Solvent Accessibility of the Phycocyanobilin Chromophore in the α Subunit of *C*-Phycocyanin: Implications for a Molecular Mechanism for Inertial Protein-Matrix Solvation Dynamics

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ABSTRACT: The solvent environment of the phycocyanobilin chromophore bound by the α subunit of *C*-phycocyanin was probed in buffered binary solvent systems consisting of water and methanol, acetonitrile, or acetone. The focus of the work was on determining whether the inertial phase of the solvent response observed previously in the α subunit from femtosecond transient hole-burning spectroscopy [Riter et al. (1996) *J. Phys. Chem. 100*, 14198−14205] involves solvent dipoles in the bulk. Continuous absorption and fluorescence spectra at room temperature show that addition of the nonaqueous solvent results in a change in the tertiary structure of the protein so that the phycocyanobilin chromophore is unclamped and allowed to assume a cyclic conformation. At low concentrations of nonaqueous solvent, we observe a conformational equilibrium characterized by a cooperative binding of nonaqueous solvent. The phycocyanobilin chromophore exhibits a nonshifted absorption and fluorescence spectrum characteristic of its native, extended conformation in the state with bound water molecules. In the state with bound solvent molecules, the phycocyanobilin chromophore exhibits an absorption spectrum that reports a cyclic configuration, and its fluorescence is essentially quenched. The absorption and fluorescence spectra exhibit a solvatochromic response in this state, indicating that the chromophore is now exposed to the bulk solvent. Far-UV circular dichroism spectra evidence an abrupt loss of 10% of the α-helical character in the nonaqueous solvent concentration regime that results in exposure of the chromophore to the bulk. These results show that the ultrafast solvation response previously detected in the α subunit in aqueous media from femtosecond transient hole-burning spectroscopy arises solely from protein-matrix solvation dynamics.

The energy landscape theory advanced by Frauenfelder, Sligar, and Wolnyes (1991) predicts the presence of a vast number of minima on a protein's conformational potential energy surface. This theory suggests that proteins share many of the dynamical properties of glasses, especially at low temperatures (Elber & Karplus, 1987; Iben et al., 1989). At physiological temperatures, the theory would anticipate a slow solvation response, so that the dynamic Stokes shift (Simon, 1988) of the fluorescence or stimulated emission of an intrinsic chromophore would be observed on a time scale extending over hundreds of picoseconds or longer. Indeed, work by Pierce and Boxer (1992) and by McLendon, Mukamel, and co-workers (Bashkin et al., 1990) on solvation dynamics in myoglobin showed that the dynamic Stokes shift of the fluorescence of an extrinsic probe spans several orders of magnitude of delay, out to at least the nanosecond regime.

There are a variety of results, however, that suggest that proteins exhibit a regime of fast internal solvation motions that would not necessarily be expected under the glassy picture. Loppnow and co-workers (Fraga et al., 1996; Loppnow & Fraga, 1997) recently obtained mode-specific vibrational reorganization energies for the blue-copper center in plastocyanin from an analysis of resonance Raman excitation profiles. The homogeneous line shape for the ligand-to-metal charge-transfer transition obtained from the coupled vibrational modes is convolved with a Gaussian line shape arising from fast internal protein-matrix motions on the <200-fs time scale. A similar conclusion can be reached from direct measurements of protein-matrix-induced electronic dephasing. The results of three-pulse stimulated photon-echo peak shift experiments by Fleming and co-workers (Joo et al., 1996) on the purple bacterial light-harvesting system LH2 led to the suggestion that the protein matrix surrounding the bacteriochlorophyll *a* chromophores exhibits a fast phase of response on the <100-fs time scale.

