Journal of Muscle Research and Cell Motility **25**: 95–102, 2004.

Engineering lysine reactivity as a conformational sensor in the *Dictyostelium* myosin II motor domain

MIHÁLY KOVÁCS[1,*,†], JUDIT TÓTH[1,†], ANDRÁS MÁLNÁSI-CSIZMADIA[1], CLIVE R. BAGSHAW[2] and LÁSZLÓ NYITRAY[1]

[1]*Department of Biochemistry, Eötvös Loránd University, H-1117 Budapest, Pázmány P. sétány 1/C, Hungary;* [2]*Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK*

[†]*Current address: Laboratory of Molecular Cardiology, National Heart, Lung and Blood Institute, 9000 Rockville Pike, Bethesda, MD 20892, USA*

Received 19 May 2003; accepted in revised form 6 August 2003

Abstract

Lys84 of skeletal muscle myosin located at the interface between the motor and neck domains has long been utilized as a useful chemical probe sensing motor domain conformational changes and tilting of the lever arm. Here we report the first site-directed mutagenesis study on this side chain and its immediate chemical environment. We made *Dictyostelium* myosin II motor domain constructs in which Lys84 was replaced by either a methionine or a glutamic acid residue and another mutant containing an Arg704Glu substitution. By following trinitrophenylation of the mutant constructs, we first unambiguously identify Lys84 as the reactive lysine in *Dictyostelium* myosin. Analysis of the reaction profiles also reveals that the Lys84–Arg704 interaction at the interface of two subdomains of the myosin head has a significant effect on Lys84 reactivity, but it is not the only determinant of this property. Our findings imply that the nucleotide sensitivity of the trinitrophenylation reaction is a general feature of conventional myosins that reflects similar changes in the conformational dynamics of the different orthologs during the ATPase cycle.

Abbreviations: AMPPNP, adenosine 5′-(β, γ -imidotriphosphate); ATPγS, adenosine 5′-O-(3-thiotriphosphate); BeF_x, beryllium fluoride with undefined stoichiometry; S1, subfragment-1; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TNBS, 2,4,6-trinitrobenzene sulfonate; TNP, trinitrophenyl; Tris, tris(hidroxymethyl)aminomethane; V_i, vanadate; wt, wild-type.

Introduction

The existence of an exceedingly reactive lysine in rabbit skeletal muscle myosin was pointed out as early as 1960 (Kubo *et al.*, 1960a). This lysine was later shown to reside in the N-terminal 27 kDa portion of skeletal muscle subfragment-1 (S1 or 'head', comprising the motor (catalytic) domain and neck region of myosin) (Hozumi and Muhlrad, 1981). The crystal structure of skeletal S1 showed that the reactive lysine (Lys84) is located at the interface between the motor and neck domains (Rayment *et al.*, 1993). The reactivity of this residue towards TNBS[1] appears to be inhibited by both nucleotides (Tonomura *et al.*, 1963; Ajtai *et al.*, 1999) and actin (Muhlrad *et al.*, 2003), making it a sensitive conformational sensor within the protein molecule.

In recent years, recombinant myosin II motor domain fragments from *Dictyostelium discoideum* (Ruppel and Spudich, 1996) have become outstandingly important in the investigation of myosin structure and function for mainly two reasons. First, crystal structures of this fragment complexed with various nucleotides and nucleotide analogs have visualized a large conformational change between the so-called *open* and *closed* states (Fisher *et al.*, 1995; Smith and Rayment, 1996; Bauer *et al.*, 1997, 2000; Gulick *et al.*, 1997, 2000; Geeves and Holmes, 1999). Upon this conformational transition, profound rearrangements occur at the interface containing Lys84 (homologous to the skeletal residue with the same number), which provides explanation for its conformational sensitivity. Furthermore, kinetic studies revealed the significance of this conformational transition in motor function and provided insight into the correspondence between the ATPase intermediates and the identified structural states (Manstein *et al.*, 1991; Uyeda *et al.*, 1996; Kuhlman and Bagshaw, 1998; Sasaki *et al.*, 1998; Suzuki *et al.*, 1998; Batra and Manstein, 1999; Furch *et al.*, 1999; Malnasi-Csizmadia *et al.*, 2000, 2001b; Kovacs *et al.*, 2002). In a recent study, a similar

* To whom correspondence should be addressed: Tel.: +1-301-496-6887; Fax: +1-301-402-1542, E-mail: kovacsm@nhlbi.nih.gov

trinitrophenylation reaction profile of *Dictyostelium* myosin II constructs to that of skeletal muscle S1 has been reported (Reynoso *et al*., 2001), suggesting the possibility that Lys84 of this ortholog can also have unusual chemical properties.

