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# Tryptophan-130 is the most reactive tryptophan residue in rabbit skeletal myosin subfragment-1

Y. Michael Peyser, Andras Muhlrad and Moshe M. Werber*

*Department of Oral Biology, Hebrew University–Hadassah School of Dental Medicine, Jerusalem 91010* and **Bio-Technology General, Kiryat Weizmann, Rehovot 76326, Israel*

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Rabbit skeletal muscle myosin subfragment-1 (S-1) was reacted with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide (DHNBS) resulting in modification of 0.8 tryptophan residues per S-1. In order to assign the most reactive tryptophan of the 5 S-1 tryptophans, antibodies were raised in rabbits against bovine serum albumin modified with DHNBS. The antibodies reacted with the 27 kDa tryptic fragment of DHNBS-treated S-1, indicating that the reactive tryptophan resides on this domain. The 27 kDa fragment was isolated from DHNBS-treated S-1 and was further cleaved at a single cysteine residue by 2-nitro-5-thiocyanobenzoic acid. This cleavage resulted in two peptides, each of them containing one tryptophan. The antibodies reacted with the smaller peptide consisting of residues 122–204. The only tryptophan residing on this peptide is $Trp^{130}$, and this is therefore the most reactive tryptophan of S-1.

Myosin; Subfragment-1; Tryptophan

## 1. INTRODUCTION

Myosin subfragment-1 (S-1), the head segment of myosin, contains separate sites responsible for ATPase activity and actin interaction. Limited tryptic digestion cleaves the heavy chain of rabbit skeletal S-1 into three trypsin-resistant fragments, 27, 50 and 20 kDa, aligned in this order from the N-terminus [1]. The binding and hydrolysis of ATP by S-1 are accompanied by transient conformational changes which were discovered by monitoring the spectral characteristics of the Trp's of S-1. Morita [2] observed a UV difference spectrum upon addition of ATP or ADP to rabbit skeletal myosin; the difference is due to the perturbation of the absorption spectrum of the Trp's. Werber et al. [3] found a significant increase in the Trp fluorescence emission upon addition of various nucleotides to myosin, HMM and S-1. This Trp fluorescence enhancement was successfully used to describe the kinetic scheme of the myosin-catalyzed ATPase cycle [4,5]. Furthermore, one of the five S-1 Trp's, $Trp^{130}$, was found to react specifically with a photoaffinity analog of ATP and, based on this finding, it was suggested that this Trp is near or at the ATP binding site of S-1 [6].

The aforementioned relation of the Trp's to myosin function made it imperative to characterize the individual Trp residues in S-1. Torgerson [7] classified the five Trp's of S-1 into three life-time classes on the basis of their decay-associated spectra. We have recently used the water-soluble Trp reagent, DHNBS [8], for modifying S-1, and found that the most reactive Trp(s) reside(s) on the 27 kDa fragment [9]. This fragment contains two Trp's, $Trp^{112}$ and $Trp^{130}$ [10], and it was not clear which of these, or maybe both, was modified. In order to solve this problem we raised antibodies against the HNB moiety and by a combination of a specific chemical cleavage and immunoblotting of the isolated, HNB-labeled, 27 kDa fragment, we concluded that $Trp^{130}$ is the most reactive Trp residue in S-1.

*Correspondence address:* M.M. Werber, Bio-Technology General, Kiryat Weizmann, Rehovot 76326, Israel

*Abbreviations:* DHNBS, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide; HNB, 2-hydroxy-5-nitrobenzyl; NTCB, 2-nitro-5-thiocyanobenzoic acid; S-1, myosin subfragment-1

## 2. MATERIALS AND METHODS

### 2.1. *Preparation of protein*

Myosin was prepared from the back and leg muscles of rabbits [11]. S-1 was prepared by digestion of myosin filaments with chymotrypsin [12].

### 2.2. *Modification of S-1 by DHNBS*

S-1 was modified at 0°C at pH 6.0 in 20 mM histidine hydrochloride buffer, by adding a 10-fold molecular excess of DHNBS over S-1 [9]. After incubation of S-1 with DHNBS for 10 min, the reaction was quenched by adding 5 mM DTE, and the excess reagent was removed by dialysis.

### 2.3. *Preparation of the 27 kDa tryptic fragment of S-1 heavy chain*

The 27 kDa fragment was prepared from the tryptic digest of DHNBS treated S-1, essentially by the method of Muhlrad [13] with the exception that urea was removed by dialyzing the 27 kDa fragment against 10 mM ammonium acetate, 0.6 mM EDTA and 3 μg/ml leupeptin. A second dialysis was performed against 10 mM ammonium acetate + 0.5 mM EDTA, and finally the pure 27 kDa fragment was lyophilized.



