Does intrinsic fluorescence reflect conformational changes in the Ca^{2+}-ATPase of sarcoplasmic reticulum?

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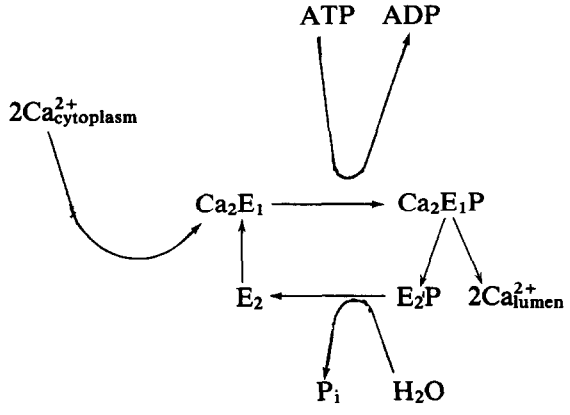
We have investigated the kinetics of the intrinsic fluorescence drop observed when ATP is added to purified sarcoplasmic reticulum ATPase in a potassium-free medium containing magnesium and calcium, at pH 6 and 20°C. Under these conditions, analysis of the fluorescence drop is complex. Several events contributed to the rate of the fluorescence drop initiated by turnover, including phosphorylation, conformational transition of the phosphorylated complex, and dephosphorylation. On the other hand, when 75% of total fluorescence was quenched by energy transfer to the membrane-bound ionophore A23187, the observed turnover-dependent drop in residual fluorescence mainly reflected the conformational transition of the phosphorylated ATPase. Combination of fast kinetics with the quenching of selected tryptophan residues is suggested to be a promising tool for the study of proteins containing many of these residues.

Protein conformation *Energy transfer* *Fast kinetics*

1. INTRODUCTION

Tryptophan residues in proteins may behave like intrinsic probes of the various protein states, because their fluorescence spectrum is sensitive to the environment. A typical example of this is provided by sarcoplasmic reticulum Ca^{2+}-ATPase, a protein with 13 tryptophan residues responsible for calcium transport across the endoplasmic reticulum membrane in muscle cells [1,2]. Pioneering work by Y. Dupont established that calcium binding to this ATPase enhanced its intrinsic fluorescence by a few percent; its intrinsic fluorescence was also found to be sensitive to various ligands, and stopped-flow fluorometry made a significant contribution to clarifying the kinetics of this enzyme (see a review in [2]), whose simplified reaction scheme is described in scheme 1 below [3,4].



Scheme 1.

From the beginning, the most widely held assumption was that the Ca^{2+}-induced change in

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sarcoplasmic reticulum fluorescence reflected a general conformational change of the polypeptide chain. However, fluorescence probes environmental changes in the vicinity of fluoropheric groups [5], and such changes are likely to be most pronounced near binding or reaction sites, without necessarily extending to other parts of the molecule as the result of intra- or interpeptidic allosteric effects [6].

An example of a situation in which both these possibilities might be involved is the fluorescence drop observed when ATP initiates the ATPase catalytic cycle. Assignment of this drop to a specific step in the reaction cycle has aroused some controversy, and different candidates have been put forward by authors working under different experimental conditions as the reaction steps responsible for the fluorescence drop: they include Ca_2E_1 to Ca_2E_1P phosphorylation [7], Ca_2E_1P to Ca_2E_2P simple isomerization [8], and the Ca_2E_1P to E_2P Ca^{2+}-releasing transition [9]. We report here stopped-flow measurements of the ATP-induced fluorescence drop recorded under conditions which select different subpopulations of fluorophores through the quenching of certain tryptophan residues by energy transfer or through selection of those residues whose spectra are enhanced rather than shifted. For identical ATP additions, the kinetic patterns were very different. This study emphasizes the caution necessary in the interpretation of fluorescence kinetic data when several fluorophores are involved. On the other hand, it also suggests how structural information about the Ca^{2+}-ATPase, obtained from sequence determinations [1], can be combined with selective quenching of tryptophan residues, as recently explored by a few groups [10–12], to study the molecular mechanism of Ca^{2+}-ATPase function.

