1997).

Photoisomerization of the chromophore, conversely, is known to be the early event in the process of light detection and transduction by sensory rhodopsins in *Halobacterium salinarum* (Birge, 1990, and references therein; Hoff et al., 1997, and references therein) and by photoactive yellow protein in *Ectothiorhodospira halophila* (Genick et al., 1997, and references therein).

Among photochromic pigments, spiropyrans have been intensively utilized to shape photonic devices (Suzuki et al., 1994; Hibino et al., 1994), and have been regarded as possible models of biological photoreceptors (Pieroni and Fissi, 1992; Inouye, 1994; Kinoshita, 1995; Willner and Rubin, 1996; Willner, 1997; Willner and Willner, 1997). Nitrospiropyran monolayer electrodes, in particular, have been shown to provide efficient systems for detection and

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amperometric transduction of optical signals (Lion-Dagan et al., 1994; Willner et al., 1996).

As a matter of fact, when spiropyrans are inserted in polypeptide chains, their photoisomerization induces a conformational variation in the polymer (see, e.g., Fissi et al., 1993, and references therein), and in all of the photobiological processes initiated by a chromophore photoisomerization, from vision (Stryer, 1996, and references therein) to photomorphogenesis (Kendrick and Kronenberg, 1994), the step subsequent to the early photochemical event is a conformational variation of the macromolecule that the chromophore is bound to. In the case of phytochrome A, for instance, in which a photochromic tetrapyrrole is bound to a protein matrix, the $P_r \rightarrow P_{fr}$ phototransformation is accompanied by a photoreversible increase in the α-helix content of the apoprotein conformation (Sommer and Song, 1990; Deforce et al., 1994).

The model system utilized throughout our experiments is a nitrospiropyran-containing poly(L-glutamic acid), dissolved in hexafluoro-2-propanol (HFP), in which the photoisomerization of photochromic units from the "open" merocyanine form to the "closed" spiropyran form (see Fig. 1) yields a reversible random coil to α-helix transition of the polypeptide chain (Fissi et al., 1993).

To better understand the spectroscopic behavior of our photoreceptor model system, an introductory study of samples containing "free" nitrospiropyran in HFP was also carried out by means of absorption and fluorescence measurements. These first results, together with those obtained from absorption, fluorescence, and circular dichroism (CD) measurements on the nitrospiropyran-containing poly(L-glutamic acid), provide evidence for intermolecular interactions between "open" merocyanine forms and contribute to clarification of the mechanism of light-induced structural changes observed in these photochromic polypeptides.

MATERIALS AND METHODS

Preparation of samples

"Free" nitrospiropyran and nitrospiropyran-containing poly(L-glutamic acid) (average molecular weight 250,000) were both prepared following a

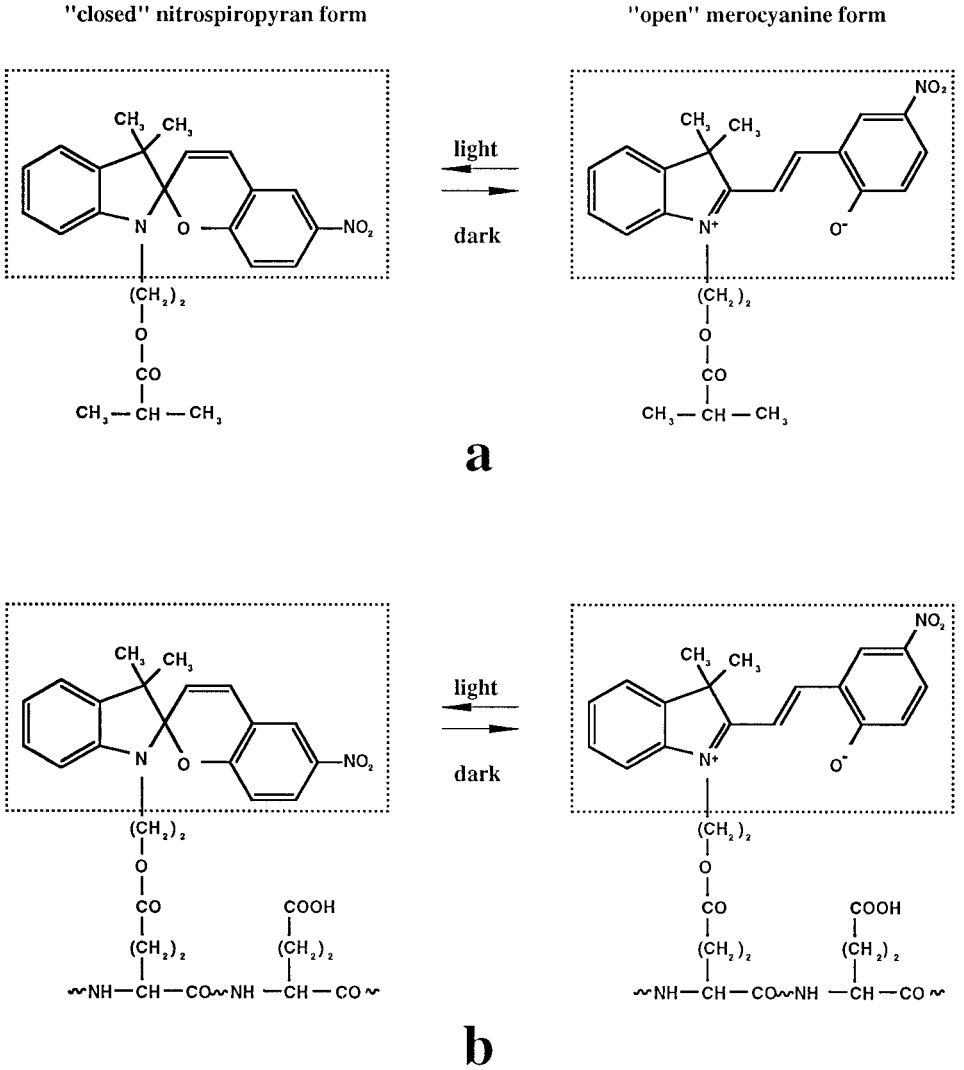

FIGURE 1 Chemical structure and photochromic behavior in hexafluoro-2-propanol (HFP) of nitrospiropyran. (*a*) "Free." (*b*) Bound to poly(L-glutamic acid).

previously described procedure (Fissi et al., 1993). Their chemical structure and photochromic behavior in hexafluoro-2-propanol (HFP) are illustrated in Fig. 1. In the course of this paper, the term "local" concentration of the photochromic group (moles of nitrospiropyran molecules per moles of L-glutamic acid residues) will denote the percentage of polypeptide lateral chains substituted by nitrospiropyran molecules.

