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Fluorescence heterogeneity of tryptophans in Na,K-ATPase: evidences for temperature-dependent energy transfer

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Abstract

The intrinsic fluorescence emission kinetics of Na,K-ATPase, a large membrane protein containing 16 tryptophan residues, was studied by time-resolved techniques. The lifetime distributions recovered by the Maximum Entropy Method exhibit a strong dependence on the emission wavelength at temperatures between 37°C and −70°C. From the 'blue' edge of the fluorescence emission spectrum up to the maximum of emission, the lifetime distribution at room temperature is the result of four broad peaks which cover the time range 0.3–7 ns. With increasing emission wavelength, these peaks move to longer lifetimes and the peak at shorter times are suppressed at the red edge, while the longest component (6–7 ns) becomes dominant. With decreasing temperature, the number of lifetime components is reduced for the benefit of the long one. At cryogenic temperatures, the emission decay in the red-edge of the fluorescence spectrum consists of one major slow component (6–7 ns) and a fast one (0.5 ns) associated with a negative pre-exponential term. This is a characteristic feature of an excited-state reaction. The temperature dependence of this fast component and the fluorescence anisotropy decay at low temperature in the red-edge, indicate that this excited state reaction may be accounted for a unidirectional inter-tryptophan fluorescence energy transfer from 'blue' populations of donors to 'red' populations of acceptors. This is also illustrated by the time-resolved emission spectra. In the blue edge of the fluorescence emission spectrum, moreover, the time course of the anisotropy decay suggests the existence of homo-transfer of excitation energy involving 'blue' tryptophan residues. The steady-state anisotropy excitation spectrum in vitrified solvent agrees with this suggestion. These different energy transfer mechanisms may be used as structural probes to detect more accurately conformational changes of the protein elicited by effectors and ion binding or release.

Keywords: Protein dynamics; Na,K-ATPase; Ultraviolet fluorescence; Time-resolved spectroscopy; Lifetime distributions; Dielectric relaxations; Excited-state energy transfer

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1. Introduction

Fluorescence methods are extensively used in the studies of protein structure and dynamics. While results of steady-state spectroscopic measurements were usually interpreted in terms of structural positions of tryptophans with respect to other residues and protein surface [1], time-resolved fluorescence allows a correlation of the observed emission decay with photophysical and photochemical processes that affect the excited state tryptophan (Trp): static and dynamic quenching by electron and/or proton transfer, dielectric relaxations, excitation energy transfer between tryptophans or from tryptophans to cofactor groups [2]. The promising possibility to obtain dynamic information which may be associated with protein functions stimulated a rapid development of these experimental techniques and the methods of data analysis of fluorescence decays. More and more sophisticated models are being developed. Since very often their applications to the same set of primary data result in different interpretations, it is essential that the first steps of analysis are performed with a minimum set of a priori hypotheses, especially concerning the number of parameters necessary to describe the fluorescence decays. The introduction of the Maximum Entropy Method (MEM) to the analysis of lifetime distributions [3,4] has proven to be such an useful approach in the analysis of complex decay kinetics. The success of this method in the analysis of photochemical reactions [5] and dielectric relaxations [6] extends the background of its application in protein research [7–17].

The complexity of the Trp fluorescence emission decays of multi-tryptophan proteins was the reason to concentrate studies mostly on proteins containing only a single tryptophan. Even in these cases, however, the fluorescence emission of Trp exhibits usually complex multi-exponential decays [18]. So far there is no general agreement on the origin of this phenomenon but several mechanisms are accepted: tryptophan conformers in the ground state [19], fluorescence quenching by neighbouring groups [20], excited state reactions like dipolar relaxation [21] and coexistence of several local conformations of the protein [22]. If the tryptophan is shuttling between these conformations, the lifetime distribution may contain information about the residence time in each of them.

The heterogeneity of tryptophan fluorescence decay in single Trp-containing proteins complicates the interpretation of time-resolved emission data in multi-tryptophan proteins. In order to reduce the complexity of the fluorescence emission, usually selected Trp residues were substituted by other residues using genetic engineering [11,23,24] or selective fluorescence quenching was applied [25]. These approaches cannot be considered as general strategies since it is technically difficult to perform all possible substitutions of tryptophans and be sure that these changes maintain the protein structure genuine. In addition, each tryptophan may exhibit its own time-dependent reactions which are associated with specific quenching or wavelength shifts. Moreover, transfer of excitation energy between tryptophans may occur which not only may average the emission probabilities of individual chromophores but can result also in a redistribution of these probabilities by a directed flow of excitation energy [2]. In such cases, the attempts will not be successful to resolve the emission parameters of individual tryptophans. Therefore, in case of multiple emissions, time and spectral resolution should be utilized to separate at least groups of Trp emitters sharing similar environments and thus showing similar spectral properties.

In the present work, we have studied the intrinsic fluorescence emission of Na,K-ATPase, a complex membrane protein, constituted as $\alpha\beta$ protomers, each of them containing 16 Trp residues. The primary and secondary structures of the protein have been established, but its three-dimensional structure is still unknown. The steady-state fluorescence emission spectrum of Na,K-ATPase exhibits a maximum at 340–342 nm. The width of fluorescence band is 59 nm, which implies that the heterogeneity of tryptophan emission is not large [26]. These properties suggest that either the tryptophan residues which exhibit the highest quantum yields are predominantly located in moderately polar environments or that an effective energy transfer occurs from more buried Trps to those located in polar environments. A third possibility was previously proposed [27] that some polar amino acid side chains may exhibit fast intramolecular dynamics which allows dielectric relax-

ations in the environment of excited tryptophans and leads to an averaging of the fluorescence emission spectra.

In a previous paper [27], an unusually strong dependence of the steady-state fluorescence spectra of Na,K-ATPase with temperature was described in an extended range from physiological temperatures of 37–40°C down to −120°C. Cooling to temperature as low as −120°C led to a gradual blue shift of 11 nm (from 340 to 329 nm). These results were interpreted as indication of the existence of intramolecular dynamics on the nanosecond time scale which have their origin in dielectric relaxations around the tryptophan residues. This dynamics should be slowed down at low temperatures, which results in an emission from unrelaxed state possessing higher energy. This interpretation was supported by the existence of a red-edge effect (shifts of fluorescence spectra on excitations at the long-wavelength edge [2]). As expected for dielectric relaxations and in accordance with the studies of indole and tryptophan in model environments [2], the red-edge effect observed for Na,K-ATPase was significant, and it increased substantially on cooling. Time-resolved measurements showed a large increase of the mean fluorescence lifetime as a function of the emission wavelength [27], in agreement with this interpretation. However, another likely excited state process was not considered in this previous publication, which could account also for the interpretation, namely excitation energy transfer between Trp residues. In addition, modification of the spectral dependence of the lifetime distributions on temperature could not be excluded to be involved in the spectral shift.

The present research is focused on a detailed analysis of the wavelength and temperature dependence of the intrinsic fluorescence decay kinetics of Na,K-ATPase in order to discriminate between these different mechanisms. This is coupled with the perspective to obtain more specific structural and dynamic information from the spectroscopic properties of the Trp residues which may be used to monitor conformation and dynamic changes occurring on interaction of the protein with effectors and ions. Synchrotron radiation was used as pulsed light source for data acquisition by the single-photon counting technique. Lifetime distributions were recovered by Maximum Entropy Method. This procedure allowed us not only to suggest a new interpretation of the time-resolved fluorescence data obtained for Na,K-ATPase but also to achieve better insight into dynamic properties of the protein.

2. Materials and methods

2.1. Materials and general strategy of experiments

Na,K-ATPase was prepared from outer medulla of rabbit kidneys using procedure C of Jørgensen [28]. The sample consists of flat membrane fragments which were purified from the native cell membranes. They consist of Na,K-ATPase with a purity of > 98% and membrane lipids (about 0.8 mg phospholipid and 0.2 mg cholesterol per mg protein). The molecular weight of the protein is approximately 140 kDa and the average molecular mass of the lipid is 800 Da. Detergent (SDS) is present only in negligible traces. The membrane fragments have diameters in the range of 0.5–1 μm and a thickness of 10–12 nm. The specific ATPase activity, determined as described elsewhere [27], was between 1800 and 2300 μmol Pi/h and mg protein at 37°C. The samples of Na,K-ATPase-rich membrane fragments were used after short-term storage in the frozen form at −70°C in buffer (25 mM imidazole sulfate, pH 7.5, 1 mM EDTA, 10 mg/ml sucrose). Incubation in 65% w/w glycerol did not result in significant change of ATPase activity, as it was shown in the standard activity test.