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2.4. *Preparation of anti-HNB antibody*

BSA whose Trp's were labeled with HNB (HNB-BSA) was used as antigen for immunization. Modification was performed by adding a 200-fold molar excess DHNBS over BSA in 20 mM histidine buffer, pH 4.3. The reaction was stopped after 10 min incubation at 0°C by DTE added in two-fold molar excess over DHNBS. After 30 min the solution was mixed with complete Freund's adjuvant in an 1:1 ratio. The concentration of BSA in the mixture was 2 mg/ml. Rabbits were immunized subcutaneously at two sites with 2.4 mg HNB-BSA. Six weeks later a booster injection was added (0.5 mg of HNB-BSA in incomplete Freund's adjuvant in 1:1 ratio, final volume 1 ml), which was repeated again after 6 weeks. Finally, seven days later the blood containing the antiserum was collected.

2.5. *Chemical cleavage of 27 kDa fragment*

The method of Sutoh [14] was used to cleave the 27 kDa fragment at $Cys^{112}$ with NTCB. Briefly, to 1 mg 27 kDa fragment in 1 ml 20 mM borate buffer, pH 8.0, and 6 M urea, 40 μl 0.1 M NTCB in 33% dimethyl formamide was added. After 1 h of incubation at 25°C, the pH of the reaction was raised to 9.0 by NaOH, and the reaction mixture was further incubated at 55°C for 3 h. (To obtain a good cleavage it was necessary to incubate the reaction mixture at 37°C overnight or at 55°C for 3 h.) The reaction was quenched by adding an 80-fold molar excess of 2-mercaptoethanol over NTCB.

2.6. *SDS-PAGE and immunoblotting*

Electrophoretic analysis of samples was performed on gradient slab gels. For immunoblots, protein bands were electrophoretically transferred from SDS-PAGE to a nitrocellulose membrane and either stained by amido black stain or immunostained by antibodies. The following antibodies were used: anti-HNB; 'anti N-terminus' monoclonal antibody, whose epitope is located near to the N-terminus of the S-1 heavy chain [15]; 'anti 142-148' polyclonal antibody which was raised against a synthetic peptide containing the $Tyr^{142}$-$Gln^{148}$ sequence of the 27 kDa fragment [16]. The reacting protein bands were visualized by alkaline phosphatase-conjugated second antibodies.

## 3. RESULTS

Anti-HNB antibody was used to detect the location of the most reactive Trp (towards DHNBS) in S-1. The antibody was raised against DHNBS-modified BSA in rabbits. Care was taken to modify only the Trp residues of BSA by performing the reaction at low pH (pH 4.3), which [8] prevents the modification of thiol groups. By using the resulting HNB-BSA conjugate for immunization of rabbits, a high titer antiserum was obtained. Good immunostaining was observed with DHNBS-modified S-1 in Western blots, even after an 8000-fold dilution of the antiserum (result not shown).

In order to detect the most reactive Trp in S-1, we modified S-1 by a 10-fold molar excess of DHNBS resulting in the incorporation of 0.8 HNB per S-1 [9]. The DHNBS-treated S-1 was subjected to limited tryptic digestion and the resulting peptides were separated on SDS-PAGE and analyzed by Western blot using the anti-HNB antiserum (fig.1). In the amido black-stained membrane (fig.1A) the three tryptic fragments of the S-1 heavy chain (27 kDa, 50 kDa and 20 kDa) together with the 75 kDa intermediate (made of the 27 kDa and 50 kDa fragments) as well as the light chains (25 kDa and 16 kDa) are easily recognizable. Of all these peptides only the 27 kDa and 75 kDa reacted strongly with

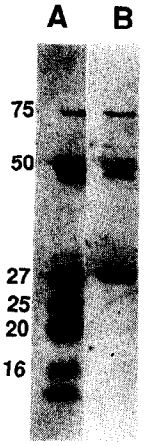


Fig.1. Reaction of the anti-HNB antibody with trypsin digested HNB S-1. Nitrocellulose membrane stained by amido black (A); immunostained by anti-HNB antibody (B). Vertical numbers refer to apparent molecular masses in kilodaltons. For molecular masses of the tryptic fragments of S-1 see [22].

the anti-HNB antibody (fig.1B). This indicates, in agreement with our previous findings [9], that the 27 kDa fragment contains the most reactive Trp residue of S-1. The 27 kDa fragment contains $Trp^{112}$ and $Trp^{130}$ [10] and it was necessary to separate them in order to identify the most reactive residue. The separation was achieved by cleaving the polypeptide chain between the two Trp's at $Cys^{122}$ with NTCB, which cuts peptide bonds specifically at the amino end of cysteine residues [14,17]. Since the 27 kDa fragment contains only one cysteine, $Cys^{122}$ [10] the NTCB treatment of the isolated 27 kDa fragment yields two peptides, a larger one (residues 1–121) containing the N-terminus and a shorter one (residues 122–204) containing the C-terminus of the 27 kDa fragment. Following NTCB treatment the 27 kDa fragment, which had been isolated from DHNBS-modified S-1, was electrophoresed on SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane, which was cut into four pieces. One piece was stained by amido black (fig.2A) and the other three were immunostained by 'anti N-terminus', 'anti-HNB' and 'anti 142–148' antibodies; fig.2B, fig.2C and fig.2D, respectively. Of the two peptides (14 and 11 kDa) resulting from the NTCB cleavage, only the smaller 11 kDa peptide reacted with the anti-HNB antibody. Since this peptide stretches from $Cys^{122}$ to the C-terminus of the 27 kDa fragment and contains only one Trp, $Trp^{130}$, it is clear that this Trp is the most reactive. The reaction of the other two antibodies in the immunoblots supports the electrophoretic mobility-based identification of the NTCB peptides and contributes to the identification of the reactive Trp. The 'anti N-terminus' antibody reacts with the larger 14 kDa peptide identifying it as residues 1–121, whereas the 'anti 142–148' antibody reacted with the smaller 11 kDa peptide identifying it as residues 122–204. Since the latter peptide reacted also with the

