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Structure and dynamics of proteins encapsulated in silica hydrogels by Trp phosphorescence

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Abstract

This report establishes the conditions for monitoring the intrinsic Trp phosphorescence of proteins encapsulated in silica hydrogels and demonstrates the usefulness of the delayed emission for examining potential perturbations of protein structure-dynamics by the silica matrix. Phosphorescence measurements were conducted both in low temperature (140 K) glasses and at ambient temperature on the proteins apo- and Cd-azurin, alkaline phosphatase and liver alcohol dehydrogenase together with the complexes of liver alcohol dehydrogenase with coenzyme analogs ADPR and H_2NADH. While spectral shifts and broadening indicate that alterations of the Trp microenvironment are more marked on superficial regions of the macromolecule the decay kinetics of deeply buried chromophores show that the internal flexibility of the polypeptide in two out of three cases is significantly affected by silica entrapment. Both the intrinsic lifetime and the bimolecular acrylamide quenching constant confirm that, relative to the aqueous solution, in hydrogels the globular fold is more rigid with azurin, looser with alcohol dehydrogenase and substantially unaltered with alkaline phosphatase. It was also noted that large amplitude structural fluctuations, as those involved in coenzyme binding to alcohol dehydrogenase or thermally activated in alkaline phosphatase, were not restricted by gelation. Common features of the three silica entrapped proteins are pronounced conformational heterogeneity and immobilization of rotational motions of the macromolecule in the long time scale of seconds.

Keywords: Sol–gel; Wet gels; Protein-structure; Dynamics; Phosphorescence

Abbreviations: Az, apoazurin; LADH, horse liver alcohol dehydrogenase; AP, alkaline phosphatase; Trp, tryptophan; ADPR, adenosine (5′) diphospho (5)-β-D ribose; H_2NADH, 1,4,5,6,-tetrahydronicotinamide adenine dinucleotide; *kq*, Bimolecular quenching constant; τ, intrinsic phosphorescence lifetime.

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1. Introduction

In the last decade the entrapment of functionally active proteins within a porous three-dimensional network formed by inorganic silicate matrices has received considerable attention for its numerous potential applications in diverse fields such as biocatalysts, biosensors and immunodiagnostics [1–4] (and references within). Besides practical applications, confinement of proteins within a rigid cage is appealing also for more basic studies. Confinement prevents self-aggregation reactions and is, therefore, attractive in fundamental investigations of the factors affecting the stability of the native fold [5]. Furthermore, by limiting the space to extended configurations of the polypeptide, the solid matrix may also serve to stabilize the more compact native structure [6].

Although most enzymes maintain some catalytic efficiency, the state of proteins entrapped in silica sol–gel matrices is still poorly characterized. It has been pointed out that several factors may contribute to perturb the native fold [7–10]. Among them the direct adsorption of the macromolecule to the inorganic matrix, its covalent linkage to it, the altered properties of water in the pores and harsh polymerizing conditions. According to spectroscopic techniques based on CD and IR the secondary structure remains largely native like although in the case of myoglobin and α-lactalbumin partial unfolding was reported [5]. More subtle perturbations of tertiary and quaternary structure are not as readily detected but may be inferred indirectly from the change in ligand binding affinity [1,3], heterogeneous enzyme kinetics [10,11] Trp fluorescence [3,9] and restrictions on the allosteric R-T transition of hemoglobin [12,13]. A related question is how entrapment of biopolymers in rigid cages, which are characterized by a relatively high solvent viscosity [14–16], may affect protein dynamics both with respect to the flexibility/conformational freedom of the polypeptide and the rotational mobility of the whole macromolecule. To date there is no direct measure of the internal flexibility of proteins in hydrogels. The R-T conformational isomerization in hemoglobin is drastically slowed down [12,13] but the kinetics of ligand rebinding suggest that the gel does not perturb the structural fluctuations involved in that process [7]. Absorption and fluorescence of several proteins hinted at native conformations within a rigid polymer cage in which segmental motions are largely unaffected but global movements, such as unfolding and rotation, are restricted [4,9]. However, other analysis of silica entrapped cytochrome *c*, glucose oxidase, [17,18] human and bovine serum albumin [19] and green fluorescence protein [20] have concluded that the protein environment is similar to the aqueous solution and that practically unhindered molecular rotations occur.

The sensitivity of Trp phosphorescence to the nature [21] and to the dynamical properties [22–28] of the Trp environment provides a potentially attractive approach to address issues regarding perturbations of the tertiary structure, the degree of gel induced structural heterogeneity as well as the effects of entrapment on both the internal flexibility and the rotational mobility of the macromolecule up to the long, ms/s, time scale. The present investigation first addresses the feasibility of monitoring the phosphorescence emission from proteins embedded in wet gels. Then, it examines the effects of encapsulation on the internal dynamics of proteins by employing two complementary methods: one is based on the sharp dependence of the intrinsic phosphorescence lifetime of Trp (τ_0) on the flexibility of the polypeptide around the chromophore [22]. The second method determines the bimolecular phosphorescence quenching rate constant (kq), governing the diffusion of acrylamide through the globular fold to the site of the triplet probe [25]. Lastly, the rotational mobility of the macromolecule within the gel cage is determined from the anisotropy of the phosphorescence emission of internal, rigidly held Trp residue [26]. The proteins chosen for this enquiry are monomeric apoazurin (Az), dimeric liver alcohol dehydrogenase (LADH) and dimeric alkaline phosphatase (AP). They exhibit a long ($\sim$1 s) phosphorescence lifetime even in fluid solutions at ambient temperature, and in each case the emission originates from a single well identified Trp residue [23], a feature that is important for evaluating the homogeneity of the protein sample inside silica gels. We find that under suitable conditions protein

phosphorescence is readily detected in wet silica gels. The triplet state probe reveals that structural perturbations by the gel are more pronounced in superficial regions of the macromolecule although changes in segmental flexibility are found even for internal, rigid cores of the globular structure. Other common features of entrapped proteins are a marked conformational heterogeneity and a complete lack of rotational freedom inside the gel cage.