Work in our laboratory on energy transfer in the cyanobacterial light-harvesting proteins allophycocyanin and *C*-phycocyanin led us to be interested in obtaining a direct knowledge of vibrational equilibration and protein-matrix solvation dynamics in a single-chromophore-containing protein system of known structure. We characterized the dynamics of the open-chain tetrapyrrole chromophore, phycocyanobilin, that is bound by the α subunit of *C*-phycocyanin so that dynamics involving energy transfer processes between the phycocyanobilin chromophores in the intact, trimeric *C*-phycocyanin (Riter et al., 1996a) and allophycocyanin (Edington et al., 1995, 1996, 1997) systems might be discerned in comparison. The dynamic Stokes shift of the stimulated emission exhibited by the α subunit is characterized by two components, a fast component on the

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<100-fs time scale accounting for most of the response and a slower component on the >10-ps time scale that was not characterized. In discussing this result, we made an analogy to the biphasic solvation response of small polar solvents: a Gaussian-shaped inertial solvation response involving librational, free-streaming motions of nearby solvent molecules (on the <100-fs time scale) precedes a response on a longer time scale involving rotational diffusion. The reader can find a very useful discussion of solvation dynamics in the recent review by Stratt and Maroncelli (1996). The inertial phase of protein-matrix solvation in the α subunit was supposed to involve very short range motions of nearby amino acid side chains and perhaps included water molecules. A diffusional solvation response was supposed to arise from longer-range, conformational motions that would be anticipated to occur over a much longer (>10-ps) time scale (Riter et al., 1996b). It is important to note that the assignment of the solvation response observed in the α subunit to the protein matrix was made exclusively on the basis of the large separation in time between the time scales for the inertial and diffusional response. In bulk water, Fleming and coworkers observed that the diffusional response occurs on the <1-ps time scale, only an order of magnitude slower than the <100-fs time scale for the inertial phase of response (Jimenez et al., 1994; Rosenthal et al., 1991).

Because of the possible general importance of the discovery of an inertial phase of protein-matrix solvation dynamics, we are currently engaged in further studies of protein-matrix solvation in the α subunit of *C*-phycocyanin in order to determine the molecular details of the solvation mechanism. In this paper, we consider the possibility that water molecules in the bulk or on the surface of the protein contribute to the detected ultrafast response. An examination of the solvent-accessible surface (Richards, 1977) adjacent to the phycocyanobilin chromophore, as determined using the conformation of the α subunit in the X-ray crystal structure for the trimeric aggregation state of *C*-phycocyanin (Schirmer et al., 1985, 1986, 1987), suggests that water molecules can approach the chromophore within 0.2 nm; however, the orientation of the pyrrole rings with respect to the solvent-accessible surface leads to the suggestion that the π electrons are not accessible to bulk solvent dipoles.

In this paper, we test this conclusion using solvent-perturbation spectroscopy. We look in particular for a solvatochromic response of the continuous absorption and fluorescence emission spectra (Cantor & Schimmel, 1980; Kamlet et al., 1981; Lakowicz, 1983) as the bulk solvent environment around the protein is titrated with the less-polar solvents methanol, acetonitrile, and acetone. We show that the absorption and fluorescence emission spectra exhibited by the phycocyanobilin chromophore in the α subunit are not at all shifted by a change in the bulk solvent polarity until the protein adopts a perturbed conformation that allows the chromophore to fully escape its binding pocket (though still attached covalently to the polypeptide) and to be exposed to the bulk solvent. Circular dichroism (CD) spectra in the far-UV show that a 10% loss in α-helical character abruptly occurs as the chromophore is exposed to bulk solvent.

EXPERIMENTAL PROCEDURES

Sample Preparation. α Subunits of *C*-phycocyanin were isolated from the AN112 mutant of the cyanobacterium *Synechococcus* PCC 6301 using previously described methods (Glazer & Fang, 1973b). *C*-Phycocyanin trimers were purified by ion-exchange chromatography on a DEAE-cellulose column (Glazer & Fang, 1973a) followed by a second separation on a DEAE-BioGel A column (Bio-Rad). Isolated α subunits were obtained by denaturing the trimers in urea-containing buffer solutions and then separating the α and β polypeptides on a Bio-Rex-70 ion-exchange column (Bio-Rad), as previously described by Glazer and Fang (1973b). Following prolonged dialysis against a 5-mM phosphate buffer solution at pH 7, the renatured isolated α subunits were stored in the dark in the same buffer solution at 4 °C. The same procedure was used to prepare the samples used in the previously reported femtosecond transient hole-burning experiments (Riter et al., 1996b). For experiments in this paper, stock solutions of the α subunits were prepared at a concentration corresponding to an absorbance of 1.2−1.4 cm^{-1} at 620 nm. Solutions of the α subunits in binary water/nonaqueous solvent mixtures were prepared by adding an aliquot of the α subunit stock solution to the prepared solvent mixture. These solutions were used immediately at room temperature for continuous absorption, fluorescence, or circular dichroism measurements. The absorption and fluorescence spectra were stable in these binary solvent mixtures over several hours, and the changes observed from spectra obtained in 100% aqueous solutions were manifested immediately.

