HEAT, PHOSPHORUS NMR AND MICROCALORIMETRY IN RELATION TO THE MECHANISM OF FILAMENT SLIDING

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SUMMARY

For muscle heat measurements the methods available are sensitive and rapid, and the heat is related to the chemical changes in a manner that provides a firm outline for understanding the mechanism of contraction. For example linear dependence of the shortening heat on the sarcomere length has shown that the rate of turnover of cross-bridges increases during shortening. However, heat is bound to lack *specificity.*

In order to cope with this problem, various methods such as rigorous chemical analyses, phosphorus NMR and microcalorimetry have been introduced. As a result of ultra-rapid freezing and chemical analysis by D. R. Wilkie (Gilbert, Kretzchmar, Wilkie and Woledge, 1971), the energy balance discrepancy between (heat + work) and the amount of phosphocreatine (PCr) split emerged, i.e. the unexplained enthalpy.

Calcium ions move from the sarcoplasmic reticulum to the calcium-receptive proteins in the sarcoplasm during contraction. In an attempt to find the cause of the unexplained enthalpy, microcalorimetry of calcium binding to calcium-receptive proteins has been performed. The results have shown that calcium ions dislocated between sites within the sarcoplasm on activation may produce about 1/3 of the unexplained heat. In addition calcium pump should operate by consuming PCr to relocate the calcium after the contraction.

Time-resolved phosphorus NMR has also shown that a certain amount of PCr splitting continues during early minute of recovery period after the contraction without Pi released. This delayed splitting of PCr is most likely caused by the kinetic properties of the contractile proteins and can explain another 1/3 ofthe unexplained enthalpy.

The mechanism of how muscle is regulated is another important question. Studies of calcium binding to calcium-receptive proteins in the sarcoplasm by using titration

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microcalorimetry has shown that troponin C has a characteristic single calcium-binding site that is most likely to be involved in the regulation of contraction.

INTRODUCTION

In 1959 A. V. Hill (see Hill, 1965) wrote "chemical methods are usually extremely slow and insensitive... particularly since most chemical estimations in living cells involve the destruction of the material itself. For measuring heat, however, the methods available are very sensitive and can be made very rapid. The heat is related to the chemical exchanges, not always indeed in a specific way, but at least in a manner which provides a firm outline that must not be overstepped and can be filled in as knowledge accrues."

Following A. V. Hill much effort has been made to improve sensitivity in heat measurements. However heat measurements are bound to lack *specificity*. In order to cope with this problem, various methods have been introduced. Wilkie introduced, in addition to a rigorous chemical approach, phosphorus NMR (Dawson, Gadian and Wilkie, 1979), and Woledge introduced microcalorimetry in the field (Woledge, 1973). Since then several novel findings and associated advances in understanding have been attained. I would like to discuss what problems have been solved and what problems remain.

ENERGY BALANCE STUDIES AND THE EMERGENCE OF THE UNEXPLAINED ENTHALPY

Wilkie introduced a novel hammer apparatus for freezing muscles rapidly. This was to cope with the above non-specificity problem by using rigorous chemical analysis. As a result a gap emerged in energy balance (the unexplained enthalpy, Gilbert et al., 1971).

The result was that the break-down of PCr was not large enough to account for the heat produced during the first few s of isometric contraction. By the end of a 15 s tetanus as much as 42 mJ/g remained unaccounted for. Gilbert et al. (1971) have suggested some process which could produce about 42 mJ/g during the early part of a tetanus and which is not reversed during a whole tetanus. One is binding of calcium ions to proteins, and the conformational changes of the proteins. 42 mJ/g muscle is equivalent to 105 kJ per mole of moved calcium, by assuming 0.4 μmole calcium moved between sites within the sarcoplasm per g muscle (Winegrad, 1970).

Break-down of PCr continues during the minute following relaxation, but without heat production. Similarities have been noticed between the unexplained enthalpy and the labile part of the maintenance heat production, and are discussed, together with differences, by Woledge, Curtin and Homsher (1965).