2. Experimental section

All chemicals were of the highest purity grade available from commercial sources and were used without further purification. Tetramethyl orthosilicate (TMOS), ADPR and alkaline phosphatase from *Escherichia coli* were purchased from Sigma Chemical Co (St. Louis, MO). Liver alcohol dehydrogenase from horse (LADH) was supplied by Boehringer (Mannheim, Germany). Copper-free azurin (Az) from *Pseudomonas aeruginosa* was a gift from Prof. Desideri, University of Roma (Tor Vergata, Italy). Cd-azurin (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 $CdCl_2$ [29]. 1,4,5,6-Tetrahydronic nicotinamide adenine dinucleotide (H_2NADH) was synthesized as described before [30]. Acrylamide (>99.9% electrophoresis purity) was from Bio-Rad Laboratories (Richmond, CA). Spectroscopic grade propylene glycol was from Merck (Darmstadt). Water, doubly distilled over quartz, was purified by using a Milli-Q Plus system (Millipore Corp., Bedford, MA). All glassware used for sample preparation was conditioned in advance by standing for 24 h in 10% HCl suprapur (Merck, Darmstadt).

2.1. Preparation of sol–gels

The silica sol was prepared using a standard protocol [1]. Typically 7.61 g TMOS, 1.69 ml of water and 0.11 ml of 0.04 N HCl were mixed and sonicated for 15 min. The sonicated sol (60 μl) was first mixed with 20 mM Hepes buffer pH 7.5 (60 μl) and, subsequently, added to the protein solution in the same buffer (30 μl). For CdAz, 0.1 mM $CdCl_2$ was added to the buffer. For all proteins, the final concentration ranged from 5 to 8 μM. To slow down the polymerization process all solutions were kept in ice. After mixing, the sol–gel was placed to set at ambient temperature in appositely constructed Teflon cuvettes with a $3\times3\times10$ mm^3 trough at the bottom. When gelation was complete (~2–5 min) 1 ml of Hepes buffer was layered on top of the gel to remove the methanol produced during polymerization. The washing was repeated at regular intervals (six times) during the first day. Later, the monolith was removed from the Teflon mould and placed in a test tube with 2 ml of buffer. During the first week of aging the gel was washed with 2 ml of buffer two times a day and after that the gel was stored in buffer until used. In the case of Az, the procedure for the initial 2 days was slightly modified to avoid leakage of the small protein before complete gelation. During the first 2 days the volume of washing buffer was reduced (200 μl) and the time of soaking was cut to only ~10 min each time, after which most of the supernatant was removed. In this way over 90% of the protein remained permanently trapped. During aging and successive storage the gels were maintained at 4 °C a temperature at the proteins in solution are stable for several weeks. All phosphorescence measurements were conducted with gels from 1 to 2 weeks old, although it was found that the emission is largely unaltered during the first month.

To test for possible structural perturbations by the temporary accumulation of free methanol, the phosphorescence spectrum and lifetime of each protein was recorded in aqueous solution before and after the addition of 20% methanol. We found practically no alteration of the phosphorescence features by methanol. Likewise, the enzymatic activity of LADH and AP, after standing a few hours in 20% methanol and successive dialysis, showed no detectable losses.

2.2. Sample preparation for phosphorescence measurements

For phosphorescence measurements at ambient temperature it is paramount to rid the solution of

all O_2 traces. Protein gel monoliths (roughly of dimensions $2.5\times2.5\times8$ mm^3) were placed in appositely constructed quartz cuvettes (round, 4 mm I.D. cells) and were deoxygenated by repeated cycles of mild evacuation followed by inlet of pure nitrogen as described before [24]. Relative to aqueous protein samples satisfactory deoxygenation required longer equilibration times, typically 4–5 h.

The acrylamide quenching rate constant (kq) was obtained as described before [25] from measurements of the phosphorescence decay at various acrylamide concentrations according to the equation:

$$1/\tau = (1/\tau_0) + kq[\text{acrylamide}]$$

where τ_0 and τ are the phosphorescence lifetime in the absence and in the presence of a given [acrylamide] in the solution surrounding the gel. At each acrylamide concentration the gels ($V_{tot} \sim 50$ μl) were allowed to equilibrate in a large volume (~ 3 ml) of quencher solution for 16–24 h, a time that was found sufficient for acrylamide to reach a constant concentration inside the gel. In estimating the quenching constant the acrylamide concentration inside the pores of the silica glass was assumed to equal that in the external solution. In general, Stern–Volmer plots were constructed with only three acrylamide concentrations, chosen so that the largest concentration caused up to a roughly four-fold reduction of the lifetime.

Gels of LADH complexed to ADPR or to H_2NADH were obtained in two ways: one consisted in forming the complex before gelation; the other in soaking for 24 h already formed LADH gels in a buffer containing the coenzyme analogs. In both procedures, the final concentration was 600 μM for ADPR and 200 μM for H_2NADH, values that according to the respective K_d of these complexes in solution would lead to over 90% saturation.

2.3. Fluorescence and phosphorescence measurements

All luminescence measurements were conducted on home made instrumentation. Briefly, for emission spectra, continuous excitation is provided by a Cermax xenon lamp (LX150UV, ILC Technology, Sunnyvale, CA) whose output is selected (6 nm bandpass) by a 0.23-m double grating monochromator (SPEX, Model 1680, Spex Industries, Edison, NJ) optimized for maximum stray light rejection. The emission collected at 90° from the excitation is dispersed by a 0.25 m grating monochromator (Jobin-Yvon, H-25) set to a bandpass of 1 nm. A two positions light chopper intersects either the excitation beam only (fluorescence mode) or both excitation and emission beams in alternate fashion in such a way that only delayed emission gets through to the detector (phosphorescence mode). A low-noise current preamplifier (Standford, Model SR570, Sunnyvale, CA) followed by a lock-in amplifier (ITHACO Model 393, Ithaca, NJ) 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 Instrumentation Inc., Tucson, AZ). 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, Milan, Italy) ($\lambda_{ex} = 293$ nm) with a pulse duration of 5 ns and a typical energy per pulse of 0.5–1 mJ. The phosphorescence emitted at 90° from the excitation is selected by an interference filter (DTblau, Balzer, Milan, Italy) with a transmission window between 410 and 450 nm. A gating circuit 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, in the case of continuous excitation by the xenon lamp, a mechanical shutter (Uniblitz VS 25, Vincent Associates, Rochester, NY), that opens in approximately 4 ms, blocks light during the excitation period from reaching the photomultiplier. As for spectral measurements, the photocurrent signal is amplified, digitized and multiple sweeps averaged by the same computer-