2. EXPERIMENTAL

Sarcoplasmic reticulum vesicles and deoxycholate-extracted ATPase were prepared and assayed as described [13,14]. Changes in intrinsic fluorescence were followed either with a Perkin Elmer MPF 44A fluorometer or a Durrum stopped-flow apparatus, using an excitation wavelength of 295 nm. To detect the fluorescence response we used either a Balzer interference filter with peak transmission at 332 nm or a Schott WG 320 cut-off filter [13]. The experiments were performed in media, containing 150 mM Mes-Tris (pH 6.0) and 20 mM Mg^{2+}, with other additions as noted in the text to figures. This medium has previously been used for a kinetic study of Ca^{2+}-release associated with the Ca_2E_1P to E_2P transition (Champeil and Guillain, submitted). In more physiological media (potassium containing, pH neutral or slightly alkaline) E_2P intermediates are generally not formed in sufficient quantity in steady state to permit the study of the Ca_2E_1P to E_2P Ca^{2+}-releasing transition [15].

3. RESULTS

3.1. *A23187-quenched tryptophans: ligand-induced changes in residual fluorescence*

The experiments reported here were part of a wider investigation in which sarcoplasmic reticulum vesicles were first incubated with the Ca^{2+} ionophore calcimycin (A23187) to prevent them from accumulating calcium. We found that this ionophore effectively quenched ATPase tryptophan fluorescence (fig.1A), a typical fluorescence energy transfer situation [16,17]. After addition of large amounts of ionophore (e.g. 4% w/w), 1 or 2 min were required before residual fluorescence stabilized to its final level. In the absence (fig.1B, upper tracings) or presence (fig.1B, lower tracings) of the quenching ionophore, we recorded the fluorescence changes induced by addition of the following ligands: calcium and ATP, which initiate the ATPase reaction (left tracings), inorganic phosphate, which in the absence of calcium induces phosphoenzyme formation, especially if water activity has been reduced by including a significant proportion of dimethylsulfoxide (center tracings), and ATP alone, which in the absence of calcium binds to the ATPase in a non-covalent way (right tracings). The left tracings show that in the presence of the ionophore, the Ca^{2+}-induced fluorescence enhancement was reduced to a greater extent than the fluorescence level itself, suggesting that the quenched fluorophores contributed preferentially to the Ca^{2+}-induced signal. In contrast, the fluorescence enhancement observed after adding 0.1 mM ATP to the Ca^{2+}-deprived E_2 ATPase (right tracings) and the fluorescence drop observed after adding 0.01 mM ATP to the Ca^{2+}-saturated