It has been hypothesized that the unexplained energy is produced by the binding of calcium ions to parvalbumins (see for example Woledge, Curtin and Homsher, 1985). Concentration of parvalbumins in the sarcoplasm (0.4 μmole/g, Gosselin-Rey and Gerday, 1977) is considerably higher than that of troponin (0.07 μmole/g, Ebashi, Endo and Ohtsuki, 1969). Calcium binding to parvalbumins of frog muscle produces heat of 28

kJ/mole (average of two major isotypes at 5°C, Tanokura and Yamada, 1987), therefore calcium binding to parvalbumins would produce heat of 22 mJ per g muscle. This amount of heat corresponds to about a half of the unexplained enthalpy.

A comparison of total myoplasmic calcium and total calcium-binding capacity indicates that about a half of the calcium-binding sites remain unsaturated during a prolonged tetanus (Hou, Johnson and Rall, 1991). Moreover, kinetic analyses of calcium movement in the sarcoplasm during contractile activation have shown that the calcium pump of the sarcoplasmic reticulum may operate in parallel with the calcium binding to parvalbumins (Hou, Johnson and Rall, 1993). Therefore the above figure is likely to be an overestimate.

Another possible explanation of the unexplained enthalpy is delayed PCr splitting. PCr splitting that continues after contraction has ended has long been known to exist during the minute after relaxation (see Gilbert et al., 1971). It is very likely that the post-contractile splitting of PCr is coupled to the reversal of an unidentified exothermic process occurring early in the contraction. A quantitative estimate of the amount of the delayed splitting of PCr by time-resolved phosphorus NMR in living muscles is described in the later section of this article.

ACTIVATION HEAT

Heat production during isometric tetanic contractions depends both on time after the contraction is initiated, and on muscle lengths (see Woledge et al., 1985). The rate of heat production that is proportional to filament overlap is most certainly derived from actin-myosin interaction.

The activation heat, measured in twitch contractions with muscles stretched to lengths where active force development is nearly abolished, is very likely to be related to activation processes by calcium (Smith, 1972; Homsher, Mommaerts, Ricchiuti and Wallner, 1972). It can be deduced from studies on repriming of the activation heat that this component of heat most likely represents calcium release from the sarcoplasmic reticulum and subsequent interaction with troponin on the thin filament to activate contraction (Rall, 1980).

Calcium release process from the sarcoplasmic reticulum is most likely thermo-neutral because heat production during the ATP-driven calcium uptake by sarcoplasmic reticulum is small except for that of ATP hydrolysis (Kodama, Kurebayashi and Ogawa, 1980). Therefore, the fastest part of the activation heat at least should be caused by calcium binding to troponin.

Enthalpy change of calcium binding to calcium-specific sites of troponin is 74 kJ/mole calcium bound (Yamada, Mashima and Ebashi, 1976, see Fig. 1). Yamada et al. (1976) have discussed that 0.1 μmole of calcium ions per g of muscle, which is closely involved on contractile activation (Weber and Herz, 1963), would produce 7.4 mJ per g of muscle. This is more than necessary to explain the activation he at, which is about 4 mJ/g muscle maximally. Therefore the activation heat most likely represents the amount of calcium released by the sarcoplasmic reticulum on activation.