All samples were dissolved in HFP (Merck), prepared in red safe light, and stored in the dark.

The dark-reversible photoisomerization of the dye from the "open" to the "closed" form (see Fig. 1) was induced by irradiating the samples by means of a 1-kW Xe ORIEL lamp, coupled to a Balzers K-50 interference filter, centered at 500 nm, with a bandwidth at half-height of ~50 nm. Under these irradiation conditions 1 min was enough to fully photoconvert the merocyanine "open" form to the spiropyran "closed" form. The back-reaction was obtained by keeping the sample in the dark at 25°C for ~20 h. No fatigue of the photocycle was observed.

All temperature-controlled measurements were performed with a Pharmacia Biotech MultiTemp III thermostat.

Absorption and fluorescence measurements

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

Fluorescence emission and excitation spectra were recorded by means of a Perkin Elmer LS 50B spectrofluorometer. Excitation spectra were corrected by using the fluorescence excitation spectrum of the quantum counter rhodamine B in ethylene glycol (3 g/liter) (Lakowicz, 1983). 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.

In our experimental conditions neither the analyzing nor the excitation light beams, respectively, for absorption and fluorescence measurements induced misleading isomerization of dark-adapted nitrospiropyran.

Neither the "free" nor the polypeptide-bound "closed" form of nitrospiropyran showed any detectable fluorescence emission under our experimental conditions.

Circular dichroism measurements

CD spectra were recorded by means of a JASCO J-500A spectropolarimeter. CD intensities are expressed in terms of molar ellipticity values, $[\Theta]$ ($deg \cdot cm^2 \cdot dmol^{-1}$), 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 (Jackson et al., 1991).

RESULTS AND DISCUSSION

In HFP, in the dark, both "free" and polypeptide-bound nitrospiropyran gives colored solutions due to the presence of the "open" merocyanine form. Irradiation with visible light, or just exposure to sunlight, causes a complete bleaching of the solutions, resulting from the formation of the "closed" spiropyran form (Fig. 1).

"Free" dye in HFP

The optical absorption spectra of the "closed" spiropyran form and the "open" merocyanine form are reported in Fig. 2 and are in full agreement with previous findings of Fissi et al. (1993). In Fig. 2 the two spectra are shown together with those of intermediates between the two forms recorded during dark-recovery of the irradiated solution (the spectrum of the last intermediate was recorded 6 h after the sample was put in the dark; as already mentioned, 100% "closed" → "open" isomerization in the dark takes ~20 h at 25°C). The optical absorption spectrum of the irradiated "closed" form shows a band at 270 nm and a weaker one at 360 nm; the dark-kept "open" form has an absorption band at 400 nm, with a shoulder at 500 nm and a less intense band at 310 nm.

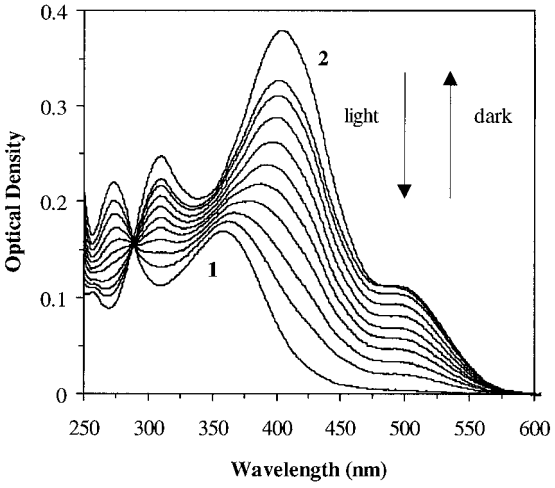

FIGURE 2 "Free" dye in HFP: optical absorption spectra of irradiated "closed" spiropyran form (*1*), of dark-adapted "open" merocyanine form (*2*), and of the intermediates recorded during the thermal decay in the dark at 25°C. Isosbestic point wavelength = 290 nm.

Fluorescence measurements show that excitation at 400 nm of a dark-kept sample ("open" form) gives rise to a fluorescence spectrum with two bands, at 600 nm and 500 nm (Fig. 3 *a*), with the relative fluorescence quantum yield (Φ_f) for the emission at 600 nm markedly depending on the excitation wavelength (Table 1). Relative Φ_f values are, in fact, quite constant (0.012–0.014) for λ_{ex} = 290, 310, and 400 nm, whereas for λ_{ex} = 360 and 500 nm significantly higher values are found (0.025 and 0.072, respectively). This dependence of the relative fluorescence quantum yield on the excitation wavelength suggests that different absorbing and fluorescing species are present in the range 250–600 nm.

