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# FRET-based methods to study ATP-dependent changes in chromatin structure

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## Abstract

DNA packaging into chromatin imposes several levels of regulation on the central nuclear processes of DNA replication, recombination, repair and transcription. ATP-dependent chromatin remodeling enzymes play a critical role in this regulation by altering the accessibility of nucleosomal DNA. Remodeling can result in large-scale changes in chromatin, such as the formation of heterochromatin, or smaller changes in exposure or occlusion of specific DNA regions. To understand the mechanisms of chromatin remodeling, we report a FRET-based method to follow remodeling of a single histone octamer on DNA. This technique provides a non-perturbing, solution-based approach to quantitatively track the movement of DNA with respect to the octamer in real-time. The method can easily be altered to examine other conformational changes within the nucleosome, and is applicable to study the enzymatic activity of several classes of chromatin-remodeling complexes.

### Keywords

chromatin; ATP-dependent chromatin-remodeling; nucleosome; FRET; histone; real-time; SNF2h

## 1. Introduction

When eukaryotic DNA is packaged into chromatin, it is not only compacted into a small dense volume, but also becomes less accessible for transcription, replication, recombination and DNA repair. Hence understanding how DNA access is regulated is key to understanding most nuclear processes. DNA accessibility is reduced even in the smallest level of packaging, the nucleosome. The nucleosome contains 147 base pairs of DNA wrapped around an octamer of histone proteins [1]. There are two main classes of enzymes that regulate DNA access in chromatin. One class uses ATP to alter the conformation of a nucleosome, while the other class covalently modifies histone residues (for a review, see [2]). It has been hypothesized that specific combinations of covalent modifications may form a histone code that can then be recognized by certain downstream regulatory proteins [3]. In contrast, ATP-dependent chromatin-remodeling complexes can modify DNA accessibility by directly altering histone-DNA contacts on the nucleosome.

While the list of new members of ATP-dependent remodelers is growing, the mechanisms by which these enzymes function are not well understood. To study ATP-dependent chromatin-remodeling complexes, a variety of biochemical methods have been developed to monitor the

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movement of the histone octamer relative to DNA. These include restriction enzyme accessibility assays, cross-linking assays, electrophoretic mobility shift assays and various enzymatic and small molecule-based mapping methods. While a wealth of information has been gained using these techniques, the techniques do not easily allow detection of fast steps or transient intermediates, both of which are crucial for obtaining mechanistically relevant information. To address these limitations, we have developed a FRET-based approach to follow the movement of DNA with respect to the histone octamer in real-time. FRET (fluorescence resonance energy transfer) can be used to measure biologically relevant changes in distance using two fluorophore labels. Upon excitation of the donor dye, energy is transferred to the acceptor dye, resulting in increased acceptor fluorescence and quenching of the donor emission. The FRET efficiency is inversely proportional to distance between the two dyes and hence can be used to monitor movement of one dye relative to the other using the following equation:

$$E = \frac{R^6}{R^6 + r^6} \tag{1}$$

where R is the Förster radius and r is the distance between the two dyes. Based on the Förster radius of specific dye pairs, changes in distance of the order of 1–10 nm can be monitored by FRET [4].

Here we describe the preparation and use of mononucleosomal substrates for FRET studies. This detailed procedure is used to observe the change in FRET between DNA and the histone octamer on nucleosomes as a function of SNF2h remodeling. SNF2h is the core ATPase subunit for several different human ATP-dependent chromatin-remodeling complexes [5]. Previous work has shown that SNF2h moves mononucleosomes away from DNA ends [6]. Here we follow SNF2h remodeling of labeled nucleosomes in a quantitative, real-time manner. This solution-based method allows for continuous data collection on a short time-scale. The protocol below measures bulk steady-state reactions, but can be modified to follow rapid kinetics or single molecule experiments.

