Structural Perturbations of Azurin Deposited on Solid Matrices as Revealed by Trp Phosphorescence

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ABSTRACT The phosphorescence emission of Cd-azurin from *Pseudomonas aeruginosa* was used as a probe of possible perturbations in the dynamical structure of the protein core that may be induced by protein-sorbent and protein-protein interactions occurring when the macromolecule is deposited into amorphous, thin solid films. Relative to the protein in aqueous solution, the spectrum is unrelaxed and the phosphorescence decay becomes highly heterogeneous, the average lifetime increasing sharply with film thickness and upon its dehydration. According to the lifetime parameter, adsorption of the protein to the substrate is found to produce a multiplicity of partially unfolded structures, an influence that propagates for several protein layers from the surface. Among the substrates used for film deposition, hydrophilic silica, dextran, DEAE-dextran, dextran sulfate, and hydrophobic octodecylamine, the perturbation is smallest with dextran sulfate and largest with octodecylamine. The destabilizing effect of protein-protein interactions, as monitored on 50-layer-thick films, is most evident at a relative humidity of 75%. Stabilizing agents were incorporated to attenuate the deleterious effects of protein aggregation. Among them, the most effective in preserving a more native-like structure are the disaccharides sucrose and trehalose in dry films and the polymer dextran in wet films. Interestingly, the polymer was found to achieve maximum efficacy at sensibly lower additive/protein ratios than the sugars.

INTRODUCTION

The native, biologically active fold of water-soluble proteins is the result of a delicate balance between intra- and intermolecular interactions with the aqueous solvent, an equilibrium that can be broken down when the macromolecule is brought to an interface (adsorption) or when the solvent is replaced in part by other macromolecules (aggregation). These strains on the native structure are expected to be further amplified upon the removal of water (dehydration). Protein adsorption/aggregation is a common and spontaneous process, often deleterious and unwanted, i.e., in medicine, dentistry, and the food industry, but quite useful in fields like the development of biocompatible material (Andrade, 1985), biosensor assemblage (Arnold and Meyerholf, 1988) and immunodiagnostic test system design (Giacomelli et al., 1999). However, in the fabrication of protein-based molecular devices, a major limitation is the uncontrolled loss of biological activity that is often associated with proteins at interfaces and/or incorporated within large molecular aggregates, assemblies from which water is often removed to increase their shelf life. Naturally, progress in preservation of the native biological function requires knowledge of the underlying structural perturbations. The main problems to overcome by current diagnostic tools are very low signals, due to small protein contents and/or optically inhomogeneous media. To date, issues of adsorbed protein structure have been addressed by visible-UV absorption (Pachence et al., 1990; Anderson and Robertson, 1995), fluorescence (Edmiston and Saavedra, 1998; Buijs and Hlady, 1997), circular dichroism (Billsten et al., 1995) and infrared (Girard-Egrot et al., 1997; Ball and Jones, 1995) spectroscopy, besides calorimetry (Haynes and Norde, 1995) and electro-acoustical techniques (Höök et al., 1998). In spite of these efforts, until now little has been known about the structural perturbations that characterize proteins deposited as dehydrated films on solid matrices.

Recently, the phosphorescence emission of tryptophan (Trp) residues was shown to provide an intrinsic probe of protein structure able to operate under highly scattering conditions and with sufficient sensitivity for detecting a single protein layer (Gabellieri and Strambini, 2000). It was pointed out that both the phosphorescence spectrum and the lifetime are indicators of the integrity of the native structure, the unfolded state being characterized by the lack of spectral resolution as well as by considerably shorter decay kinetics. Another important aspect of the decay kinetics is the potential ability to provide insight on the conformational homogeneity of the protein sample, information not commonly available with most biophysical methods insofar as they report on the average protein structure. In this report, Trp phosphorescence is used as an intrinsic probe of protein conformation to inquire into the structural perturbations that may be induced by protein-sorbent interactions (adsorption process) and protein-protein interactions (aggregation process) as water is removed and thin protein films are formed on solid supports. The sorbent surfaces examined are hydrophilic, negatively charged silica, and silica covered with neutral and charged dextran polymers or with hydrophobic octodecylamine (ODA). The ability of compounds, such as the disaccharides sucrose and trehalose and the aforemen-

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tioned dextran polymers, which are commonly used as stabilizing additives, in attenuating the negative effects of protein-protein interactions, is also tested.

The model protein system chosen for this study is azurin from *Pseudomonas aeruginosa.* It is a monomeric protein of known 3D structure (Adman et al., 1978), available in mutated forms and sufficiently small (14 kd) to be amenable to theoretical simulations by molecular dynamics techniques. The polypeptide contains a single Trp residue (W48) located in the central core of the globular structure at least 6.5 Å from the aqueous interface (Adman et al., 1981). Its phosphorescence in neutral aqueous solutions is long-lived (640 ms) and reasonably homogeneous, an indication of conformational uniformity in the phosphorescence time scale (Strambini and Gabellieri, 1991; Hansen et al., 1996). Both the deep location of the chromophore and homogeneous phosphorescence decay are fundamental requisites for a rigorous interpretation of phosphorescence data. The former guarantees inaccessibility to quenching reactions by trace impurities in the medium and, as a consequence, any variation in the phosphorescence lifetime will report unambiguously an alteration of the protein structure deep down to the core of the macromolecule. The latter allows the explicit interpretation of non-exponential decays in terms of medium-induced conformational heterogeneity. Lastly, the recent construction of azurin monolayer films on gold electrodes (Friis et al., 1999) and the demonstration of their potential in mediating interfacial electron transfer (Chi et al., 2000) have aroused a direct, practical interest on the characterization of its structure at solid interfaces.