We performed a series of time-resolved experiments at temperatures of 37, 19, 2, −48 and −70°C in aqueous buffer, pH 7.5, in the presence of 65% w/w of glycerol. In order to check the effect of glycerol, we also performed the same experiments in aqueous buffer without glycerol at temperatures > 0°C. The data with and without glycerol demonstrated similar profiles of lifetime distributions and the same tendencies with respect to their dependence on temperature and emission wavelength. In total, seven series of experiments were performed, and in each of them fluorescence decay kinetics was measured with a constant excitation wavelength of 295 nm and analyzed as function of the emission wavelength. The fluorescence decay data were collected

over the emission spectrum from 305 to 395 nm in steps of 10 nm. These data were also used for construction of time-resolved spectra and analysis of time-resolved anisotropy. In addition, we measured steady-state anisotropy as a function of excitation wavelength.

2.2. Steady-state anisotropy measurement

Steady-state anisotropy was obtained with an SLM 8000 spectrofluorometer operating in the T-format mode. Excitation wavelength was selected by a single holographic grating monochromator (bandwidth 1 nm) and the fluorescence light was selected through one interference filter Schott 336 (bandwidth 10 nm). The protein samples were dissolved in a glycerol/buffer mixture (65% glycerol w/w) at a concentration of 3 μM. The temperature was maintained at -46°C with a Hüber HS 60 cryothermostat. The steady-state anisotropy at one emission wavelength was calculated as function of the excitation wavelength λ after appropriate background subtraction:

$$A(\lambda) = \frac{I_{vv}(\lambda) - G(\lambda) \times I_{vh}(\lambda)}{I_{vv}(\lambda) + 2 \times G(\lambda) \times I_{vh}(\lambda)} \quad (1)$$

where $G(\lambda)$ is a correction factor defined as:

$$G(\lambda) = \frac{I_{hv}(\lambda)}{I_{hh}(\lambda)} \quad (2)$$

and $I_{vv}(\lambda)$, $I_{vh}(\lambda)$, $I_{hv}(\lambda)$, $I_{hh}(\lambda)$ are the polarized fluorescence intensities at the excitation wavelength λ after corresponding background subtraction. The first and second subscripts refer to the orientation (vertical or horizontal) of the excitation and emission polarizers, respectively.

2.3. Time-resolved fluorescence measurements and data analysis

Fluorescence intensity and anisotropy decays were obtained by the time-correlated single photon counting technique from the $I_{vv}(t)$ and $I_{vh}(t)$ components recorded on the experimental setup installed on the SB1 window of the synchrotron radiation machine Super-ACO (Anneau de Collision d'Orsay), which has been described previously [16]. The excitation wavelength was selected by a double monochromator (Jobin Yvon UV-DH10, bandwidth 4 nm). A MCP-PMT Hamamatsu (model R3809U-02) was used as photo multiplier. Time resolution of the data-acquisition system was about 20 ps per channel and the data were stored in 2048 channels. Automatic sampling cycles including 30 s accumulation time for the instrument response function and 90 s acquisition time for each polarized component were carried out to collect a total number of $2–4 \cdot 10^6$ counts during the fluorescence intensity decay. Temperature regulation was achieved either with a liquid circulating cryostat (Haake or Hüber HS 60) or in a variable temperature Janis cryostat VPF-100 (Janis Research, Wilmington, MA) for measurements at -70°C.

Analyses of fluorescence intensity decays as sums of exponentials were performed by the maximum entropy method [3,4]. They will be summarized in the following.

The fluorescence intensity decay is reconstructed from the polarized fluorescence decays by adding the parallel and twice the perpendicular components:

$$T(t) = I_{vv}(t) + 2\beta_{corr} I_{vh}(t) = \int_0^\infty \alpha(\tau) \exp(-t/\tau) d\tau \quad (3)$$

where β_{corr} is the correction factor [29] taking into account the difference of transmission of the polarized light components by the optics and $\alpha(\tau)$ is the lifetime distribution.

The recovered distribution $\alpha(\tau)$ which maximizes the entropy function S:

$$S = \int_0^\infty \left[\alpha(\tau) - m(\tau) - \alpha(\tau) \log \frac{\alpha(\tau)}{m(\tau)} \right] d\tau \quad (4)$$

is chosen.

In this expression, $m(\tau)$ is the starting model and $\alpha(\tau)$ is the target distribution. In every analysis, a flat map over the explored (τ) domain is chosen for $m(\tau)$, since no a priori knowledge about the final distribution is available. The analysis is bound by the constraint:

$$\sum_{k=1}^{M} \frac{\left(I_k^{calc} - I_k^{obs}\right)^2}{\sigma_k^2} \quad (5)$$

where I_k^{calc} and I_k^{obs} are the k-th calculated and observed intensities. σ_k^2 is the variance of the k-th point [29]. M is the number of (independent) observations of the fluorescence intensity at times t.

The center τ_j of a single class j of lifetimes over the $\alpha(\tau_i)$ distribution is defined as:

$$\tau_j = \frac{\sum_i \alpha_i(\tau_i)\tau_i}{\sum_i \alpha_i(\tau_i)} \quad (6)$$

the summation being performed on the significant values of the $\alpha(\tau_i)$ for the j class. C_j is the normalized contribution of the lifetime class j.

The mean excited state lifetime $\langle\tau\rangle$ is calculated as:

$$\langle\tau\rangle = \frac{c_j\tau_j}{\sum_j c_j\tau_j} \quad (7a)$$

2.4. Time-resolved emission spectra: collection and analysis

Time-resolved emission spectra were reconstructed in each experimental conditions from 10 individual decays as a function of the emission wavelength from 305 nm up to 395 nm (bandwidth 5 nm) in 10 nm steps. The decays were usually cumulated up to 10^4 or more counts in the peak channel. Each individual curve was fitted with the MEM program using the negative amplitude option [6]. The integral of each decay curve was normalized to the corresponding steady-state fluorescence emission wavelength recorded on the same instrument with identical experimental conditions. Steps of 25 ps were used for the spectral shift construction.

3. Results

The fluorescence emission heterogeneity of a multi-tryptophan protein may be of static and/or dynamic origins. The static part is related to the location of the Trp residues which are distributed over the protein structure of varying physicochemical properties. The Trp residues may be exposed to dissimilar polarities and to tertiary contacts with various amino acid moieties or peptide bonds which are electron and/or proton acceptors from the excited state of Trp [30–32]. The dynamic effects are due to environment relaxation, dynamic quenching and possible interconversions of conformational substates like in single-tryptophan proteins [33–39]. Excited state reactions like resonant energy transfer between the similarly or differently embedded chromophores (called homo- or hetero-transfer) [40–42] may occur between close Trp residues. Dielectric relaxation of protein dipole around the Trp excited state should also be considered as a likely possibility. Different approaches may be utilized to discriminate between the different mechanisms for dynamic effects. A temperature decrease should reduce the dynamic heterogeneity while the static heterogeneity would not be affected. On the other hand, the static heterogeneity may depend on the emission wavelength if it is linked to different environments which affect the fluorescence emission spectrum. Relaxational mechanisms can be detected and characterized by measurements at different emission wavelengths and may be separated from fluorescence energy hetero-transfer by low-temperature measurements. Homo-transfer cannot be revealed by lifetime measurements but by fluorescence anisotropy measurements at low temperatures, when depolarization due to rotational motions is inhibited. All the results presented in this paper are focused on the study of the temperature-dependent evolution of the emission decay parameters at different emission wavelengths in order to scrutinize the complex photophysics of the Trp emission in this protein.

3.1. An unusually strong dependence of the mean fluorescence lifetime on emission wavelength

One of the findings of the previous paper [27] was a strong dependence of the position of the steady-state fluorescence spectrum of Na,K-ATPase on temperature. The emission maximum was shifted from 338–340 nm at 35–40°C to 334–335 nm at 0°C and to 330 nm at −100°C. This observation led to the suggestion that among the different types of mechanisms, the dielectric environmental relaxation could be dominant. If this assumption is correct, the mean fluorescence lifetime should increase as a function of

the emission wavelength because the excitation energy of the chromophore decreases and the emission spectrum shifts to longer wavelengths as function of time [43]. In other words, the chromophore populations which emit at short wavelengths decrease faster, resulting in a shorter decay time while the populations which emit at long wavelengths exhibit longer average decay times [43,44]. This effect is expected to be maximal in the middle of relaxation range, i.e., when the dielectric relaxation time τ_R is close to the mean fluorescence lifetime $\langle\tau\rangle$.

In the present studies, the mean fluorescence lifetime values increase steeply over the emission spectrum (Fig. 1). This dependence is so strong that it devaluates results measured at a single emission wavelength only or with broad bandwidth filters (which is frequently done in research with other proteins). Significant variations of $\langle\tau\rangle$ as function of the emission wavelength were observed for other proteins previously [44,45], but the reported dependencies were much smaller than those observed in the present study with the Na,K-ATPase. This variation is at first sight in agreement with the proposed relaxation model.