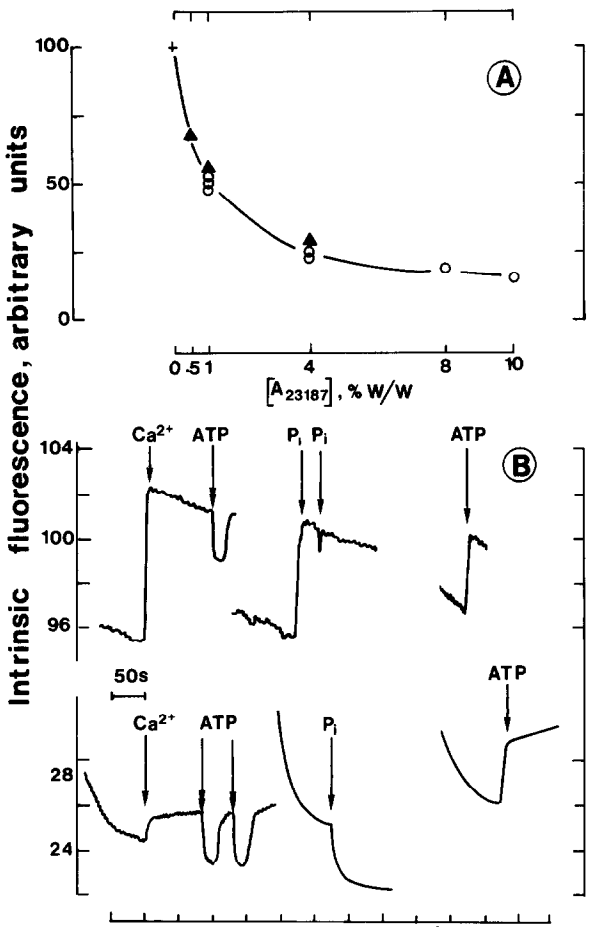

Fig.1. Intrinsic fluorescence in the presence of the ionophore A23187. (A) Quenching by A23187 of the intrinsic fluorescence of native sarcoplasmic reticulum vesicles (▲) and purified ATPase (○). The medium contained 150 mM Mes-Tris, 20 mM Mg^{2+}, 0.1 mM Ca^{2+} and 0.1 mg/ml protein (pH 6, 20°C). Excitation wavelength, 295 nm; emission wavelength, 330 nm. (B) Fluorescence changes induced by adding various ligands to purified ATPase in the absence (upper recordings) or presence (lower recordings) of 4% (w/w) A23187. The medium contained 150 mM Mes-Tris, 20 mM Mg^{2+} and 0.1 mg/ml ATPase (20°C, pH 6). Left traces: 0.5 mM EGTA was included in the medium; 0.7 mM Ca^{2+} was then added (single arrow), followed by 0.01 mM ATP (double arrow). Center traces: 2 mM EGTA and 20% (v/v) DMSO were included in the medium; 2 mM P_i was then added (arrow). Right traces: 0.5 mM EGTA was included in the medium; 0.1 mM ATP was then added (arrow). Optical conditions as in (A). The traces have been corrected for the dilution-induced drops.

Ca_2E_1 ATPase (left tracings) were virtually unchanged in the presence of ionophore, suggesting that most of the fluorophores responsible for these ATP-induced changes had not experienced an efficient energy transfer to the ionophore.

The fluorescence levels observed during turnover (left tracings) are consistent with independent studies of [^{32}P]ATP and ^{45}Ca binding to the ATPase (Champeil and Guillain, submitted), which suggested that the predominant species under these conditions was the calcium-free and nucleotide-free phosphoenzyme, E_2P (cf. fluorescence levels in the left tracings with those in the center and right tracings), and that a significant amount of the unphosphorylated E_2 species was also present under these turnover conditions (pH 6, no potassium, high magnesium, low ATP, 20°C).

3.2. *Stopped-flow investigation of the ATP-induced fluorescence drop*

From previous chemical quenching and rapid filtration experiments performed under the same conditions (Champeil and Guillain, submitted), we had established that after ATP had been added to Ca^{2+}-saturated ATPase (in the Ca_2E_1 state, see scheme 1), phosphoenzyme was formed very rapidly (30 s^{-1} at 0.005 mM ATP) while the Ca^{2+}-releasing isomerization from Ca_2E_1P to E_2P occurred at a slower rate, with an observed rate constant of about 6 s^{-1}; millimolar concentrations of ATP accelerated this isomerization rate 2-fold. Here, the ATP-induced drop in fluorescence was measured under identical conditions in the presence of ionophore, for ATP concentrations ranging from 0.005 to 10 mM (fig.2). The recordings in fig.2 show that the ATP-induced fluorescence drop occurred at a much slower rate than the phosphorylation reaction and was accelerated by millimolar concentrations of ATP, corresponding to low affinity binding of ATP to E_1P. These characteristics suggest that the fluorescence data predominantly monitor the Ca_2E_1P to E_2P transition under these conditions.