The marked difference between the fluorescence excitation spectra for the two emissions at 500 nm and 600 nm (Fig. 3 *b*) confirms the presence of two fluorescing species. As the corrected excitation spectrum is proportional to the absorption spectrum, these results indicate that the species fluorescing at 500 nm strongly absorbs at 400 nm and, more weakly, at 310 nm, whereas the species emitting at 600 nm has an absorption spectrum with a pronounced band at 500 nm and a weaker one at 360 nm. The superposition of the two excitation spectra, multiplied by an appropriate factor, makes it possible to "build" the absorption spectrum corresponding to a mixture of the two species (Fig. 3 *b*); the

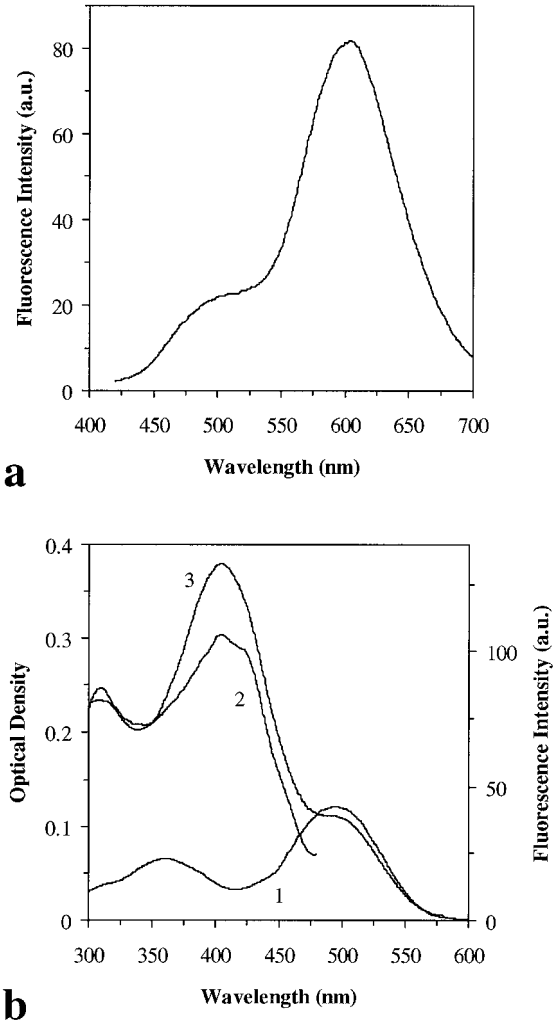

FIGURE 3 Merocyanine form (dark-kept) of the "free" dye in HFP. (*a*) Fluorescence emission spectrum, excitation wavelength = 400 nm. (*b*) *Curve 1*: Fluorescence excitation spectrum for fluorescence emission at 600 nm (right *y* axis); *curve 2*: fluorescence excitation spectrum for fluorescence emission at 500 nm (right *y* axis); *curve 3*: optical absorption spectrum (left *y* axis).

absorption band at 360 nm is not resolved because of its weakness.

In agreement with previous findings of Fissi et al. (1993), the structure of the absorption spectrum of the "open" form was found to notably depend on temperature as well as on dye concentration. Guglielmetti (1990) and Fissi et al. (1993, and references therein) suggested that the different profiles of the absorption spectra, measured at different temperatures and/or dye concentrations, were due to the formation of aggregates between molecules in the "open" form.

To further investigate this phenomenon, a set of fluorescence measurements have been performed while the sample temperature was varied from −5°C to 35°C. Fig. 4 illustrates the effects of temperature on absorption and on fluorescence emission spectra of the "open" form of "free" dye in HFP. At −5°C the absorption spectrum shows a band at 310 nm and one at 400 nm, with a broad weak shoulder at 500 nm, whereas in the fluorescence emission spectrum two bands of comparable intensities, at 500 nm and at 600 nm, are present. As the temperature increases to 35°C, the two absorption bands at 310 nm and at 400 nm appear to be moderately faded, whereas the shoulder at 500 nm becomes

TABLE 1 Fluorescence quantum yields of the dark-adapted "free" dye (merocyanine form) in HFP for fluorescence emission at 600 nm (A) and of the dark-adapted polypeptide-bound 85 mol% photochromic units (merocyanine form) in HFP for fluorescence emission at 620 nm (B)

λ_{ex} (nm)	A Φ_f	B Φ_f
290	0.012	0.069
310	0.014	0.065
360	0.025	0.067
400	0.010	0.064
500	0.072	0.067

Quantum yield values here reported are relative to those of hypericin.

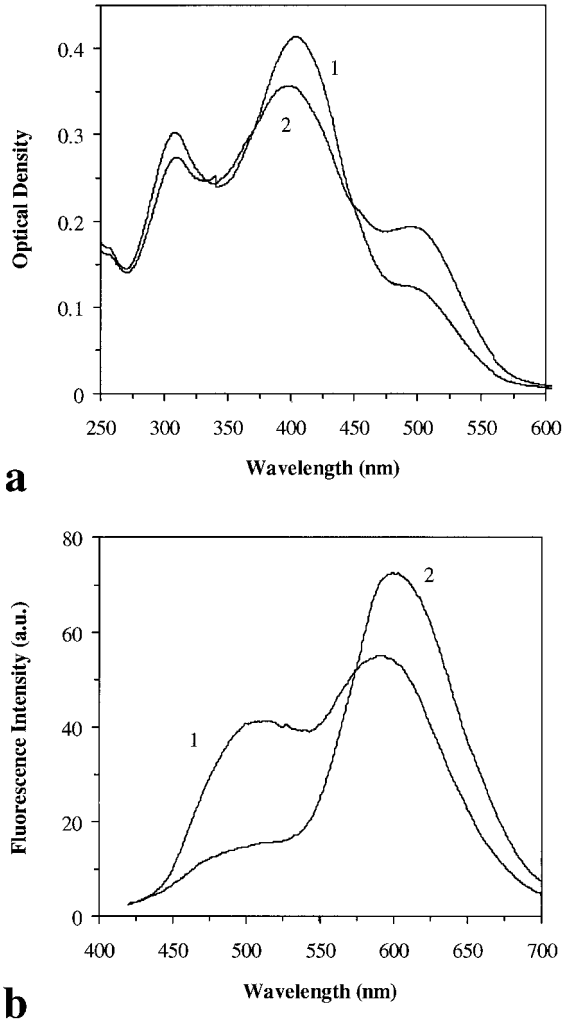

FIGURE 4 Merocyanine form (dark-kept) of the "free" dye in HFP. (*a*) Optical absorption spectra at −5°C (*1*) and 35°C (*2*). (*b*) Fluorescence emission spectra at −5°C (*1*) and 35°C (*2*) (excitation wavelength = 400 nm).

much more pronounced. At 35°C, the fluorescence emission at ~500 nm is strongly reduced and appears as a weak short-wavelength shoulder of the intense band at 600 nm.