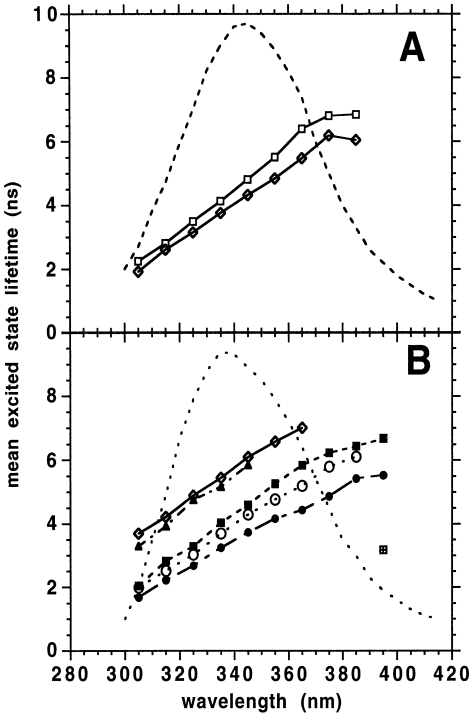


Fig. 1. Variation of the mean excited state lifetime value $\langle\tau\rangle$ over the fluorescence emission spectrum for Na,K-ATPase at different temperatures. Excitation wavelength: 295 nm. (A) In buffer without glycerol. (◇) 19°C, (□) 2°C. (B) In 65% w/w glycerol. (●) 37°C; (○) 19°C; (■) 2°C; (▲) −48°C and (◇) −70°C; (□) 2°C excitation 305 nm. Dotted line: steady-state fluorescence spectrum at excitation 290 nm at 24°C in buffer without glycerol.

However, an unexpected result was that this strong dependence on the emission wavelength persists at all temperatures studied. The variation of the $\langle\tau\rangle$ values occurred over the emission spectrum with the same slope at different temperatures (Fig. 1). The relative increase of $\langle\tau\rangle$ at the blue edge was of around 50%, whereas it is of only 30% at the red edge. These results are in contradiction to the simple model of dielectric relaxations [21,43,44]. According to this model, the relaxation should produce an average effect of the chromophore properties and at high temperatures, lifetime values independent of the emission wavelength should be observed. This may be expected because in such a case the dielectric relaxations are faster than the emission decay which occurs from an already relaxed population of emitters. At low temperatures, which is the other extreme, this wavelength dependence of the mean lifetime should also be small, since the populations of tryptophan residues are unrelaxed with respect to their environment and the excited state lifetime is not long enough to allow exchange of energy with the environment during the duration of the excited state. A steep wavelength dependence should be observed in the intermediate case when the energy of the excited state decreases during its lifetime [42,43]. Since such a temperature dependence was not observed (Fig. 1), we have to propose that the underlying phenomenon is not a simple dipole relaxation and other mechanisms should then be considered.

The contribution of the spectral heterogeneity of the tryptophan residues should be first examined. It is related to their lifetime heterogeneity and to their position within the protein: tryptophans emitting at longer wavelengths may exhibit longer lifetimes and, conversely, Trps with short-living excited states may emit at short wavelength. Some examples of such heterogeneity are discussed in the literature. Spectacular in this respect is the case of horse liver alcohol dehydrogenase. Two tryptophans, one buried in the protein and one exposed on the surface of the

molecule possess fluorescence lifetimes of 3.8 and 7.0 ns, respectively [45]. The difference between the fluorescence maxima of both amino acids (324 and 337 nm) results in a variation of $\langle\tau\rangle$ over the fluorescence spectrum from 4 to 6 ns. A recent example is provided by the immunophilin domain of FKBP59 [16], which possesses 2 Trp residues, one buried in the immuno-suppressor binding site, the second located on the protein surface. The mean lifetime is changed by a factor of 2 from the blue to the red edge of the fluorescence emission spectrum, due to specific quenching of the buried Trp. In the case of Na,K-ATPase with the relatively narrow width of its fluorescence spectrum, the spectral heterogeneity should not exceed that of alcohol dehydrogenase or immunophilin. However, the observed effect was much greater. Therefore, it cannot be excluded that the effect of heterogeneity of emission is reinforced by some other processes.

The next possibility to explain the strong variation of $\langle\tau\rangle$ with emission wavelength, is an excited-state energy transfer directed from Trp residues emitting at short wavelengths to those emitting at long wavelengths (see Ref. [2], Chap. 9). In this case, the transfer will depopulate temporarily the first group of emitting tryptophans and will reduce their lifetimes, while the time-dependent increase of the number of emitters at longer wavelengths may exhibit a substantial increase of effective lifetimes.

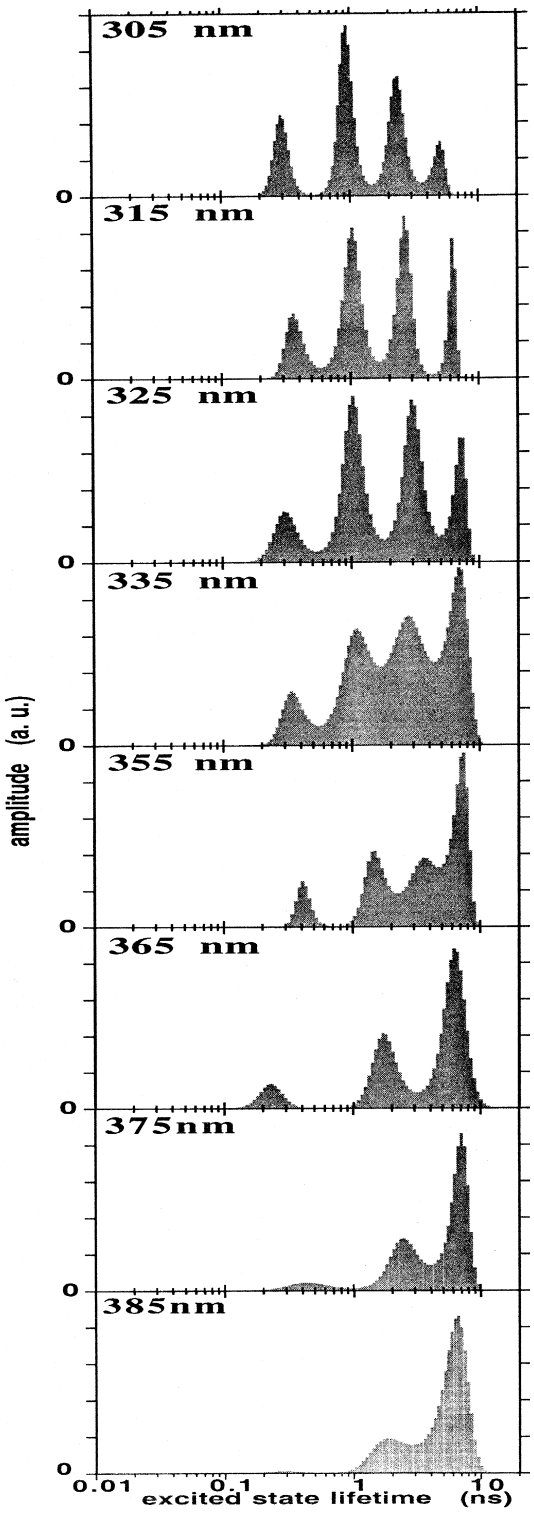
Based on $\langle\tau\rangle$ values only, we are not able to make a decision between the discussed three possibilities. Moreover, all three effects may be present simultaneously. Accurate analysis of the fluorescence decays at different emission wavelengths and as a function of temperature may allow a specification of the underlying mechanisms.

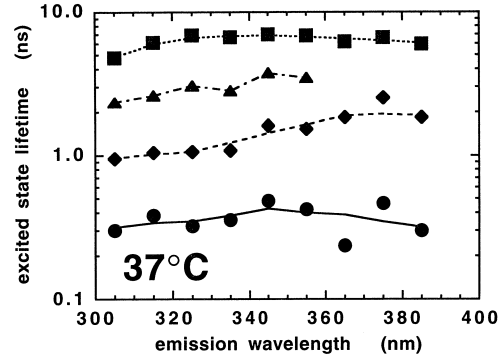
3.2. Trp fluorescence emission heterogeneity: excited state lifetime distributions as function of emission wavelength and temperature

Fluorescence decay measurements with Na,K-ATPase were performed in the temperature range from 37 down to -70°C, in cryo-protective solvents (glycerol/water 65% w/w) at different emission wavelengths. Fluorescence anisotropy measurements were performed under these conditions. In addition, we will describe first the results obtained for fluorescence intensity decays. The anisotropy data will be presented and discussed in a further section.