In contrast, when identical experiments were repeated in the absence of ionophore, fluorescence behaved in a different and more complex way (fig.3A): at a low ATP concentration (0.005 mM) the drop in fluorescence was somewhat slower than in the presence of ionophore; at intermediate con-

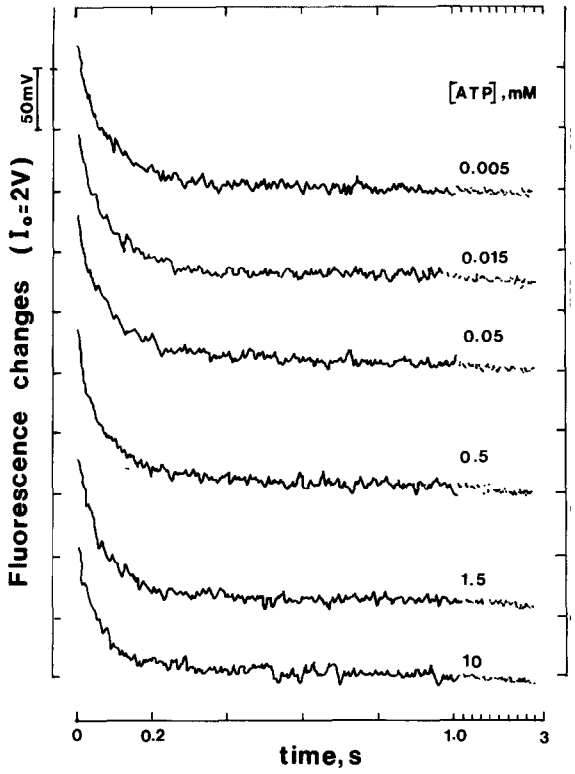

Fig.2. Stopped-flow recordings of the fluorescence drop induced by adding various concentrations of ATP to purified ATPase in the presence of 4% (w/w) A23187. The Balzer interference filter was used. Medium as in fig.1A.

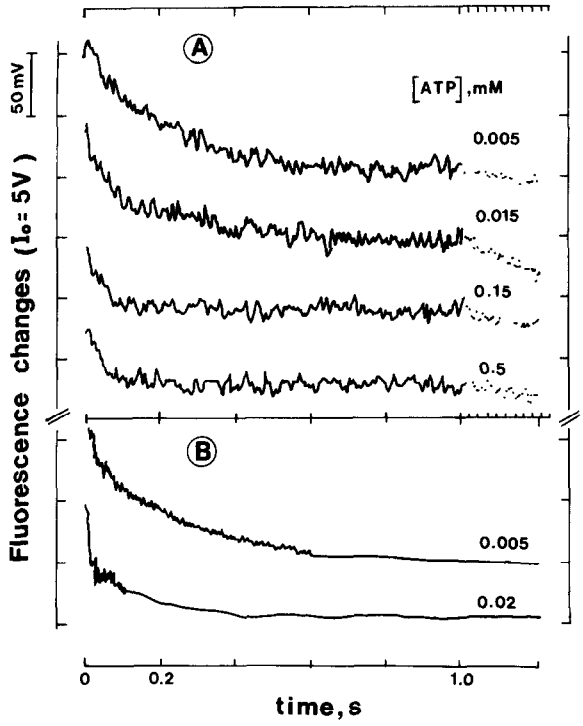

Fig.3. Stopped-flow recordings of the fluorescence drop induced by adding various concentrations of ATP to purified ATPase in the absence of ionophore. Either the Balzer interference filter (A) or a cut-off filter (B) was used.

centrations, the amplitude was reduced and a fast phase became visible which was much faster than 6 s^{-1}. This pattern was even more apparent when a cut-off filter was used to detect the emitted fluorescence. Such a filter emphasizes the contribution of those fluorophores whose spectrum is enhanced rather than shifted. With this filter, we found that the fluorescence change clearly revealed a faster phase (about 100 s^{-1} at 0.02 mM ATP, see fig.3B), which might correspond to the phosphorylation reaction per se.