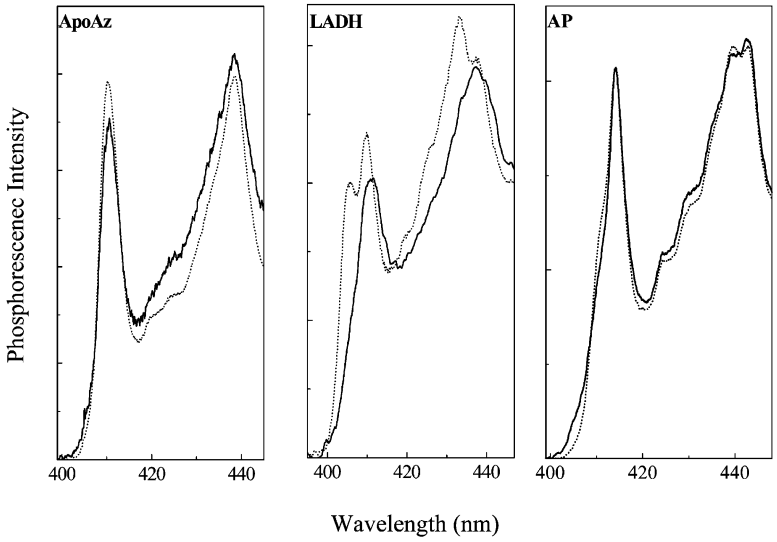

Fig. 1. High-resolution Trp phosphorescence spectra, in the region of the 0,0 vibrational band, of wet-gel encapsulated proteins (—) soaked in propylene glycol (PG)/buffer (50 mM Hepes pH 7), at 140 K. The spectrum of each protein, free in the solvent (----), is shown for comparison. $\lambda_{ex}=290$ nm. The small Tyr component was subtracted from each spectrum.

scope system. All phosphorescence decays were analyzed in terms of a sum of exponential components by a non-linear least-squares fitting algorithm (Global Unlimited, LFD, University of Illinois). No signal deterioration was observed on successive laser pulses.

Anisotropy (r) measurements were carried out by inserting linear polarizers, Polaroid type HNP'B, in both excitation and emission beams. To get the largest limiting anisotropy (r_0), the excitation wavelength was set to the red edge of the absorption spectrum while the emission was centered on the 0,0 vibronic band of the phosphorescence spectrum. The anisotropy was calculated in the usual way from the formula:

$$r=(I_v-GI_h)/(I_v+2GI_h)$$

where I_v and I_h are the emission intensities polarized parallel and perpendicular, respectively, to the vertically polarized exciting beam. The correction factor G is the ratio of the vertically to horizontally polarized emission intensities obtained with horizontal excitation.

3. Results

3.1. Low temperature Trp phosphorescence from proteins in wet gels

In low temperature glasses, the phosphorescence spectrum of Trp exhibits a pronounced vibronic structure with a relatively narrow 0,0 vibrational band. The wavelength of the 0,0 band ($\lambda_{0,0}$) is related to the polarity of the indole environment [21] and its bandwidth (BW) reports on the structural homogeneity of the site. Often, two or more Trp residues in the same protein exhibit well resolved 0,0 vibrational bands [31], in which case their emission can be studied individually.

Protein containing hydrogels were soaked in glass forming propylene glycol/buffer (50:50, V/V) and their phosphorescence emission was monitored at 140 K. When compared to that of the protein free in the same solvent distinct alterations were observed in the Trp phosphorescence spectrum of each protein (Fig. 1). For single Trp Az, encapsulation of the metal free protein causes a red shift (0.5 nm) and broadening of the 0,0 band (the BW increasing from 5.8 to 7.0 nm). This

implies a rearrangement of the structure about buried Trp-48 and a concomitant increase in conformational heterogeneity. Interestingly, the spectrum of the more stable CdAz is less affected by encapsulation in that the red shift is less than 0.2 nm and the change in BW is merely from 5.7 to 5.9 nm (data not shown).

LADH has two Trp residues per subunit, with distinct 0,0 vibrational bands centered at 405.5 nm, for solvent exposed Trp-15, and at 410.6 nm, for deeply buried Trp-314 [31]. Upon gel encapsulation the blue band shifts to the red so that the two components of the spectrum are no longer resolved (Fig. 1). From the spectral red shift of Trp-15 we infer that its aromatic ring is no longer exposed to the aqueous phase ($\lambda_{0,0}$ of solvent exposed Trp is 406 nm). Selective excitation of buried Trp-314, at 305nm [31], emphasizes also that the spectrum of the internal residue is red-shifted by 1.0 nm (data not shown).

AP has three Trp residues per subunit, two of which are on the surface of the macromolecule and one (Trp-109) is deeply buried [32]. Compared to the protein in solution, the spectrum in wet gels exhibits a less intense shoulder at 410 nm. This shoulder is assigned to one of the superficial residues [33] and, therefore, the spectral alteration indicates that the emission of this component is either red shifted or quenched. At warmer temperatures, above 220 K, where the remaining phosphorescence is due entirely to Trp-109 the $\lambda_{0,0}$ was found to be 414.2 nm both in solution and in hydrogels. Overall, high-resolution spectral data indicate that alterations are particularly pronounced for Trp residues on the surface of these macromolecules implying that their microenvironment undergoes considerable changes on encapsulation. In comparison, the structure about buried Trp residues is less affected by gel entrapment although, in the case of apoazurin, spectral broadening attests to a certain degree of heterogeneity also on the internal structure.

