9-Anthroylnitrile Binding to Serine-181 in Myosin Subfragment 1 As Revealed by FRET Spectroscopy and Molecular Modeling†

Krisztina Szarka,‡ Emöke Bódis,§ Balázs Visegrády,|| Miklós Nyitrai,‡ Ferenc Kilár,|| and Béla Somogyi*,‡,§

Research Group for Fluorescence Spectroscopy, Office for Academy Research Groups Attached to Universities and Other Institutions, and Department of Biophysics, Faculty of Medicine, University of Pécs, P.O. Box 99, H-7601 Pécs, Hungary, and Central Research Laboratory, Faculty of Medicine, University of Pécs, Pécs, Hungary

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ABSTRACT: It has been shown that one of the 12 serine residues within the 23 kDa segment of myosin subfragment 1 can be covalently modified with a fluorescent probe 9-anthroylnitrile (ANN) [Hiratsuka, T. (1989) *J. Biol. Chem. 264* (30), 18188−18194]. To identify the exact binding site of the probe, the distances between the bound ANN as donor and acceptors in known positions (Lys-553 or Cys-707) of the myosin head were determined by using fluorescence resonance energy transfer. Comparison of the spectroscopic results with distances obtained from the atomic model of subfragment 1 revealed that ANN binds to Ser-181. The result was in good agreement with the assumptions of Andreev and co-workers [Andreev, O. A., et al. (1995) *J. Muscle Res. Cell Motil. 16* (4), 353−367]. This conclusion was further supported by protein modeling calculations. The results presented herein might bring ANN into the focus when the molecular mechanism and effects of the binding of ATP and its subsequent hydrolysis are studied.

The conformational changes occurring within the myosin head play a key role during muscle contraction (for a review, see ref *1*). These conformational changes are coupled to the binding and hydrolysis of ATP, which occurs in the catalytic domain of S1.[1] The chemical energy released during the hydrolysis is converted to mechanical energy and transmitted to the distal part of the myosin head so that the rotation of the long C-terminal α-helix, the putative lever arm, could occur (*2*, *3*). It seems reasonable to assume that the conversion of chemical energy to mechanical energy happens within the close vicinity of the nucleotide-binding site. Accordingly, characterization of intramolecular events around the nucleotide-binding site during muscle contraction is important for a better understanding of the molecular basis of chemomechanical transduction. On the basis of X-ray diffraction data, the atomic model of S1 (*4*) and its complex with actin (*5*) become available in the early 1990s, providing a framework for interpreting experimental results.

A powerful approach to obtaining information about the intramolecular events occurring in S1 that underlie muscle contraction is fluorescence spectroscopy. The application of fluorescence methods requires the presence of intrinsic and/or extrinsic fluorophores. The latter type is usually introduced into the protein matrix by chemical modifications. Such a labeling procedure was described by Hiratsuka (*6*), applying ANN to covalently modify S1. The author showed that ANN binds to a serine residue located in the 23 kDa tryptic segment (Asp-4−Glu-204) (*2*) of S1 (*6*). One of the characteristic features of ANN labeling was that it was not possible to increase the ratio of the concentration of bound ANN to the concentration of S1 to >1, suggesting that only one of the 12 serines was modified (*6*). When the fact that there are very few fluorescent reagents which are suitable for labeling the 23 kDa tryptic segment of S1 is considered (*6*), the labeling procedure described by Hiratsuka gained major importance. However, the exact location of the ANN-binding serine residue within the 23 kDa segment remained unknown. Andreev and co-workers (*7*) also made likely the localization of the binding site of ANN in S1. According to their assumption, four serines (Ser-156, Ser-158, Ser-173, and Ser-181) of the 12 in the 23 kDa fragment could be candidates for labeling. According to Cremo and co-workers Ser-181 can be specifically modified in the presence of nucleotide (*8*). Andreev and co-workers supposed that the same serine residue was modified with ANN because in both cases the presence of nucleotide is required for modification.

