# STUDY OF THE STRUCTURE OF HMM · VANADATE COMPLEX

Katalin AJTAI, L. SZILÁGYI and E. N. A. BIRÓ
*Department of Biochemistry, Eötvös Loránd University, VIII. Puskin utca 3, 1088 Budapest, Hungary*

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## 1. Introduction

Vanadate ($H_2VO_4^-$) an analogue of phosphate ion inhibits several phosphate hydrolysing enzymes [1]. The reaction of myosin ATPase with $V_i$ has been examined in [2–4]. In [4], a slowly developing inhibition was described: as ATP splitting in the presence of $V_i$ progresses, a stable irreversible complex forms containing 1 mol ADP and 1 mol $V_i$/mol active site. This complex is enzymatically inactive. The results in [4] (the essential role of nucleotide for the effect of $V_i$, the inhibition of ATPase activity and the lack of fluorescence enhancement after addition of ATP to the HMM · ADP · $V_i$ complex) suggest that both ADP and $V_i$ occupy the active site of myosin ATPase in the ternary complex.

This work aimed to gain structural information on this stable complex to obtain new insight into the structure and function of the myosin ATPase.

Our results show that the tryptic digestion pattern of the HMM · ADP · $V_i$ complex differs from that obtained by digestion of HMM without any addition and is identical with the digestion pattern obtained in the presence of high excess of ATP or ADP. The HMM · ADP · $V_i$ ternary complex does not bind to F-actin, a further similarity of its structure to that of the HMM · ADP · $P_i$ intermediate in the ATPase reaction.

## 2. Materials and methods

ATP, ADP, $NH_4VO_3$ and chymotrypsin were from Sigma (St Louis), [$^{14}$C]ADP was from Amersham (Bucks). All other reagents were of analytical grade from Reanal (Budapest). HMM was prepared according to [5] and actin according to [6]. Protein concentrations were estimated either spectrophotometrically using $A^{1\%}_{280} = 6.48$ for HMM, $A^{1\%}_{290} = 6.3$ for actin or using the biuret method according to [7]. HMM · ADP · $V_i$ complex was formed and isolated essentially as in [4]. $V_i$ was determined by the method in [8] as applied [4]. HMM treated as described below was separated before further experiments from the excess of reagents by Sephadex G-50 gel filtration. Control proteins were kept in the same conditions, as the treated samples.

HMM was fragmented with trypsin as in [9,10]. Proteolysis was at a 1:50 trypsin/HMM ratio (w/w) at 20°C. Medium contained 3 mg HMM/ml, 90 mM NaCl, 20 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$ and in part of the experiments 10 mM EDTA. Concentrations of substrates and analogues were: 5 mM phosphate/pyrophosphate/ADP or ATP, respectively. At various times aliquots were taken from the digestion mixtures. The polypeptide fragments were separated by slab SDS–PAGE as in [11].

Actin · HMM formation was studied by preparative ultracentrifugation. Samples containing 2.5 mg HMM, 2 mg actin, 0.1 M NaCl, 20 mM Tris–HCl (pH 8) in 4 ml were ultracentrifuged at 4°C (after 10 min incubation at room temperature) for 2 h at $8 \times 10^4 \times g$. Pellets and supernatants were separated, $V_i$ was determined and SDS–PAGE done on each fraction. [HMM] were calculated from the densitometric tracings of the stained gels. Concentrations are expressed in % of the total HMM or of the sum of $V_i$ measured in the pellet + supernatant, respectively.

*Abbreviations:* HMM, heavy meromyosin; $V_i$, vanadate; $P_i$, phosphate; SDS–PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; A · HMM, acto-HMM; $M_r$, relative molecular mass; $LC_1$ and $LC_3$, light chains of HMM



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## 3. Results and discussion

The amount of vanadate and ADP incorporated in HMM agreed well with [4]: HMM contained 0.8–1.0 mol $V_i$, 0.75 mol [$^{14}$C]ADP/active site (HMM 3.6 × $10^5$ $M_r$).

Tryptic digestion of HMM is a sensitive method for detection of conformational changes induced by substrate [9,10]. To elucidate the conformation of HMM · ADP · $V_i$ complex, tryptic digestion pattern of this complex was compared to that of native enzyme digested in the presence of substrate analogs (phosphate, pyrophosphate, ADP, ATP) both in the presence of mono- and di-valent cations. The digestion patterns of the samples at two different times are shown in fig.1.