Analysis of Aggregation State. We used HPLC size-exclusion chromatography on a Rainin Hydropore-5-SEC column to confirm the previous determination that the subunits are monomeric, at least as isolated using our procedures and under the aqueous solution conditions used in our previous work (Riter, 1997; Riter et al., 1996b). Figure 1 shows examples of chromatograms obtained from the α subunit preparations and from a gel filtration standard (Bio-Rad) containing thyroglobulin (670 kDa), bovine γ globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B-12 (1.35 kDa). The elution profile was obtained with in-line absorption detection at 280 nm to detect total protein content or at 620 nm to detect the phycocyanobilin chromophore. The column was eluted with 25 mM phosphate buffer at pH 7.0 at a flow rate of 0.25 mL/min. The column was injected with α subunit solutions at the same concentration (0.4 absorbance for a 1-mm cell, as measured at 620 nm) used in our previous femtosecond spectroscopic experiments (Riter et al., 1996b).

Figure 1 shows that the main peak in the elution profile of the α subunit solutions elutes at almost the same retention time obtained for the myoglobin component of the gel-filtration standard. The α subunit preparations exhibit two peaks in the elution profile: a main peak corresponding to 18 ± 5 kDa and a much smaller peak at 37.0 ± 10 kDa. The determination of the apparent molecular weight was based on the peak retention time and the linear log(molecular weight) vs retention time profile obtained from the standards. The standard deviations that we report are based on the elution profiles obtained from at least three runs. Our results indicate that our isolation and storage conditions greatly favor the monomeric aggregation state, with only a minor (∼5%) amount of the dimer present.

We should point out here that Glazer, Fang, and Brown (1973) used studies of sedimentation coefficients obtained

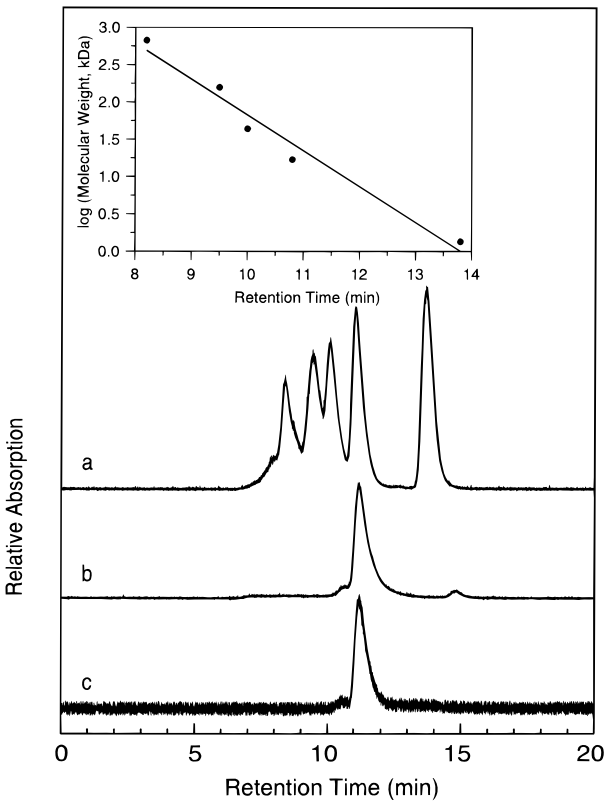

FIGURE 1: HPLC size-exclusion analysis of the α subunit preparations. Traces b and c show the elution profiles obtained for α subunit preparations detected at 280 and 620 nm, respectively. The inset shows the molecular weight calibration curve obtained from the elution profile measured for the gel-filtration standards (trace a).