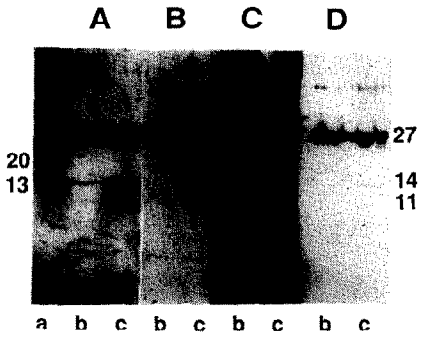


Fig.2. Immunoblot of NTCB-treated HNB 27 kDa fragment. Nitrocellulose membrane stained by amido black (A); immunostained by 'anti-HNB' (B); by 'anti 142–148' (C); and by 'anti N-terminus' (D) antibodies; (a) molecular weight markers (cytochrome *c*, 13 kDa, and soybean trypsin inhibitor, 20 kDa); (b) HNB 27 kDa fragment; (c) NTCB-treated HNB 27 kDa fragment. Numbers on the sides refer to apparent molecular masses in kDa.

anti-HNB antibody all the results lead to the conclusion that the only Trp of this peptide, $Trp^{130}$, is the most reactive Trp residue of S-1.

## 4. DISCUSSION

In this communication it is shown that the most reactive Trp – towards DHNBS – in S-1 is $Trp^{130}$. This conclusion is in agreement with our previous findings that the DHNBS reactive Trp(s) reside on the 27 kDa fragment, that their fluorescence is red-shifted [9], and that the 27 kDa Trp's are accessible to acrylamide [14], while the rest of the S-1 Trp's which are located on the 50 kDa fragment are hardly accessible to this polar quencher [18]. On the basis of its high reactivity and accessibility to acrylamide, $Trp^{130}$ can be assigned to the 'long life-time' Trp class of Torgerson [7]. Accordingly, it has a long fluorescence life-time, a red-shifted fluorescence emission spectrum and it is located in a relatively polar environment on the surface of the molecule [7,9,13].

It was shown by Okamoto and Yount [6] that a photosensitive analog of ATP did covalently label the same $Trp^{130}$. On the basis of this finding, it was assumed that $Trp^{130}$ is in the vicinity of the binding site of the purine moiety of ATP or that this residue is part of the binding site. Because of its closeness to the binding site it was also believed that $Trp^{130}$ may be the 'ATP- sensitive' Trp, whose UV spectrum is red-shifted [2] and fluorescence emission is enhanced [3] upon binding and hydrolysis of the nucleotide. However, in light of our recent findings that the introduction of one HNB per S-1 (shown here to reside on $Trp^{130}$) does not affect actin-activated ATPase, and causes only minor changes in the $K^+$(EDTA)- and $Ca^{2+}$-activated ATPase activities of S-1 [9], it seems improbable that $Trp^{130}$ is directly involved in the binding of ATP or in its hydrolysis. It is much more plausible that ATP attaches to the 'consensus' binding site stretching from $Gly^{178}$ to $Val^{186}$ [10], since it was shown [19] that $Ser^{180}$ within this stretch can be specifically photooxidized by UV irradiation of the MgADP-vanadate-S1 complex. Similarly, it seems improbable that $Trp^{130}$ is the 'ATP-sensitive' Trp, since we have previously shown [9] that upon introduction of 2.2 HNB residue per S-1, no significant change was observed in the fluorescence enhancement caused by addition of MgATP. Furthermore, it had been shown both in peptides [20] and proteins [21] that the fluorescence intensity of Trp residues which are in contact with purine moieties of ATP is not enhanced but rather decreased, due to the quenching of these fluorophores. The 'ATP-sensitive' Trp(s) are probably located in the 50 kDa fragment, since it was shown by Torgerson [7] that the 'ATP-sensitive' Trp(s) belong to the intermediate life-time class and that the Trp's of this class are not quenchable by acrylamide. According to our results [18], the non-quenchable Trp's reside on the 50 kDa fragment.

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