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* To whom correspondence should be addressed. Telephone: +36-72-536-260. Fax: +36-72-536-261. E-mail: somogyi.publish@aok.pte.hu.

‡ Office for Academy Research Groups Attached to Universities and Other Institutions, University of Pécs.

§ Department of Biophysics, Faculty of Medicine, University of Pécs.

|| Central Research Laboratory, Faculty of Medicine, University of Pécs.

[1] Abbreviations: S1, myosin subfragment 1; ANN, 9-anthroylnitrile; 5-IAF, 5-(iodoacetamido)fluorescein; 6-IAF, 6-(iodoacetamido)fluorescein; FHS, 6-(fluorescein-5-carboxamido)hexanoic acid succinimidyl ester; FRET, fluorescence resonance energy transfer; DMF, dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MEA, 2-mercaptoethanol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; TRIS, tris(hydroxymethyl)aminomethane; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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The knowledge of the exact position of S1-bound ANN would substantially increase the precision of the interpretation of experimental data obtained by the use of this extrinsic fluorophore. Although the results of Andreev and his colleagues provided estimates regarding the position of the bound ANN, the exact location was not determined. Therefore, our goal was to determine the location of the serine residue that covalently binds ANN.

In this study, we have determined the distance between ANN as a FRET donor and either of two appropriate acceptor molecules attached to two known locations in S1. The experimental results were compared with the atomic model of S1 (*4*). Relying on the three-dimensional structure of S1, we also carried out protein modeling (ANN docking). According to the results, ANN binds to Ser-181, as a residue that is part of the nucleotide-binding site in S1 (*4*, *9*). Therefore, it seems to be very likely that in future works the characterization of ANN fluorescence in various myosin conformational states of the contractile cycle, the mechanism of conversion of chemical energy to mechanical energy, and the propagation to the lever arm may be better understood.

MATERIALS AND METHODS

Reagents. TES, $MgCl_2$, NaCl, KCl, MOPS, NaOH, glycine ethyl ester, α-chymotrypsin, trypsin, PMSF, EDTA, Na_2HPO_4, EGTA, MEA, DMF, TRIS, DTT, and pyronin Y were obtained from Sigma Chemical Co. (St. Louis, MO). ATP was obtained from Merck. ANN, FHS, 5-IAF, and 6-IAF were purchased from Molecular Probes (Eugene, OR). TEMED and the protein assay kit were from Bio-Rad, and SDS was from U.S. Biochemical.

Protein Preparation. Rabbit skeletal muscle myosin was prepared as described previously (*10*). S1 was prepared by chymotrypic digestion of myosin (*11*). The labeling of S1 with ANN, FHS, or IAF was performed according to previously published procedures (*6*, *12*, *13*).

Concentrations of S1 were determined from absorption data using an extinction coefficient $A^{1\%}_{1cm}$ of 0.745 at 280 nm (*14*). When S1 was labeled with fluorophores, appropriate corrections were made to take into account the absorption of the probes at 280 nm. The concentrations of ANN, FHS, and IAF were determined using absorption coefficients of 8400 M^{-1} cm^{-1} at 361 nm (*6*), 68 000 M^{-1} cm^{-1} at 495 nm (*12*), and 55 000 M^{-1} cm^{-1} at 496 nm [determined at pH 7.0 based upon the work of Takashi (*15*)], respectively. The labeling ratio was calculated as a ratio of the dye concentration to the protein concentration.

ATPase Assays. Labeled S1 was routinely characterized by measuring the K^+/EDTA and Ca^{2+}-ATPase activities by determination of the extent of phosphate release (*16*). The assays were performed at room temperature in 50 mM Tris-HCl (pH 8.0), 0.6 M KCl, 2.5 mM ATP, and either 10 mM EDTA or 9 mM $CaCl_2$. The ATPase activities measured simultaneously for unlabeled S1 served as a reference.