The results of the fluorescence intensity decay experiment at 37°C evidenced four well-resolved peaks in the range between 0.3 and 6.6 ns at emission wavelengths around the fluorescence maximum of 340 nm (Fig. 2). Since the lowest limit of our time resolution was about 20–50 ps, we may conclude that no essential contribution to fluorescence emission was present in the time range below 300 ps. The presence of four or less discrete lifetime peaks could indicate at a first sight that most of the 16 tryptophans of the Na,K-ATPase molecule are not emitting (statically quenched). It is more likely, however, that the lifetimes of the emitting tryptophans are grouped within discrete classes exhibiting close barycenter values, which are in fact not very different from those found in many single-tryptophan proteins in which the Trp residue is either solvent-exposed [7,11,13–16] or buried in the protein matrix [10,16,17]. In fact, we observed lifetimes of 0.3, 1–1.5, 3 and 5–6 ns in the many different proteins that have been studied. Thus, according to these observations at a single emission wavelength, a significant heterogeneity of emission exists in Na,K-ATPase, but it is not more spectacular than in many other single-tryptophan proteins.

A much larger emission heterogeneity was revealed, however, when the emission wavelength was varied. The magnitudes and positions of the discrete peaks of the lifetime distributions changed as a function of the emission wavelength (Fig. 3). With increasing emission wavelength, the amplitude of the long-living component was largely increased and reached 80% of the relative amplitudes while that of the short-living one correspondingly decreased. However, the lifetime of the long component was not constant through the emission spectrum: it was shifted to higher values by increasing the emission wavelength from 305 to 325 nm (Fig. 3). This means that the long-living excited state population itself is heterogeneous. Probably this is also the case for the two intermediate lifetimes which tend to display similar values of 3–4 ns at the maximum of emission and in the red region of the fluorescence spectrum. In the red edge of the emission spectrum, the lifetime distribution was much simpler than in the blue edge (Fig. 2).





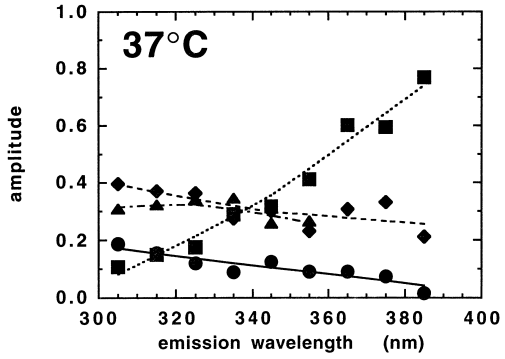
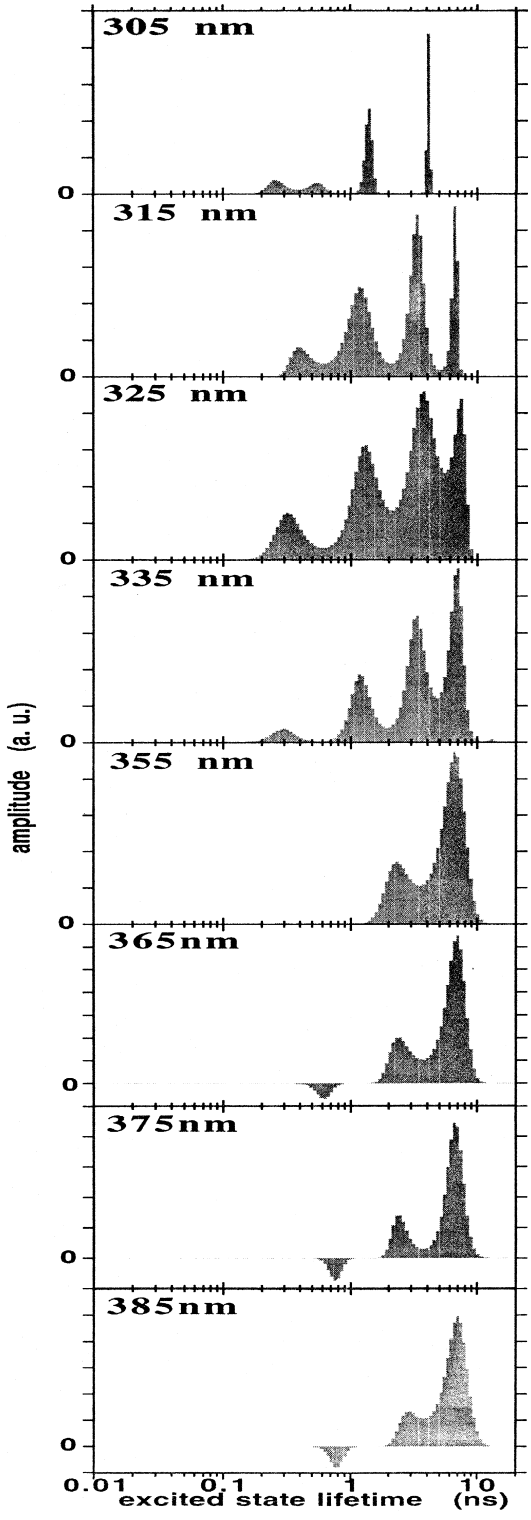


Fig. 3. Variation of the barycenters of the lifetime peaks and of their amplitudes as a function of emission wavelength at 37°C. The same symbols are used respectively for the center and amplitude values of each lifetime class.

Therefore, the steep gradual increase of the mean lifetime over the emission spectrum (as depicted in Fig. 1) is the result of an interplay of at least four lifetime components, their variation in position and in relative intensity over the emission spectrum. The observed emission wavelength dependence of the lifetime distribution emphasizes the much larger emission heterogeneity of this multi-tryptophan protein as compared to single-tryptophan proteins. This suggests the existence of several classes of Trp according to their location within the protein.

At 20°C, similar features were observed (data not shown). A significant evolution occurred to the

Fig. 2. MEM recovered excited state lifetime distributions for Na,K-ATPase at 37°C (65% w/w glycerol) as a function of emission wavelengths (indicated). Excitation wavelength 295 nm.



wavelength-dependent lifetime profiles when the temperature was decreased to 2°C (Fig. 4). The behavior of the long-living component followed the same trend as at higher temperature. In the blue edge, this component displayed values of ~4.8 ns whereas it increased up to ~6–7 ns and stayed constant at long wavelengths (Fig. 5). The associated amplitude followed a complicated pattern: first it decreased until 325 nm (at the emission wavelength where the lifetime value changed) and then increased to reach a high value of ~80–90% of the amplitudes in the red edge of the fluorescence emission spectrum.

The two intermediate lifetime populations appeared as composite. In the blue edge, they displayed values of 0.5–1 ns and 1–3 ns for each population respectively whereas in the middle of the spectrum and in the red edge their values became similar (or one of them disappeared) (Fig. 5).

The short-living component showed a decrease in amplitude upon increasing the emission wavelength as it was found at 37 and 20°C. Its value increased with the emission wavelength (Fig. 5). This means again that several short-living excited state populations are coexisting. In addition, a new feature could be observed: the appearance of a well-resolved negative component (~800 ps) at emission wavelengths above 365 nm, when the lifetime changed. Red edge excitation showed that the lifetime distribution changed dramatically: instead of a negative, a strong positive component was observed at shorter times (Fig. 6).

The existence of a component in the fluorescence-lifetime distribution with negative amplitude indicates that at a particular emission wavelength the number of excited molecules increases temporarily as a function of time. This can be induced by a time-dependent shift of the emission spectra of excited tryptophan species to longer wavelengths. This reaction may be either a dielectric relaxation or an excited-state energy transfer. The first process should be suppressed by lowering the temperature. The second mechanism could also be

Fig. 4. MEM recovered excited state lifetime distributions for Na,K-ATPase at 2°C (65% w/w glycerol) as a function of the emission wavelength. Excitation wavelength 295 nm.