To return to the experiments performed in the presence of ionophore, closer examination of fig.2 suggests that the tracings at low ATP concentrations were also slightly biphasic (at 0.005 mM ATP, for instance, the best fit was obtained with two exponentials whose rate constants differed by a factor of 4 (8 and 34 s^{-1}), the larger amplitude corresponding to the slower rate, which is probably the one related to isomerization.

4. DISCUSSION

4.1. *Assignment of the ATP-induced fluorescence drop to a particular step in the reaction cycle*

Once the rate constants for the various steps depicted in scheme 1 have been separately measured [15], and after steady-state determination of the fluorescence level of individual species in the reaction cycle (E_2, Ca_2E_1, E_2P, see fig.1B), the fluorescence drop observed under discriminating conditions by the use of an interference filter and in the presence of ionophore (fig.2) can be seen to reflect mainly the destabilization of the calcium-ATPase complex which follows phos-

phorylation (i.e. what we refer to here as the Ca^{2+}-releasing isomerization from Ca_2E_1P to E_2P); other steps in the cycle apparently contribute to the signal to a limited extent only. In contrast, detailed analysis of the kinetics of the fluorescence drop observed in the absence of ionophore at different ATP concentrations (fig.3) probably requires further consideration of both the slow dephosphorylation step (hence the slightly slower drop in the absence of ionophore) and the fast phosphorylation and ATP binding steps (hence the clearly multiphasic drop at increasing ATP concentrations). Moreover, under different experimental conditions, the relative importance of the changes related to phosphorylation and isomerization, respectively, may also vary [7–9]. In practical terms, this means that the kinetics of the ATP-induced drop in total fluorescence is too complex to be easily related to one specific conformational event.

4.2. *Optical selection of tryptophan residues and three dimensional description of the ATPase polypeptide chain*

On the other hand, the main outcome of the present investigation is the demonstration that addition of the quenching ionophore greatly simplifies analysis of the fluorescence drop (fig.2) and that the various tryptophan residues in the protein evidently do not contribute equally to the fluorescence changes induced by different ligands (fig.1). A recent prediction of the secondary structure of skeletal muscle ATPase [1] suggested that all tryptophan residues except two are embedded in the transmembrane region, and that all except one are situated close to the polar water-membrane interface, a location where energy transfer to the membrane-bound ionophore can be expected to take place efficiently (from preliminary calculations, we found that the Förster radius, i.e. the critical distance for energy transfer from tryptophan residues to ionophore, was R_O = 24 Å). It might therefore be speculated that the tryptophan residue at position 552 in the nucleotide binding domain is far enough from the membranous phase to escape quenching by the ionophore, thus making significant contribution to the signals observed in the presence of ionophore (figs 1B and 2). If this is the case, this residue evidently does not contribute much to the Ca^{2+}-induced fluorescence enhancement (fig.1B). Note that this same residue is probably also quenched by trinitrophenyl-nucleotides [18].

In conclusion, the present experiments suggest that studies of tryptophan quenching [10–12,18–22] could be pursued not only to investigate lipid-protein interactions [20–22] or the depth at which ATPase tryptophans are embedded [10–12], but also to identify the various tryptophan residues on the basis of their sensitivity to different ligands after selective quenching of other tryptophan residues. Brominated or spin labelled lipophilic quenchers might be useful for this purpose, provided their localization is not uncertain, as is the case, for instance, for fatty acid probes which may bind to non-annular sites on the ATPase [22]. Experiments are in progress along this line. We hope that combination of stopped-flow fluorometry with increasingly selective quenching agents will provide greater insight into the relation between protein structure and the molecular mechanism of ATPase activity.

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