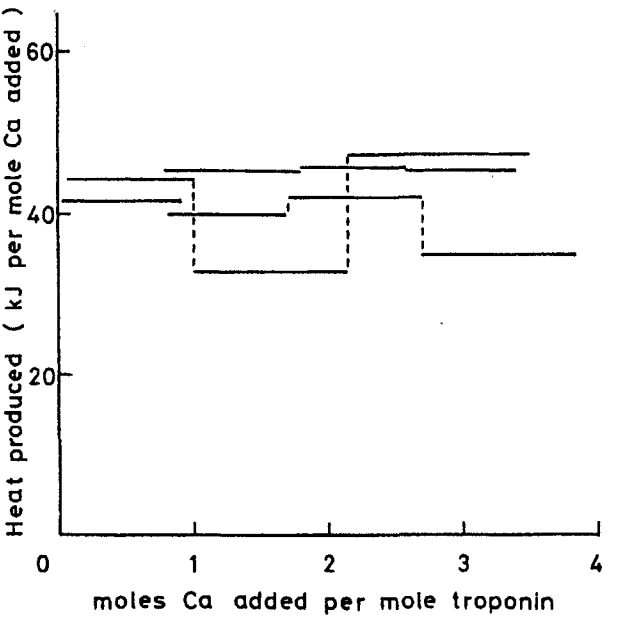

Fig. 1. Heat produced per mole of Ca added to troponin (rabbit skeletal) in 1 mM Mg (10°C). Reproduced with permission from Yamada, Mashima and Ebashi, 1976.

DEPENDANCE OF SHORTENING HEAT ON SARCOMERE LENGTH

During shortening the heat rate is higher than in the periods when the muscle is isometric. The shortening heat is thought to represent a greater rate of cross-bridge turnover during the shortening. The concept of independent force-generator indicates that the shortening heat is expected to be directly proportional to the extent of filament overlap. At longer muscle lengths allowance has to be made for the thermoelastic heat absorption on releasing pre-stretched muscles, which is likely to be caused by parallel elastic structures. It has been shown that the shortening heat at a rapid isovelocity releases linearly depended on the sarcomere lengths as the isometric tension did in the sarcomere length range between 2.2 and 3.7 μm (Irving and Woledge, 1981 ; Kometani and Yamada, 1983).

It has also been shown that in the shortening at near V_{max} there is a significant discrepancy between the observed and the explained enthalpy (Homsher, Yamada, Wallner and Tsai, 1984). This discrepancy disappears after the shortening and is not seen in the shortening at a slower speed (1/2 V_{max}).

Kodama and Yamada (1979) proposed a model based on results from kinetic and calorimetric studies of myosin and actomyosin ATPase reaction, and showed that could account for such separation of the energy output, i.e. (heat + work), from the high-energy phosphate splitting by shortening muscles. The model predicts that a significant amount of heat is produced, in addition to that from ATP splitting, when isometrically

contracting muscle is allowed to shorten and the transition from isometric to isotonic contraction occurs (see Figs. 2 and 3).

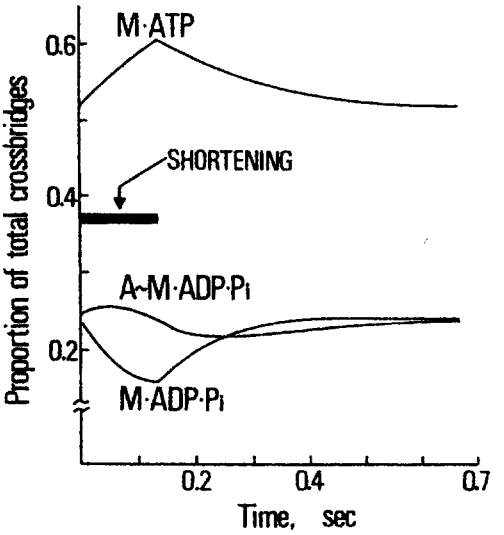

Fig. 2. Change in proportion of cross-bridge states during shortening followed by isometric contraction based on a kinetic model of actomyosin ATPase reaction. Reproduced with permission from Kodama and Yamada, 1979.

TIME-RESOLVED PHOSPHORUS NMR

Phosphorus NMR has become available for the non-destructive measurements of phosphorus compounds in intact muscle early in 1970s. Wilkie and colleagues have developed the method of using NMR further, for the study of phosphorus compounds in living muscle during rest, contraction and recovery (Dawson et al., 1977). This was inspired by serious doubt on the then current biochemical description of the situation based on studies with disrupted tissue, even if ultra-rapid freezing was employed as described above.