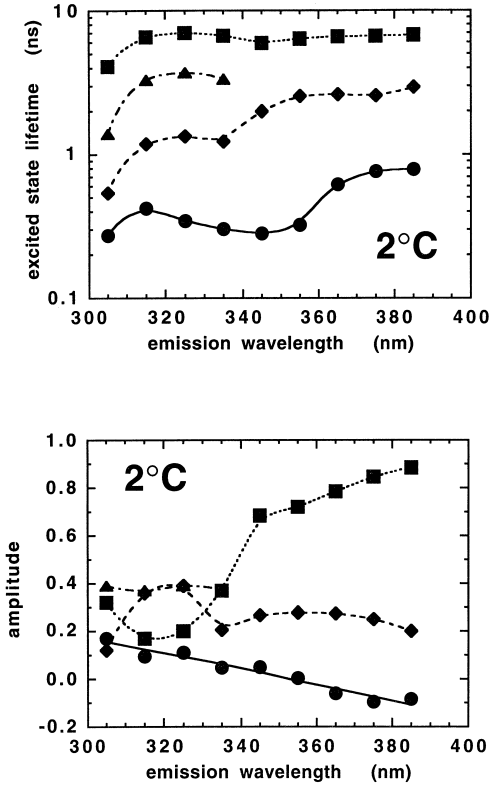


Fig. 5. Variation of the barycenters of the lifetime peaks and of their amplitudes as a function of emission wavelength at 2°C. The same symbols are used respectively for the center and amplitude values of each lifetime class.

indirectly affected by either temperature-dependent conformational changes or by thermal-induced spectral shift related to dielectric relaxation.

When the temperature was lowered to −48°C (data not shown) and further to −70°C, the number of the resolved peaks in the lifetime distributions at the emission maximum decreased further to three (Fig. 7) and the peaks became sharper. The evolution of the wavelength dependency of the lifetime distributions showed similar tendencies as observed at higher temperatures. The lifetime of the long-living component increased as function of the emission wavelength in the blue edge of the fluorescence emission and stayed at a constant value of about 7 ns above 330 nm (Fig. 8). The amplitude of this component decreased up to 325 nm and increased for longer wavelength. At the red edge, it represented the only emissive component. This confirms that several long-living populations of lifetimes were present. At least two such families can be separated which are different by their emission wavelengths: a blue population of Trp residues (located in hydrophobic regions) that displays an average long lifetime of about 4.8 ns and a red population (located in polar regions) with a longer lifetime of about 7 ns. Both long-living components were only slightly sensitive to temperature. This indicates that their respective environments are rigid in the nanosecond time scale.

The amplitude of the intermediate lifetime had a maximum at about 325–330 nm and vanished at long emission wavelength. Therefore, the lifetime profile in the red edge of the fluorescence emission spectrum became unexpectedly simple at these low temperatures: it consists of only one long positive and one short negative lifetime component. The negative component is substantial, at wavelengths between 365 and 385 nm, it included between 30 and 40% of the amplitudes (Table 1). Its value remained approximately unchanged as function of the emission wavelength (Fig. 8). If the fit is processed without negative component, the chi-square increases from

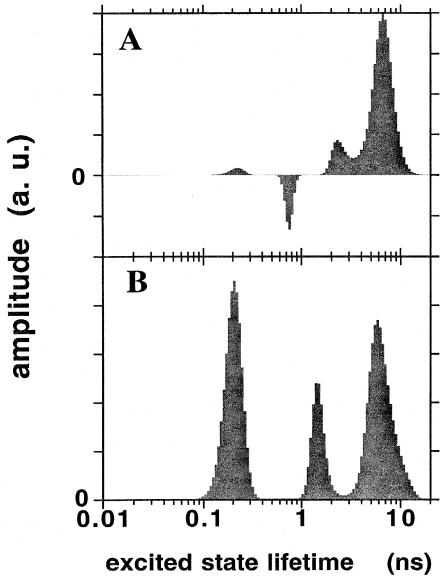
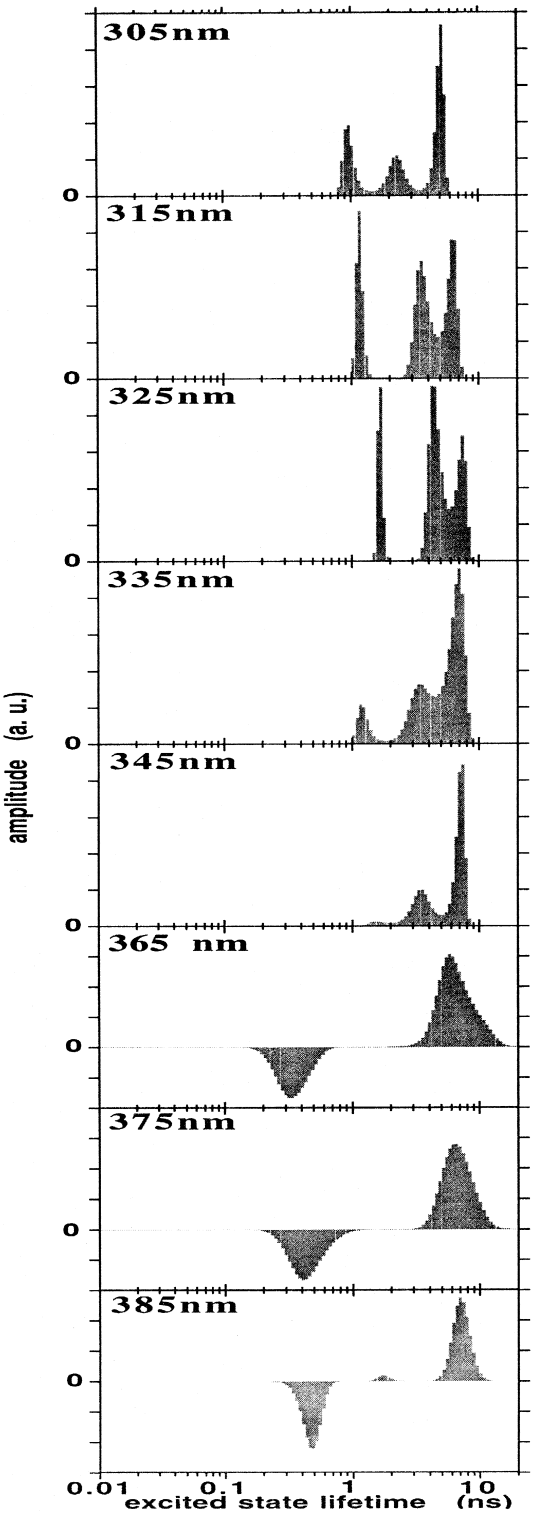


Fig. 6. MEM recovered excited state lifetime distributions at 2°C (65% glycerol) upon (A) excitation at 295 nm and (B) red edge excitation at 305 nm (emission wavelength: 385 nm).



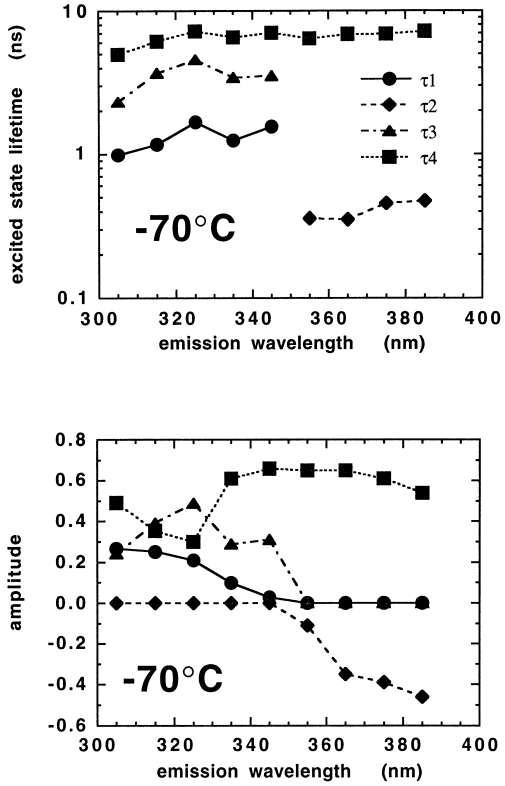


Fig. 8. Variation of the barycenters of the lifetime peaks and of their amplitudes as a function of emission wavelength at −70°C. The same symbols are used respectively for the center and amplitude values of each lifetime class.

1.0 to 4–5, depending on the temperature (data not shown).

These observations show that deep freezing did not suppress the excited state process which gave rise to the short-living component with negative amplitude. Molecular relaxations should be canceled out at these temperature. On the contrary, the fluorescence energy transfer between tryptophans should not be suppressed. Its efficiency can be increased substantially due to a combination of several factors acting in the same direction. A reduction of temperature can increase the quantum yields of energy transfer donors, suppresses the molecular relaxations and,

Fig. 7. MEM recovered excited state lifetime distributions for Na,K-ATPase at −70°C (65% w/w glycerol) as a function of the emission wavelength. Excitation wavelength 295 nm.