MATERIALS AND METHODS

All chemicals were of the highest purity grade available from commercial sources and were used without further purification. Trehalose and sucrose were purchased from Pfanstiehl (Waukegan, IL). Dextran (Dx; Mw = 500,000), DEAE-dextran (DEAE-Dx; Mw = 500,000), and dextran sulfate ($DxSO_4^-$; Mw = 500,000) were from Sigma (St. Louis, MO). Copper-free azurin from *P. aeruginosa* was a gift of Prof. A. Desideri, University of Rome Tor Vergata (Rome, Italy). The Cd^{2+} substituted protein (CdAz) was used in all experiments, because Cu^{2+} quenches completely the phosphorescence emission of native azurin (Strambini and Gabellieri, 1991). CdAz was prepared by the addition of a slight excess of $CdCl_2$ to 2.1 mg/ml copper-free azurin solution and then dialyzed overnight into 0.1 mM $CdCl_2$ (Strambini and Gabellieri, 1991). Doubly distilled MilliQ (Millipore, Bedford, MA) water was used throughout. All plastic tips and glassware used for sample preparation were conditioned in advance by standing at least 24 h in 10% HCl Suprapure (Merck, Darmstadt, Germany).

Amorphous CdAz films were prepared by depositing the protein, alone or mixed with a stabilizing additive, on a quartz slide either on the clean silica surface or on a substrate layer adsorbed to the quartz slide. Quartz slides (8 × 15 mm^2, 0.7 mm thick) were cleaned by strong oxidation in HNO_3/EtOH (0.5 h), then conditioned in 10% HCl suprapure (24 h) and thoroughly rinsed with MilliQ water. The substrate layer, consisting of either Dx, DEAE-Dx, or $DxSO_4^{--}$, was adsorbed on the quartz slide by first soaking it for 12 h in the appropriate solution (0.1 mg/ml, 10 mM potassium phosphate buffer, pH 7) and then drying it under N_2 flow. Subsequently, the protein plus stabilizer layer is added on it by spreading 30 μl of a solution containing CdAz and additive all in 0.1 mM $CdCl_2$, 10 mM potassium phosphate, pH 7. Depending on the film thickness the protein concentration ranged between 0.01 and 0.5 mg/ml (amounts corresponding to 2.5 and 125 mg/m^2, respectively), which are equivalent to roughly 1 and 50 layers, respectively (assuming spherical shape, close packing and a specific volume o f 0.73 cm^3g^{-1}). Water was first removed from the film by N_2 flow (0.5 h) followed by moderate vacuum application (0.5 h). To remove more tightly bound water molecules, the vacuum was increased to 0.05 millibar for at least 6 h at ambient temperature. At the end of this procedure the relative humidity of the sample doesn't exceed 5%, and we refer to these samples as dry. On visual inspection, the films appear uniform, although those containing sucrose or trehalose are more transparent. Before phosphorescence measurements, dry films are deoxygenated in an appositely constructed, vacuum tight, cuvette by first applying high vacuum (10 min) and then filling the cuvette with pure N_2. Hydrated protein films were prepared by equilibrating for 24 h dry films with the vapor pressure of saturated salt solutions at 20°C. Relative humidity (RH) of 33%, 55%, and 75% was obtained from the vapor pressure of saturated $MgCl_2$, $Mg(NO_3)_2$, and NaCl solutions, respectively (Wexler and Hasegawa, 1972). Before introducing the film in the phosphorescence cuvette, the latter was thoroughly deoxygenated by repeated vacuum/pure N_2 flushing cycles. The cuvette was then transferred to a nitrogen box, where a small vessel containing a saturated solution (0.2 ml) of one of the above salts plus dithionite (10 mM) was placed at its bottom. Finally, the cuvette containing the salt solution and the protein film above it was closed vacuum tight. The film was examined every 2 h until the phosphorescence lifetime settled to a stable plateau, indicating that vapor equilibration and oxygen removal by dithionite was complete. This time ranged from less than 2 h for 33% RH up to about 6 h for 100% RH. No further changes in the phosphorescence properties were observed during three days. The completeness of O_2 removal by this procedure was checked by the extent of quenching of liver alcohol dehydrogenase phosphorescence whose intrinsic lifetime (τ_0 = 600 ms in 50 mM TRIS:Cl, pH 7.5) and O_2 quenching constant ($k_{O2} = 3.6 \times 10^{-7}$ $M^{-1}s^{-1}$) are known (Strambini, 1987). The results show that a solution of liver alcohol dehydrogenase in the same cell exhibits a lifetime that is indistinguishable from τ_0, implying that $[O_2] < 10^{-8}$ M.