## 2. Assembly of fluorescently-labeled nucleosomes

### 2.1 Design of nucleosome constructs

Using the crystal structure as a guide, we assess the region of interest on the nucleosome for appropriate fluorophore attachments sites [7]. Two examples of suitable FRET nucleosomes are diagramed in Figure 1. Both constructs contain DNA labeled with Cy3 (red) and histone octamer labeled with Cy5 (green) at residue 120 on histone H2A. The construct in Figure 1A is designed to follow the movement of the DNA end at the exit site of the nucleosome. In Figure 1B the nucleosome is designed to follow the movement of internal DNA relative to the exit site. Given the composition of the histone octamer, there are two labeled histones per octamer. Thus the dye attachment site on the histone should be designed such that only one dye acts as an efficient donor or acceptor.

### 2.1 Purification of labeled DNA

Mononucleosomes can be highly positioned using the 601 positioning sequence defined by Lowary and Widom [8]. DNA is labeled using an HPLC-purified fluorescently conjugated PCR primer (Integrated DNA Technologies, IBA GmbH). Large-scale PCR is carried out in a 96-well plate, followed by ethanol precipitation and purification by native gel electrophoresis. A 5% polyacrylamide gel (acrylamide/bisacrylamide, 29:1, w/w in 1x TBE) with large loading wells is ideal for gel purification. Upon separation of the PCR product and primers, the product band is then excised from the gel, crushed and soaked overnight in TE. The slurry is put over a 0.2 micron filter to separate the DNA from gel pieces. The filtrate is reprecipitated and the resulting DNA pellet is resuspend in TE. The DNA concentration is measured by absorbance.

### Labeling and purification of labeled histones

*Xenopus* histone H3 is the only histone that has a native cysteine residue (at position 110). This is mutated to alanine using the QuikChange Site-Directed Mutagenesis Kit **(**Stratagene) to prevent attachment of the dye to this position. The dye attachment site in the relevant histone is then introduced by site-specific cysteine mutagenesis. The modified histone is then individually bacterially expressed, purified and lyophilized, as described previously [9].

The lyophilized histone is resuspended to 0.1 mM in Labeling Buffer (20 mM Tris, pH 7.0, 7 M Guanidinium HCl, 5 mM EDTA) at room temperature. The histone is incubated for 2 hours after addition of 0.5 M, pH 7.0 TCEP (Pierce) to a final concentration of 1.25 mM. Maleimide-conjugated Cy5 (GE Healthcare) is dissolved to 100 mM in anhydrous DMF and added to the histone at 3 mM final concentration. The reaction is kept in the dark at room temperature for 3 hours, and is subsequently stopped by adding beta-mercapto-ethanol to a final concentration of 80 mM. A small aliquot of the reaction is saved for quantification later. Unreacted dye is removed from the remaining reaction by three successive rounds of concentration and dilution at room temperature using a Microcon unit with a 10 kDa MW cut-off (Millipore). Each round consists of concentration to one-third the original volume followed by resuspension to the original volume with Labeling Buffer. A fresh Microcon unit is used for each round.

The concentration of the labeled histone after the Microcon step can be determined by running the histone next to an equal volume of material prior to the microcon step on a 15% SDS gel. Since the concentration of the histone before microcon treatment is known, this is used to determine the concentration of the histone after microcon treatment by comparing the fluorescence signals for the two samples. Prior to loading on the gel, it is necessary to dilute an aliquot of the sample ~ 50-fold in water to prevent the guanidium in the reaction mixture from causing precipitation on the SDS gel. The gel is scanned with a Typhoon Variable Mode Imager (GE Healthcare) at the appropriate excitation and emission wavelength. An optional control is to perform the labeling with a histone lacking any cysteine residues to ensure that labeling is specific to the single desired cysteine.

### Assembly of histone octamers and nucleosomes

Histone octamers are assembled by mixing appropriate amounts of the labeled histone in Labeling Buffer with other histones dissolved in Refolding Buffer (7 M Guanidinium HCl, 20 mM Tris, pH 7.5, 10 mM DTT), as described previously [9]. It is also possible to assemble octamers with two different, singly-labeled histones to follow conformational changes between individual histones within an octamer. Octamers are assembled and purified, and subsequently assembled into nucleosomes with the labeled DNA as described [9].