Tryptic Digestion of S1. To control whether the dye binds to a serine of the 23 kDa segment of S1 only (*6*), limited cleavage of S1 was performed. ANN-bound S1 in 20 mM TRIS and 50 mM NaCl (pH 8.0) was incubated with 0.02 mg/mL trypsin for 10 min at 25 °C. The sample was added to a solubilizing solution and 20 mM DTT in boiling water for 2 min.

Gel Electrophoresis. The tryptic-digested sample was analyzed by SDS−PAGE using molecular mass markers of rabbit psoas, β-lactoglobulin, bovine albumin, and pepsin. The method was carried out by the system of Laemmli (*17*) using 12% acrylamide gels. For observation of the fluorescence bands, gels were washed with methanol and acetic acid and photographed. After photographs had been taken, gels were stained with Coomassie Blue to compare the digested fragments with the marker.

Fluorescence Measurements. Fluorescence was measured with a Perkin-Elmer LS50B luminescence spectrometer. To calculate the FRET efficiency, the fluorescence intensities of the donor (ANN) were recorded in the presence and in the absence of acceptors (FHS or IAF). The S1 concentration was adjusted to 3, 6, or 9 μM in these experiments. The measurements were carried out under rigor conditions in 25 mM TES, 80 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, and 4 mM MEA (pH 7.0). The excitation monochromator was set to 350 nm. The fluorescence intensity of ANN was monitored at 400−470 nm with optical slits adjusted to 3 nm. The contributions of fluorescence by either of the applied acceptor molecules to the measured fluorescence intensity can be excluded over this wavelength range. Due to the fact that the optical density of the samples was not negligible at the excitation and emission wavelengths, the fluorescence intensities were corrected for the inner filter effect, using the following equation:

$$F_c(\lambda) = F_{obs}(\lambda) \times 10^{OD_{ex}+OD_{em}(\lambda)} \quad (1)$$

where $F_c(\lambda)$ is the corrected fluorescence intensity, $F_{obs}(\lambda)$ is the measured fluorescence intensity, and OD_{ex} and $OD_{em}(\lambda)$ are the optical densities of the samples at the excitation and emission wavelengths, respectively. To determine the FRET efficiency (E), the under-curve areas of the emission spectra were determined and used for the calculation. The apparent transfer efficiency (E_{obs}) was calculated as

$$E_{obs} = 1 - (F_{DA}/F_D) \quad (2)$$

where F_{DA} and F_D are the fluorescence intensities of the donor molecule in the presence and absence of the acceptors, respectively. Since the acceptor labeling ratio was usually less than 1, the FRET efficiency was corrected as

$$E = E_{obs}/\beta \quad (3)$$

where E and E_{obs} are the corrected and observed FRET efficiencies, respectively, and β is the actual acceptor labeling ratio. Throughout this article, these corrected values are presented. If the FRET efficiency (E) is known, the distance between the donor and the acceptors can be calculated from the following equation:

$$E = R_o^6/(R_o^6 + R^6) \quad (4)$$

where R_o is Förster's critical distance, defined as the donor−acceptor distance at which the FRET efficiency is 0.5. To calculate R from E, the value of R_o needs to be obtained using the following equation:

$$R_o^6 = (8.79 \times 10^{11}) n^{-4} \kappa^2 \Phi_D J \quad (5)$$

where n is the refractive index of the medium, κ^2 is the orientation factor, Φ_D is the fluorescence quantum yield of the donor in the absence of the acceptor, and J is the overlap integral given in M^{-1} cm^{-1} nm^4. The value of the refractive index (n) and the orientation factor κ^2 were taken to be 1.4 and $^2/_3$, respectively. The overlap integral is defined as

$$J = \int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda / \int F_D(\lambda) \, d\lambda \quad (6)$$

where $F_D(\lambda)$ describes the fluorescence emission spectra of the donor and $\epsilon(\lambda)$ describes the absorption spectra of the acceptor as a function wavelength. To determine the fluorescence quantum yield of the donor molecule, the fluorescence emission spectra of ANN-bound S1 were compared with that of a reference substance, quinin sulfate. The fluorescence quantum yield of quinin sulfate was taken to be 0.53 (in 0.1 N H_2SO_4) in these calculations (*18*).