We have carried out a site-directed mutagenesis study on the reactive lysine region of the *Dictyostelium* myosin II motor domain. By substitution of Lys84 with other side chains, we unambiguously show that this residue is the one with unusually high reactivity. We also demonstrate that the nearby positive charge of the conservative Arg704 side chain is only one of the factors that contribute to Lys84 reactivity. Nucleotide sensitivity of the kinetics of Lys84 trinitrophenylation was also detected and thus it appears to be a general feature of conventional myosins, which makes it a useful chemical probe in solution studies.

Materials and methods

DNA constructs, protein expression and purification

The DNA construct containing the coding sequence for the W501+ (W36F, W432F, W584F) mutant of the M761 *Dictyostelium* myosin II motor domain cloned into the pDXA-3H expression vector (Manstein *et al*., 1995) was constructed as described earlier (Malnasi-Csizmadia *et al*., 2000). The plasmid for the W501+/R704E mutant was produced from the pDXA/W501+ construct using PCR-based mutagenesis with the replacement of the BglII-BpiI cassette within the motor domain coding region. The R704E mutation was introduced with the downstream primer 5′-ATCGGTGGCTTTTTGTGAG-TCTTCAGCGTCTCTTGGAACGTTTGGAGCT-AATAAATAGTATTCTTTGACGAAATCGGC-3′ (mutant triplet underlined). The upstream primer used was 5′-CAACAAGATCTCGAACTTTGCTTC-3′. The W501+/K84M and W501+/K84E DNA constructs were created by means of a BsaBI–EcoRI cassette replacement using 5′-TCAAAAAGGATGATGCCAATCAACGT-AATCCAATCATGTTCGATGGTGTCG-3′ (K84M) and 5′-TCAAAAAGGATGATGCCAATCAACGT-AATCCAATCGAGTTCGATGGTGTCG-3′ (K84E), respectively. The downstream primer used was 5′-GAT-TGGAATTCTCTTGAATGGATTGACGGC-3′. All constructs were verified by DNA sequencing.

Transformed *Dictyostelium* cells were cultured and the recombinant proteins were prepared as described previously (Manstein and Hunt, 1995; Malnasi-Csizmadia *et al*., 2000). Protein concentrations were determined using Bradford Reagent (Sigma). Purity of the preparations was checked by 9% SDS-PAGE.

Nucleotides and nucleotide analog complexes

ATP (special quality, vanadate-free) was from Roche Molecular Biochemicals. Care was taken that ATP was not exhausted during the experiments. Other nucleotides and reagents were purchased from Sigma Chemical Co. The motor domain-ADP·AlF_4 complex was prepared by incubation of 4 μM motor domain, 50 μM ADP, 3 mM NaF, 50 μM $AlCl_3$ for at least 120 min.

Steady-state ATPase measurements

Steady-state MgATPase activities were measured from A_{340} using a pyruvate kinase-lactate dehydrogenase linked assay (Trentham *et al*., 1972). Buffer conditions were 20 mM TES, 40 mM NaCl, 2 mM $MgCl_2$, pH 7.5. Assays were performed at 20°C in the presence of 1 mM ATP, 200 μM NADH, 400 μM PEP, 10 U/ml pyruvate kinase and 20 U/ml lactate dehydrogenase (Sigma).

Trinitrophenylation kinetics

The progress reaction of the motor domain with TNBS was followed by recording changes in absorbance at 345 nm at 25°C in a thermostated 1 cm path length cell of a Shimadzu UVPC2000 spectrophotometer for at least 3000 s. 100 μM TNBS was added to protein samples of 4 μM. The concentration of TNP–Lys was calculated using $\varepsilon_{345} = 14{,}500\ M^{-1}\ cm^{-1}$. Buffer conditions were as described in the previous section.

Fluorescence measurements

Fluorescence measurements were performed at 25°C in a Spex FluoroMax spectrofluorimeter. Tryptophan was excited at 295 nm (1 nm bandwidth) with a Xe lamp and emission at 345 nm (5 nm bandwidth) was monitored through a monochromator. Buffer conditions were as described in the Section 'Steady-state ATPase measurements'.

Kinetic analysis

Fitting of the experimental data to mathematical functions was done using Origin 6.0 (Microcal Software). Kinetic simulations and global fitting of kinetic parameters were performed with Gepasi v3.21 (© Pedro Mendes, Virginia Bioinformatics Institute).