## 3. Use of FRET to study ATP-dependent chromatin-remodeling

### 3.1 End point emission scans

All fluorescence studies are conducted on an ISS K2 fluorometer. Variable parameters such as excitation/emission wavelength, temperature, filters, number of iterations and step size depend on each individual experiment. For example, when conducting emission scans on nucleosomes containing a Cy3-Cy5 pair, samples were excited at 520 nm with a 400 nm cutoff filter and emission spectra collected from 540 to 710 nm with a 495 nm cutoff filter (Melles Griot). 10 iterations were collected at 30° C with a step size of 1 nm. In Figure 2, the end-labeled nucleosome from Figure 1A was remodeled with the ATP-dependent chromatin remodeler SNF2h. Based on previous data, it is known that SNF2h moves mononucleosomes away from the ends of DNA (Fig. 2A) [6]. Before remodeling, the nucleosomes displayed robust FRET (Fig. 2B, orange), since the two dyes were close together. Following remodeling by SNF2h, the donor emission increased and the acceptor became quenched (Fig. 2B, blue), indicating that the two dyes had moved apart. This is consistent with movement of the octamer away from the DNA end.

### 3.2 Kinetics measurements

Either donor un-quenching or acceptor quenching can be measured in real-time. In Figure 3A, the nucleosome shown in Figure 1A was remodeled by SNF2h. Samples were excited at 520 nm and collected at the Cy3 (565 nm) or Cy5 (668 nm) peak intensity, blue and red, respectively. The data in Figure 3B follows Cy3 unquenching of the nucleosome shown in Figure 1B as a function of SNF2h remodeling. Reactions were initiated by addition of ATP, and data were collected at 30° C for at least 10 minutes and sampled once per second.

A detailed protocol of the experiment is below:

1. Prepare the ATP mix (40 l):
    - **a.** 16 l BC100 (20 mM Hepes, pH 7.9, 100 mM KCl , 0.2 mM EDTA, 20% glycerol) with 1 mg/ml FLAG peptide (Sigma)
    - **b.** 8 l Nucleosome Buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 20% glycerol, 0.1% NP-40)
    - **c.** 8 l BC100 without glycerol
    - **d.** 6 l 100 mM $MgCl_2$ (3 mM final)
    - **e.** 2 l 120 mM Mg.ATP (2 mM final)
2. Set up the reaction mix on ice, keep in the dark (80 l):
    - **a.** 16 l 15 mM $MgCl_2$ (3 mM final)
    - **b.** 16 l BC100 buffer
    - **c.** 16 l 37.5 nM nucleosomes in Nucleosome Buffer (5 nM final)
    - **d.** 32 l 1.125 M SNF2h in BC100 with 1 mg/ml FLAG (300 nM final)
3. Immediately after addition of the enzyme in (2d), incubate both the ATP stock and reaction mix at 30° C for 5 minutes in the dark.
4. Add the 80 l reaction mix to a pre-warmed cuvette and perform an emission scan at 30° C as described in Section 3.1.
5. Begin kinetics measurement of the 80 l reaction mix. Verify that the fluorescence intensity is stable over at least 10 seconds. Pause the data acquisition, manually mix in the 40 l of pre-warmed ATP mix, pipetting up and down at least five times. Avoid introducing air into the sample. After thorough mixing, resume data recording. The time elapsed between the start of mixing and continuation of data acquisition should be accurately recorded, as this lag will be critical when fitting the data (usually about 10–15 seconds).
6. Upon completion of the reaction, a final emission scan is performed to ensure the proper change in donor and acceptor fluorescence intensities.

The final concentrations for the reaction are as follows: 12 mM Hepes, pH 7.9, 4 mM Tris, pH 7.5, 60 mM KCl, 3 mM $MgCl_2$, 0.32 mM EDTA, 12% glycerol, 0.02% NP-40, 0.4 mg/ml FLAG peptide, 2 mM Mg.ATP, 5 nM nucleosomes, 300 nM SNF2h.