by analytical ultracentrifugation to show that the subunit preparations they produced were predominantly dimeric. We have no explanation for the apparent discrepancy between the two determinations of the aggregation state; however, the renaturation and subsequent storage conditions used in our laboratory were intentionally chosen to be more than 50-fold diluted from the point-of-use experimental conditions, mentioned above, in order to favor retention of the monomer. The samples used in the studies reported in this paper were obtained in the same way as the samples used in our previous femtosecond spectroscopy work, so it is appropriate to base an assessment of those results on the solvent-perturbation studies presented in this paper.

Spectroscopy. Fluorescence emission spectra were obtained at room temperature with either a ISS model PC 1 photon-counting spectrofluorometer in Professor John Desper's laboratory (Department of Chemistry, Vanderbilt University) or with a home-built diode-array spectrometer. The spectra presented in Figure 3 were obtained with the ISS spectrofluorometer, while the relative quantum-yield measurements were performed with the diode-array instrument.

The diode-array spectrofluorometer employs excitation light selected from the emission of a 100-W quartz-halogen lamp (ISA model AH-10) by a 0.25-m monochromator taken from a Durrum stopped-flow instrument. Emission is collected by a singlet quartz lens and passed to an Acton Research SpectraPro 150 spectrograph by a plastic fiber bundle. The spectrograph was operated in first order with a 300-groove/mm grating so that the entire fluorescence range was imaged on a Tracor-Northern model TN-1223-4I diode-

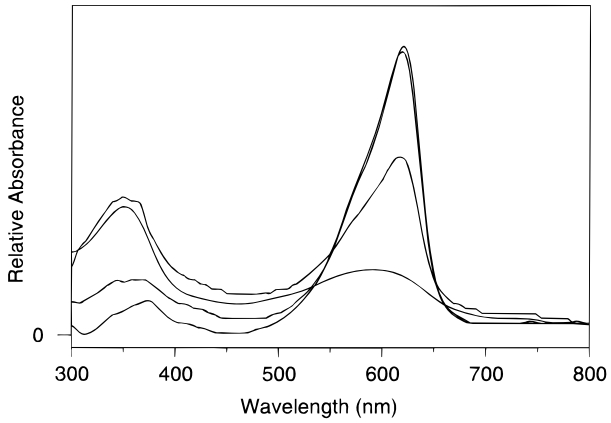

FIGURE 2: Continuous absorption spectra obtained at 22 °C with solutions of the α subunit of *C*-phycocyanin. The presence of methanol in the solution at 37.5%, 50%, and 100% (v/v) causes the 620-nm absorption band to decrease, in the concentration order given, from the maximum intensity observed in the absence of methanol. The 350-nm absorption band increases in intensity as methanol is added to the solution.

array detector, which was operated at room temperature. The fluorescence spectra used for quantum-yield measurements were obtained as the difference of the spectra obtained with equivalent exposure intervals of the diode-array detector with the emission from a solution containing α subunits and that of an equivalent blank containing the same solvent mixture. The spectra were not corrected for the response of the diode array or for the transfer function of the fiber bundle and spectrograph. Downloading of fluorescence spectra to an Apple Macintosh IIsi computer was mediated by LabVIEW (National Instruments) routines.

Absorption spectra were obtained with a Hitachi U-2000 spectrophotometer, which was controlled by LabVIEW routines. Circular dichroism (CD) spectra were obtained at room temperature with a Jasco J-720 instrument using Jasco software running under Microsoft Windows on a 80386 microcomputer. The CD spectra were obtained with a 1.0-mm fused silica cell with solutions of the α subunit that exhibited a 0.6 cm^{-1} absorption at 620 nm. The CD spectra were obtained as the average of 10 scans, each taken with 1-nm steps at a scan rate of 50 nm/min and a band pass of 2 nm.

RESULTS

Effect of Solvent Polarity on the Absorption Spectrum. Figure 2 shows a series of continuous absorption spectra obtained with solutions of the α subunit of *C*-phycocyanin at room temperature to which methanol was added. Comparable series of spectra (not shown) were obtained in the presence of acetone and acetonitrile.