Results

Design of mutants

The W501+ mutant of the *Dictyostelium* myosin II motor domain contains the substitutions W36F, W432F and W586F to yield a single tryptophan protein with advantageous spectroscopic properties that still retains the enzymatic properties of the wild-type enzyme (Malnasi-Csizmadia *et al*., 2000). In the present study, we introduced the mutations K84M, K84E and R704E into the W501+ motor domain to investigate lysine reactivity towards TNBS. By replacing Lys84 with a roughly isosterical methionine or an oppositely charged

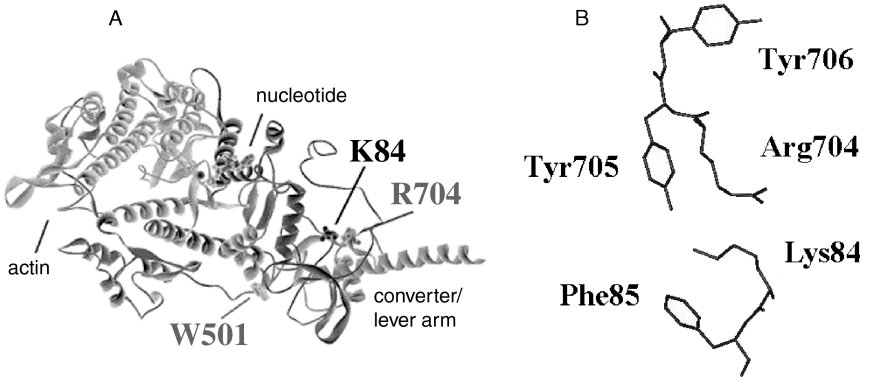

Fig. 1. (A) Ribbon representation of the *Dictyostelium* myosin II motor domain (Kliche *et al.*, 2001) in the open structure showing the position of the actin and nucleotide binding sites as well as the converter/lever arm region and amino acid positions Lys84, Trp501 and Arg704. (B) Relative positions of Lys84, Arg704 and the nearby aromatic residues in the open conformation of *Dictyostelium* myosin II motor domain ($ADP \cdot BeF_x$ complex (Fisher *et al.*, 1995)). Lys84 and Arg704 are homologous to Lys84 and Arg723 of rabbit fast skeletal muscle myosin. Homologous positions to Phe85 and Tyr705 are also occupied by aromatic residues in skeletal myosin (Tyr85 and Tyr724, respectively).

Table 1. Steady-state basal MgATPase activities of the *Dictyostelium* myosin II motor domain mutants used in this study. Experimental conditions are described in Materials and Methods.

Construct	Steady-state ATPase activity (s^{-1})
wt (Malnasi-Csizmadia *et al.*, 2000)	0.06 ± 0.01
W501+	0.05 ± 0.01
W501+/R704E	0.17 ± 0.03
W501+/K84M	0.08 ± 0.01
W501+/K84E	0.05 ± 0.01

glutamic acid residue, we investigated the effect of the elimination of this lysine on the reaction of the motor domain with TNBS. These mutations can provide the opportunity for direct identification of Lys84 as the most reactive lysine in *Dictyostelium* myosin. Involvement of a nearby positive charge in altering the pK and hence the reactivity of Lys84 was proposed in earlier studies (Muhlrad and Takashi, 1981; Ajtai *et al.*, 1999). The positive charges of Lys84 and Arg704 in the *open* conformation of the *Dictyostelium* myosin II motor domain are in close proximity (0.6 nm, Figure 1), which implies that the latter side chain can have a significant effect on Lys84 reactivity. We investigated this possibility by changing Arg704 to an oppositely charged residue in the W501+/R704E mutant.

Steady-state ATPase activities

The steady-state MgATPase activities of the constructs used in this study are listed in Table 1. It appears that although K84 is almost invariable in class II myosins and R704 is even more conserved (in classes I, II, III, IV, V, VII, VIII, X, XI, XV and XVI), an amino acid substitution at these positions does not greatly affect the ATPase activity of myosin. The slightly higher basal ATPase rates of W501+/K84M and W501+/R704E compared to the wild-type protein may reflect a subtle modulation of the enzymatic activity resulting from a change at the interface of the N-terminal 25 kDa