Fluorescence and Phosphorescence Measurements from Protein Films

A fluorometer/phosphorimeter appositely constructed to monitor protein luminescence from thin films was described elsewhere (Gabellieri and Strambini, 2000). Briefly, for emission spectra, continuous excitation is provided by a Cermax xenon lamp (LX150UV, ILC Technology, Sunnyvale, CA) whose output is selected (λ_{ex} = 290 nm, 6 nm bandpass) by a 0.23 m double grating monochromator (SPEX, Mod.1680, Spex Industries, Edison, NJ) optimized for maximum stray light rejection. The exciting radiation, polarized by a Glan-Thomson linear polarizer, is focused on the protein film held with its normal at 30° from the exciting beam direction. The emission from the front surface, collected at 90° from the excitation, is passed through an horizontally placed linear polarizer (Polaroid type HNP'B film) and dispersed by a 0.25 m grating monochromator (Jobin-Yvon, H-25) set to a bandpass of 3 nm. A two-position light chopper intersects either the excitation beam only (fluorescence mode) or both excitation and emission beams in alternating fashion in such a way that only delayed emission gets through to the detector (phosphorescence mode). During phosphorescence measurements the polarizers are removed. A low-noise current preamplifier (Mod. SR570, Standford, Sunnyvale, CA) followed by a lock-in amplifier (Mod. 393, ITHACO, Ithaca, NY) operated at the chopper frequency are used to amplify the photomultiplier (EMI 9635QB) current. The output is digitized and stored by a multifunction board (PCI-20428W, Intelligent Instrumentation Inc., Tucson, AZ) utilizing visual Designer software (PCI-20901S Ver. 3.0, Intelligent In-

strumentation). Spectra are acquired at a scan rate of 0.5 nm s^{-1} and with a time constant (lock-in amplifier) of 125 ms.

For phosphorescence decays, pulsed excitation is provided by a frequency-doubled, Nd/Yag-pumped dye laser (Quanta Systems, Milano, Italy; λ_{ex} = 292 nm) with a pulse duration of 5 ns and a typical energy per pulse of 1 to 2 mJ. An interference filter (DTblau, Balzer, Milan, Italy) with a transmission window between 400 and 450 nm selects the phosphorescence emitted at 90° from the excitation. A gating circuit (Kao and Verkman, 1996) that inverts the polarity of dynodes 1 and 3 for up to 1.5 ms after the laser pulse protects the photomultiplier (Hamamatsu R928, Hamamatsu, Japan) from the intense excitation-fluorescence light pulse. Alternatively, a mechanical shutter (Uniblitz VS 25, Vincent Associates, Rochester, NY), that opens in about 4 ms, blocks light during the excitation pulse from reaching the photomultiplier. As for spectral measurements, the photocurrent signal is amplified and digitized, and multiple sweeps are averaged by the same computer-scope system. Although the phosphorescence decay of CdAz films is in general highly non-exponential, indicative of a wide distribution of lifetime components, analysis of decay curves was carried out in terms of three discrete exponential components by a nonlinear least squares fitting algorithm, implemented by the program Global Analysis (Global Unlimited, Laboratory for Fluorescence Dynamics, University of Illinois, Urbana, IL). Lifetime data from independent sets of experiments are quite reproducible, deviations from the mean value generally do not exceed 10%.

RESULTS AND DISCUSSION

Phosphorescence characteristics of CdAz in the aggregated solid state

The emission from relatively thick (50 layers), dry (RH ≅ 5%) azurin films exhibits a spectrum that is better resolved and slightly blue-shifted compared to that of the isolated protein in solution (Fig. 1). The spectrum is similar, although less resolved, to that obtained in rigid, low temperature glasses and, therefore, indicates that spectral relaxation is partly inhibited. The phosphorescence decay kinetics is slower and highly heterogeneous relative to the solution (Fig. 2). The average lifetime (τ_{av}) is about 870 ms, but there are components ranging from 130 to 2650 ms (Table 1). Shortening of the lifetime is generally taken to indicate enhanced flexibility of the polypeptide about the chromophore even if, in principle, static quenching interactions with side chains (Cys, His, and Tyr) in proximity of the chromophore may also contribute to or dominate the decay rate. For azurin, static quenching could take place with Cys112 if its separation of 8.5 Å were considerably reduced as a result of distortions of the native structure. However, the observation that heterogeneous lifetime is a general feature of all of the other four proteins examined (Gabellieri and Strambini, 2000), plus the finding that at low temperature (below 160 K) the decay kinetics of dry azurin films becomes uniformly long-lived, suggest that short lifetimes in dry films are of prevalently dynamic origin. The direct correlation between τ and solvent viscosity, observed in studies with model compounds (Strambini

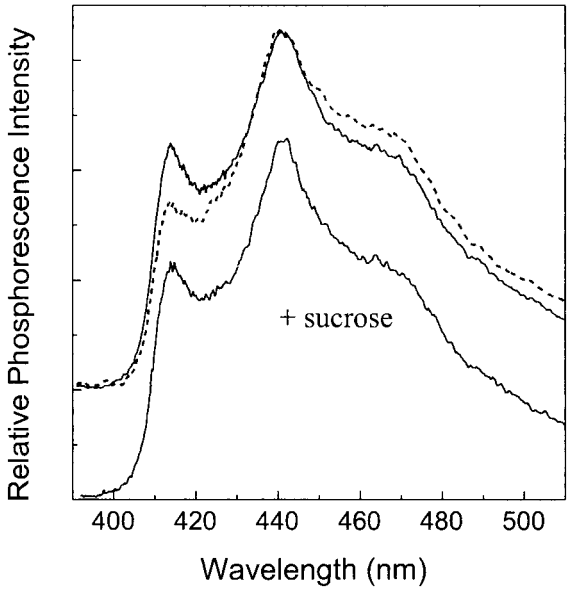

FIGURE 1 Phosphorescence spectrum of 50-protein-layer films of CdAz and of 20 protein layers CdAz/sucrose (1:40, w:w) films. λ_{ex} = 290 nm, T = 20°C. The phosphorescence spectrum of azurin in dilute aqueous solution at 20°C (*dashed line*) is shown for comparison.