We have developed a method to improve the time-resolution from 7 min (Dawson et al., 1977) to 16 s to study living muscle contraction (Yamada and Tanokura, 1983; Kawano, Tanokura and Yamada, 1988). Contraction and recovery of bull-frog skeletal muscles were studied by using the methods described above in contractions of various durations at 5°C. The amount of PCr split coincided well with Pi appeared except for the initial few minutes following relaxation. During the early recovery period ΔPCr was significantly smaller than ΔPi (temporal separation between ΔPi and ΔPCr). The difference was 0.35 mmole/kg immediately after 2 s and longer tetanic contractions (Fig. 4). It is notable that this figure is comparable to the number of myosin heads in the muscle (0.28 mmole/kg, Ebashi, Endo and Ohtsuki, 1969).

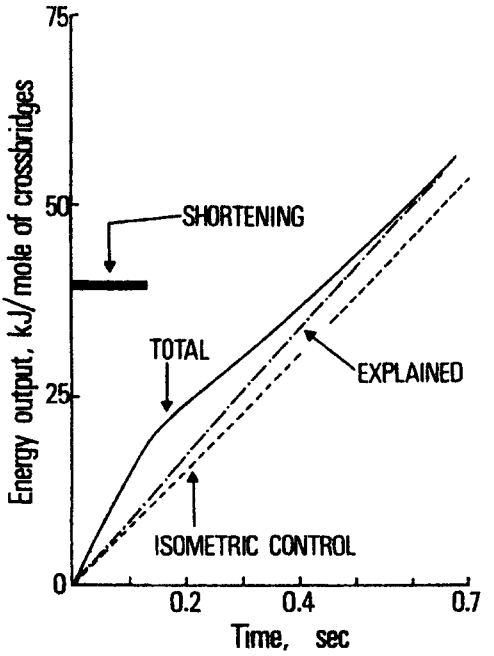

Fig. 3. Time course of energy output by muscle that is allowed to shorten and then returned to isometric contraction based on the enthalpy profile of actomyosin ATPase reaction. Reproduced with permission from Kodama and Yamada, 1979.

This phenomenon may be related to the repeated observations that certain amount of high-energy phosphate is utilized after contraction (see Gilbert et al., 1971). It may thus be related to the results of energy balance studies in which a significant fraction of the (heat + work) cannot be explained by PCr splitting (unexplained enthalpy, Gilbert et al., 1971; see Woledge et al., 1985). Operation of calcium pump may not produce such a separation of Pi and PCr.

This phenomenon suggests a delayed release of ADP from myosin heads in the early recovery period. This possibility seems compatible with the fact that the rate of release of ADP from rabbit myosin S1 is known to be markedly temperature dependent and can be very slow at low temperatures (Bagshaw, 1975).

Enthalpy change of PCr splitting has been estimated to be –34 kJ per mole by *in vitro* calorimetry under the conditions inside muscle cells (Woledge, 1973). Enthalpy change of PCr in muscle has been reported to be –46 kJ per mole (Gilbert et al, 1971). Therefore, the delayed splitting of PCr (0.35 mmole/kg) would produce heat of 12–16 mJ per g muscle. Because PCr break-down continues without heat production after contraction (Gilbert et al., 1971), the same amount of heat should have been produced without PCr splitting early during contraction (the unexplained enthalpy), and is reversed during early recovery period associated with PCr splitting without heat exchanges. The