In the case of the "closed" form (light-adapted samples), no effect of temperature or nitrospiropyran concentration has been observed on the absorption spectrum, and in any case the fluorescence emission is not measurable.

The above-described results not only confirm that the capacity of photochromic molecules in the "open" merocyanine form to aggregate increases with temperature (Guglielmetti, 1990), but also indicate that both the monomer and the aggregate fluoresce: the fluorescence spectrum of the monomer, which absorbs at 310 nm and 400 nm, is centered at 500 nm, and that of the aggregate, absorbing at 360 nm and 500 nm, is centered at 600 nm.

To ascertain the nature of the aggregate, absorption spectra of "open" merocyanine form at different dye concentrations were measured, normalizing for the total concentration of nitrospiropyran in solution, derived from [Mc], the concentration of the light-adapted "closed" form. The "closed" form, in fact, is surely monomeric, and its concentration can be calculated by means of the extinction coefficient at 355–360 nm (ϵ = 11,200 $M^{-1}\cdot cm^{-1}$ (Fissi et al., 1993)). This normalization procedure emphasizes any modification of the absorption spectrum structure due to the equilibrium shift between possible different absorbing species.

With increasing concentration, the equilibrium shifts from the monomer to the aggregate, and the relative absorbances at 500 nm and at 360 nm rise, whereas the relative absorbance at 400 nm, due to the monomer, correspondingly decreases (Fig. 5).

Assuming that the aggregate is a dimer, as suggested by Lenoble and Becker (1986), and not a complex of higher stoichiometry, the absorbance for the dark-kept sample is

$$A(\lambda) = \epsilon_{Mo}(\lambda)\cdot[Mo] + \epsilon_{D}(\lambda)\cdot[D] \tag{1}$$

where $\epsilon_{Mo}(\lambda)$ and $\epsilon_{D}(\lambda)$ are the molar extinction coefficients for the monomer and the dimer, and [Mo] and [D] are their respective concentrations. None of these quantities are known, and only [Mc] can be calculated. $A(\lambda)$ can be expressed as

$$A(\lambda) = f(\epsilon_{Mo}(\lambda), \epsilon_{D}(\lambda), [Mc]) \tag{2}$$

where $\epsilon_{Mo}(\lambda)$ and $\epsilon_{D}(\lambda)$ are parameters to be estimated. If K is the equilibrium constant of the process

$$M_o + M_o \leftrightarrows D \tag{3}$$

causing two monomers to give origin to a dimer, the relations

$$[D] = K\cdot[Mo]^2 \tag{4}$$

$$[Mc] = [Mo] + 2[D] \tag{5}$$

can be written.

Substitution of Eq. 4 into Eq. 5 yields an equation of the second order in [Mo] depending on [Mc], the solution of which, substituted into Eq. 4, gives [D] as a function of [Mc]. These two expressions transform Eq. 1 in

$$A(\lambda) = \left(\epsilon_{Mo}(\lambda) - \frac{\epsilon_{D}(\lambda)}{2}\right)\cdot\frac{1}{4K} \cdot\left(\sqrt{1 + 8K\cdot[Mc]} - 1\right) + \frac{\epsilon_{D}(\lambda)}{2}\cdot[Mc] \tag{6}$$

This equation is too intricate to obtain reliable estimates of the parameters K, ϵ_{Mo}, ϵ_{D} with best fitting procedures. However, if at a specific wavelength λ_0 the optical density values $A(\lambda_0)$ are linearly dependent on [Mc] (and in our case this condition is fulfilled at 440 nm; Fig. 5), a simplified expression for $A(\lambda_0)$ can be obtained:

$$\frac{A(\lambda_0)}{[Mc]} = \text{cost} \Leftrightarrow \begin{cases} \epsilon_{Mo}(\lambda_0) = \dfrac{\epsilon_{D}(\lambda_0)}{2} \\ A(\lambda_0) = \epsilon_{Mo}(\lambda_0)\cdot[Mc] = \dfrac{\epsilon_{D}(\lambda_0)}{2}\cdot[Mc] \end{cases} \tag{7}$$

where $A(\lambda_0)$ does not depend on K.

The best fit (χ^2 test) of data for absorption values at 440 nm (Fig. 6) gives the values of ϵ_{Mo} and ϵ_{D} for this wavelength to a fairly good approximation (ϵ_{Mo}(440 nm) = ϵ_{D}(440 nm)/2 = 1.6×10^4 $M^{-1}\cdot cm^{-1}$). These values for the extinction coefficients at 440 nm allow us to obtain the

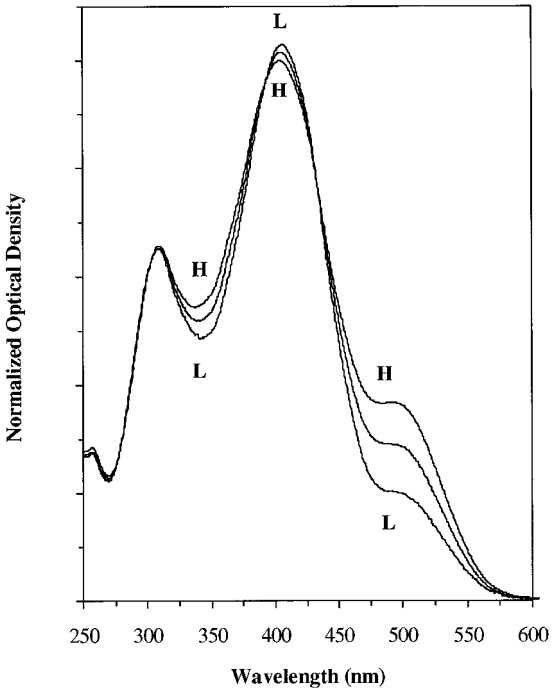

FIGURE 5 Optical absorption spectra of dark-adapted, merocyanine form of the "free" dye in HFP at different concentrations (H: high, [Mc] = 60 μM; L: low, [Mc] = 5 μM). Spectra are normalized for the total dye concentration in solution, derived from [Mc], the concentration of the light-adapted "closed" form. The monomeric "closed" form concentration can be calculated by means of the extinction coefficient at 355–360 nm (ϵ = 11,200 $M^{-1}\cdot cm^{-1}$; Fissi et al., 1993).