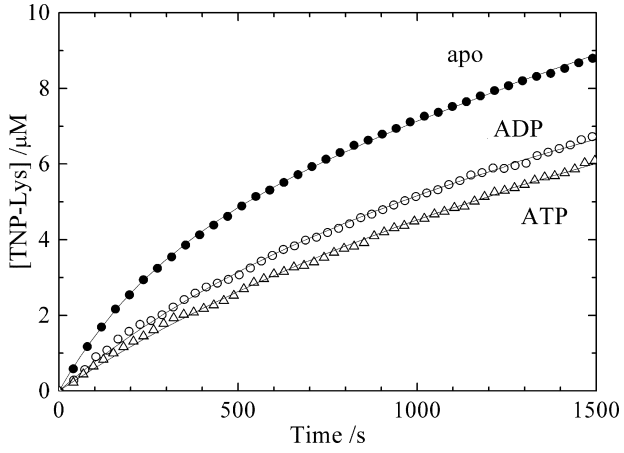

Fig. 2. Progress of TNP–Lys formation during the reaction of W501+ (4 μM) with TNBS (100 μM), as calculated from changes in absorbance at 345 nm. Symbols denote experimental traces obtained in the absence of nucleotide (solid circles), in the presence of 0.2 mM ADP (open circles) and 1.5 mM ATP (open triangles). Reactions were carried out at 25°C in 20 mM TES, 40 mM NaCl, 2 mM $MgCl_2$, pH 7.5. Simulated traces based on the best fits to the experimental data are shown as solid lines. Second order rate constants used in the simulations were $k_{fast} = 33.9\ M^{-1}\ s^{-1}$, $k_{slow} = 0.17\ M^{-1}\ s^{-1}$ (*apo*); $k_{fast} = 15.5\ M^{-1}\ s^{-1}$, $k_{slow} = 0.11\ M^{-1}\ s^{-1}$ (ADP); $k_{fast} = 11.2\ M^{-1}\ s^{-1}$, $k_{slow} = 0.10\ M^{-1}\ s^{-1}$ (ATP). See Results for model description.

subdomain and the converter region as it has been pointed out by fluorescence and transient kinetic studies (Malnasi-Csizmadia *et al.*, 2002).

Kinetics of TNP-Lys formation

Trinitrophenylation of the lysyl side chains of the myosin motor domain was followed spectrophotometrically after mixing the protein with trinitrobenzene sulfonate (TNBS). In the case of W501+, the reaction had a fast and a slow component (Figure 2). The fast reaction was essentially completed during the time of the experiment, while only the early onset of the slow reaction was observed. During the recording time of the reactions (3000 s), less than 15% of the initial amount of

TNBS was exhausted as calculated from the total change in absorbance. The initial concentration of TNBS used in the experiments (100 μM) was 25 times greater than protein concentration. Therefore, if we assume the existence of one fast-reacting and 56 (identical) slow-reacting lysines in the motor domain, then it is appropriate to fit the experimental traces to a single exponential (fast reaction) with a linearly increasing baseline (slow reaction) because trinitrophenylation of the fast-reacting lysine takes place under pseudo-first order conditions. However, the slow phase did not appear to be linear and therefore the arbitrarily chosen boundaries of the fitting range (and thus the relative weighting of the two phases) heavily influenced both the amplitude of the fast phase and the value of the obtained pseudo-first order rate constant (k_{fast}). The same was observed when attempting to obtain k_{fast} from the difference of the slope of the trace at $t = 0$ ($k_{fast} + k_{slow}$) and after completion of the fast reaction (k_{slow}) as in (Ajtai *et al.*, 1999) because both slopes varied widely with the fitting range selected.

We attempted to solve this problem and to obtain rate constants containing less error coming from fitting artifacts by applying a global fitting approach using a simple reaction scheme containing two competitive irreversible reactions (Scheme 1):

$$1: \quad F + T \overset{k_{fast}}{\rightarrow} P$$

$$2: \quad S + T \overset{k_{slow}}{\rightarrow} P$$

Scheme 1

The model still contains the assumption that there is one fast-reacting residue in the protein (denoted as 'F'), while all other 56 lysyl side chains ('S' for slow) have identical reactivities towards TNBS (marked as 'T'). Both reactions lead to the formation of a TNP–Lys side chain ('P' for product) whose concentration can be calculated from the A_{345} records using $\varepsilon_{345} = 14{,}500\ M^{-1}\ cm^{-1}$. During the fitting iterations, the second order rate constants k_{fast} and k_{slow} were varied to obtain the best fit to the experimental data. The fitted rate constants are shown in Table 2.