During the calculation of R_o, one problem comes from the determination of κ^2, because the orientation factor cannot be determined experimentally. Its value changes between 0 and 4. Provided that the fluorophores do not attach to the protein matrix rigidly, but their dipole moments have random steric distribution and they rotate quickly, $\kappa^2 = ^2/_3$, an average value of these distributions. Indeed, in the case of labeling of proteins, the rotational movement of fluorophores is not completely random, but in general it is assumed that the value of $^2/_3$ is proper in determining the Förster distance.

Identification of the Serine Groups that Bind ANN. FRET experiments provided distances between ANN (as a donor) and acceptors on either Lys-553 (FHS) or Cys-707 (IAF). The location of the ANN-binding serine residue was determined from these distances mapped onto the three-dimensional atomic model (*4*) of S1 [atomic coordinates were obtained from the Protein Data Bank (Brookhaven National Laboratory, Upton, NY), entry 2MYS]. On the basis of the FRET results, the combined deviation (D) of the experimental data from the distances obtained using the atomic model of S1 was calculated as follows:

$$D = [(d^{IAF})^2 + (d^{FHS})^2]^{1/2} \quad (7)$$

where d^{IAF} and d^{FHS} are the differences between the donor−acceptor distances determined by the method of FRET and obtained from the atomic model in the case of IAF (Cys-707) and FHS (Lys-553), respectively. The greater the combined deviation, the smaller the probability of ANN binding to the particular serine. Accordingly, the probability of ANN binding to the given serine residue is proportional to the inverse of the combined deviation, D^{-1}. Such an estimation of binding probability is not directly related to any biochemical parameter that describes the reactivity of a serine with ANN. Rather, D provides a theoretical framework for the spatial interpretation of FRET data.

Protein Modeling and Calculations. The probability of ANN binding was further characterized by applying protein modeling in the case of the 12 serines located in the 23 kDa segment of S1. The energy minimization was performed using Sybyl 6.5 software (Tripos, Inc.) on an Indy computer (Silicon Graphics). Throughout the calculations, we used the Tripos force field (*19*), Kollman all atom charge (*20*), and 100 (in the first minimization) or 200 (in the final minimization) iterations.

Hiratsuka showed that the labeling characteristics of S1 and its 23 kDa segment with ANN were identical, and a similar result was obtained when trypsin-digested S1 or intact S1 was incubated with ANN (*6*). Therefore, for the sake of simplicity, only the 23 kDa segment (amino acids 4−204) was considered in the calculations. A protein−ANN complex was constituted for each of the 12 serine residues using the atomic model of S1 (*4*). To obtain these complexes, a covalent link was established between ANN and the investigated serine residue. In lieu of information about the true orientation of the probe relative to the protein matrix, the aim of the first energy minimization was to find the most probable orientation of ANN. Three initial structures (labeled here with the number 1, 2, or 3) of each protein−ANN complex were determined by rotating ANN around the O−C bond of the serine−ANN complex by 120°. To estimate the most likely orientation of the probe, the first energy minimization was carried out allowing only ANN to change while the structure of the protein was kept fixed. Following the initial energy minimization, the complexes with lowest energies were taken as the starting structures for the final energy minimization. With both the probe and the protein matrix being allowed to change, the final energy calculations were carried out and the obtained data were analyzed with respect to the probability of binding of ANN.

RESULTS AND DISCUSSION

The fluorescent probe ANN is one of the few fluorescent reagents suitable for labeling the 23 kDa tryptic segment of S1 (*6*). While this fluorophore has previously been used to study the conformational changes in the myosin head or to identify the 23 kDa segment (*7*, *21*, *22*), its location within the 23 kDa segment has been unclear. The aim of this study was to determine the exact binding site of ANN so that the interpretation of fluorescence spectroscopic results obtained by using this probe could be more precise and confident.