3.2. Room-temperature phosphorescence (RTP) from proteins in hydrogels

In fluid solutions long-lived phosphorescence (0.1–1 s lifetimes) is detected exclusively from internal Trp residues and the decay rate, when not influenced by internal quenching reactions with proximal Cys, His or Tyr side chains, as is the case with the proteins examined here, is related to the local flexibility of the polypeptide [23].

Long-lived phosphorescence from Az, LADH and AP at ambient temperature was readily detected in wet gels. An example of raw data is given in Fig. 2, which reports the spectrum and decay kinetics of AP at 20 °C. Before dwelling on the effects of silica entrapment on the RTP emission we notice that the gel exhibits background phosphorescence that under certain conditions may give a non-negligible contribution. As indicated in the right hand panel of Fig. 2, the gel background is relatively weak under continuous excitation and with relatively long phosphorescence lifetimes (the steady state intensity is proportional to the lifetime) but makes a substantial contribution on pulsed excitation. For typical micromolar protein concentrations, the gel background can reach amplitudes up to 50–60% of the total initial phosphorescence intensity. This background cannot be spectrally discriminated from Trp phosphorescence and attempts to diminish it through alternative gel preparation procedures have failed. The spurious emission is characterized by a markedly non-exponential decay with lifetime components ranging from 3 to 50 ms. It is partially quenched by atmospheric oxygen but is rather insensitive to temperature or prolonged excitation. With the present experimental set up blank subtraction using an empty gel was not sufficiently reproducible because the background intensity varied from gel to gel and, perhaps, also with the kind of protein incorporated. Naturally, this unwanted component complicates the analysis of dilute protein samples when the phosphorescence lifetime is comparable to or shorter than the background. Because of this limitation, the present report restricts the analysis of protein phosphorescence decays to the time range for which the background is negligible, i.e. only to lifetimes distinctly longer than the main gel component. For shorter lifetimes we merely estimate their expected amplitude (α_S) utilizing the constancy of the phosphorescence to fluorescence intensity ratio, P_0/F, between solution and gel and the observation that the empty gel is not

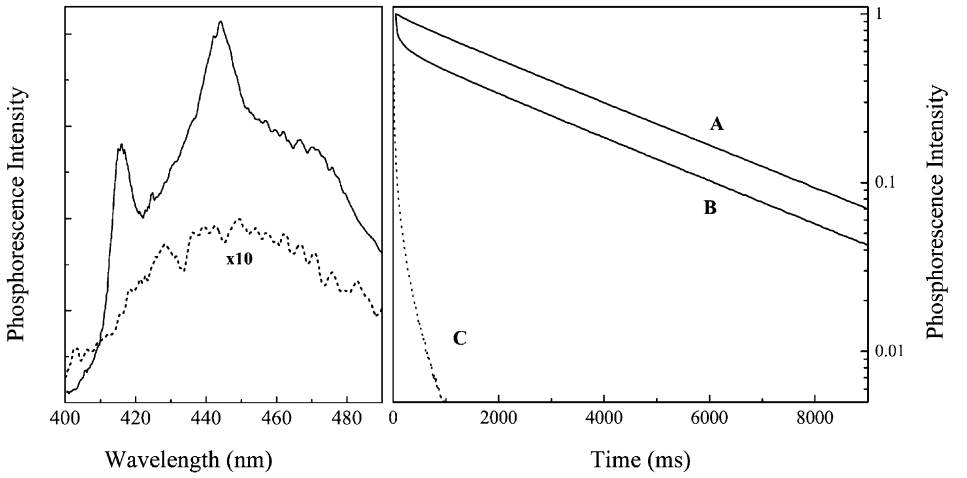

Fig. 2. Phosphorescence emission from AP in hydrogels soaked in buffer, at 20 °C. (Left panel): spectrum (—) shown together with the empty gel background (----) 10 fold amplified. $\lambda_{ex} = 290$ nm. (Right panel): phosphorescence decay upon steady-state (a) and laser pulsed excitation (b) ($\lambda_{ex} = 293$ nm). The decay of the gel background obtained with pulsed excitation (c) is also included (…).

fluorescent $[P_0/F \text{ (solution)} = (\alpha_S + \alpha_L) \; P_0/F \text{ (gel)}]$ where P_0 is the phosphorescence intensity extrapolated to time zero, F the integrated fluorescence intensity obtained in the same excitation pulse and α_L is the amplitude of the phosphorescence intensity with lifetimes longer than the gel background].

Pulsed phosphorescence decays of Az, LADH and AP in hydrogels, at 1 and 20 °C, are compared to the corresponding decays in buffer in Fig. 3. The average lifetime, $t_{av,} = \sum \alpha_i \tau_i$, of the emission that is free from the gel background and its amplitude, $\alpha_{L,}$ are collected in Table 1. For AP, the decay at 1 °C is strictly exponential, after the short-lived contribution of the gel, and the lifetime is identical, within the experimental error, to that in solution. Furthermore, the long lifetime component accounts for the entire protein sample as α_L is practically 1. Increasing the temperature to 20 °C, the average lifetime remains essentially the same as in solution but the decay becomes slightly non-uniform. Based on the lifetime of Trp-109 the AP sample in wet gels is homogeneous at 1 °C but exhibits evidence of conformational heterogeneity at ambient temperature.

Perturbations of the phosphorescence lifetime by the gel are marked with Az and LADH. For Az, the decay becomes distinctly heterogeneous in the gel matrix and the average lifetime increases by 50% at 1 °C, and 75% at 20 °C. At the higher temperature, however, α_L is only 0.56 implying that 44% of the intensity decays within the time range of the gel background so that the lifetime of this fraction is considerably smaller than that of the protein free in solution. The lifetime multiplicity of azurin shows that in wet gels the protein adopts a variety of conformations. When translated in terms of structural flexibility we see that at 1 °C the majority of protein molecules have a more compact, rigid structure compared to the native state. At 20 °C, however, a sizable fraction of the sample exhibits a more flexible internal core which is consistent with a looser globular fold. Practically, the same lifetime variations are observed with CdAz in spite of the greater thermal stability of the latter as evidenced by the 30 °C increase in melting temperature [35].