Table 1
Tryptophan fluorescence decay parameters as a function of temperature in the red edge of the fluorescence emission spectrum of Na,K-ATPase (excitation wavelength: 295 nm, bandwidth: 4 nm, emission wavelength: 385 nm, bandwidth: 5 nm)

Temperature	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)
(°C)	(C_1)	(C_2)	(C_3)
37	0.30	1.84	5.97
	0.02)	(0.21)	(0.77)
19	0.79	2.41	6.85
	(−0.10)	(0.27)	(0.64)
2	0.79	2.94	6.73
	(−0.07)	(0.17)	(0.76)
−48	0.74	−	6.48
	(−0.18)	−	(0.82)
−70	0.47	1.77	7.19
	(−0.45)	(0.03)	(0.53)

The amplitudes C_i for each component τ_i were normalized using their absolute values according to: $C_i = (C_i)/(\Sigma_i|C_i|)$.

in turn, may shift the donor fluorescence spectra to shorter wavelengths. Therefore, the overlap integral between the donor emission and the acceptor absorption spectra may be increased. Temperature reduction may increase also the protein density and thus decreases the donor–acceptor distance. These parameters enter into the well-known Förster equation [40–42], which determines the energy transfer rate k_T. There are several experimental arguments that favor the interpretation of these data in terms of energy transfer between unlike chromophores (hetero-transfer). They are developed in the following.

On the assumption that there is a single donor and a single acceptor possessing individual exponential decay rates and that the probability of energy transfer can be considered to be time-independent, the emission decay of the donor remains mono-exponential,

$$I_{D(t)} = I_0 \exp\left(-t/\tau_D^{ET}\right) \quad (7b)$$

but the lifetime τ_D is decreased to the value τ_D^{ET} in correspondence with the energy transfer rate, k_T:

$$\tau_D^{ET} = \left(\tau_D^{-1} + k_T\right)^{-1} \quad (8)$$

If the energy transfer efficiency is increased by lowering the temperature, one should observe correspondingly a reduction of the donor lifetime instead of the usual increase due to less efficient non-radiating processes at low temperatures. This reduced lifetime is exactly what we observed in the extreme blue edge of the fluorescence emission spectrum for the three longer lifetimes when the temperature was reduced from 37 to 2°C (Table 2). At lower temperatures, the lifetime values increased again. This behavior suggests that these components belong probably to the 'blue' tryptophans which are energy transfer donors. The energy transfer becomes more and more efficient at low temperatures and compensates the normal lifetime increase due to the reduced efficiency of the radiationless transitions. At cryogenic temperatures, the latter mechanism dominates the former one. At longer emission wavelengths (315–325 nm), the temperature effect displayed a more 'classical' behavior: the lifetime was increased with a temperature decrease. This suggests that other 'blue' Trp groups were detected which did not participate in the energy transfer process. Acrylamide-quenching experiments performed at the blue edge

Table 2
Tryptophan fluorescence decay parameters as a function of temperature in the blue edge of the fluorescence emission spectrum of Na,K-ATPase (excitation wavelength: 295 nm, bandwidth: 4 nm, emission wavelength: 305 nm, bandwidth: 5 nm)

Temperature (°C)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_4 (ns)	$\langle\tau\rangle$ (ns)
		(C_1)	(C_2)	(C_3)	(C_4)
		[I_1]	[I_2]	[I_3]	[I_4]
37	0.31	0.96	2.45	5.05	1.69
	(0.18)	(0.41)	(0.33)	(0.08)	
	0.04]	[0.24]	[0.48]	[0.24]	
19	0.14	0.61	1.92	4.75	1.85
	(0.05)	(0.36)	(0.39)	(0.18)	
	[<1%]	[0.12]	[0.41]	[0.47]	
2	0.27	0.54	1.39	4.09	2.04
	(0.17)	(0.12)	(0.39)	(0.32)	
	[0.02]	[0.03]	[0.28]	[0.67]	
−48	−	0.43	1.46	4.47	2.83
		(0.20)	(0.27)	(0.53)	
		[0.03]	[0.14]	[0.83]	
−70	−	0.99	2.31	4.99	3.30
		(0.27)	(0.24)	(0.49)	
		[0.08]	[0.17]	[0.75]	

The barycenter values τ_i and the relative surface area α_i of each peak in the lifetime distribution are given. The values of the mean lifetime $\langle\tau\rangle$ were calculated according to $\langle\tau\rangle = \Sigma_i C_i\tau_i$ and the fractional intensities of the species i were calculated from: $I_{(i)} = \frac{C_i\tau_i}{\Sigma_i C_i\tau_i}$

(315 nm) did show no quenching of any of these lifetime populations. This demonstrates that these 'blue' emitters are located in apolar regions inaccessible to small water-soluble solutes.

In contrast, the emission decay of the acceptor, even in this simplest case of hetero-transfer, is described by two decay times, possessing positive and negative amplitudes. Let the lifetime of the acceptor be τ_A° and $\tau_A^\circ > \tau_D$ in the absence of an energy transfer. Then the intensity decay of the acceptor can be expressed as:

$$I_{A(t)} = (A_1 + A_2)\exp(-t/\tau_A^0) - A_3\exp(-t/\tau_D^{ET}) \quad (9)$$

where A_1 is the initial amplitude of emission of the directly excited acceptor, while A_2 and A_3 are the functions of donor concentration, energy transfer rate and rates of donor and acceptor emissions. They describe the decay of accepted energy (A_2) and its uprising as a function of time due to transfer to the acceptor (A_3). In our case, we probably have several donors with different lifetimes and (as a minimum) one acceptor. In such a case, the decay should be more complicated, but the negative exponential(s) remain as characteristic feature of the acceptor decay. Observation of only one negative exponential together with one emissive decay component at the long-wavelength edge suggests that only few energy acceptors (maybe a single one) with a long lifetime, participated in the mechanism. The absence of the correspondent positive component at short wavelengths may indicate the participation of few energy donors, the other Trps being not involved.

The time constants and normalized integrated amplitudes of the 'negative' component observed in the red edge as function of temperature are listed in Table 1. The tendency is obvious that an increase of temperature led to an increase of the time constant associated with the negative amplitude, τ_1, and not to a reduction as it would be in the case of a dielectric relaxation. This reverse temperature dependence is an additional support for the assumption that the origin of the uprising kinetics observed at temperatures < 2°C is related to the kinetics of excited state energy transfer and not to the dielectric relaxation. This energy transfer is 'directed': it occurs from 'blue' emitters to one or few 'red' tryptophans which serve as energy traps. The characteristic position of the emission spectrum of these excitation energy acceptors indicates that they are in a polar environment. Experiments in which the long-living red emitters (emission wavelength: 375 nm) were quenched by acrylamide were performed in order to check their accessibility to the solvent. At 20°C in the absence of glycerol, a bimolecular quenching constant value of $8.5 \cdot 10^7$ M^{-1} s^{-1} was obtained (data not shown). This value is very low as compared to that obtained for water-accessible Trps in different proteins ($k_q \sim 3$–$4 \cdot 10^9$ M^{-1} s^{-1}) [46]. The value for Na,K-ATPase is quite different from that obtained with Ca-ATPase from sarcoplasmic reticulum, which displayed a faster bimolecular quenching constant value of $\sim 3 \cdot 10^8$ M^{-1} s^{-1} [47,48], despite its Trp residues are believed to be located mainly in hydrophobic regions constituted by the protein–lipid interfaces [47]. In case of the Na,K-ATPase, the results suggest that the Trp residues with a red-shifted emission are located in polar environments but not freely accessible to small water-soluble molecules.

3.3. Steady-state and time-resolved fluorescence anisotropy

Additional specific evidence for the existence of excited-state energy transfer between tryptophan residues can be obtained from the measurements of fluorescence anisotropy [49]. The fluorescence emission can be in fact depolarized by two processes: by rotational motions and by excitation energy transfer [50]. In the absence of rotational motions at low temperature in a vitrified solvent, the depolarization should be due to the energy transfer mechanism(s). In addition, the fluorescence anisotropy is sensitive to hetero-transfer and to homo-transfer as well, but it is extremely useful to detect the existence of the latter process and to measure the transfer rate [12,51]. In fact, the homo-transfer process cannot in fact be detected by lifetime measurements since this mechanism does not affect the fluorescence emission kinetics; in contrast to the hetero-transfer which is responsible for the negative components observed in the decays measured in the red edge of the fluorescence emission spectrum.

The results of the steady-state fluorescence anisotropy measurements over the excitation spectrum at −48°C are presented in Fig. 9. The results obtained with Na,K-ATPase were compared with data from the model tryptophan derivative NATA (*N*-acetyltryptophanamide) in dilute solution of glycerol. In the conditions of this experiment, NATA exhibited neither chromophore rotations nor excited-state energy transfer and can serve as a reference for the absence of these effects.