Characterization of the Samples. Relying on the absorption data, we found the labeling ratios to be 0.4−0.6, 0.7−0.8, and 0.9−1.0 for ANN, FHS, and IAF, respectively. Labeled S1 was characterized by measuring the K^+/EDTA ATPase and Ca^{2+}-ATPase activities. Labeling S1 with ANN at a ratio of 0.6 (ANN/S1) decreased both the K^+/EDTA ATPase and Ca^{2+}-ATPase activities to 60% of their values measured for the unlabeled protein, in agreement with previous results (*6*). The effects of modification of S1 with the acceptors (FHS and IAF) were also in agreement with the data published earlier for Lys-553 (*12*), or for Cys-707 (*13*). ANN-bound S1 was digested as described by Hiratsuka (*6*). The three main generations of tryptic peptides were separated via SDS−PAGE. The ANN fluorescence appeared only in the 23 kDa peptide of S1, and no fluorescence was observed at the 20 and 50 kDa peptides, in accordance with the observation of Hiratsuka (*6*).

During the application of a double-modified protein, the first modification might influence the specificity of the label applied in the second one. One way to monitor this phenomenon is to change the order of the labeling procedure of the donor and acceptor. The labeling ratio was found to be the same in case of previously labeled and unlabeled S1. The labeling ratio of ANN never increased to more than 100%, which indicates that the labeling was specific.

Table 1: FRET Parameters Characteristic of the Applied Donor−Acceptor Pairs Situated in S1[a]

	FHS	IAF
E (%)	69.2 ± 6.3	76.7 ± 2.7
J ($\times 10^{-14}$ M^{-1} cm^{-1} nm^4)	14.5 ± 0.3	16.3 ± 0.2
R_o (Å)	38.9 ± 0.2	39.7 ± 0.3
R (Å)	33.8 ± 1.1	32.5 ± 1.0

[a] E is the FRET efficiency. J is the overlap integral. R_o is the Förster's critical distance. R is the donor−acceptor distance. The donor was ANN, and the acceptor was FHS on Lys-553 or IAF on Cys-707. The presented standard deviations were calculated from the results of three independent experiments.

Localization of the ANN-Binding Serine Residue with FRET Spectroscopy. Localization of the ANN binding site in S1 was carried out by using FRET spectroscopy. The efficiency of energy transfer between ANN and acceptors (FHS or 5-IAF) in known positions in S1 was determined using eqs 2 and 3 (Table 1). The calculation of the distance between the donor (ANN) and the actual acceptor (IAF or FHS) required the determination of spectral parameters characteristic of the applied fluorophores. The quantum yield of donor (Φ_d) was 0.157. The overlap integrals (eq 6) characteristic of the ANN−IAF or ANN−FHS pairs were also determined (Table 1). The knowledge of these spectral parameters allowed the determination of the donor−acceptor distances from FRET efficiency (eq 4). The distance between ANN and FHS was found to be 33.8 ± 1.1 Å ($R^{Lys-553}$), and the value of this parameter was 32.5 ± 1.0 Å ($R^{Cys-707}$) when measured between ANN and IAF. The value of this latter distance was found to be identical in the case of 5-IAF and 6-IAF. The links established between the protein and 5-IAF or 6-IAF are not the same, and therefore, they are expected to have different orientations of dipoles relative to that of ANN. The observation that the measured distance between ANN and IAF was not sensitive to the displacement of 5-IAF with 6-IAF indicates that the use of an orientation factor value of $^2/_3$ was appropriate.