Greater lifetime heterogeneity in hydrogels than in solution is also observed for the phosphorescence of LADH. Unlike azurin, for this protein all lifetime components are shorter-lived than in solution, indicating a reduced compactness of the intersubunit region hosting the phosphorescence probe. Overall, the intrinsic phosphorescence life-

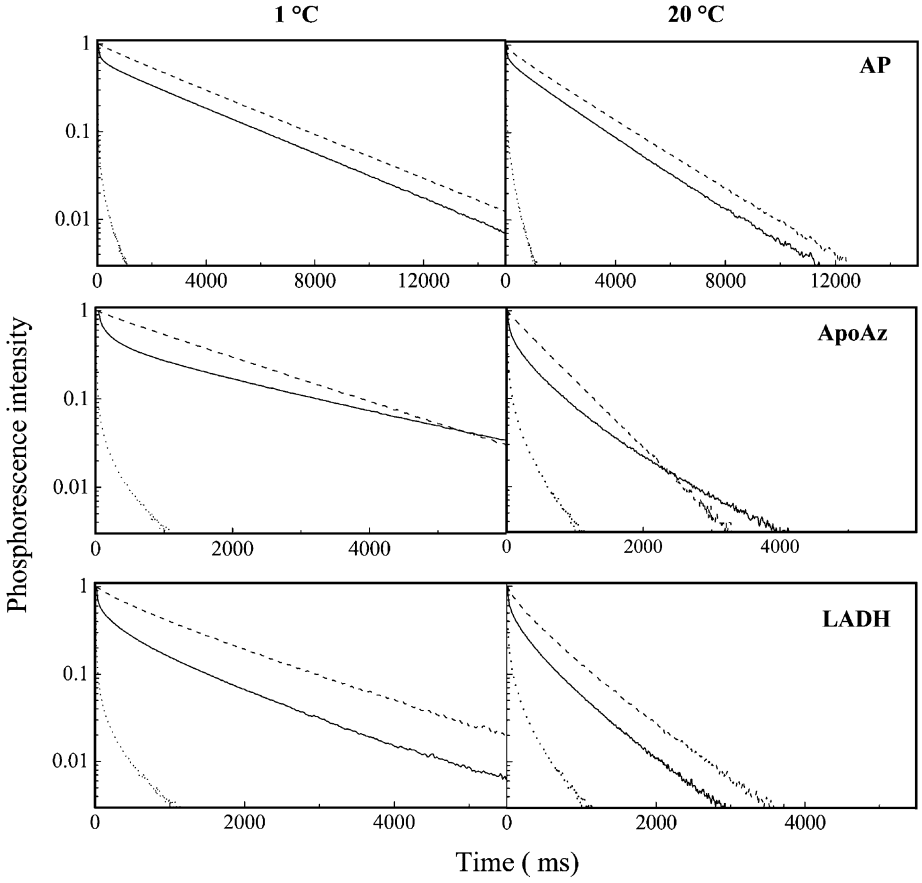

Fig. 3. Comparison between the laser pulsed phosphorescence decay of the proteins in buffer (Hepes 50 mM pH 7.0) (----) and encapsulated in wet-gel (—), at 1 and 20 °C. The short decay of the gel background is also included (…).

time of the proteins examined in this study demonstrates that their encapsulation in wet gels tends to stabilize a range of different conformations of the macromolecule which remain distinct in the second time scale of phosphorescence and for which the internal flexibility of the polypeptide is either damped or enhanced relative to the native state in solution.

Large amplitude structural fluctuations in globular proteins generally set in above room temperature. They are characterized by a sharp gain in conformational flexibility and a concomitant reduction of the phosphorescence lifetime [36]. To test for a potentially inhibitory effect of the gel matrix on these large amplitude fluctuations, the lifetime of AP, the only protein for which Trp phosphorescence could be easily discriminated from the gel background well above room temperature, was measured up to 60 °C. The results show that between 20 and 60 °C τ decreases 16-fold both in gel and in solution and, therefore, this parameter gives no evidence of gel-induced dampening of structural fluctuations in AP.

LADH undergoes a substantial structural isomerization upon binding of the nicotinamide coenzyme or coenzyme analogs [30]. In response to ligation of the analogs ADPR and H_2NADH the phosphorescence lifetime of LADH increases by at least two fold [37]. It should be noted that the delayed emission from the coenzyme complexes is exclusively Trp phosphorescence as no phosphorescence is detected from the coenzymes in fluid

Table 1
Time-resolved phosphorescence decay parameters in wet gels

Protein	T, °C	α_L[b]	τ_{av}[b] (ms)	τ_{av}/τ_{sol}
AP	1	1.00	3600	1.05
«	20	1.00	2080	1.01
ApoAZ	1	1.00	2250	1.36
«	20	0.56	980	1.81
CdAz	1	1	2300	1.39
«	20	0.55	1032	1.91
LADH	1	0.62	1015	0.64
«	20	0.59	450	0.72
LADH :ADPR	1	0.65	1622	1.03
LADH :ADPR[a]	1	0.60	1700	1.08
LADH:H_2NADH	1	0.63	2190	0.89
LADH:H_2NADH[a]	1	0.58	2141	0.87

[a] These complexes were formed by diffusion of the coenzyme analogue from the soaking buffer (data of Fig. 4).
[b] The standard deviation (S.D.) in these parameters is less than 6%.

solutions [37]. To verify if LADH entrapped in wet gels can still undergo these structural rearrangements the two complexes were formed both prior to and after gelation, in the latter case by diffusion of the coenzyme analogs from the outside buffer through an LADH containing gel. The results show that the characteristic lengthening of τ of the complexes in solution is observed also in wet gels (Fig. 4) and that the change in lifetime is independent of whether the complexes are formed before or after gelation (Table 1). These findings confirm that the analogs can diffuse to every protein-containing pore and, furthermore, demonstrate that entrapped LADH maintains sufficient conformational freedom to undergo the characteristic isomerization.