As has been extensively discussed previously in the excellent comprehensive reviews by Scheer (1981) and by Glazer (1985), the α subunit's phycocyanobilin chromophore exhibits two absorption transitions in the UV/visible region of the spectrum. The $\pi \rightarrow \pi^*$ transition populating the lowest singlet state S_1 gives rise to a broad absorption band with a maximum near 620 nm. A second absorption band with a maximum in the 350-nm region of the spectrum arises from transitions to the S_2 and possibly higher singlet $\pi \rightarrow$

[1] Abbreviations: CD, circular dichroism; HPLC, high-pressure liquid chromatography; UV, ultraviolet/visible.

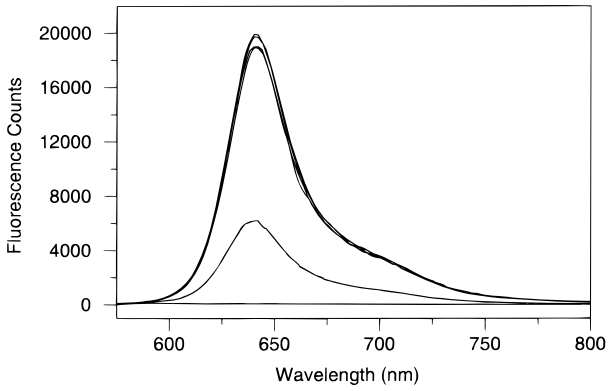

FIGURE 3: Fluorescence emission spectra obtained at 22 °C with excitation at 565 nm with solutions of the α subunit of *C*-phycocyanin. The presence of methanol in the solution at 12.5%, 25%, 37.5%, 50%, and 62.5% (v/v) causes the 640-nm emission band to decrease, in the concentration order given, from the maximum intensity observed in the absence of methanol. The excitation and emission spectrographs were used with 8- and 4-nm band-pass settings, respectively.

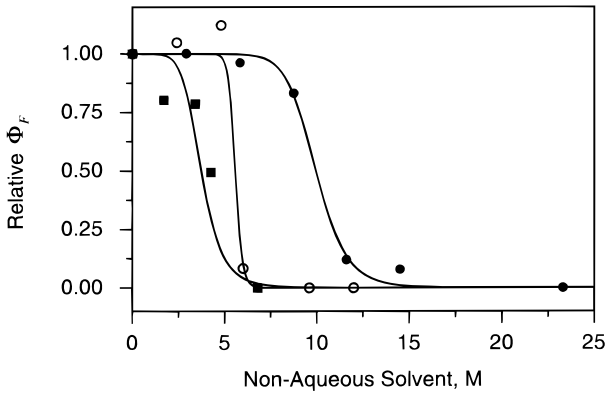

FIGURE 4: Dependence of the relative fluorescence quantum yield obtained with excitation at 620 nm for solutions of the α subunits of *C*-phycocyanin as methanol (●), acetonitrile (○), or acetone (■) is added. The smooth curves drawn through the data points were determined by nonlinear least-squares fits to eq 2. The fit parameters are discussed in the text.

π^* states. In the native configuration observed in aqueous media, the visible transition is about ten times stronger than the transition in the UV region; this intensity ratio arises from the extended configuration of the phycocyanobilin tetrapyrrole. Addition of the nonaqueous solvents causes a change in the structure of the phycocyanobilin-binding pocket in some way that causes a substantial change in the configuration of the chromophore. The UV band grows in intensity at the expense of the visible band as the nonaqueous solvents are added. This kind of intensity change has been associated with the assumption of a hemihelical, porphyrin-like configuration (Scheer, 1981), though the exact stereochemical configuration cannot be unambiguously determined from just the intensity pattern. It should be noted that the spectrum observed from the α subunit in the fully denatured state that arises in 8-M urea solutions [the condition used to separate the α and β subunits from each other from native $(\alpha\beta)_3$ trimers] is quite similar to the spectrum reached in the limit of 100% nonaqueous solvent.