Table 2. Second order rate constants (k_{fast} and k_{slow}) of Lys84 trinitrophenylation in the absence of nucleotide and in the presence of nucleotides and nucleotide analogs

Construct	Ligand	k_{fast} ($M^{-1}\ s^{-1}$)	k_{slow} ($M^{-1}\ s^{-1}$)	R
W501+	apo	33.9 ± 2.2	0.17	3.6
	ADP	15.5 ± 1.0	0.11	2.6
	ATP	11.2 ± 0.7	0.10	2.1
	ADP·AlF_4	11.9 ± 0.6	0.07	2.9
	AMPPNP	9.5 ± 1.3	0.09	2.5
W501+/R704E	apo	26.0 ± 1.0	0.11	4.1
	ADP	9.3 ± 0.8	0.06	2.6
	ATP	8.4 ± 1.3	0.02	6.1
	ADP·AlF_4	7.3 ± 0.6	0.04	3.4
	AMPPNP	6.8 ± 0.4	0.05	2.3
W501/K84M, W501/K84E	All states			<2

The value R [$= k_{fast}/(56 \times k_{slow})$] is also given to illustrate the separability of the two phases and the confidence of the k_{fast} values. Experimental conditions are described in Materials and Methods. See Results for details of data analysis and modeling.

Figure 2 shows examples of experimental (symbols) and simulated (lines) time courses of TNP-Lys formation on mixing 4 μM W501+ motor domain with 100 μM TNBS in the absence of nucleotide and in the presence of ATP and ADP. In all cases, the values obtained for k_{fast} and k_{slow} were highly covariant. The reliability of these rate constants depends on how well the two phases are separated i.e. the ratio of the rates of the fast and slow phases at the beginning of the reaction, which can be calculated as $R = k_{fast}/(56 \times k_{slow})$ (see Table 2). In the experiments described herein, this value was either in a range close to the limit of separability of the two phases ($R = 2$–7), or values of $R < 2$ indicated that even though the fit did produce two different values for k_{fast} and k_{slow}, these are unreliable and the reaction can be treated as monophasic. There was no case where the classification of the lysine residues into more than two groups of different reactivity would be reasonable or of practical importance.

We have carried out reactions of the W501+, W501+/R704E, W501+/K84E and W501+/K84M mutants with TNBS in the absence of nucleotide (*apo* state) and in the presence of different nucleotides and nucleotide analogs (ADP, ATP, ADP·AlF_4 and AMPPNP). Figure 3 shows traces obtained in the absence of nucleotide. The reaction had fast and slow components in both W501+ and W501+/R704E, showing the existence of at least two groups of lysines with different reactivities. The two phases were well separated. The rate constant extracted

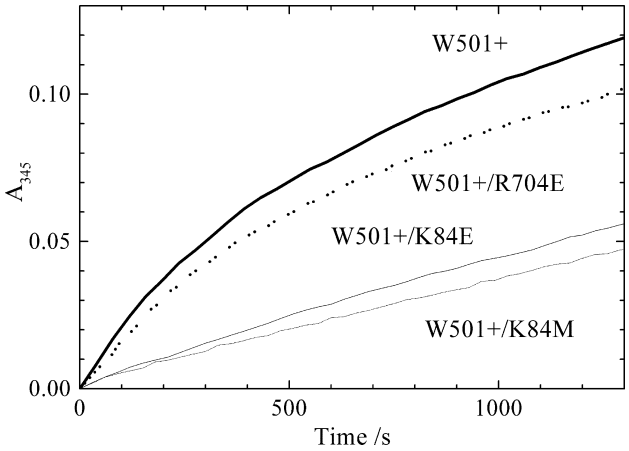

Fig. 3. Reaction of the W501+ (thick solid line), W501+/R704E (thick dotted line), W501+/K84E (thin solid line) and W501+/K84M (thin dotted line) mutants with TNBS. Reactions were started by adding 100 μM TNBS to 4 μM protein samples and followed by monitoring absorbance at 345 nm. Traces shown in this figure were recorded in the absence of nucleotide (*apo* state). In the case of W501+ and W501+/R704E, reactions were biphasic. The fast phase was absent in W501+/K84E and W501+/K84M. Reaction conditions were as in Figure 2.

from the fast phase was significantly lower in the W501+/R704E mutant than in W501+ (Table 2). The difference, however, was not as profound as would be expected if the high reactivity of Lys84 was solely due to the spatial proximity of the positive charge of Arg704. Therefore, other significant factors must contribute to the unusual chemical properties of the Lys84 ε-amino group.

In contrast to W501+ and W501+/R704E, the fast phase was absent in W501+/K84E and W501+/K84M, and the reaction was monophasic (Figure 3). These data apparently show that mutation of Lys84 eliminates the fast-reacting side chain of the motor domain. The presence of various nucleotides did not alter the reaction profile of these mutants significantly (data not shown). Thus, similarly to skeletal muscle myosin, Lys84 can be identified as the reactive lysine residue in *Dictyostelium* myosin II.