3.3. Acrylamide quenching of protein phosphorescence in wet gels

In addition to τ_0, an alternative and complementary way to assess the flexibility of globular proteins is by monitoring the diffusion of small quenching molecules through the protein matrix to the site of the chromophore. In particular, quenching of protein phosphorescence by acrylamide was shown to be a sensitive indicator of their flexibility, the bimolecular quenching rate constant, kq, derived from the gradient of the lifetime Stern–Volmer plot $[1/\tau = 1/\tau_0 + kq(\text{acrylamide})]$ providing a measure of the average acrylamide diffusion coefficient inside the macromolecule [25].

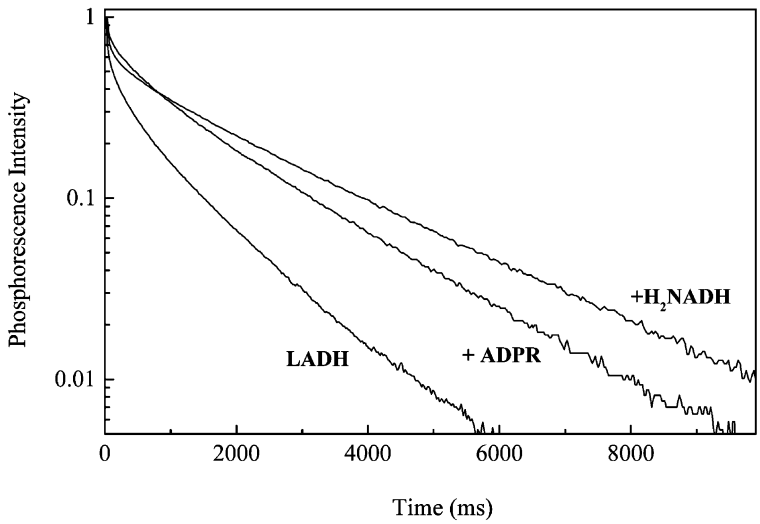

Fig. 4. Phosphorescence decay of LADH in hydrogels before and after equilibration of the gel with a buffer (Hepes 50 mM pH 7.0) containing either 600 μM ADPR or 200 μM H_2NADH. Measurements were carried out at 1 °C.

The addition of acrylamide to protein gels reduced the phosphorescence lifetime of each protein examined. To minimize the interference from the gel background, experiments were conducted at a temperature (1 °C for Az and LADH and at 20 °C for AP) and at acrylamide concentrations for which protein phosphorescence could still be distinguished from the gel background. While the decay of AP was a single exponential at all acrylamide concentrations that of LADH and Az, which are initially heterogeneous, were found to remain so even when the quencher considerably reduced the average τ. For convenience, lifetime Stern–Volmer plots were constructed from the average lifetime obtained from a biexponential fit of phosphorescence decays. Consequently, the value derived for kq is an average quantity. In the evaluation of kq it was assumed that the acrylamide concentration in the aqueous pores of the gel is the same as that in the outside buffer. The results indicate that, relative to the proteins in buffer, the magnitude of kq decreases from 2.0 ± 0.3 to 0.74 ± 0.2 M^{-1} s^{-1} for Az, increases from $0.4(\pm 0.1) \times 10^4$ to $1.5(\pm 0.2) \times 10^4$ M^{-1} s^{-1} for LADH and remains practically unaltered at 0.1 ± 0.03 M^{-1} s^{-1} for AP. The roughly three-fold reduction/enhancement of kq with Az/LADH is significantly larger than the 10–20% experimental error and implies that in hydrogels acrylamide migration through the globular fold of these proteins is slowed down in the case of Az but facilitated in the case of LADH. No significant effects of encapsulation were found with the very compact AP. Significantly, these alterations of kq ?correlate with the change in internal flexibility (a tighter/looser structure for Az/LADH) inferred from τ_0.

3.4. Phosphorescence anisotropy and protein rotational mobility in wet gels

The phosphorescence anisotropy was measured with continuous excitation because under these conditions the short-lived emission from the gel makes a negligible contribution to the total phosphorescence intensity. The steady-state phosphorescence anisotropy, r, of each protein was measured in wet gels both soaked in 50:50 propylene glycol/buffer, which forms a viscous solution at low temperature, and in buffer. Measurements in rigid solutions (low temperature glasses) yield the limiting anisotropy, r_0, which depends on both the protein and the optical quality of the gel. The latter is probably the cause of a 20% lower r_0 of LADH in hydrogels than in solution, a phenomenon reported also in fluorescence polarization studies of other proteins [3].

Fig. 5 compares the thermal profile of r for AP free in solution and in hydrogels. At approximately 200 K, the glass softens into a fluid solution and the increased tumbling rate of the free protein results in a complete loss of its steady-state anisotropy. Comparative studies with other globular proteins show that the rotational correlation time, θ, derived by approximating the macromolecule to a rigid sphere ($\theta = V\eta/kT$, V = volume of the sphere, η = solvent viscosity and k = the Boltzmann constant) is in agreement with the hydrodynamic volume of the hydrated macromolecule [26,38]. In contrast to the behavior in solution, the anisotropy of AP in wet gels remains largely unchanged, decreasing by less than 10% even at the highest temperature examined (2 °C). The same anisotropy is confirmed when the gel is placed in pure buffer (2 °C). In the spherical approximation [$r_0/r = (\tau/\theta + 1)$], values of $r/r_0 = 0.94$ and $\tau = 3$ s yield $\theta > 10$ s, from which it is evident that any rotational motion of AP in wet gels is fully blocked even in the remarkably long time scale of seconds. This conclusion applies equally to Az, CdAz and LADH (Table 2), which exhibit practically the same degree of immobilization as AP, even if the size of azurin is six times smaller than that of LADH or AP. A comparable immobilization was observed only for LADH in the crystalline state [39]. It should be noted that these conclusions, based on an intrinsic probe deeply buried in the rigid core of the macromolecule, are in net contrast with the nanoseconds/sub-nanoseconds rotational correlation times inferred from some fluorescence polarization studies of labeled proteins [17,18].