A closer examination of a series of spectra such as that shown for methanol in Figure 1 indicates that at least two steps occur in the change in the configuration of the chromophore. At low concentrations of the nonaqueous solvent [<37.5% (v/v) for methanol and acetone and <20% (v/v) for acetonitrile], an apparent isosbestic point forms near 540 nm as the visible band decreases in intensity. The peak maximum in the visible region is unshifted from the 620-nm maximum that is observed in aqueous media in this regime. At higher concentrations of the nonaqueous solvent, the shape of the visible absorption band changes to a broader profile, yielding a second apparent isosbestic point near 520 nm, and the maximum shifts to ∼595 nm. In this higher concentration regime, the UV absorption band is more intense than the visible band.

Effect of Solvent Polarity on the Fluorescence Emission Spectrum. Figure 3 shows a series of continuous fluorescence emission spectra obtained with α subunit preparations showing the effects of adding methanol. Comparable series of spectra (not shown) were obtained with acetone and acetonitrile. The spectra were obtained with excitation at 565 nm, but identically shaped spectra were obtained with excitation at 620 nm at the peak of the visible absorption band. The fluorescence spectra indicate that addition of the nonaqueous solvent causes a decrease in the fluorescence yield at low concentrations of nonaqueous solvent [<37.5% (v/v) for methanol and acetone and <20% (v/v) for acetonitrile], but the position of the fluorescence maximum is unshifted from 640 nm, the position of the maximum in aqueous media. With methanol present at 37.5% (v/v), the fluorescence quantum yield is ∼80% of its native value [$\Phi_F = 0.72$ for the α subunit of isolated from *Agmenellum quadruplicatum* (Sauer et al., 1987)]. As additional methanol is added, the fluorescence quantum yield abruptly drops further, to less than 10% of its native value. A similar response is observed in acetonitrile and in acetone.

The sigmoidal shape of the response of the fluorescence quantum yield as a function of the addition of nonaqueous solvent is shown in Figure 4. The data points are approximately described by a Hill-type model involving a cooperative binding of nonaqueous solvent:

$$\alpha + nS \overset{K}{\rightleftharpoons} \alpha S_n \qquad (1)$$

In binding *n* nonaqueous solvent molecules *S*, the α subunit is converted to a conformation in which the phycocyanobilin chromophore's fluorescence is quenched by nonradiative decay. The decreased fluorescence quantum yield encountered with the phycocyanobilin chromophore with denatured phycobiliproteins can be attributed to radiationless decay paths arising from an increased mobility of the chromophore in its binding pocket, as has been discussed for denatured forms of the phycobiliproteins (Glazer, 1988; Glazer et al., 1985; Scheer, 1981). The relative fluorescence quantum yield $\Phi_{F,rel}$ is described in terms of the equilibrium constant *K* by

$$\Phi_{F,rel} = 1 - ([S]^n/(K + [S]^n)) \qquad (2)$$

The "equivalence point" (at $\Phi_{F,rel} = 0.5$) in the titration of the fluorescence quantum yield is reached at 3 M in acetone, at 6 M in acetonitrile, and at 10 M in methanol. In all three solvents a sigmoidal response is observed, with $n \cong 6$ in acetone, 10 in methanol, and 25 in acetonitrile, where the sharpest response is observed. The equilibrium constant $K \cong 6 \times 10^3$ in acetone, 6×10^{21} in acetonitrile, and 7×10^{11} for methanol. The parameters listed here were used to