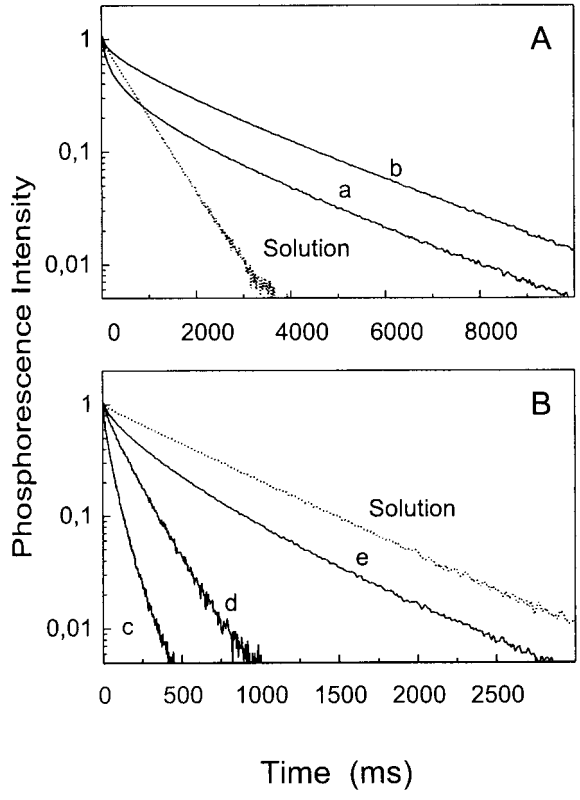

FIGURE 2 Examples of Trp phosphorescence decays from CdAz films. (*A*) Dry films formed by (*a*) 50 layers protein and (*b*) 20 layers protein plus sucrose (1:40,w:w). (*B*) Films hydrated to 75% RH (*c*) 50 layers protein, (*d*) 20 layers protein plus sucrose, and (*e*) 20 layers protein plus dextran (1:40, w:w). The phosphorescence decay of azurin in dilute aqueous solution (*dotted line*) is shown for comparison. λ_{ex} = 292 nm, T = 20°C.

TABLE 1 Lifetime components (τ_i) and corresponding amplitude (α_i) obtained from fitting the phosphorescence decays of CdAz films in terms of three discrete exponential components

No. of protein layers	RH (%)	Substrate	Additive	τ_1 (α_1) (ms) (%)	τ_2 (α_2) (ms) (%)	τ_3 (α_3) (ms) (%)	τ_{av} (ms)
50	Dry	Silica	—	132 (0.49)	832 (0.30)	2647 (0.21)	870
50	33	Silica	—	93 (0.44)	554 (0.38)	1551 (0.18)	530
50	75	Silica	—	36 (0.60)	91 (0.37)	331 (0.03)	65
50	100	Silica	—	16 (0.49)	94 (0.23)	268 (0.28)	103
20	Dry	Silica	Sucrose	197 (0.18)	1017 (0.30)	2731 (0.52)	1760
20	Dry	Silica	Trehalose	203 (0.21)	1055 (0.33)	2679 (0.46)	1623
20	Dry	Silica	Dextran	132 (0.39)	816 (0.36)	2360 (0.25)	935
20	Dry	Silica	Dx-SO_4^{--}	96 (0.60)	557 (0.28)	2101 (0.13)	487
20	Dry	Silica	DEAE-Dx	228 (0.20)	1147 (0.34)	2808 (0.47)	1755
20	75	Silica	Sucrose	30 (0.21)	152 (0.73)	311 (0.06)	139
20	75	Silica	Trehalose	22 (0.14)	129 (0.83)	299 (0.03)	963
20	75	Silica	Dextran	81 (0.22)	333 (0.57)	683 (0.21)	351
20	75	Silica	Dx-SO_4^{--}	28 (0.22)	118 (0.72)	345 (0.06)	112
20	75	Silica	DEAE-Dx	11 (0.24)	140 (0.66)	295 (0.10)	124
1	Dry	Silica	—	23 (0.60)	134 (0.34)	628 (0.07)	103
1	Dry	ODA	—	19 (0.67)	101 (0.27)	411 (0.05)	62
1	Dry	DEAE-Dx	—	21 (0.62)	125 (0.29)	718 (0.08)	107
1	Dry	Dx-SO_4^{--}	—	60 (0.67)	325 (0.24)	1929 (0.09)	292
1	Dry	Dextran	—	22 (0.54)	126 (0.34)	727 (0.12)	142
1	33	Silica	—	15 (0.73)	103 (0.22)	543 (0.05)	61
1	33	ODA	—	12 (0.72)	75 (0.22)	359 (0.06)	47
1	33	DEAE-Dx	—	14 (0.71)	96 (0.22)	709 (0.07)	81
1	33	Dx-SO_4^{--}	—	15 (0.65)	106 (0.25)	695 (0.10)	106
1	33	Dextran	—	11 (0.67)	78 (0.24)	364 (0.09)	59

and Gonnelli, 1995) indicates that, on average, the azurin structure is more rigid in the dry solid state than in solution (τ = 640 ms), a phenomenon that is generally observed upon dehydration of polypeptides (Gregory, 1995). What is remarkable in these films is that the lifetime in different azurin molecules can vary up to at least 20-fold. This implies that in a largely dehydrated state, protein-protein interactions give rise to a multiplicity of distorted protein conformations, many of which exhibit a looser, more flexible internal structure than that of the native state in solution, a condition attainable only through partial unfolding of the polypeptide.