### 3.3 Data fitting and interpretation

Data are best fit to single or multiple exponentials using a program such as Kaleidagraph, Berkeley Madonna or MATLAB (Fig. 3). Variables to fit include rate constants as well as fluorescence values for the starting material, any intermediates and the end product. The fluorescence intensity obtained from the fit for the starting material should be very close to the value obtained from dilution of the reaction mix with the ATP mix. For example, in the above experiment, the starting fluorescence intensity should equal 2/3 the fluorescence intensity before addition of ATP (80 l diluted to 120 l final). The first data point and actual starting value may be very different since the reaction may have proceeded partially between manual mixing and data acquisition. Thus one must also correct for this lag at every time point. Lastly, R-values of 0.99 are routinely obtained.

The rate constants obtained from fitting the data only represent the change in fluorophore distances that is detectable by FRET, which is based on the Förster radius of the fluorophores. Thus there may be portions of a remodeling reaction that are not measurable using a particular FRET pair. The fit of data to multiple exponentials may indicate the presence of intermediates in the remodeling reaction or the presence of two populations of nucleosomes, which react with different rates. By moving the Cy3 dye to an internal location on the DNA, as shown in Figures 1B and 3B, it is possible to identify intermediates in the reaction. During SNF2h remodeling, the nucleosome starts with low FRET, transiently increases to high FRET intensity, and then returns to low FRET. This indicates that the Cy3 and Cy5 dyes transiently come close together, suggesting the presence of an intermediate in which part of the DNA has moved across the histone octamer. Finally, while FRET cannot distinguish the exact distances between dyes due to orientation effects, this technique can reliably detect changes in distance between dyes.

## 4. Concluding Remarks

The FRET-based method described above allows for the direct visualization of mononucleosome remodeling in real-time. This technique is well suited for quantitative analysis of remodeling kinetics in solution. By changing the location of the FRET pairs, remodeling can be monitored at several different locations on the nucleosome. Furthermore, this technique can be applied to directly compare the remodeling rates for several different ATP-dependent chromatin-remodeling complexes.

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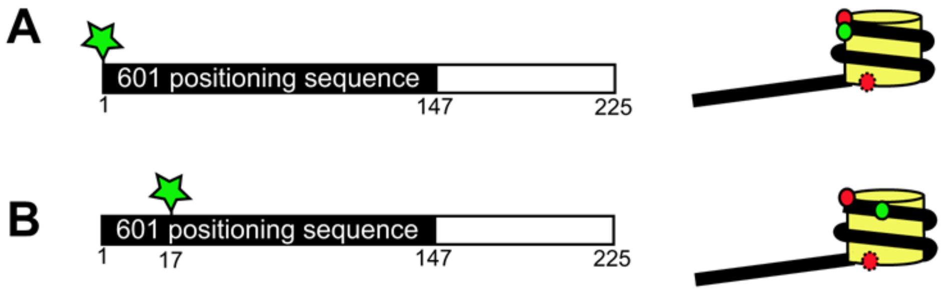
A
601 positioning sequence
1
147
225
B
601 positioning sequence
1
17
147
225

**Fig 1.**

Schematic of DNA and nucleosome constructs. **(A)** and **(B)** Left, labeled Cy3 DNA containing the 601 positioning sequence. Right, DNA assembled on a histone octamer with H2A residue 120 mutated to cysteine and labeled with Cy5. (A) is an end-labeled construct, and (B) is an internally-labeled DNA construct.

