69. The Enthalpy Change Accompanying the Binding of Calcium to Troponin Relating to the Activation Heat Production of Muscle

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The term "activation heat" was introduced by Hill (1949) to describe part of the twitch heat produced by muscle, and was ascribed to the processes of activating contraction. Recently the essential feature of Ca ions regulating the contraction has been clarified (Ebashi & Endo, 1968). Accordingly the activation heat has been supposed to be the thermal sign of the movement of Ca ions following the stimulation (Woledge, 1971). Indeed, Smith (1972) and Homsher *et al.* (1972) have shown that even in a highly stretched frog semitendinosus muscle, where practically no overlap between the thick and thin filaments has remained, a substantial fraction of the initial heat is still produced. Since the discovery of troponin by Ebashi & Kodama (1965) as the Ca-receptive protein located along the thin filament it has been well established that Ca released from the sarcoplasmic reticulum is bound to troponin as the first step to activate the contractile system (Ebashi & Endo, 1968). As troponin binds Ca very rapidly and very strongly when it is released from part of the sarcoplasmic reticulum, thermal changes associated with this process could mainly be responsible for the production of the activation heat in muscle. The present work was undertaken to determine enthalpy changes accompanying the binding of Ca to troponin *in vitro* and to examine whether the heat of troponin-Ca binding reaction is in exact balance with the activation heat production in muscle.

Calorimetric observations have been made mostly with a modified Rhesca rotation microcalorimeter, the principal design of which is based on Tian-Calvet's conduction microcalorimeter (Calvet & Prat, 1963). Troponin was extracted from rabbit skeletal muscle following the procedure of Ebashi *et al.* (1971). To remove bound Ca, troponin (about 10 mg/ml) was passed through a column of Dowex-Al chelating resin. After passing the column, the level of Ca bound was

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Fig. 1. Heat produced in kJ per mole of Ca added to troponin in the presence of 1 mM MgCl_2 , 0.1 M KCl, 40 mM carnosine (pH 7.0-7.2) at 10°C . Ca ions were introduced to Ca-free troponin in 3 steps and the average heat production in each step has been drawn against the changes in the level of the apparent saturation of Ca to the troponin sites (moles Ca added per mole of troponin).

reduced to less than 0.1 mole per mole troponin.

Heat of binding of Ca to troponin in the presence and absence of Mg ions. In Fig. 1 are shown heats produced by introducing Ca ions to troponin in the presence of 1 mM MgCl_2 (in kJ per mole of Ca added) against the apparent saturation of Ca to the troponin sites (moles Ca added per mole of troponin) at 10°C . Ca ions were introduced to Ca-free troponin in 3 steps, each consisting of $0.5 \mu\text{mole}$. Before initiating the reaction, $0.5 \mu\text{mole}$ Ca in 1 ml solution was in the smaller compartment and 40 to 50 mg troponin in 10 ml solution in the main compartment of the chamber. Both solutions contained 0.1 M KCl, 1 mM MgCl_2 and 40 mM carnosine at a pH ranging from 7.0 to 7.2. The pH of both solutions were brought as close as possible to each other within about 0.05 unit. Since it has been known that each troponin molecule has two classes of Ca-binding sites, with their intrinsic binding constants of $1.3 \times 10^6 \text{ M}^{-1}$ and $5 \times 10^4 \text{ M}^{-1}$ respectively, each class consisting of two equivalent sites (Ebashi *et al.* 1968), about 90 percent of the Ca introduced in the first step should be bound almost exclusively to the first class of sites having the higher affinity and about 60 percent of that in the last step almost exclu-

Fig. 2. pH changes of troponin solutions (5 mg/ml) by introducing $0.5 \mu\text{mole}$ CaCl_2 at different pH levels in the presence of 1 mM MgCl_2 and 0.1 M KCl at 10°C . "Ca" indicates the introduction of $0.5 \mu\text{mole}$ Ca, total Ca-binding capacity of troponin being about $2 \mu\text{moles}$.

sively to the second class. Thus enthalpy changes associated with the binding of Ca to troponin could be estimated at -39 kJ (-9.3 kcal) per mole of Ca bound to the first class and -74 kJ (-17.7 kcal) per mole of Ca bound to the second class of sites, from the results in Fig. 1. Moreover, enthalpy changes of troponin-Ca interaction for the first class of sites were less in the absence than in the presence of Mg. If we could justifiably adopt the method of extremely stretching muscles to separate the true activation heat from that part derived from the actin-myosin ATPase activity, leaving the activation processes intact, then the activation heat would be around $2.6\text{--}4.2 \text{ mJ}$ ($0.6\text{--}1.0 \text{ mcal}$) per gram muscle (Smith, 1972; Homsher *et al.* 1972). As one gram muscle contains about $0.07 \mu\text{mole}$ troponin (Ebashi *et al.* 1969), one gram muscle would have a total of $0.28 \mu\text{mole}$ Ca-binding sites, assuming 4 sites per troponin molecule. According to Weber & Herz (1963) the amount of exchangeable Ca is on average $1.6 \mu\text{moles}$ per gram of myofibrils and a removal of $1 \mu\text{mole}$ Ca per gram myofibrils is sufficient to fully inhibit the superprecipitation and ATPase activity in the presence of 4 mM MgATP. Assuming there are 0.1 gram of myofibrils per gram muscle, $1 \mu\text{mole}$ Ca per gram myofibrils would be equivalent to $0.1 \mu\text{mole}$ Ca per gram muscle. Thus $0.1 \mu\text{mole}$ Ca bound to troponin per gram muscle, or the heat production of 3.9 mJ (0.93 mcal) per gram muscle, in view of the enthalpy change of -39 kJ per mole Ca bound to the first class of sites, would be required for the complete activation of contraction. This fits in well with the upper limit of the activation heat.

Proton exchanges of troponin on Ca binding. To examine whether protons were released from troponin on Ca binding, changes in pH were measured following the addition of Ca to Ca-free troponin. Ca ions were introduced in 4 steps, each consisting of 0.5 μ mole Ca in 50 μ l, Ca-binding capacity of troponin being about 2 μ moles. As seen in Fig. 2-A, the pH of the troponin solution slightly increased in each step, when Ca ions were introduced at the neutral pH in the presence of Mg. In contrast to this, troponin at pH 5.5 showed a prominent release of protons, becoming less prominent in the second step and not observed in the subsequent steps (Fig. 2-B). The acidification was titrated back with NaOH, indicating that about 0.4 μ mole protons were released by the first introduction of 0.5 μ mole Ca. Thus at the neutral pH the release of protons on Ca binding to troponin and their subsequent interaction with the buffer system present would not affect the observed heat appreciably.

Changes in the sedimentation pattern. Sedimentation pattern has been known to be altered by the level of Ca bound to troponin (Wakabayashi & Ebashi, 1968). The pattern was very broad in the absence of bound Ca, recovering gradually towards the original single peak when Ca was introduced in steps. The changes in the profile induced by the Ca deprivation became more prominent in the absence of Mg ions. From these results it is apparent that the large aggregates of troponin molecules dissociate towards the original single molecules when Ca ions are introduced to Ca-free troponin. A possibility exists therefore that the dissociating reaction of the associated troponin molecules has a positive enthalpy change, thus absorbing some heat.

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