For Na,K-ATPase, much lower steady-state fluorescence anisotropy values were observed than for NATA in the major part of the excitation spectrum and especially in the specific region of Trp absorption (> 290 nm). The depolarization of the protein fluorescence was almost temperature-independent. A slightly lower anisotropy at 19°C than at −46°C may be the result of some contribution of tryptophan rotational motions (Fig. 9). Therefore, the major depolarization effect cannot be caused by large rotational motions of the tryptophan residues but should be assigned to the second depolarization mechanism: the excitation energy transfer.

At the long-wavelength edge of excitation spectrum (305 nm), the fluorescence emission of Na,K-ATPase tended to match the high anisotropy value of 0.3 which is observed for NATA (Fig. 9). This behavior is a manifestation of the so-called Weber red-edge effect in excitation-energy homo-transfer [52], due to the failure of transfer at the low-energy edge of electronic spectrum. This effect was observed in many systems by inhomogeneous broadening of the spectra and slow mobility of chromophore environment (Ref. [2], Chap. 9). In agreement with this observation, it can be reminded that the fast time constant affected by a negative amplitude disappeared when excitation wavelength was performed at 300 nm (Fig. 6). Therefore, we can conclude that the tryptophan environments are rigid on the nanosecond time scale at −46°C and at 20°C. This confirms further that in the red edge of the fluorescence emission spectrum at temperatures lower than 2°C the negative component observed in the fluorescence decay kinetics is not the result of a time-dependent spectral shift due to dipolar relaxation but the result of an energy transfer.

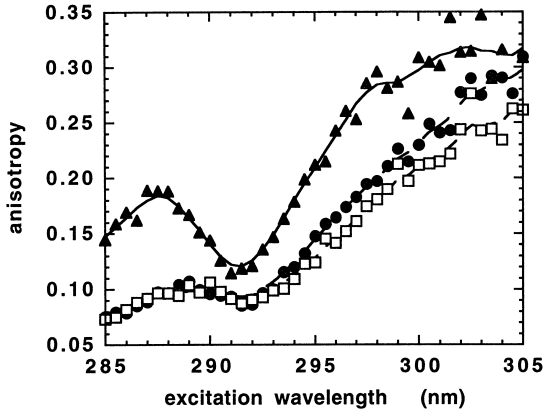
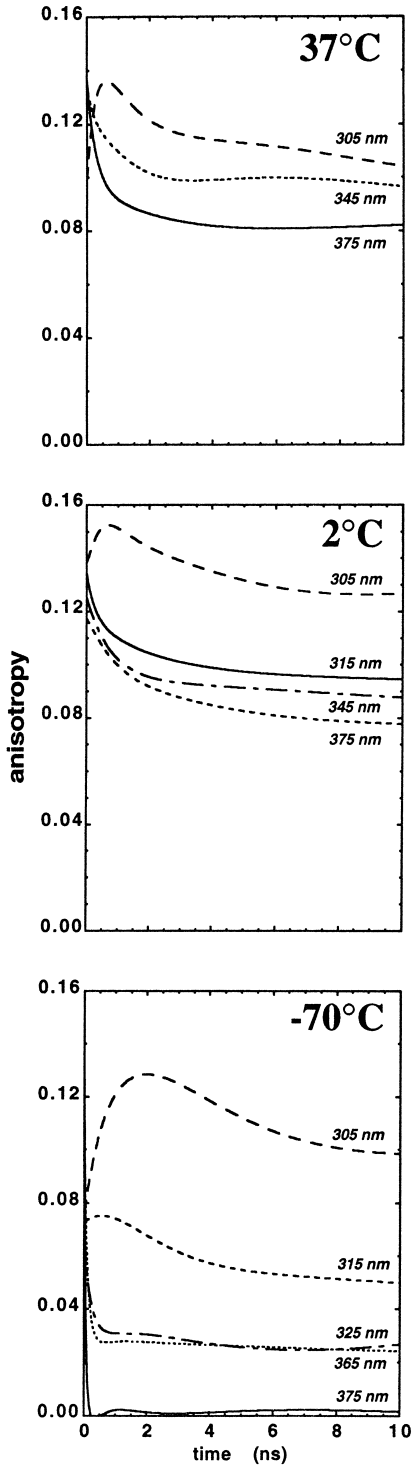


Fig. 9. Steady-state excitation–polarization spectra of Na,K-ATPase at 20°C (□) and −46°C (●) in buffer containing 65% w/w glycerol and of *N*-acetyltryptophan amide (NATA) (▲) at −46°C in neat glycerol. Emission wavelength 340 nm.

In order to describe qualitatively the energy transfer mechanisms in more details and to detect the existence of energy transfer between like-chromophores (homo-transfer), impulse anisotropy decays were measured at different emission wavelengths and at three temperatures (37, 2 and −70°C). Data for selected emission wavelengths are represented in Fig. 10.

The impulse fluorescence anisotropy decay measured at −70°C at the red edge of the emission spectrum exhibited an extremely fast time constant that led to a complete depolarization within 0.2 ns. According to the semi-quantitative model of the fluorescence depolarization by electronic energy transfer in donor–acceptor pairs of like and unlike chromophores [51], a complete depolarization demonstrates that we are dealing with an hetero-transfer and not with an homo-transfer. This means that the spectral properties of the donor(s) and of the acceptor(s) are different and so are their environments. However, in such a case the initial anisotropy at time zero should display a value of 0.016, the Agranovich's and Galanin's result [41]. A much higher value was observed for the Na,K-ATPase. This indicates that the acceptor(s) is (are) probably excited directly. Therefore, the anisotropy at the longest emission wavelength gave evidence for a highly efficient energy transfer from donor Trp residues emitting in the blue edge (with shorter excited-state lifetimes) to acceptor Trp residues emit-



ting in the red edge (with longer excited-state lifetimes). According to the model vanishing anisotropy can only be reached if fluorescence decays of acceptor and intrinsic donor are much slower than energy transfer [51], i.e., the transfer process exceeds the fluorescence emission efficiency. This assumption is verified qualitatively since the long-living acceptor(s) which emit in the red, exhibited lifetimes of ~ 7 ns. The shortest lifetimes measured in the blue region of the emission spectrum displayed values of ~ 1 ns. The inter-chromophore distances for these Trp couples should be of the order or smaller than the R_0 value of 10–15 Å which was determined for inter-Trp energy transfer [1,12]. This efficient energy hetero-transfer applies probably to few Trp residues.

The impulse fluorescence anisotropy decays calculated at shorter emission wavelengths (365, 345 and 325 nm), which exhibited also a fast decay, showed a plateau with a positive value of the anisotropy higher than that at longer emission wavelength. This may be due to the increasing influence of Trp residues not involved in the hetero-transfer process as the emission wavelength is moved to shorter values. The anisotropy decay pattern at the extreme blue edge (315 and 305 nm) were very different. Both showed a more and more pronounced uprising part followed by a slow decline to a plateau value which was higher at 305 than at 315 nm. At 305 nm, the plateau value was 0.1, close to half of the intrinsic anisotropy value of NATA when no depolarizing processes were present. This indicates the existence of an homo-transfer mechanism of depolarization between blue Trps [12,51]. This could be explained by the existence of clusters of 'blue' Trp residues located in those hydrophobic regions of the protein which are either in contact with the phospholipid hydrocarbon chains in transmembrane helices or located in the protein core or at oligomer interfaces. In the simplest case of one donor and one acceptor, the homo-transfer process should lead,

Fig. 10. Impulse fluorescence anisotropy decays of Na,K-ATPase at different temperatures and at selected emission wavelengths. Excitation wavelength: 295 nm. The impulse anisotropy response functions were calculated from the separate deconvolution of the vertical and horizontal fluorescence decays. Temperatures and emission wavelengths are indicated.

however, to a decrease of the anisotropy to the plateau value of $A_0/2$ but not to a biphasic behavior as observed with the Na,K-ATPase. Such a time course of the anisotropy decay may be interpreted qualitatively by a spectrum of transfer rates in which fast rates are associated with short lifetimes and conversely slow transfer rates with longer lifetimes.

At higher temperature when probably internal motions of small amplitudes start to contribute to the depolarization, the results were very different at long emission wavelengths. The decay of anisotropy was much slower and reached a high plateau value at about 0.07–0.10. This is a strong argument to consider that the hetero-transfer of excited-state energy as described above was much less efficient at these higher temperatures. A possible interpretation is that some temperature-induced conformational changes of the protein (or phase behavior of the phospholipid membrane) occurs that bring some few Trp residues in hydrophilic regions closer to Trp residues located in more hydrophobic parts of the protein at low temperature, or that there exists a very efficient quenching of the acceptor which is strongly dependent on temperature. It may also suggest that the efficiency of the energy transfer is increased at low temperature because of thermally induced spectral blue-shift associated with dielectric relaxation. In the extreme blue region of the fluorescence spectrum (305 nm), the anisotropy showed an uprising part as found at lower temperatures. A likely interpretation of this observation would be the persistency of the homo-transfer processes in addition to molecular rotations. These motions have to be strongly restricted in any case since the anisotropy curves did not reach values below 0.07.