4. Discussion

This report demonstrates that long-lived Trp phosphorescence can be readily detected from

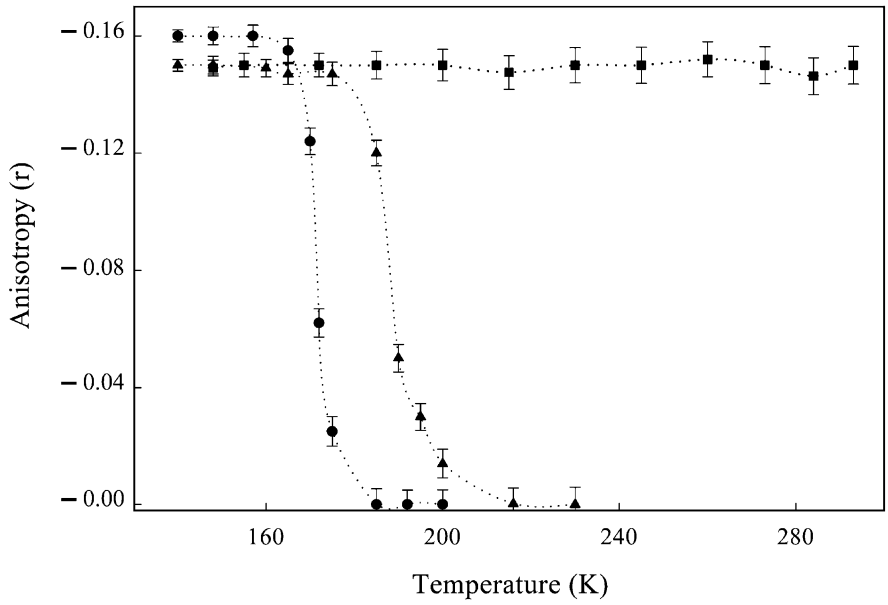

Fig. 5. Phosphorescence anisotropy-temperature profile for AP, free in solution (▲) and in hydrogels (■), in 50:50 PG/buffer. The anisotropy profile of the Trp derivative NATA (●) in the same solvent is included for comparison.

proteins entrapped in silica sol–gel matrices. It also points out that, unfortunately, the gel itself contributes a non-negligible delayed background emission, a complication that is not generally encountered with the much more intense fluorescence signal. The gel background is not easily subtracted from Trp phosphorescence and, therefore, poses some limitations in the characterization of dilute proteins samples with relatively short triplet lifetimes (<20 ms). A preliminary report of the supposed phosphorescence of Ca parvalbumin in hydrogels did exhibit a spectrum and decay kinetics that are, in all respects, very similar to the background reported in this study [40]. We found that the gel luminescence can be excited even at 315 nm, outside the absorption spectrum of Trp and, therefore, its contribution can potentially be estimated by a double excitation experiment. At this stage it is not known whether the spurious emission is due to impurities in the TMOS stock or if chromophoric centers are generated in the silica matrix during the polymerization process. It was observed that the gel background increased several fold on the formation of xerogels, particularly during the last stages of shrinkage (to below one fifth of the original volume) and water removal.

4.1. How native are protein structures in hydrogels?

Trapping of proteins in silica matrices may alter the native fold and in cases of marginally stable macromolecules it has led to loss of secondary structure and unfolding [5]. Changes of tertiary structure are more difficult to detect but may be equally important for the possible deleterious effects on the catalytic efficiency of enzymes. We have addressed the issue of changes in tertiary structure exploiting the sensitivity of the intrinsic phosphorescence emission to alterations of the polypeptide/solvent configuration about Trp residues. Low temperature high-resolution spectra have shown that the Trp residues, of LADH and AP, lying on the surface of the macromolecule in contact with the solvent undergo a remarkable red shift upon silica entrapment. This suggests that their environment is less polar in the silica matrix,

Table 2
Steady state anisotropy parameters of proteins in wet gels

Proteins	λ_{ex}, nm	λ_{em}, nm	r_0 (160 K)	r/r_0 (293 K)	τ_{av}, s (293 K)	θ_{rot}, s (293 K)
AP	295	413.7	0.146	0.94 ± 0.04	2.08	>10
ApoAz	295	412.8	0.092	0.99 ± 0.05	0.98	>19
CdAz	295	412.8	0.092	0.97 ± 0.03	1.03	>15
LADH	300	412.7	0.120	1.00 ± 0.05	0.45	>28

an effect that may in part be ascribed to a decrease in the extent of hydration but not to the direct contact of the indole ring with the inorganic polymer as, at neutral pH, the latter is negatively charged. Because small spectral changes occur also with deeply buried W48 of Az and W314 of LADH, the structural alterations of superficial regions are presumably responsible for the modification of these buried Trp sites. Certainly, the changes detected by internal residues entail that subtle reorganizations of the structure occur over large regions of the macromolecule.

Another salient feature of silica entrapment is the emergence of conformational heterogeneity. This was inferred from both the broadening of the vibrational bands in the spectrum of single Trp Az, and more generally, from the multiplicity in phosphorescence lifetimes of the internal Trp residue in each protein studied. The extent of the perturbation varies among proteins and might be related to stability of the native fold. Indeed, spectral broadening in Az decreased upon binding of Cd to apoazurin (Tm increased from 65 to 95 °C) [34]. Likewise, the smallest difference in lifetime between solution and hydrogel, as well as the smallest lifetime heterogeneity, was found with AP, which is the most thermally stable of the proteins examined.