The actual alterations in the structure of azurin might be larger than indicated by the lifetime in the dry state because, in absence of water, segmental flexibility is largely inhibited and proteins tend to form rather rigid matrices irrespective of their 3D structure. In the dry state, mobility is only weakly coupled to the type of secondary structure or location (degree of burial) of the probe and, consequently, Trp phosphorescence is relatively long-lived and only moderately sensitive to protein conformation. On hydration of the polypeptide, however, conformational dynamics is largely restored and τ is then expected, as for proteins in solution, to discriminate more sharply between native and perturbed structures. Hydration of azurin did not significantly affect its phosphorescence spectrum but, as shown in Figs. 2 and 3, decreased drastically the average lifetime. The decay remains distinctly non-exponential up to 100% RH and the changes are stable for at least 3 days. It must be noted that, whereas the decrease of τ_{av} confirms the large gain in structural flexibility expected for hydrated proteins, the lifetime in wet films is considerably smaller than the 640 ms of azurin in solution. Thus, both the magnitude of τ_{av} and the wide distribution in lifetimes attest to long-lasting, unfolding-like distortions of the globular fold in the solid state that persist even at large degrees of hydration. Interestingly, Fig. 3 shows that the tendency to smaller lifetimes is re-

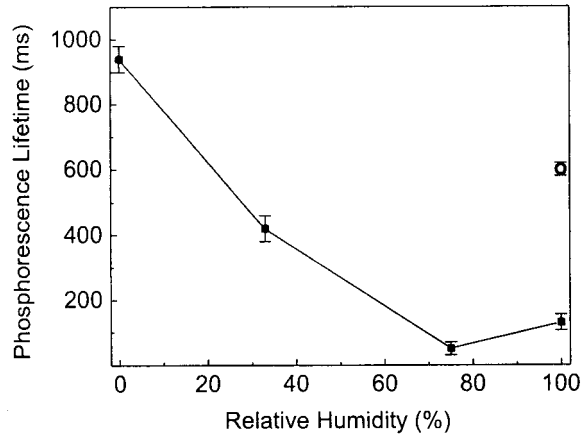

FIGURE 3 Average phosphorescence lifetime of 50 protein layer CdAz films at increasing relative humidity. The phosphorescence lifetime of CdAz in dilute solution is also indicated (○).

versed on approaching 100% RH, although τ_{av} does not increase to the solution value. Eventually, when the protein film is dissolved in water, azurin recovers the unperturbed lifetime. This finding clearly implies that the observed perturbations are reversed when water-protein interactions fully replace protein-protein interactions in the aggregate.

Influence of the substrate on the phosphorescence lifetime

Decreasing the amount of protein deposited on the film a greater fraction of macromolecules will come into direct contact with the solid support and the protein structure will be increasingly affected by protein-substrate interactions. The phosphorescence emission of azurin films, containing an amount of protein equivalent to monolayer coverage, exhibits a largely unaltered spectrum but drastically shorter decay kinetics, relative to 50 layers of films. On a silica substrate, the average lifetime of dry films is 103 ms, the decrease from 870 ms with 50 layers, indicating a large increase in the flexibility of the protein core. The decay is again highly heterogeneous and over 90% of the sample possesses a lifetime drastically shorter than that of the protein in solution or in thick films (Table 1). Note that as we have no control on the thickness homogeneity of the protein layer, conformational multiplicity may in part arise from macromolecules that are not in direct contact with the sorbent. The observation, however, that a predominant fraction of the sample exhibits a shorter lifetime than in solid azurin aggregates (thick films) suggest that most macromolecules experience the substrate perturbation.

In an attempt to attenuate the negative influence of the substrate on the protein structure, quartz supports were pretreated with either hydrophilic dextran polymers (Dx, DEAE-Dx and $DxSO_4^{--}$) or with the surfactant ODA that on quartz forms a hydrophobic surface, as confirmed by the large contact angle of water droplets. According to τ_{av}, neutral and negatively charged dextrans reduce the flexibility of adsorbed proteins, dextran sulfate being the most efficient (Table 1 and Fig. 4 *A*). ODA, on the other hand, has an opposite effect, the interaction of the protein with the hydrophobic surface being more destabilizing relative to quartz, a behavior not unusual with water-soluble proteins in contact with non-polar surfaces (Wu et al., 1993).

The strain on the protein structure induced by contact with the substrate is even more apparent in hydrated films. The results, reported in Table 1, show that at 33% RH τ_{av} is roughly twofold smaller than in the dry state, and, on approaching 100% RH, the phosphorescence decay becomes too short-lived for detection. This behavior is observed with all substrates. At 33% RH (Fig. 4 *A*) the average lifetime is largest with dextran rather than with dextran sulfate, and ODA is no longer destabilizing relative to silica the general trend showing that in the wet state the differences among these substrates are attenuated. By and large,