Addition of phosphate or pyrophosphate, both in the presence (A) or absence (B) of divalent cations has no influence on the course of proteolysis (fig.1). Lanes 2 and 3 do not differ from the control (lane 1). The heavy chain fragments [9] are present: 27, 50 and 60 × $10^3$ $M_r$ in the presence (A) or 27, 50, 20 and 37 × $10^3$ $M_r$ in the absence (B) of divalent cations (i.e., presence of EDTA). In contrast if ATP or ADP is added, new fragments of 47 and 21 × $10^3$ $M_r$ are formed. The presence of nucleotides opens up new trypsin-sensitive points, which allow the 50 → 47 and 27 → 21 × $10^3$ $M_r$ degradations as in [10]. The splitting of HMM · ADP · $V_i$ complex follows this second type of proteolysis. As the above influences of the nucleotides result from conformational changes during nucleotide binding, we may assume that the stable ternary complex has a similar conformation to that of the HMM · ADP or HMM · ATP complexes. $V_i$ in the complex seems to have an indirect influence on proteolysis by fixing ADP to HMM. Fig.1, lane 6 was obtained by digestion of the isolated ternary complex while lanes 4 and 5 show digestions in the presence of excess nucleotides.

Our proteolysis experiments support the view [4]

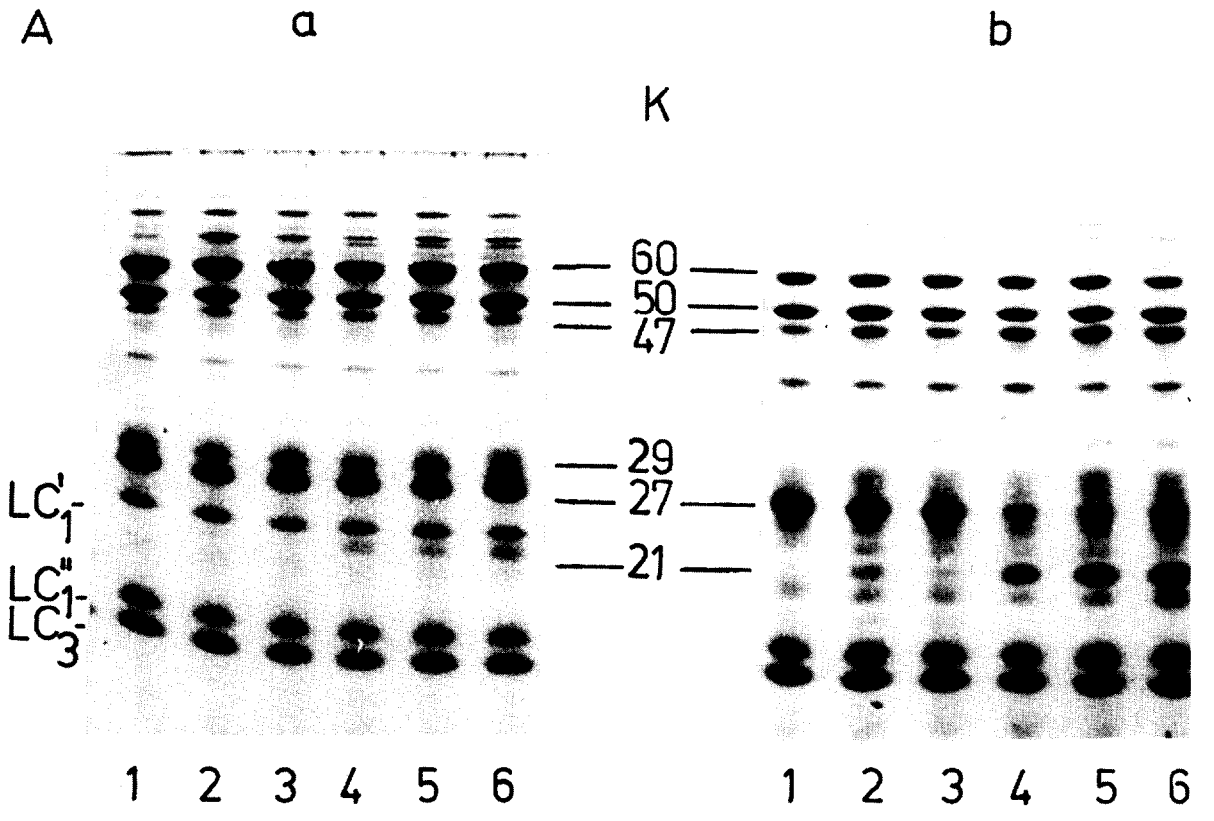


Fig.1. Slab SDS–PAGE of HMM and HMM · $V_i$ · ADP complex digested by trypsin under different conditions: (A) digestion in the presence of 5 mM $MgCl_2$.

Table 1
Protein and vanadate concentrations of pellets and supernatants of actin binding experiments

| | Acto · HMM | | Acto · HMM · ADP · $V_i$ | |
|---|---|---|---|---|
| | Pellet | Supernatant | Pellet | Supernatant |
| HMM | 91 | 9 | 31 | 69 |
| $V_i$ | – | – | 12 | 88 |

that the ternary complex is analogous to one of the intermediates of the ATPase reaction. This assumption is further supported by the following actin-binding experiments with this complex.