The known acceptor locations (Lys-553 for FHS and Cys-707 for IAF) were used as reference points in the three-dimensional structure of S1 (*4*) to localize the binding site of the donor (Figure 1). The schematic model of S1 was constructed using the atomic coordinates (*4*). In this representation, spherical surfaces were generated around the Lys-553 or Cys-707 residue. The spheres were constructed with the appropriate FRET distances ($R^{Lys-553}$ and $R^{Cys-707}$) as radii (Figure 2). The intersection of the two spheres was a circle determining the set of points, which are located a distance of $R^{Lys-553}$ from Lys-553 and a distance of $R^{Cys-707}$ from Cys-707. When an ideal case is considered, when the error of distance determination can be neglected, this circle includes the location of ANN. In a real situation, the experimental errors are not negligible and spatial determinations are approximate. Thus, the serine residue closest to the intersection of the spheres is the most likely binding site of ANN (Figure 2a). As indicated in Figure 2a, of the 12 serines in the 23 kDa segment, Ser-181 was situated closest to the intersection of the two spheres, suggesting that this is the most probable binding site of ANN. This serine residue is one of the four which was proposed by Andreev and co-workers to be the potential binding site of the dye (*7*).

To estimate the probability with which ANN binds to each of the 12 individual serines, the combined deviation (D_i) of

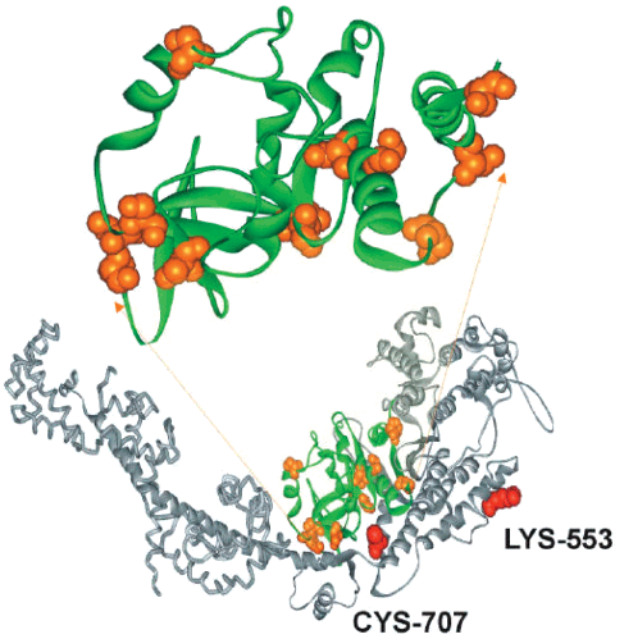

FIGURE 1: Schematic representation of S1 (*4*). The 23 kDa segment is labeled with green, and the 12 serine residues within this segment are shown in orange. The positions of Lys-553 (FHS) and Cys-707 (IAF), which were the acceptor labeling sites in this study, are indicated with red surfaces.

Table 2: Comparison of FRET Results with the Atomic Model of S1

serine position	Lys-553 distance[a] (Å)	Cys-707 distance[a] (Å)	Lys-553 deviation[b] (Å)	Cys-707 deviation[b] (Å)	D^{-1} ($\times 10^2$ Å^{-1})[c]
20	63.4	30.1	29.6	−2.5	3.367
36	56.6	25.7	22.8	−6.8	4.202
37	54.9	23.8	21.1	−8.7	4.367
46	41.1	23.2	7.3	−9.3	8.475
54	56.8	31.7	23.0	−0.8	4.348
79	49.1	17.4	15.3	−15.2	4.629
119	42.7	10.4	8.9	−22.1	4.202
157	49.7	22	15.9	−10.5	5.263
159	46.9	23.8	13.1	−8.7	6.369
174	39.2	22.5	5.4	−10.0	8.772
181	33.6	28.3	−0.2	−4.3	23.256
202	56.1	41.6	22.3	9.0	4.167

[a] The distances between the given serine residue (left column) and the acceptor binding sites (Lys-553 or Cys-707) obtained from the atomic model of S1. [b] Taking into account that ANN is located 33.8 Å from the Lys-553 and 32.5 Å from the Cys-707 residues, as revealed by FRET experiments, the deviations of FRET results from the model distances are also presented. [c] D^{-1} is the inverse of the calculated combined deviation (eq 7) obtained by comparing the FRET distances and the ones obtained from the atomic model of S1.

the measured FRET distances from the ones obtained by using the atomic coordinates (*4*) was determined (see eq 7) (Table 2). According to these results (Figure 2b), the most probable binding site of the ANN is Ser-181. This result was predicted by Andreev and co-workers (*7*). With the knowledge that Ser-181 can be specifically modified in the presence of nucleotide (*8*), they proposed that the same serine residue is the binding site of ANN.