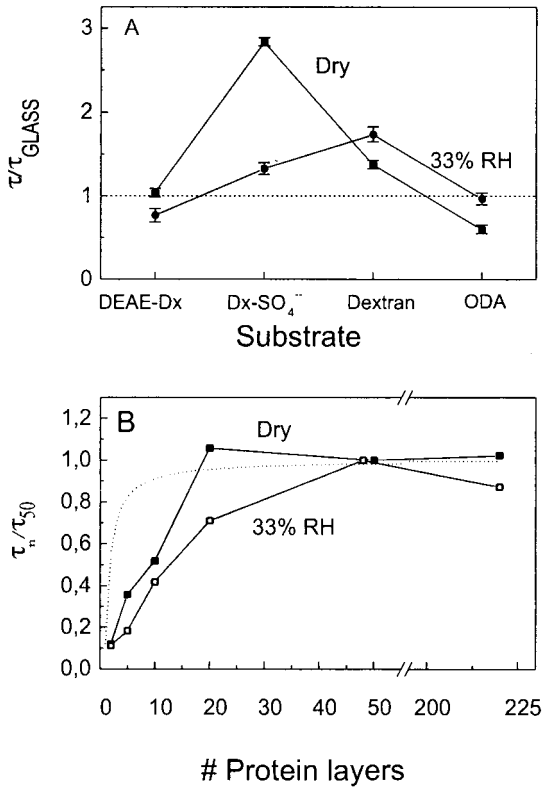

FIGURE 4 (*A*) Effect of the substrate (silica) on the Trp phosphorescence lifetime of dry (■) and 33% hydrated (□) CdAz monolayer films. (*B*) Thickness range of the substrate effect. The dotted curve represents the average lifetime predicted for a sample where only the phosphorescence lifetime of the adsorbed layer would be affected by the substrate.

the above results on azurin films emphasize that in the water-poor solid state, the structure-destabilizing effect of protein adsorption is greater relative to protein self-aggregation. These findings are consistent with a substantial loss of secondary and tertiary structure, as revealed by circular dichroism on adsorbed proteins (Norde and Favier, 1992), compared to a less drastic perturbation of the globular fold found by infrared spectroscopy on protein powders (Carpenter and Crowe, 1989).

To test the distance range of the substrate-induced structural perturbations, films with an increasing number of protein layers were constructed. The average phosphorescence lifetime as a function of film thickness is shown in Fig. 4 *B*. The lifetime increases monotonically with film thickness and reaches a plateau (τ_{av} = 870 ms) only beyond 10 layers. According to τ_{av}, full recovery from the destabilizing influence of the substrate is even more gradual at 33% RH. The dotted line in the plot of Fig. 4 *B* represents the hypothetical value of τ_{av} predicted for dry samples in the case in which only the phosphorescence lifetime of the adsorbed layer would be affected by the substrate. The discrepancy with experiment is quite evident and emphasizes that under these conditions the substrate perturbation

propagates, on average, to several protein layers from the surface.

Effect of stabilizing additives on the phosphorescence emission of CdAz films

The effectiveness of various stabilizing agents was compared at a constant protein/additive ratio of 1:40 (w:w) on films 20 protein layers thick, dry and equilibrated with 75% RH. Control tests with single protein layer films yielded analogous results. The additives employed were the disaccharides sucrose and trehalose and the polymers Dx, DEAE-Dx, and $DxSO_4^{--}$. Incorporation of these compounds does in general affect the phosphorescence lifetime but not the phosphorescence spectrum (see, e.g., sucrose in Fig. 1). The average lifetime of each film, reported in Fig. 5, indicates that in the dry state only sucrose and trehalose, by doubling the magnitude of τ_{av}, have a significant compacting effect on azurin structure. The dextran polymers have either no effect or, in the case of $DxSO_4^{--}$, actually enhance its flexibility. On partial hydration of the films (75% RH), however, all additives exert a net refolding action on the polypeptide. Interestingly, the large, sevenfold increase in lifetime found with uncharged Dx shows that, in hydrated films, this compound is by far the most effective among them. Note that the structuring effects of the additives inferred from the phosphorescence lifetime are also supported by an increased homogeneity of the phosphorescence decay. As shown in Fig. 2, the decay of azurin becomes increasingly more exponential in the presence of sucrose (dry) or Dx (75% RH). These findings suggest that the additives, by isolating the protein from contacts with other proteins and/or with the substrate, reduce the multiplicity of distorted structures caused by these specific interactions.

To inquire on the dependence of these effects on the amount of additive incorporated, films were constructed with an additive/protein ratio varying from 1 to 40. The average lifetime of Dx- or sucrose-stabilized films, equilibrated at 75% RH, is shown in Fig. 5 *B*. These lifetime profiles point out that dextran, as opposed to sucrose, is effective at relatively small weight ratios, reaching almost maximum efficacy when the polymer is 10 times in excess of the protein. Considering the respective molecular weights of polymer and protein, one finds that the utmost stabilizing action is already achieved at a dextran:azurin molar ratio of roughly 1:3. By contrast, the effectiveness of sucrose sets in more gradually and at higher weight ratios, so much so that a plateau is apparently not reached within the range of compositions tested.

The different behavior of the two compounds is probably a reflection of distinct stabilizing mechanisms. A protective action by the sugars is not unexpected, because both sucrose and trehalose are known to stabilize the folded structure of proteins in solution (Lee and Timasheff, 1981; Xie and Timasheff, 1997) as well as to help prevent irreversible inactivation of enzymes during the process of lyophilization and long-term storage (Carpenter and Crowe, 1989). In the dehydrated state, this ability of sugars appears to be related to their versatility in satisfying H-bonds formation with surface groups of the macromolecule, normally bound to water molecules, without imposing great adjustments of the native structure (Carpenter and Crowe, 1989; Prestelski et al., 1993). Furthermore, by forming rigid glasses at low water contents, they presumably quench structural fluctuations and help maintain the compact globular state in the protein by inhibiting unfolding-like conformational transitions. The large increase in phosphorescence lifetime by these sugars at low water contents and the relatively large amounts needed to produce these effects are probably related to bulk properties of these compounds, such as multiple crystalline phases and glass-forming behavior (Crowe et al., 1996). With regard to large polymers, although they appear with increasing frequency as excipients in protein stabilizing formulations (Gibson et al., 1993), little is known of the underlying protecting mechanism. They are chosen on an empirical basis, and their effectiveness is often protein-specific. From the results obtained here, the influence of dextran polymers on azurin films differ from that of