3.4. Time-resolved spectra as a function of temperature

Construction of time-resolved spectra is the usual method to analyze the rates of dielectric relaxations [53,54] and also to detect excited state inter-conversions [55]. Time-resolved spectra were obtained from the emission wavelength dependence of fluorescence decays at 37, 2 and −70°C (Fig. 11). At these temperatures, the position of the steady-state spectra corresponded to high and low points observed in the previously published temperature dependence [27]. It

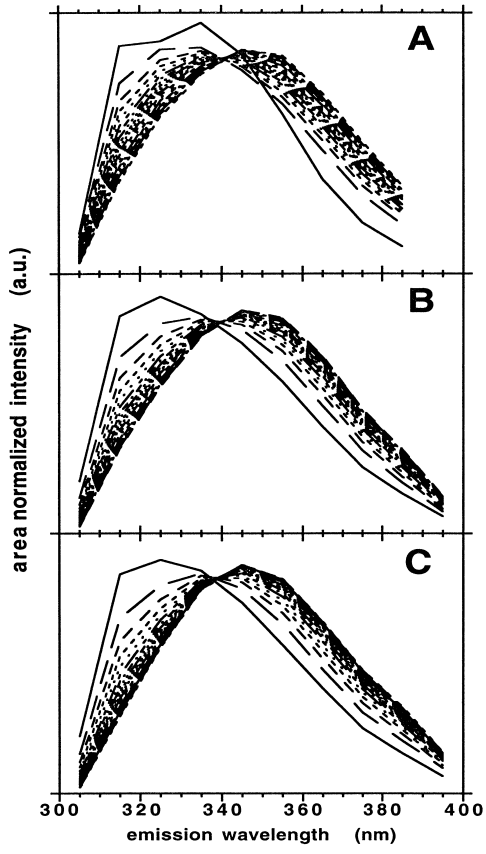


Fig. 11. Time-resolved fluorescence spectra for Na,K-ATPase at 37°C (65% w/w glycerol) calculated from the decay curves with steps of 10 nm in the range 305–395 nm. The spectrum at the shortest wavelengths (solid line) corresponds to time zero, and each next spectrum (dashed lines) corresponds to time increase by 200 ps. (A) −70°C, (B) 2°C and (C) 37°C.

can be seen that at the three temperatures a large similar shift to longer wavelength occurs (Fig. 11).

In case of dielectric relaxations, a gradual time shift of the emission spectra should be obtained when molecular motions are allowed [56–58]. In our case with the low spectral resolution of 10 nm between decay measurements, it can be seen that the major effect was an exchange between two main excited state populations, one close to 325 nm and the other near 345 nm. From the spectra (Fig. 11), which were normalized to the same total intensity, one cannot see a progressive shift with temperature. In addition, the maxima of emission seemed to be approximately constant at all three temperatures. This

makes molecular relaxations as major mechanism improbable. Finally, with the spectral resolution of 10 nm, we can observe an iso-emissive point at around 340 nm for all three temperatures.

The most likely interpretation of these data has to consider spectral and temporal heterogeneity of emission. We observed that the components which emit at shorter wavelengths, decay faster than components which emit at longer wavelengths. Therefore, the relative contribution of these 'blue' components will decrease with time and a time-dependent shift of the emission spectrum to longer wavelengths will be observed [59]. This was observed for Ca-ATPase: the short-living components are dominant in the blue edge and the long-lived ones are emitting in the red edge [60]. A hetero-transfer of excitation energy between tryptophans with emission at short-wavelength to a tryptophan emitting at long wavelength should reinforce the effect in the case of Na,K-ATPase at low temperature as well as dielectric relaxation at higher temperature.

4. Discussion and conclusions

One of the striking observations in this work is that it is possible to photo-select in the red edge of the emission spectrum the decay kinetics of few Trp residues in a protein that contains 16 Trp residues per protomer. Only two major components at room temperature and even only one at cryogenic temperature are present in this spectral region. The anisotropy decays at low temperature showed also photo selectivity and proved the existence of clusters of Trp residues within the protein structure. Both sets of data suggest that a few 'red' emitters are located on protein/water interfaces. The fact that we observe more or less discrete peaks in the lifetime distribution with defined barycenter values is not surprising because such 'classical' values were seen in many single-tryptophan proteins. Some of the tryptophans may also be quenched statically by neighboring groups of atoms in the protein structure.

The excitation-energy transfer between tryptophans appears to be the main factor which reduces the number of detectable emitters. The existence of inter-tryptophan energy transfer is the most characteristic feature of the Na,K-ATPase that is revealed by this study. Both homo- and hetero-transfer processes occur between identical chemical chromophores (tryptophan residues) but which differ only by the surrounding protein structure. In case of homo-transfer, only small differences in absorption spectra, usually less than 1–2 nm (Ref. [2], Chap. 3) should be produced by the protein structure. In case of hetero-transfer, larger differences in the environment inducing large shifts of the fluorescence emission spectra, should be generated.

At low temperatures, the excited-state energy transfer in the protein may share some similarities with the same process occurring in concentrated dye solutions in rigid polar matrices. Due to the distribution of solute–solvent configurations, which is more random than in a protein, the fluorescence spectra cover an extended wavelength range. It has been reported that in these systems the energy flow does not occur randomly between chromophores but is directed from chromophore-solvent configurations emitting at short wavelength to those emitting at long wavelengths [61–63]. As a result, the mean lifetime of the excited state increases strongly over the fluorescence spectrum with wavelength. Moreover, the time-resolved spectra shift with time to longer wavelengths. This type of energy transfer is called 'the directed transfer'. It is highly probable that we observed essentially the same phenomenon in the Na,K-ATPase at low temperature. Differences may arise due to the fact that the chromophores occupy well-defined positions in a macromolecular protein structure and the chromophores are characterized by discrete lifetimes depending on their positions.

The existence of both energy transfer mechanisms suggests the existence of structural constraints on the inter-Trp distances. The homo-transfer mechanism requires obviously that the distance between the 'blue' Trps should be of the order of ~ 10 Å to be efficiently detected and that it involves many Trp residues located in hydrophobic regions of the protein. They may be present either in a hydrophobic pocket within the protein core or in the transmembrane helices at the protein/lipid interfaces. According to models based on the primary sequence, 10 transmembrane helices are postulated for the α subunit. Out of the 12 Trp residues that it contains, two are located in the ATP-binding region, outside the

membrane domains (Trp389, Trp413), eight would be situated in the membrane region of the protein, four would be present strictly in the transmembrane helices, two of them are adjacent in the sequence (981–982). It is very likely that energy transfer can take place between these last two residues as it was shown for human epidermal growth factor and bis-Trp [13]. However, implication of only two Trps is probably not sufficient to explain the large depolarization effect that we observed. More Trp residues should be involved. The helices should be brought close enough to each other in the folding of the protein in the membrane bilayer to form Trp clusters within the lipid bilayer. The distance requirement for the hetero-transfer is less restrictive, a critical distance of ~ 15 Å has been estimated [1].

We suggest therefore that the 16 Trp residues of the Na,K-ATPase may be divided into several classes. One class includes Trp residues that participate in the fluorescence hetero-transfer that we observed more clearly at low temperature. They may be divided into two sub-classes, 'blue' donors and 'red' acceptors. The number of acceptors may be much smaller than the number of donors, but these 'red' acceptors become the dominant emitters at long wavelengths since they accept the excited-state energy from the donors and/or may be unquenched at low temperatures. The existence of such an efficient hetero-transfer occurring between Trp residues located in extremely different environments, rises the question of how the protein overall folding can allow such a proximity. The second sub-class corresponds to Trp residues in hydrophobic regions that are close enough to each other to be involved in energy homo-transfer (in this case their inter-distance should be around 10 Å [12]. This may apply to Trp residues located either in transmembrane helices or in hydrophobic core of the protein regions outside the membrane bilayer. These different mechanisms of fluorescence energy transfer may be used therefore as structural probes to detect more accurately conformational changes elicited by effectors and ion binding or release.

We propose that by combination of time and spectral resolution as well as temperature studies and anisotropy measurements more specific information about the conformational changes in the protein structure surrounding the Trp residues can be obtained. Especially when by use of ions and effectors perturbations of the intrinsic fluorescence will be measured in fast kinetic experiments at extreme emission wavelengths, new and deeper insight in the conformational changes of the Na,K-ATPase may be expected.

Acknowledgements

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