It was shown in previous studies that binding of nucleotide significantly reduces the rate of trinitrophenylation in skeletal muscle myosin (Tonomura *et al*., 1963; Ajtai *et al*., 1999). Moreover, the differences in the rate constant of the fast phase of the reaction were used as a basis for classification of different protein conformations depending on the nature of the bound nucleotide. Therefore, we assessed the conformational sensitivity of the *Dictyostelium* Lys84 side chain by monitoring the effect of different nucleotides and nucleotide analogs on its reactivity in the W501+ and W501+/R704E mutants. Similarly to skeletal muscle myosin, nucleotide binding caused a significant (55–75%) decrease in Lys84 reactivity in the case of all nucleotides and nucleotide analogs examined (Table 2). However, with the reduction of the rate of the fast phase, separation of the fast and slow phases became more difficult. That is, values of $2 < R < 7$ were obtained, and thus the magnitude of the fitting error reached the order of the difference between the values obtained in different nucleotide states (see Table 2). Hence, in the case of *Dictyostelium* myosin II, analysis of the trinitrophenylation reaction allows the firm conclusion that nucleotide binding causes a profound change in the chemical environment of Lys84 but, because of high background signal from the other slow-reacting lysines, subtle differences of Lys84 reactivity in different nucleotide complexes remain unresolved.

Monitoring the trinitrophenylation reaction via Trp501 fluorescence

Using the single-tryptophan W501+ mutant, we were able to detect interaction between a specific tryptophan side chain of known location and the TNP groups incorporated into the protein molecule. Figure 4 shows a comparison of A_{345} and Trp501 fluorescence signals obtained during the reaction of W501+ with TNBS. Trp501 showed a fluorescence quench upon formation of nearby TNP–Lys moieties in the protein molecule. Similarly to the absorbance change, the fluorescence quench also had fast and slow components. The observed pseudo-first order rate constant of the fast phase of the quench ($k_{obs} = 0.0051\ s^{-1}$) was in line with the fast rate constant obtained from the analysis of the absorbance data (cf. Table 2). The distance between Lys84 and Trp501 in the known structures of the *Dictyostelium* myosin II motor domain is in the range of 2.8–3.2 nm (Fisher *et al*., 1995; Smith and Rayment, 1995; Smith and Rayment 1996; Bauer *et al*., 1997; Gulick *et al*., 1997; Bauer *et al*., 2000; Gulick *et al*., 2000; Kliche *et al*., 2001), which is in the order of the typical Förster distances of a Trp–TNP donor–acceptor pair within a protein molecule (Lakowicz, 1999). The fast quench phase is thus likely to reflect an energy transfer interaction between the Trp501 and TNP–Lys84 side chains, whereas trinitrophenylation of (some of the) slowly reacting lysines results in a further significant quench in Trp501 fluorescence.

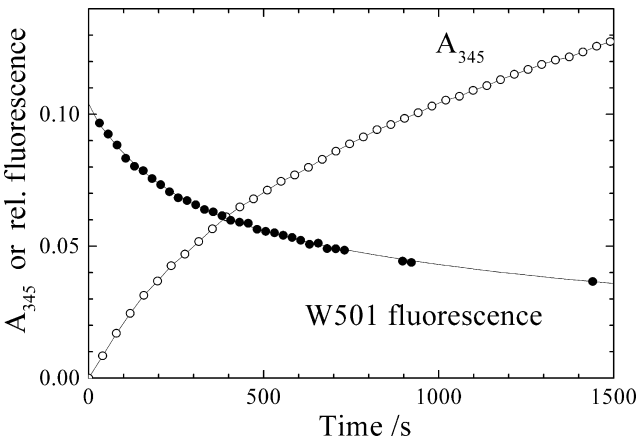

Fig. 4. Comparison of A_{345} (open circles) and Trp501 fluorescence (solid circles) signals recorded during the reaction of 4 μM W501+ and 100 μM TNBS in the absence of nucleotide. Similarly to the absorbance change at 345 nm, the observed decrease in tryptophan fluorescence was also biphasic. The fast phase is likely to be a result of TNP–Lys84 quenching of Trp501 fluorescence. The rate constant of this phase was similar to that extracted from absorbance data ($k_{obs} = 0.0051\ s^{-1}$). Reaction conditions were as in Figure 2.