Isolated HMM · ADP · $V_i$ complex was ultracentrifuged in the presence of equivalent (w/w) concentrations of F-actin. As control, untreated HMM was subjected to the same procedure. After ultracentrifugation, pellet and supernatant were analysed for the amount HMM present (section 2) and where feasible for $V_i$. About 70% of an HMM preparation containing 0.85 mol $V_i$/head remained unbound to actin (table 1). Simple statistical calculations (assuming that there is no interaction between the 2 heads) show that when 85% of the heads binds $V_i$, 72% of the HMM molecules must be blocked on both heads, 26% on one head and only 2% unmodified HMM can be present. The results correlate well if we assume that only HMM blocked by $V_i$ on both heads remains unbound. If HMM blocked on one head can still be bound to actin and the complex formation of one head with ADP and $V_i$ does not interfere with the actin binding of the other head, we would expect ~30% of HMM still to be capable to bind actin, and this is indeed found in the experiment.

The fact that HMM · ADP · $V_i$ complex cannot bind to actin agrees well with observations obtained by using more complex systems. The presence of vanadate with ADP or ATP abolished tension development in glycerinated insect flight muscle fibers [12]. A similar effect, using chemically skinned smooth muscle fibers, was observed in [13].

Myosin · ATP and myosin · ADP · $P_i$ complexes have a reduced affinity for actin as compared to the

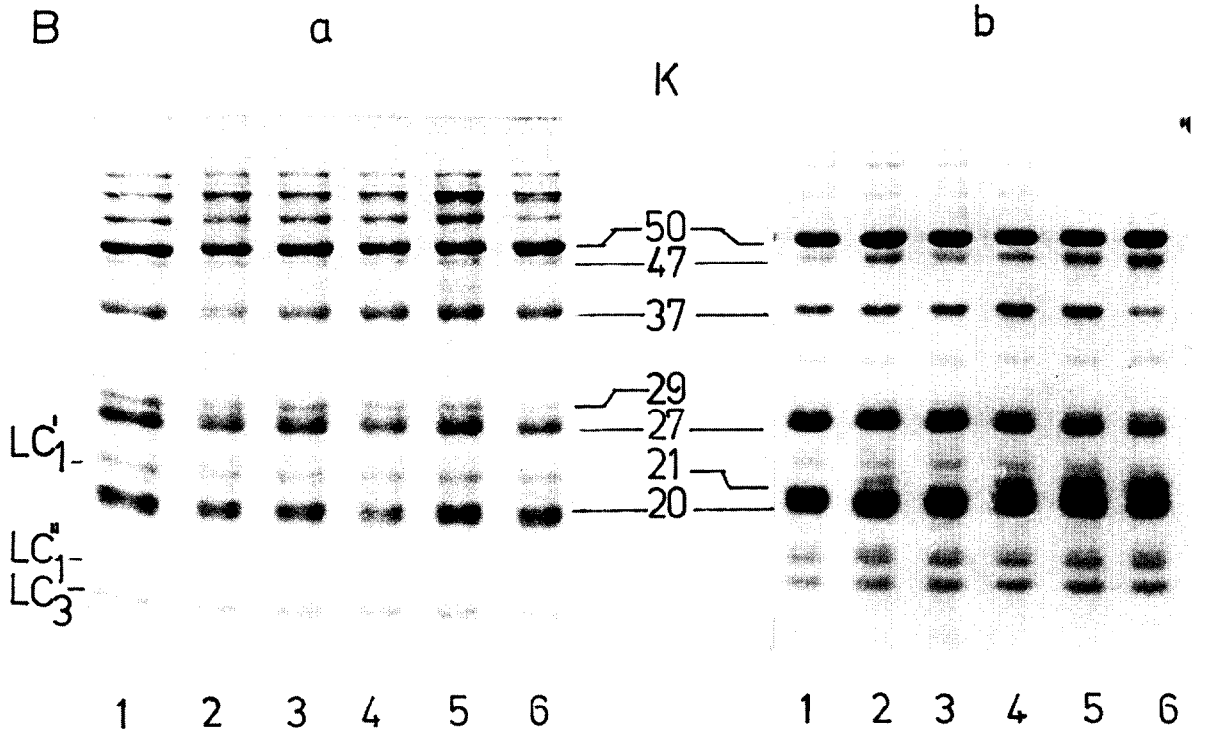


Fig.1. (B) digestion in the absence of divalent cations (10 mM EDTA); (a) 5 min, (b) 30 min digestion. Other conditions of digestion in section 2. Lanes (1–5) HMM; (6) HMM · ADP · $V_i$ complex; (1,6) no addition; (2) pyrophosphate; (3) phosphate; (4) ADP; (5) ATP added in the course of digestion; all cases at 5 mM concentration. LC' and LC'' are the tryptic fragments of $LC_1$.

affinity of myosin · ADP complex [14–16]. Direct binding experiments and X-ray diffraction data [12] strongly suggest that the conformation of the HMM · ADP · $V_i$ complex is structurally analogous to the HMM · ATP or HMM · ADP · $P_i$ conformation.

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