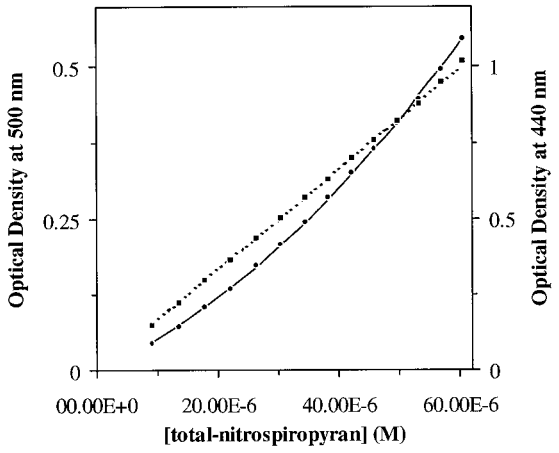

FIGURE 6 Optical densities of merocyanine form (dark-adapted) of the "free" dye in HFP versus total dye concentration, derived from [Mc], the concentration of the light-adapted "closed" form. The monomeric "closed" form concentration can be calculated by means of the extinction coefficient at 355–360 nm (ϵ = 11,200 $M^{-1}\cdot cm^{-1}$; Fissi et al., 1993). ·····, 440 nm; data points fitted with Eq. 7, $\epsilon_{Mo} = \epsilon_D/2 = 1.65 \times 10^4\ cm^{-1}\ M^{-1}$, $K = 10^3$. - - - - -, 500 nm; data points fitted with Eq. 9, $\epsilon_{Mo} = 0.44 \times 10^4\ cm^{-1}\ M^{-1}$, $\epsilon_D = 6.3 \times 10^4\ cm^{-1}\ M^{-1}$, $K = 10^3\ M^{-1}$.

order of magnitude at the other wavelengths. This additional information, too, is not enough for a good fit of our data to Eq. 6, but it is possible to make an approximation that simplifies data elaboration. Assuming that the concentration of the dimer is very low with respect to that of monomer, i.e.,

$$K\cdot[\mathrm{Mc}] \ll 1 \Rightarrow [\mathrm{D}] \ll [\mathrm{Mo}] \qquad (8)$$

expression Eq. 6 becomes

$$A(\lambda) \cong \epsilon_{Mo}(\lambda)\cdot[\mathrm{Mc}] + 2K\cdot\left(\frac{\epsilon_D}{2} - \epsilon_{Mo}(\lambda)\right)\cdot[\mathrm{Mc}]^2 \qquad (9)$$

where higher than second-order terms are not included. Fitting our data by means of this expression, we obtain the ϵ values reported in Table 2.

The resulting value of the equilibrium constant between the monomer and the dimer, $K = 10^3\ M^{-1}$, for the used concentration ($[\mathrm{Mc}]_{max} = 60\ \mu M$), fully satisfies Eq. 8, which in our experimental conditions is a good approximation.

The dimeric nature of the aggregate can be validated, under an assumption equivalent to Eq. 8, also by a modified version of Eq. 9:

$$A(\lambda) = \epsilon_{Mo}(\lambda)\cdot[\mathrm{Mc}] + nK\cdot\left(\frac{\epsilon_A}{n} - \epsilon_{Mo}(\lambda)\right)\cdot[\mathrm{Mc}]^n \qquad (10)$$

in which the suffix A denotes an aggregate of n "open" merocyanines.

As a matter of fact, fitting for different concentration values the optical densities at 500 nm and 400 nm, n comes out to be, respectively, 1.97 and 2.05, i.e., in both cases very close to 2, and again expression 9 is obtained.

From a qualitative point of view, $\epsilon_{Mo}(\lambda)$ and $\epsilon_D(\lambda)$ values, each multiplied by an appropriate factor, assuming an error of 10% in our measurements, smoothly overlay fluorescence excitation spectra (Fig. 7).

From Table 2 it also appears that at 500 nm ϵ_D is an order of magnitude higher than ϵ_{Mo}, as expected because of the low absorbance of the absorption spectrum tail of the monomer in this spectral range, whereas the value of ϵ_{Mo} at 400 nm is about half the value of ϵ_D at 500 nm, as usual in the case of a dimer (Cantor and Schimmel, 1980).

These findings allow us to spectroscopically characterize the monomer and the dimer of the merocyanine form of the "free" dye in HFP: the monomer is the species absorbing at 310 nm and 400 nm and fluorescing at 500 nm; the dimer is the species absorbing at 360 nm and 500 nm and fluorescing at 600 nm. The equilibrium constant of the dimerization process is on the order of $10^3\ M^{-1}$.

In our experimental conditions, the profiles of optical absorption spectra of the "closed" form are identical in all samples, and the absorbance is a linear function of the concentration (ϵ(355–360 nm) = 11,200 $M^{-1}\cdot cm^{-1}$ (Fissi et al., 1993)).

TABLE 2 Molar extinction coefficients of merocyanine dimeric (ϵ_D) and monomeric form (ϵ_{Mo}) in HFP

	ϵ_D ($\times 10^4\ cm^{-1}\ M^{-1}$)	ϵ_{Mo} ($\times 10^4\ cm^{-1}\ M^{-1}$)
400 (nm)	2.50	2.57
440 (nm)	3.30	1.65
500 (nm)	6.30	0.44

Values have been estimated by means of best fitting procedures (χ^2 test).