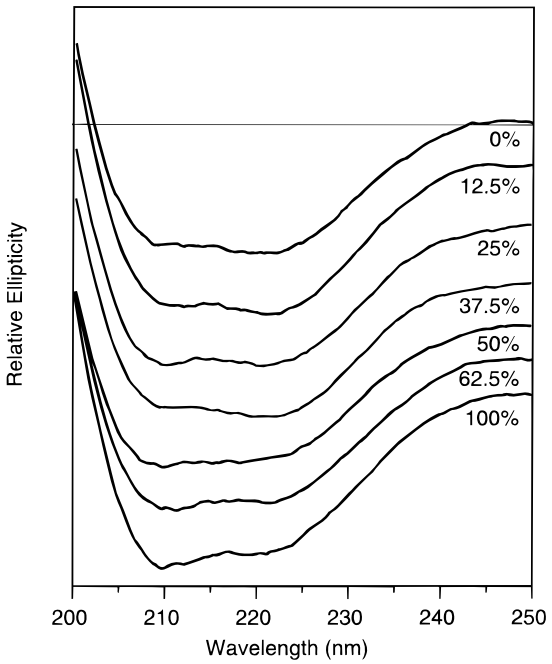

FIGURE 5: Far-UV circular dichroism spectra obtained at 22 °C with solutions of the α subunit of as methanol is added to several (v/v) percent concentrations. The spectra are displaced vertically in an arbitrary manner; the base line is shown for the 0% (v/v) spectrum.

generate the smooth curves drawn through the data points in Figure 4. The rough modeling of the data in Figure 4 is intended only to allow comparison of the relative responses exhibited by the α subunit in the three solvents. Note that the model breaks down significantly above the equivalence point; a weak fluorescence is observed even in 100% (v/v) methanol.

At solvent concentrations above the equivalence point, the maximum of the weak fluorescence observed from the α subunit shifts to the red, to near 650 nm, when methanol or acetone is present. In contrast, the fluorescence maximum observed above the equivalence point when acetonitrile is present shifts to the blue, to near 635 nm.

Effect of Solvent Polarity on the Secondary Structure. We used far-UV circular dichroism (CD) spectroscopy to study the effect on the secondary structure of the α subunit that occurs as a result of adding nonaqueous solvents. Figure 5 shows a portion of the far-UV CD spectrum that is indicative of the presence of an α-helical secondary structure (from 200−250 nm) in the presence of methanol. A very similar series of spectra (not shown) was obtained in acetonitrile; acetone exhibits a strong absorption in this region, so we were not able to obtain CD spectra in the α-helical region. The CD spectrum is dominated by a strong derivative-shaped CD band, with a maximum ellipticity observed near 190 nm and a zero-crossing near 203 nm. The spectrum in this region is quite similar to that observed previously for myoglobin (Goto et al., 1990).

The CD spectral series obtained in methanol and acetonitrile were analyzed by fitting the spectra over the 186−250-nm region to a linear combination of five basis spectra, as described by Hennessey and Johnson (1981) and reviewed by Johnson (1990). The fitted spectral region contains sufficient information to determine the α-helical content but not to discern random secondary structure from other types of secondary structure (Johnson, 1990). Figure 6 shows that

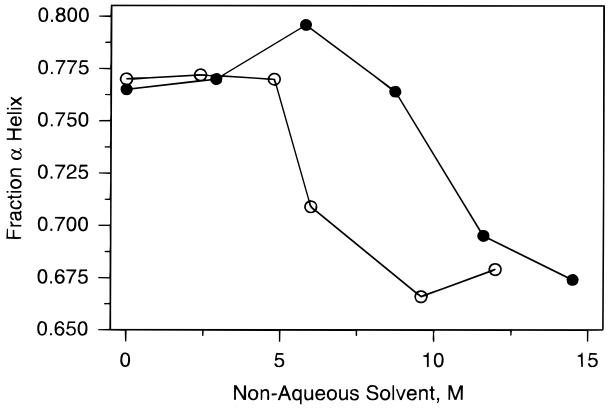

FIGURE 6: α-helical content exhibited by solutions of the subunit as methanol (●) or acetonitrile (○) is added to several concentrations, as determined by fits to far-UV circular dichroism spectra over the 186−250-nm region.

the fraction of the secondary structure exhibiting an α-helical signature is 0.77 in aqueous media and at low concentrations of methanol or acetonitrile. A relatively abrupt change in the α-helical content occurs near 6 M in acetonitrile and 10 M in methanol. The breaks occur at nearly the same concentrations that affect the fluorescence quantum yield (see figure 4). The α subunit retains a secondary structure dominated by α-helical secondary structure, even at high concentrations of the nonaqueous solvent, but the changes observed in the 205−250-nm region probably are accompanied by a collapse of the myoglobin-like tertiary structure exhibited by the α subunit in the native conformation (Schirmer et al., 1985, 1986, 1987). Similar changes were observed in the far-UV CD as myoglobin was induced to assume a molten-globule conformation upon addition of acid (Goto et al., 1990).