Discussion

Understanding the connection between the enzymatic mechanism of myosin and its mechanical function requires detailed characterization of kinetic states in solution and establishment of their relation to the crystallograpically identified conformations. Site-specific chemical reporters such as reactive cysteine and lysine residues have proven particularly powerful in solution studies (Miller *et al*., 1982; Hiratsuka, 1992; Park *et al*., 1996; Phan *et al*., 1996; Maruta *et al*., 1998; Bobkova *et al*., 1999; Nitao and Reisler, 2000; Burghardt *et al*., 2001; Peyser *et al*., 2001). The highly reactive Lys84, located in the N-terminal subdomain at an interface of the catalytic and neck domains of conventional myosins, has long been exploited as a conformational sensor (Kubo *et al*., 1960b; Tonomura *et al*., 1963; Muhlrad,

1977; Muhlrad *et al*., 1981; Muhlrad and Takashi 1981). Its rate of trinitrophenylation by TNBS was reported to be 3 orders of magnitude higher than that of the other lysyl side chains of the protein, which leads to a biphasic reaction profile (Muhlrad *et al*., 1975; Ajtai *et al*., 1999). Here we report the first site-directed mutagenesis study on this reactive lysine residue. Our experiments, in which the fast phase of the trinitrophenylation reaction is absent in *Dictyostelium* myosin II motor domain mutants lacking Lys84, clearly demonstrate that this residue is the one that has outstanding reactivity in the *Dictyostelium* myosin head, similarly to the homologous residue of skeletal muscle myosin S1. Trinitrophenylation kinetics of a motor domain construct (W501+), in which the reactive lysine region is unchanged compared to the wild-type protein, shows that the reactivity of Lys84 is even higher in *Dictyostelium* myosin than in the skeletal muscle isoform (for the latter, a pseudo-first order rate constant of $1.37 \pm 0.086 \times 10^{-3}$ s^{-1} at 100 μM TNBS has been reported (Ajtai *et al*., 1999)). However, the reaction rate of the other, slow-reacting lysines of the *Dictyostelium* motor domain is also several times higher than in skeletal S1. The ratio of the rate constants for trinitrophenylation of the fast and slow lysines is around 1000 in skeletal muscle S1 (Muhlrad *et al*., 1975), which is reduced to 200–300 in the case of the *Dictyostelium* myosin motor domain, which makes the separation of the two phases of the reaction more difficult, considering the 56-fold molar excess of the potentially slow-reacting residues.

The binding of various nucleotides to the *Dictyostelium* myosin motor domain causes a reduction in the Lys84 trinitrophenylation rate of a very similar extent to skeletal muscle S1 (55–75%, (Ajtai *et al*., 1999)). In the nucleotide-bound states, the fast and slow phases of the reaction were poorly separable and thus the errors in the k_{fast} values did not allow for the identification of different nucleotide-bound states (Table 2). In an earlier study, solution conformations of skeletal myosin S1 were grouped into two separate classes based on Lys84 reactivity (Ajtai *et al*., 1999). ADP, ATPγS and ADP·BeF_x caused smaller inhibition of the fast reaction, whereas ATP, ADP · AlF_4 and ADP · V_i fell into the other class of nucleotides with a higher extent of inhibition. The two groups were separable probably due to the higher difference between the reactivity of Lys84 and the other lysines. The pseudo-first order rate constants for the fast phase were calculated on the basis of subtracting the rate of the reaction after 30 min (regarded as v_{slow}) from the initial rate ($v_{fast} + v_{slow}$). However, the rate constants reported in (Ajtai *et al*., 1999) reflect that the fast phase was in most cases far from completion after the 30 min interval specified and thus, it made a significant contribution (up to 60% of its initial rate) to the overall rate. Therefore, this calculation underestimates the rate constant for Lys84 trinitrophenylation, especially at lower k_{fast} values, which will result in artificially larger differences between nucleotide states. Nevertheless, the two groups of nucleotides separated on this basis are in fair agreement with the results of other solution and crystallographic studies showing that the myosin head is predominantly in the *open* conformation in ADP and ATPγS, whereas ADP · AlF_4 and ADP · V_i induce the *closed* conformation, which is the most abundant state during steady-state ATP hydrolysis (Kuhlman and Bagshaw, 1998; Malnasi-Csizmadia *et al*., 2000). Another interesting feature of skeletal muscle S1 trinitrophenylation is that the rate constant of the fast phase is about 20–30% higher in Tris buffer than in other buffers at the same pH (Muhlrad and Takashi 1981), while the rate constant of the slow phase remains unaffected. This effect can contribute to the better separability of the two phases of the reaction, but the increase is not so large as to cause a significant improvement in data analysis.