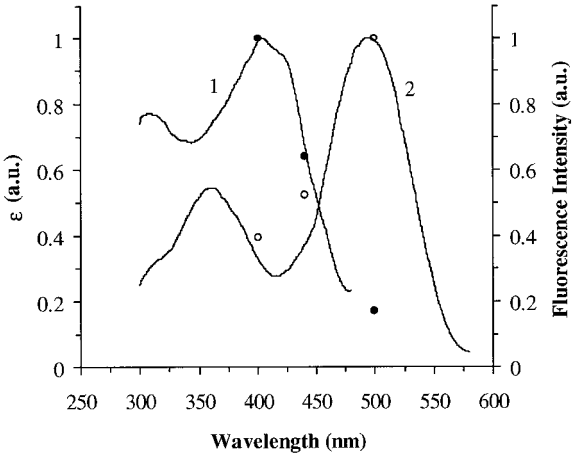

FIGURE 7 Molar extinction coefficients of merocyanine monomer (●) and dimer (○) and fluorescence excitation spectra for emission at 500 nm (*1*) and at 600 nm (*2*). Values of monomer and dimer molar extinction coefficients as well as fluorescence excitation spectra normalized to the maximum are included.

Poly(L-glutamic acid)-bound dye in HFP

Irradiated solutions of polypeptides, which contain photochromic units in the "closed" spiropyran form, show the same absorption bands at 270 nm and 360 nm as observed in the case of "free" nitrospiropyran (see Fig. 2), regardless of the number of photochromic units along the polypeptide chain ("local" concentration). Only the relative intensities of the two bands depend slightly on "local" concentration, probably because of different interactions between substituted and nonsubstituted side chains. As in the case of "free" nitrospiropyran, no fluorescence emission from the poly(L-glutamic acid)-bound "closed" form of nitrospiropyran was detected in our experimental conditions.

Absorption and fluorescence spectra of dark-adapted solutions of poly(L-glutamic acid) containing the "open" merocyanine form are reported for two representative "local" concentrations: 85 mol% and 25 mol%.

In the case of the 85 mol% sample, the absorption spectrum shows a band at 360 nm and a more intense one at 500 nm, and the fluorescence emission spectrum is made up of a single band at 620 nm (Fig. 8 *a*). Both spectra can be assimilated to the absorption and fluorescence emission profiles of the dimeric species of the merocyanine form of the "free" dye in HFP. The two bands in the fluorescence excitation spectrum, at 360 nm and at 500 nm, have exactly the same relative intensities as in the absorption spectrum (Fig. 8 *a*). This fact, together with the finding that the relative fluorescence quantum yield calculated for the emission at 620 nm is independent of the excitation wavelength (see Table 1; $*\Phi_f = 0.064-0.069$), indicates that in the 85 mol% sample a unique absorbing and fluorescing species exists. It is therefore reasonable to infer that, when inserted in the polypeptide chain at high "local" concentration, in the dark, all "open" merocyanine species are in the dimeric form.

In agreement with such a conclusion are the results of a set of absorption measurements of dark-kept (dye in the "open" form) 85 mol% samples at different total concentrations of the photochromic units. They show that the optical density linearly increases with concentration (data not shown) and allow us to calculate the molar extinction coefficient at 500 nm. The value $\epsilon(500\text{ nm}) = 5.7 \times 10^4\ M^{-1}\ cm^{-1}$, assuming an error of 10%, is very close to the $\epsilon_D(500\text{ nm})$ value reported in Table 2.

The absence of any precipitate in dark-kept solutions, finally, indicates that merocyanine dimerization occurs only between molecules inserted in the same polypeptide chain and not among units bound to different macromolecules.

In the case of the 25 mol% sample, the fluorescence emission spectrum is again quite similar to the fluorescence emission spectrum of merocyanine dimers (a single band at ~620 nm), whereas the optical absorption spectrum consists of an intense band at 400 nm with a pronounced shoulder at 500 nm, possibly resulting from the presence in the sample of both the monomer and the dimer (Fig. 8 *b*). Such a hypothesis is supported by the structure of the fluorescence excitation spectrum for emission at 620 nm, which, on the one hand, is not proportional to the absorption spectrum and, on the other hand, reminds one of the fluorescence excitation spectrum of the 85 mol% sample, even though the two bands at 360 nm and 500 nm have approximately the same intensities and the band at 360 nm is broader than in the 85 mol% sample (Fig. 8). Therefore, in dark-kept polypeptides at low dye "local" concentration, both the monomer and the dimer are present, but only the dimer can be detected by fluorescence emission spectroscopy.

A dipole-dipole Förster-type resonant energy transfer (Förster, 1967) from the monomer (donor) to the dimer (acceptor) can satisfactorily explain why in low "local" concentration samples only the dimer fluorescence emission at 620 nm is revealed, whereas no monomer fluorescence emission at 500 nm is detectable.