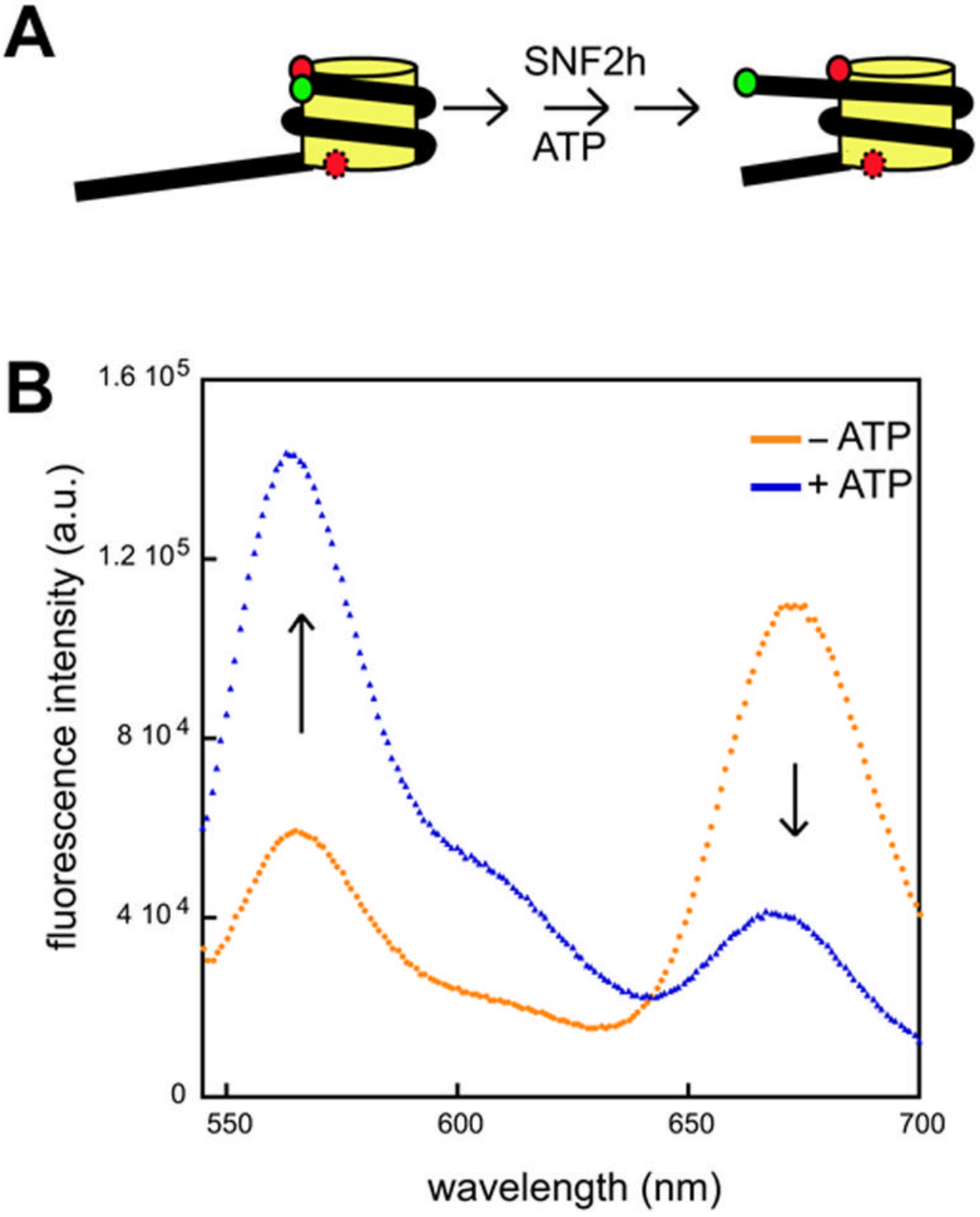

**Fig 2.**

Emission scan of nucleosomes before and after remodeling. **(A)** Schematic of the change in dye positions as a function of SNF2h remodeling of the end-labeled nucleosome. **(B)** 5 nM nucleosomes were incubated with 450 nM SNF2h for 30 min in the absence (orange) or presence (blue) of 2 mM ATP at 30° C. Samples were excited at 520 nm, the absorption maximum of Cy3.