Heterogeneity in structure of entrapped proteins has also been inferred from non-uniform enzyme kinetics [10,11], broad denaturation transitions [3] and Trp fluorescence [3,10]. Presumably, multiple conformations reflect the distinct perturbation induced by the variety of pore size/shapes, pH differences among pores [41] if not from different ways of protein silica association. An alternate view has been put forth suggesting that conformational heterogeneity in silica gels arise from the freezing out of distinct conformation that are normally present in solution, owing to the high viscosity of water in the pores [14–16] and/or steric constraints imposed by the rigid polymer cage preventing rapid interconversion. An example of the latter appears to be the allosteric R-T isomerization of hemoglobin that is drastically slowed down in silica gels [12,13,20]. However, both the magnitude of τ average and the distribution in phosphorescence lifetimes suggest that the heterogeneity reported here is not merely of a dynamic nature. For Az and LADH, the average lifetime in hydrogels, which relates to the average structure of the protein, differs considerably from the value in solution. More significantly, raising the temperature the distribution in lifetimes gets wider, instead of becoming more uniform as would be expected from faster conformer interconversion. Emphasizing the direct perturbation of the native fold by the silica environment, partial removal of water from the gel during the formation of xerogels (data not shown) caused highly non-exponential decays and a dramatic reduction in lifetime for each protein.

4.2. Protein dynamics in hydrogels

Under this heading it is convenient to distinguish between small amplitude, high frequency atomic motions, large amplitude segmental motions, responsible for conformational isomerization or unfolding, and the rotational freedom of the whole macromolecule. To date, the effects of encapsulation on some of these aspects of protein dynamics are poorly characterized and still largely controversial. The prevailing picture is one in which segmental motions in both hydro and xerogels are

largely unaffected whereas global movements are restricted by the rigid polymer cage.

According to the variation of the RTP lifetime and the empirical relationship between τ and the viscosity of the medium, obtained in model studies [22], the internal flexibility of the protein examined has been affected by encapsulation but there is no general trend among them. Moreover, the same protein exhibits a great variability among different sites of the gel. Thus, the lengthening of τ average for Az in hydrogels implies an overall tightening of the core of the globular structure, where the probe is located. However, a sizable fraction of the sample shows evidence of a lifetime much shorter than in solution, an indication that in some pores of the gel the protein structure is distorted and more flexible probably through strong interactions with the silica matrix. The overall effect on the internal dynamics is opposite with LADH, where Trp-314 within the large β-sheet spanning the dimer interface shows that entrapment increases the local flexibility. On the other hand, in the rigid inner core of AP the effect is negligible. It may be argued that, as τ merely probes the local structure about Trp, the observed variability among proteins merely reflects the site to site variability of polypeptide structures. However, the interesting correlation between the changes in flexibility inferred by τ with those from the acrylamide diffusion coefficient suggests, that the observed alterations are not limited to the immediate surrounding of the probe but probably involve large domains of the globular fold. In a different context, the data on acrylamide migration also sheds light on how the gel matrix by affecting protein dynamics can modulate the accessibility of substrate/effector molecules to the active site of enzymatic proteins.

Possible restrictions on larger structural rearrangements were tested through the isomerization of LADH induced by binding of coenzyme and coenzyme analogs [30]. In solution as well as in hydrogels the complexes formed between LADH and the analogs ADPR and H_2NADH exhibit a RTP lifetime that is at least two-fold larger than that of the protein alone [37]. It was found that the addition of the analogs to LADH in hydrogels was able to produce the lifetime typical of the complex. Although the long dead time of these measurements prevents determination of the kinetics of the conformational change induced by complexation these data confirm the ability of trapped LADH to reorganize its structure and maintain its affinity for the coenzyme analogs.

The rotational freedom of trapped proteins has been debated and different conclusions have been made from past assessments. Fluorescence anisotropy studies of the green fluorescence protein [42] and of labeled BSA and HSA [19] and other analysis of silica entrapped cytochrome *c* and glucose oxidase [17,18] have concluded that the protein environment is similar to the aqueous solution and that unhindered molecular rotations occur. On the other hand, from the fully polarized fluorescence of Mg myoglobin it was deduced that its rotational correlation time in hydrogels is very long, larger than the roughly 300 ns detection limit imposed by a 10-ns lifetime [43]. The present phosphorescence anisotropy results on three different proteins demonstrate that their immobilization in wet gels is even more dramatic, the rotational constant exceeding several seconds (Table 2). Their immobilization appears also largely independent of the protein size because analogous results are obtained with Az (14 kDa), which is six times smaller than LADH (80 kDa) and AP (84 kDa). With regards to proteins in aqueous media only the crystalline state [39] afforded a comparable restriction to tumbling motions. This striking result may help to discriminate between the kinds of possible silica matrix–protein interactions and explain the puzzling observation that once the gel is formed, proteins smaller than the estimated average pore size are prevented from leaching out whereas even larger particles are able to diffuse into the gel [2,45]. Among the factors that can slow down the rotational diffusion of entrapped proteins one may list the following: a very high local viscosity of water inside the pore; steric restrictions imposed by a pore size not larger than the macromolecule; biomolecule directed templating of the developing sol–gel matrix or covalent and non-covalent (H-bonding) protein-silica surface binding interactions. The average pore diameter is estimated at over 100 Å [2,44], clearly larger than the diameter of Az (32 Å), of AP and

LADH (~60 Å). Furthermore, the ability of relatively large macromolecules to permeate wet gels [2,46] apparently rules out factors such as viscosity and pore size restrictions. For protein directed templating of the growing gel to achieve this exceptional degree of immobilization the gel must wrap around a large portion of the macromolecule to guarantee a close association between polypeptide and inorganic polymer in most protein filled pores. Extended wrapping of the protein globule by the polymer is not supported by experiment because it would permit little conformational freedom to the biopolymer and would also inhibit the free access of solutes (substrates, cofactors and quenching molecules like acrylamide) to the portion of the protein surface covered by the gel. The only alternative mechanism capable of rigidly immobilizing proteins inside the pores would appear to be the formation of either extensive H-bonding or covalent bonds between surface residues of the protein and the polymer.

In conclusion, the ability to monitor the phosphorescence emission of encapsulated proteins will offer an additional sensitive probe of their tertiary structure and conformational dynamics. This tool will be useful in the characterization of the state of polypeptides in silica glasses, their interaction with substrates/effectors molecules as well as for testing the influence of stabilizing/denaturing agents [8]. Lastly, the very long lifetime of phosphorescence will be particularly suitable to investigate slow diffusion processes and highly hindered molecular reorientations in a time scale fully complementary to fluorescence and ESR spectroscopy.

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