DISCUSSION

The addition of methanol, acetone, or acetonitrile to aqueous solutions of the α subunit causes the polarity of the bulk solvent to be lowered. Using the π^* scale for solvent polarities (Kamlet et al., 1981), methanol is the least polar solvent used in the present experiments ($\pi^* = 0.60$), with acetone [$\pi^* = 0.72$ (Maroncelli & Fleming, 1987)] and acetonitrile ($\pi^* = 0.76$) trailing water ($\pi^* \equiv 1.00$) in polarity. A solvatochromic response to a lowering of the bulk solvent polarity (owing to the addition of a solvent of lower polarity) would be to shift the maxima of the absorption (Cantor & Schimmel, 1980) and fluorescence emission spectra (Lakowicz, 1983) to the blue.

A second effect of adding methanol, acetone, or acetonitrile to aqueous solutions of the α subunit is that the tertiary structure of the protein is perturbed. The results indicate that the binding site for the phycocyanobilin chromophore is loosened as the nonaqueous solvents are added. The Hill analysis used in Figure 4 suggests that the interaction of the solvents with the α subunit is specific. In the solvent-bound state S_n (eq 1), the loosened binding site permits radiationless decay of the phycocyanobilin S_1 state.

At solvent concentrations below the equivalence point (Figures 4 and 6), the position of the absorption and fluorescence maximum is not at all affected by the addition of nonaqueous solvent. This finding indicates that the π electrons of the phycocyanobilin chromophore are isolated

from the bulk solvent until the tertiary structure is perturbed by the change in bulk solvent polarity. It is fortunate with respect to the question at hand that the protein structure is the least affected by the presence of methanol, the lowest polarity solvent used in these experiments; we were able to observe an unperturbed absorption and fluorescence emission spectrum even in the presence of a substantial amount of methanol.

At solvent concentrations above the equivalence point, however, the phycocyanobilin chromophore is exposed to solvent. The absorption spectrum observed in the presence of high concentrations of all three nonaqueous solvents is shifted significantly to the blue, as would be expected if the chromophore were entirely bathed in the lowered polarity of the bulk solvent. The weak fluorescence observed at high concentrations of nonaqueous solvent is also sensitive to the solvent; with acetonitrile, the maximum is shifted to the blue, in line with the shift in solvent polarity, but the maximum is shifted to the red in the presence of methanol or acetone. This result suggests that acetone and methanol interact in a specific manner with the phycocyanobilin chromophore in the excited state so that the general solvent effect is overwhelmed. [Note that the two-state model (eq 1) we used to approximately describe the behavior of $\Phi_{F,rel}$ as a function of the nonaqueous solvent concentration breaks down somewhere above the equivalence point. The model postulates that $\Phi_{F,rel} = 0$ in the state with bound solvent molecules, but we observe that there is a weak fluorescence at high solvent concentrations.]

The far−UV CD spectra show that the α-helical structure of the α subunit is relaxed somewhat above the equivalence point, but most of the secondary structure can be described as being α-helical in nature even in the presence of 100% (v/v) methanol. The subtle changes in the far-UV CD are comparable to those observed previously for myoglobin as the pH is lowered until a molten-globule conformation is assumed (Goto et al., 1990). It is possible that the α subunit exhibits a molten-globule-like conformation in the lower polarity solvents used in the present experiments, but the present experiments do not provide information on the details of the tertiary structure.

CONCLUSIONS

The results described in this paper show that bulk solvent dipoles do not interact significantly with the bound phycocyanobilin chromophore in the native conformation of the α subunit. The ultrafast solvation response previously detected using femtosecond transient hole-burning spectroscopy (Riter et al., 1996b), then, arises solely from short-range motions of the surrounding protein matrix. The finding that the structure of the subunit is altered when the polarity is lowered so that the fluorescence yield is affected may be of use in future studies of protein-matrix solvation dynamics in which the region of the spectral density function arising from protein conformational motions is characterized.

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