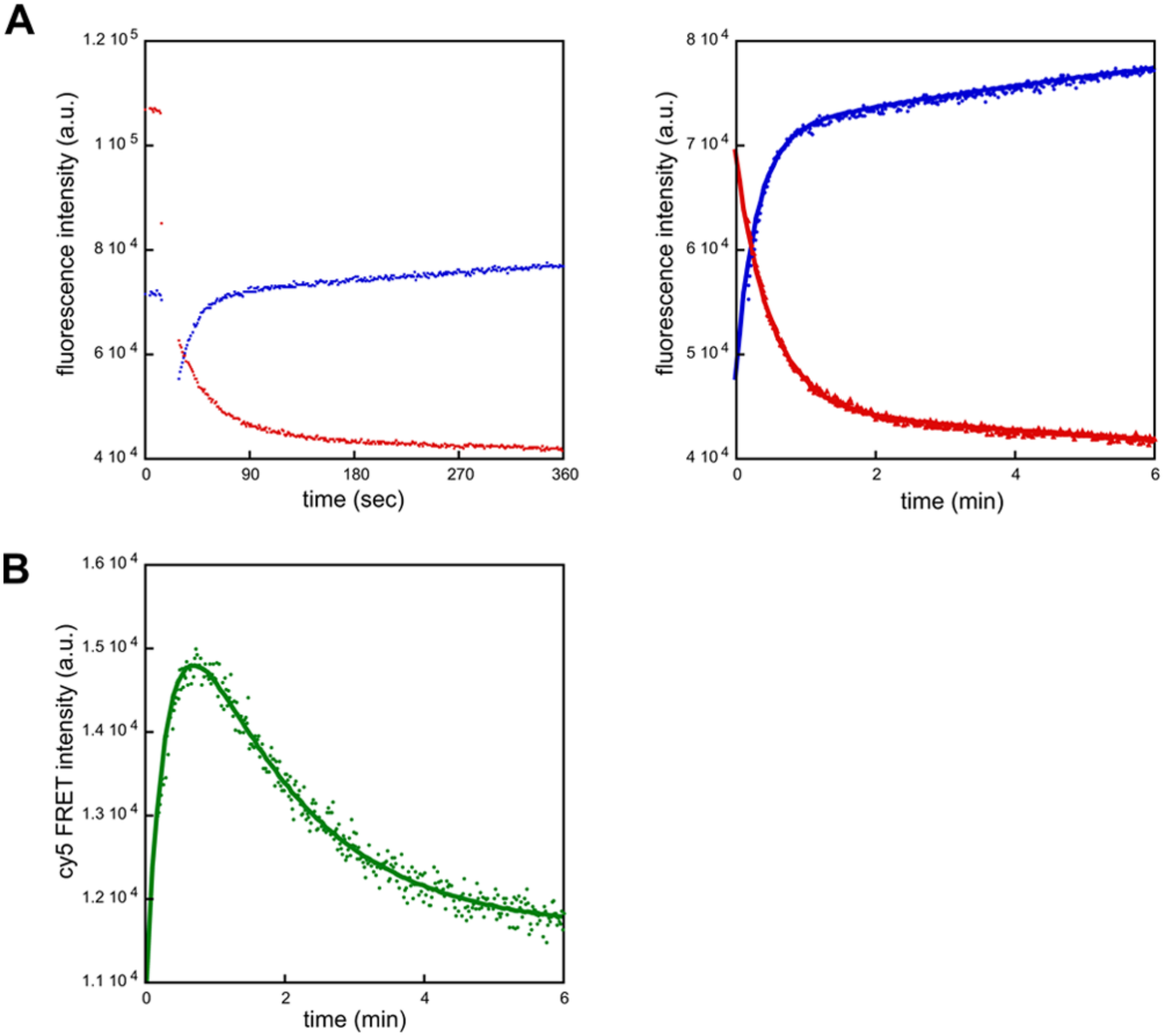

**Fig 3.**

Real-time measurement of remodeling reactions. **(A)** Left, raw data collected from remodeling of end-labeled nucleosomes (Fig. 1A) with SNF2h, following either Cy3 donor unquenching (blue) or change in Cy5 FRET intensity (red). Right**,** fit of data to two exponentials Rate constants (sec$^{-1}$) are: Cy3: first phase 3.3, second phase 0.1; Cy5: first phase 2.0, second phase 0.1. **(B)** Remodeling of internally labeled nucleosomes (Fig. 1B) following Cy3 donor unquenching. Right, fit of data to two exponentials. Rate constants (sec$^{-1}$) are: first phase 3.2, second phase 0.1. All reactions were performed at 30° C with 2 mM ATP.