The determination of molecular distances by FRET spectroscopy might involve errors, which are very difficult to identify in some cases. One source of these errors is the non-zero size of the applied fluorophores. Another source of error is the unavailability of the orientation factors (κ^2)

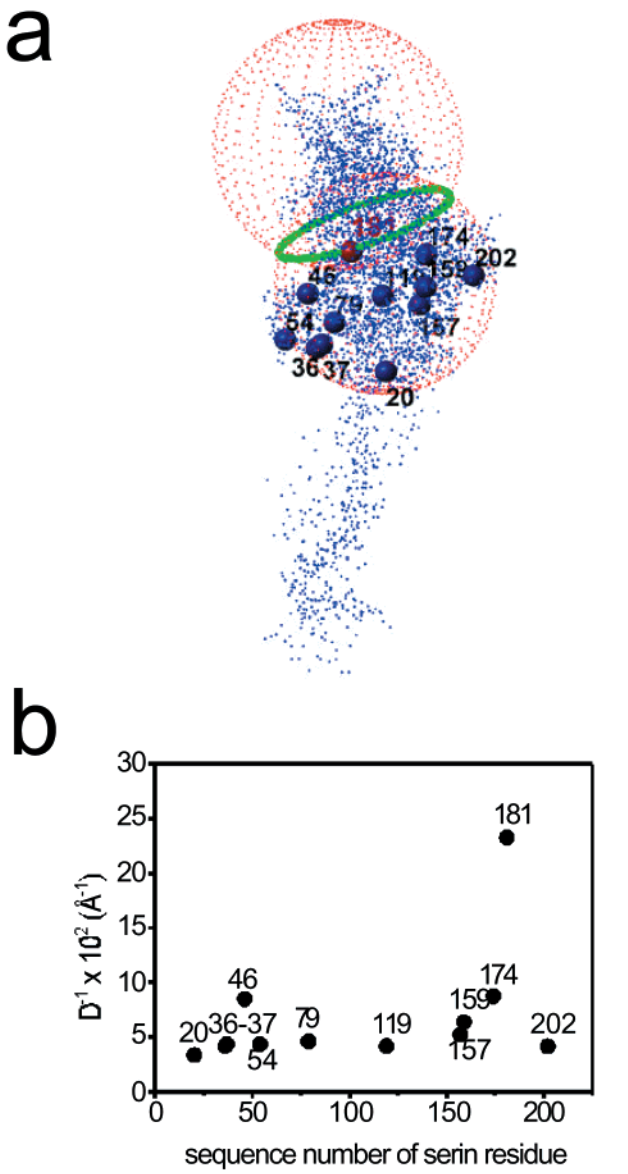

FIGURE 2: (a) Mapping the FRET results onto the atomic model of S1. With knowledge of the individual donor−acceptor distances between ANN and either FHS (Lys-553) or IAF (Cys-707) from FRET experiments, spherical surfaces were generated around the acceptor locations with the measured distances as radii. The intersection of the two spheres is shown in green. The 12 serine residues situated in the 23 kDa segment of S1 are also presented and labeled with their sequence number. (b) The inverse of the combined deviation for the 12 serine residues located in the 23 kDa segment of S1. These data were calculated from the FRET results by comparing them to the distances obtained from the atomic model of S1.

for experimental determination. The magnitude of such error is difficult to estimate due to the fact that the orientations of these fluorophores are usually not known.