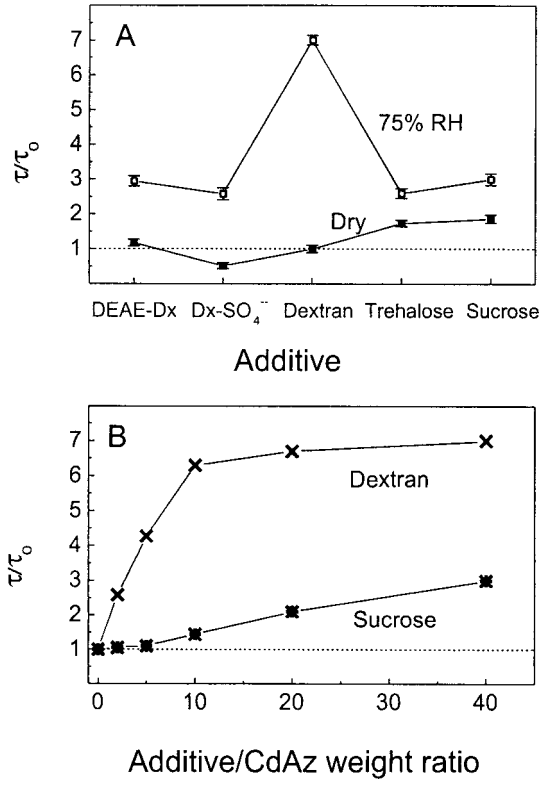

FIGURE 5 Additive effect on the phosphorescence lifetime of 20 layer CdAz films. (*A*) Change in average lifetime for dry (■) and 75% hydrated (□) films induced by the incorporation of various additives at an additive/protein weight ratio of 40/1. (*B*) Lifetime profiles of hydrated (75% RH) films at increasing of dextran and sucrose content.

sugars on at least two accounts: they are little effective in the dry state and, furthermore, do not require large amounts to reach maximum efficacy (Fig. 5). The former quality is probably related to the stiffness of the polymer in the dry state. In this state the hydroxyl groups along its backbone may not have the necessary conformational freedom to form a H-bonding template complementary to that of surface groups of the protein, the mechanism by which dextran, in the wet state, might in fact act as a stabilizer. It is remarkable, however, that this result is not achieved by positively and negatively charged dextran even if the sulfate anion and the alkylamine cation rank in the Hofmeister series among the strongest stabilizing ions (Baldwin, 1996; Cacace et al., 1997). Preliminary observations with other two proteins suggest that the greater effectiveness of neutral, over charged, dextran is not peculiar to CdAz. Evidently, charges in the polymer that are not matched by protein counterions give rise to electric fields that can impose a considerable strain on the globular fold, particularly in conditions of low dielectric constant. The other positive feature of dextran, that of reaching full effectiveness at relatively low polymer/protein weight ratios, suggests that its action derives mostly from the direct interaction between protein and surrounding dextran molecules rather than from bulk properties of the additive.

In conclusion, by monitoring alterations in the dynamic properties of the protein core, the tests conducted with CdAz as a model protein confirm that important structural perturbations occur when a protein is taken from the aqueous solution into solid matrices. They establish that in the solid state, i) both protein-substrate and protein-protein interactions concur in altering the native conformation with the production of a multiplicity of partially unfolded forms; ii) the substrate perturbation, unlike for protein adsorption in aqueous solutions, propagates far beyond the adsorbed layer and the range of this effect is even greater in hydrated films; and iii) the stabilizing action of most commonly used additives is also manifest in the solid state but, here, their efficacy may differ considerably between wet and dry films. The results obtained with azurin would suggest that in general, dextran might be the stabilizer of choice for protein-based devices working in aqueous media, whereas sugars like sucrose and trehalose should offer greater protection for long term storage of dry samples.

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REFERENCES

Adman, E. T., and L. H. Jensen. 1981. Structural features of azurin at 2.7 Angstroms resolution. *Isr. J. Chem.* 21:8–12.

Adman, E. T., R. E. Stenkamp, L. C. Sieker, and L. H. Jensen. 1978. A crystallographic model for azurin at 3 Å resolution. *J. Mol. Biol.* 123: 35–47.

Anderson, A. B., and C. R. Robertson. 1995. Absorption spectra indicate conformational alterations of myoglobin adsorbed on polydimethylsiloxane. *Biophys. J.* 68:2091–2097.

Andrade, J. D. 1985. Principles of protein adsorption. *In* Surface and Interfacial Aspects of Biomedical polymers, Vol. 2. Protein adsorption. J. D. Andrade, editor. Plenum Press, New York. 1–80.

Arnold, M. A., and M. E. Meyerholf. 1988. Recent advances in the development and analytical applications of biosensing probes. *CRC Crit. Rev. Anal. Chem.* 20:149–196.

Baldwin, R. L. 1996. How Hofmeister ion interactions affect protein stability. *Biophys. J.* 71:2056–2063.

Ball, A., and R. A. L. Jones. 1995. Conformational changes in adsorbed proteins. *Langmuir.* 11:3542–3548.

Billsten, P., M. Wahlgren, T. Arnebrant, J. McGuire, and H. Elwing. 1995. *J. Colloid Interface Sci.* 175:77–82.

Buijs, J., and V. Hlady. 1997. Adsorption kinetics, conformation, and mobility of the growth hormone and lysozyme on solid surfaces, studied with TIRF. *J. Colloid Interface Sci.* 190:171–181.