Trinitrophenylation of Lys84 causes an increase in the MgATPase activity of skeletal muscle S1 (Muhlrad, 1983), which provides an alternative method for monitoring the extent of modification. Recently, a similar effect on *Dictyostelium* myosin S1 and motor domain (S1dC) constructs has been reported (Reynoso *et al*., 2001), although the chemical modification was accompanied by a smaller change. The full-length S1 and motor domain constructs showed a marked difference in that the MgATPase activity did not increase further after about 5 min of the trinitrophenylation reaction in the case of full-length S1 (conditions: 4 μM protein, 80 μM TNBS, 22°C, pH 8.0), whereas the change did not reach a plateau even on a longer time scale in the motor domain construct. The latter feature was interpreted as Lys84 trinitrophenylation being markedly slower in the absence of the neck region. However, a closer look at the profile of the change reveals the possibility that the reaction is biphasic in S1dC, in a rather similar way to the A_{345} profiles described in the present report, and the difference between S1dC and S1 could be mainly due to the lack of the slow phase in the latter case. These findings suggest that the modification of the slowly reacting lysines significantly alters the basal MgATPase activity of S1dC, in contrast to the full-length S1 construct.

The removal of the positive charge from the immediate vicinity of the Lys84 side chain by the R704E substitution caused a reduction in the rate of Lys84 trinitrophenylation. The reaction takes place only in the deprotonated state of the ε-amino group, and thus a shift in its pK value causes a change in its reactivity. However, the relatively small decrease (23%) caused by the R704E mutation demonstrates that Arg704 is not the only factor making Lys84 outstandingly reactive. This is also indicated by the fact that, in the *closed* conformation (e.g. in ADP · AlF_4 or ADP · V_i) where subdomain movements of the motor domain put these two residues far apart and thus their interaction is abolished, Lys84 reactivity is not drastically lower (50% difference, (Ajtai *et al*., 1999)) than in the *open* state where they can interact. Studies on the ionization of Lys84 of skeletal muscle myosin showed that the pK of this side chain is about 9.0, only one pK unit lower than

that of other lysyl groups of the protein (Muhlrad and Takashi 1981). This shift alone cannot explain an increase by three orders of magnitude in reactivity. Two nearby aromatic side chains in skeletal muscle myosin (Tyr84 and Tyr724) are occupied by aromatic residues in *Dictyostelium* myosin, too (Phe85 and Tyr705, respectively, cf. Figure 1). The role of one or both of these side chains as possible proton acceptors forming a charge transfer complex with Lys84 has also been proposed (Ajtai *et al*., 1999), but this idea has not yet been tested experimentally.

The fact that the large extent of nucleotide inhibition of Lys84 trinitrophenylation and the small differences regarding the nature of the bound ligand appear to be general features of conventional myosins brings into focus a fundamental problem concerning the mechanism of energy transduction. Although the crystal structures of various myosin fragments in the absence of nucleotide have been solved (Rayment *et al*., 1993; Bauer *et al*., 2000; Houdusse *et al*., 2000), it is likely that these structures do not represent the solution conformation of *apo*-myosin. All of the known nucleotide-free structures are essentially identical to the *open* nucleotide-bound state. This seems to be inconsistent with experimentally verified solution kinetic and thermodynamic arguments stating that the irreversible step in the ATPase cycle accompanied by a large drop in free energy occurs on binding of nucleotide (Bagshaw *et al*., 1974; Bagshaw and Trentham, 1974; Kovacs *et al*., 2002), which as such is likely to involve changes in protein structure. This is also indicated by the fact that structural changes occurring on nucleotide binding can be detected by experiments utilizing reporters located further away from the nucleotide binding site, such as tryptophans (Malnasi-Csizmadia *et al*., 2000, 2001a; Park and Burghardt, 2000; Yengo *et al*., 2000) and extrinsic spectroscopic probes (Thomas *et al*., 1995; Berger *et al*., 1996), disulfide crosslinks (Wells and Yount, 1980; Nitao and Reisler, 1998) and patterns of limited proteolytic cleavage (Redowicz *et al*., 1987). The largely decreased reactivity of Lys84 in the nucleotide-bound states can indicate the possibility that the reactive lysine region containing essential contacts between the motor domain and the neck region is more flexible in the *apo* state and thus, nucleotide binding can induce positioning and possibly (partial) priming of the lever arm. This idea remains to be tested experimentally. Nonetheless, the structural interface containing Lys84 undoubtedly plays a key role in the function of myosin, an enzyme designed to carry out motor function.

Acknowledgements

We thank Drs Andras Muhlrad and James R. Sellers for discussions. This work was supported by OTKA grants T043746, T032443 and M37053 to LN, the BBSRC to CRB, and the Hungarian Ministry of Education Fund (OMFB-00718/2003) to AMC.

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