Error calculations were carried out to estimate the largest error of distance determination, with which the conclusion from the FRET experiments would still hold that the binding site of ANN is Ser-181. Simulation of the experimental data was carried out with the assumption that the magnitude of the attributed relative errors increases. When any of these error levels were considered, both the increase and the decrease in the donor−acceptor distances were taken into account in all possible variations. The largest allowable error was identified when the result of these analyses first deviated from the conclusion, which was drawn from the experimental results, i.e., that Ser-181 binds ANN. According to the results of these calculations, Ser-181 is the most probable ANN binding residue assuming that the relative error attributed to the distance determinations did not exceed 12%.

Table 3: Computer Simulation Data Obtained Using the Atomic Model of S1[a]

serine position	E_o (kcal/mol)	E_f (kcal/mol)	serine position	E_o (kcal/mol)	E_f (kcal/mol)
20	20 283.7	156.7	119	20 079.3	−23.1
36	20 283.0	176.7	157	22 702.6	146.3
37	20 320.4	137.9	159	30 900.1	2032.9
46	20 118.0	−52.7	174	20 070.0	−46.5
54	20 324.4	164.3	181	19 865.7	−69.8
79	20 278.8	107.9	202	20 282.7	152.8

[a] In the different complexes, ANN was attached to individual serines within the 23 kDa segment of S1. The first energy minimization (E_0) was carried out using initial structures obtained by rotating ANN around the O−C bond of the serine−ANN complex by 120°. In this analysis, only ANN was allowed to change and the protein matrix was kept fixed so that the proper orientation of the probe relative to the protein matrix could be estimated. Subsequently, the second, final energy minimization was started from a conformation where the orientation of the probe was the most probable and provided the combined energy of the actual protein−ANN complex (E_f). The energy values after the first (E_0) and final (E_f) energy minimizations are presented for the 12 protein−ANN complexes.

A number of FRET results on different proteins and peptides show that FRET distances reflect the appropriate distances determined from crystallographic atomic models (for a review, see ref *23*), which suggests that the identification of the ANN-binding serine is reliable. This reliability is further supported in our case by the measured data, as identical distances were obtained with IAF isoforms (5-IAF and 6-IAF). This observation suggests that the assumption that $\kappa^2 = {}^2/_3$ is valid, or at least did not introduce substantial error into the FRET distances.

However, with the lack of direct evidence, one cannot exclude the possibility that the experimental error attributed to the distance determinations described here is larger than 12%. Accordingly, we concluded from these data that the ANN-binding site was Ser-181, but further support was required to strengthen this statement.

Binding Site of ANN As Revealed by Using Computer Simulation. Protein simulations were carried out to estimate the probability of ANN binding by each of the 12 serine residues of the 23 kDa segment of S1. In this analysis, the measure of the probability of binding of ANN was the energy of the protein−ANN complex determined during the energy minimization. While it is difficult to attribute physical meaning to the numerical value of this energy, the energy differences between complexes of the protein and the probe attached to different serines provide realistic information. One may assume that the energy is minimal for the complex in which ANN is docked onto the preferred serine. Therefore, the energy values characteristic of the protein−ANN complex were determined for each of the 12 serines in two steps (see Materials and Methods). According to these calculations, the energy was lowest when ANN was attached to Ser-181 (Table 3). This observation supports the conclusion that of all the serines of the 23 kDa segment, Ser-181 is the most probable binding site of ANN.

CONCLUSION

Although both the analyses of the spectroscopic data and the protein modeling were based on the atomic model of S1 (*4*), the two methods applied here could be considered independent of each other. The results of these methods were in good correlation, implying that ANN binds to Ser-181 of the 23 kDa segment of S1. This serine residue is one of the four which was proposed by Andreev and co-workers (*7*). It was shown recently that Ser-181 takes part in the binding of the nucleotide in S1 (*4*). Accordingly, our present results will probably bring this dye into the focus when the molecular mechanism and effects of the binding of ATP and its subsequent hydrolysis are studied.

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