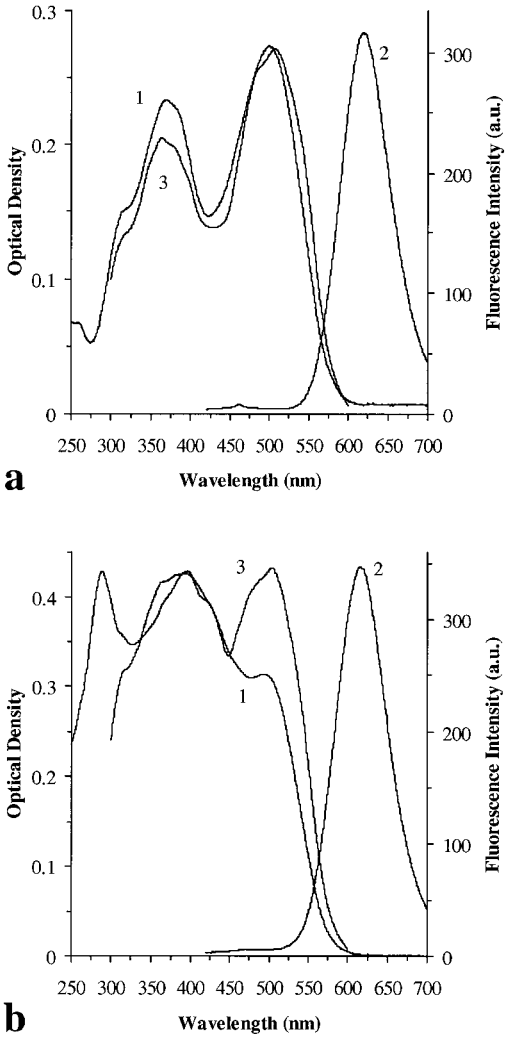

FIGURE 8 Absorption (*1*, left *y* axis), fluorescence emission (*2*, right *y* axis), and excitation (*3*, right *y* axis) spectra of dark-adapted nitrospiropyran-containing poly(L-glutamic acid) in HFP. (*a*) Nitrospiropyran "local" concentration = 85 mol%. (*b*) Nitrospiropyran "local" concentration = 25 mol%.

In the case under study, in fact,

1. The donor (monomer) fluorescence emission maximum is centered at 500 nm, where the acceptor (dimer) optical density is highest (see results on "free" nitrospiropyran).

2. The distance between the donor and the acceptor is below the R_0 threshold (50–100 Å) because both monomers and dimers are constrained to stay in the same polypeptide chain.

3. The factor of relative orientation among donor and acceptor transition dipoles can well be rather high and, in any case, much higher than in "free" dye solution, in which donor and acceptor transition dipoles are randomly oriented.

Evidence in favor of this interpretation of our results comes also from a comparison of these data with those of the temperature effect on "free" dye in the "open" form (Fig. 4). The optical absorption spectra of the 25 mol% polymer and that of "free" dye in solution at 35°C are, in fact, quite similar (Figs. 8 *b* and 4 *a*, respectively), but meaningful differences are observed in the fluorescence spectra. In the case of "free" nitrospiropyran, in which the above-mentioned conditions 2 and 3 for an efficient Förster-type energy transfer are not fulfilled, a contribution from the monomer is clearly present in the fluorescence emission spectrum (Fig. 4 *b*). In the case of poly(L-glutamic acid)-bound dye, conversely, in which the monomer → dimer energy transfer efficiently occurs, the band at 500 nm is not detectable in the fluorescence emission spectrum, and the fluorescence excitation band at 360 nm is broadened toward longer wavelengths, where the monomer absorbs (Fig. 8 *b*).

To correlate the photochromic behavior of the nitrospiropyran-containing polypeptides with their secondary structure, CD spectra have been measured of both dark-kept and light-adapted samples (dyes in the "open" merocyanine form and in the "closed" spiropyran form, respectively).

The polypeptide containing 85 mol% photochromic units, after light exposure and the consequent nitrospiropyran photoisomerization to the "closed" form, exhibits the typical CD spectrum of the α-helix. Assuming for 100% α-helix that $[\Theta]_{222\ nm} = -31{,}000$, measured for poly(methyl-L-glutamate) in HFP solution (Woody, 1977), the intensity of the 222 nm CD band observed for the nitrospiropyran-modified poly(L-glutamate) corresponds to a photoinduced variation of helical structure up to ~85%. Keeping the sample in the dark for ~20 h, thus allowing nitrospiropyran to isomerize to its "open" form, the measured CD spectrum can be attributed to a completely random coil structure of the polypeptide (Fig. 9 *a*).

Exactly the same CD spectrum, corresponding to ~85% α-helix structure, is observed in the light-adapted polypeptide containing 25 mol% photochromic units (Fig. 9 *b*). However, the CD spectrum of this dark-kept sample does not correspond to a completely random coil structure, as was the case for the 85 mol% polymer, but indicates the residual presence of ~55% α-helix structure. Therefore, whereas in polypeptides with high "local" concentration of photochromic groups the "closed" → "open" isomerization in the dark is accompanied by the complete break-up of the α-helix structure of the polypeptide, in low "local" concentration samples, CD spectra demonstrate the presence of a partially helical structure, even after the dark-isomerization of all nitrospiropyran groups.

The residual α-helix structure in dark-kept polypeptides at low dye "local" concentration can be related to the fact that in these samples also monomeric merocyanines are present. The driving force that causes the order-disorder transition of photochromic polypeptides therefore does not appear to be the dark isomerization of the dye from the "closed" to the "open" form per se, but rather the bimolecular interactions between merocyanine units yielding the

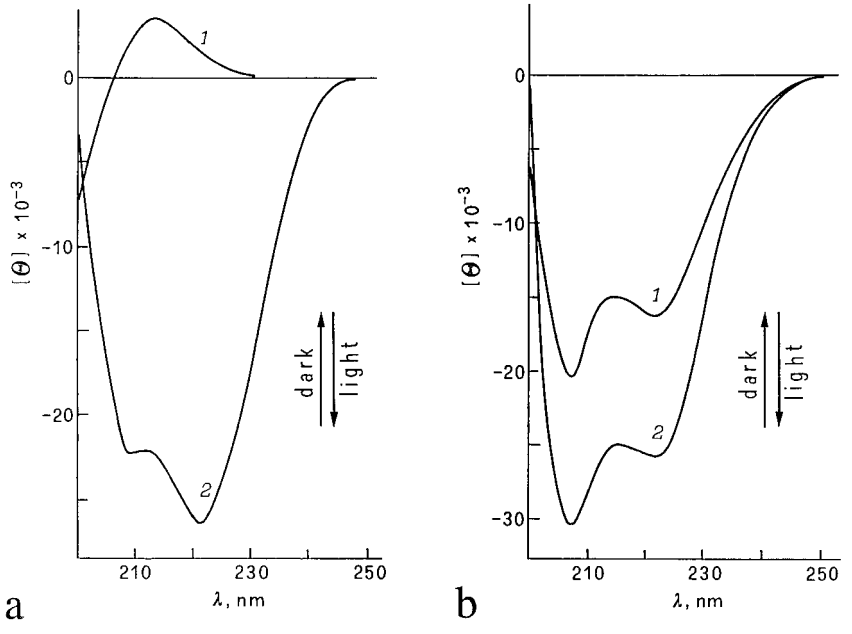

FIGURE 9 CD spectra of dark-adapted (*1*) and irradiated (*2*) nitrospiropyran-containing poly(L-glutamic acid) in HFP. (*a*) Nitrospiropyran "local" concentration = 85 mol%. (*b*) Nitrospiropyran "local" concentration = 25 mol%.