Cacace, M. G., E. M. Landau, and J. J. Ramsden. 1997. The Hofmeister series: salt and solvent effects on interfacial phenomena. *Q. Rev. Biophys.* 30:241–277.

Carpenter, J. F., and J. H. Crowe. 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry.* 28:3916–3922.

Crowe, L. M., D. S. Reid, and J. H. Crowe. 1996. Is trehalose special for preserving dry biomaterials. *Biophys. J.* 71:2087–2093.

Chi, Q., J. Zhang, J. U. Nielsen, E. P. Friis, I. Chorkendorff, G. W. Canters, J. E. T. Andersen, and J. Ulstrup. 2000. Molecular monolayers and interfacial electron transfer of *Pseudomonas aeruginosa* azurin on Au(111). *J. Am. Chem. Soc.* 122:4047–4055.

Edmiston, P. L., and S. S. Saavedra. 1998. Molecular orientation distributions in protein films: III. Yeast cytochrome c immobilized on pyridyl disulfide-capped phospholipid bilayers. *Biophys. J.* 74:999–1006.

Friis, E. P., J. E. Andersen, Y. I. Kharkats, A. M. Kuznetsov, R. J. Nichols, J.-D. Zhang, and J. Ulstrup. 1999. An approach to long-range electron transfer mechanisms in metalloproteins: in situ scanning tunneling microscopy with submolecular resolution. *Proc. Natl. Acad. Sci. USA.* 96:1379–1384.

Gabellieri, E., and G. B. Strambini. 2000. Tryptophan phosphorescence as a monitor of protein conformation in molecular films. *Biosensors Bioelectronics.* 15:483–490.

Giacomelli, C. E., M. G. E. G. Bremer, and W. Norde. 1999. ATR-FTIR study of IgG adsorbed on different silica surfaces. *J. Colloid Interface Sci.* 220:13–23.

Gibson, T. D., J. N. Hulbert, B. Pierce, and J. I. Webster. 1993. The stabilisation of analytical enzymes using polyelectrolytes and sugar derivatives. *In* Stability and Stabilization of Enzymes. W. J. J. van den Tweel, A. Harder, and R. M. Buitelaar, editors. Elsevier/North Holland, Amsterdam. 337–346.

Girard-Egrot, A. P., R. M. Morélis, and P. R. Coulet. 1997. Bioactive nanostructure with glutamate dehydrogenase associated with LB films: protecting role of the enzyme molecules on the structural lipid organisation. *Thin Solid Films.* 292:282–289.

Gregory, R. B. 1995. Protein hydration and glass transition behavior. *In* Protein-Solvent Interactions. R. B. Gregory, editor. Marcel Dekker, Inc./USA, New York. 191–264.

Hansen, J. H., D. G. Steel, and A. Gafni. 1996. Detection of a pH-dependent conformational change in azurin by time-resolved phosphorescence. *Biophys. J.* 7:2138–2143.

Haynes, C. A., and W. Norde. 1995. Structures and stabilities of adsorbed proteins. *J. Colloid Interface Sci.* 169:313–328.

Höök, F., M. Rodahl, B. Kasemo, and P. Brezezinski. 1998. Structural changes in haemoglobin during adsorption to solid surfaces: Effects of pH, ionic strength and ligand binding. *1998. Proc. Natl. Acad. Sci. USA.* 95:12271–12276.

Kao, H. P., and A-S. Verkman. 1996. Construction and performance of a photobleaching recovery apparatus with microsecond time resolution. *Biophys. Chem.* 59:203–210.

Lee, J. C., and S. N. Timasheff. 1981. The stabilization of proteins by sucrose. *J. Biol. Chem.* 256:7193–7201.

Norde, W., and J. P. Favier. 1992. Structure of adsorbed and desorbed proteins. *Colloid Surf.* 64:87–93.

Pachence, J. M., S. Amador, G. Maniara, J. Vanderkooi, P. L. Dutton, and J. K. Blasie. 1990. Orientation and lateral mobility of cytochrome c on the surface of ultrathin lipid multilayer films. *Biophys. J.* 58:379–389.

Prestelski, S. J., N. Tedeschi, T. Arakawa, and J. F. Carpenter. 1993. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophys. J.* 65:661–671.

Strambini, G. B. 1987. Quenching of alkaline phosphatase phosphorescence by O_2 and NO. *Biophys. J.* 52:23–28.

Strambini, G. B., and E. Gabellieri. 1991. Phosphorescence from Trp-48 in azurin: influence of Cu(II), Cu(I), Ag(I), and Cd(II) at the coordination site. *J. Phys. Chem.* 95:4352–4356.

Strambini, G. B., and M. Gonnelli. 1995. Tryptophan phosphorescence in fluid solution. *J. Am. Chem. Soc.* 117:7646–7651.

Wexler, A., and S. Hasegawa. 1972. Relative humidity-temperature relationships of some saturated salt solutions in the temperature range 0° to 50°C. *In* Precision Measurements and Calibrations. R. L. Bloss, and M. J. Orloski, editors. Government Printing Office, Washington, D. C., NBS Special Publication 300, 8:287–294.

Wu, H., Fan, Y., Sheng, J., and S-F Sui. 1993. Induction of changes in the secondary structure of globular proteins by a hydrophobic surface. *Eur. Biophys. J.* 22:201–205.

Xie, G., and S. N. Timasheff. 1997. The thermodynamic mechanism of protein stabilization by trehalose. *Biophys. Chem.* 64:25–43.