dimeric species. When kept in the dark, the polypeptide is constrained by the dimerization of the merocyanine moieties to stay in a disordered conformation. After irradiation, no molecular interactions occur among nitrospiropyrans photoisomerized to the "closed" form, so that the polypeptide is free to adopt its preferential conformation (α-helix).

A schematic representation of the mechanism of the conformational photoresponse is reported in Fig. 10.

CONCLUSIONS

The results of the spectroscopic study of "free" nitrospiropyran in HFP allow us to reveal, in dark-adapted solutions, the formation of merocyanine dimers, probably resulting from an electrostatic chromophore-chromophore interaction due to the presence of a strong permanent electric dipole moment in the "open" isomer. By the best fitting procedures, the equilibrium constant, K, of the dimerization process and the molar extinction coefficients, ϵ_{Mo} and ϵ_D, of the monomer and the dimer, respectively, have been calculated. The equilibrium constant K is 10^3 M^{-1}, whereas the maximum extinction coefficient of the monomer is $\epsilon_{Mo}(400\ nm) = 2.6 \times 10^4\ M^{-1}\cdot cm^{-1}$, and that of the dimer is $\epsilon_D(500\ nm) = 6.3 \times 10^4\ M^{-1}\cdot cm^{-1}$. Each of these values is given with a maximum experimental error of 10%.

The dimerization process between the "open" merocyanine units inserted in the same polypeptide chain has been shown to be responsible for the α-helix $\rightarrow$ random coil conformational variation of the macromolecule. Under these circumstances, varying the "local" concentration of the photochromic units and, therefore, the yield of the dimerization process, it is possible to obtain artificial photosensors in which the extent of the order $\rightarrow$ disorder transition of the polypeptide chain can be modulated by means of nitrospiropyran "local" concentration.

Concerning the functional properties of this system as an artificial model of a biological photoreceptor, it is worth pointing out that at low nitrospiropyran "local" concentration (less than 40 mol%), the light-induced variation of the secondary structure does not occur along the entire polypeptide chain. However, the consequences of even a tiny conformational variation of a macromolecule in a biological photoreceptor structure can be enough to trigger the chain of biochemical and biophysical events, which allow the light signal to be perceived and transduced in living organisms. As a matter of fact, in the case of phytochrome A, the light-dependent conformational changes involved in triggering the signal transduction leading to photomorphogenesis correspond to a ~3% increase in α-helical folding of the apoprotein (Sommer and Song, 1990; Deforce et al., 1994).

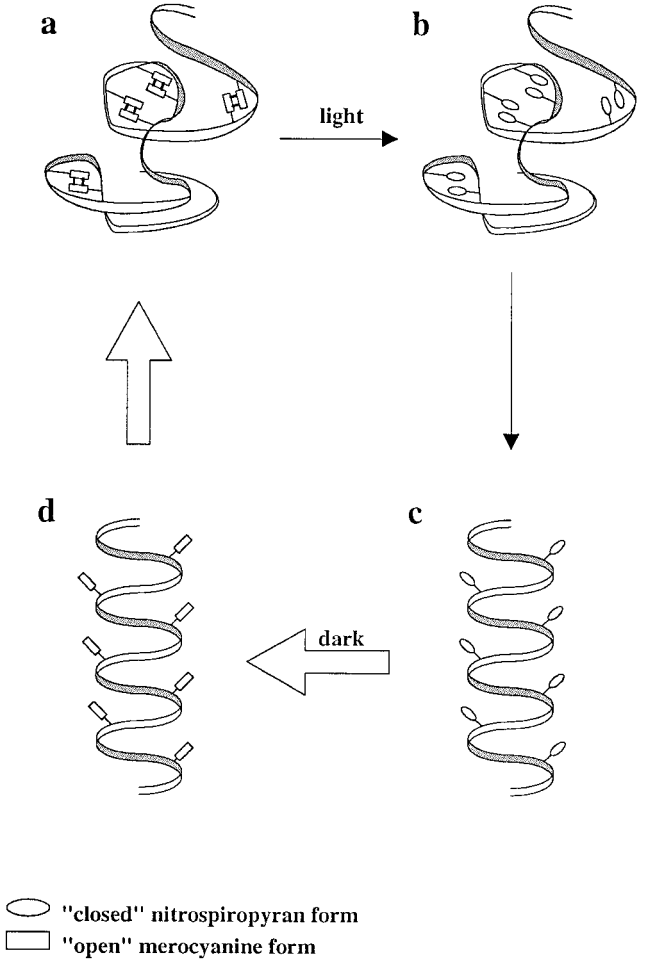

FIGURE 10 Schematic representation of the mechanism of the conformational photoresponse of nitrospiropyran-containing poly(L-glutamic acid) in HFP. Dark-kept photochromic dye molecules, isomerized to the "open" form, are present as dimers (see text), and the polypeptide adopts a random coil structure (*a*). After irradiation, dye molecules photoisomerize to the "closed" form (*b*), and poly(L-glutamic acid) adopts an α-helix structure (*c*). Back in the dark, dye molecules progressively isomerize to the "open" merocyanine form, which dimerizes and causes the α-helix $\rightarrow$ random coil conformational variation of the polypeptide chain (*d*).

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