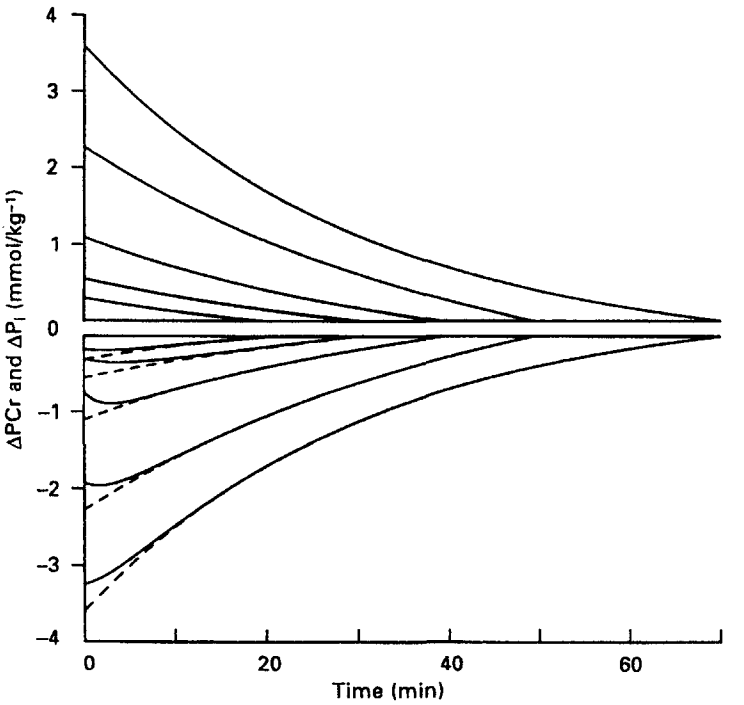

Fig. 4. Results summarizing phosphorus NMR studies on the effect of duration of contraction in bull-frog semitendinosus muscles. Changes in Pi and PCr immediately after the contraction, and time course of their recovery are shown in tetanic contractions of 0.2, 0.5, 2, 5 and 10 s duration at 5oC. Reproduced with permission from Kawano, Tanokura and Yamada, 1988.

unknown exothermic process producing 12-16 mJ of heat per g muscle early during isometric contraction corresponds to 3/10 – 4/10 of the unexplained enthalpy.

According to the kinetic model proposed by Kodama and Yamada (1979) as described above, there could also be a shift in the cross -bridge states during the transition from rest to contraction, producing a significant amount of heat without ATP splitting. According to Kodama and Yamada (1979), a shift of cross-bridge states from M. ADP. Pi to AM. ADP. Pi and M. ATP during an isometric contraction of 1 s would produce heat of about 10 mJ/g muscle (see Fig. 2).

MICROCALORIMETRIC APPROACH TO THE CALCIUM BINDING OF TROPONIN C AS A PRIMARY STEP OF THE REGULATION OF CONTRACTION

One of the problems in the mechanism of filament sliding is its regulation. Kodama and Woledge (1976) introduced titration microcalorimetry for studying the mechanism of ATP hydrolysis by myosin, and Yamada and Kometani (1983) for studying calcium

binding of troponin C associated with the regulatory mechanism.

Contraction in all muscles is triggered by an increase in the calcium concentration to 100 ~ 1000 times the resting level, and this calcium signal is conveyed to the actomyosin system. The calcium sensitivity of the actomyosin system results from the presence of the troponin complex and tropomyosin (Ebashi, 1963). The binding of calcium to troponin C, the target protein of the complex, initiates a series of events involving the regulatory proteins of the I filament.

Troponin C is the highly specialized calcium-binding protein of the troponin complex. Troponin C has two high-affinity sites that bind calcium and magnesium competitively, and two low-affinity sites which selectively bind calcium over magnesium (Ebashi and Endo, 1968; Potter and Gergely, 1975; Potter, Seidel, Leavis, Lehrer and Gergely, 1976). Since the change in free magnesium concentration did not affect calcium sensitivity of the myofibrillar ATPase activity (Potter and Gergely, 1975), it is reasonable to assume that the low-affinity sites are the ones related to the regulation of contraction.

When troponin C of the rabbit skeletal muscle was titrated with calcium in a microcalorimeter a substantial amount of heat is produced in several phases according to the site involved (Yamada and Kometani, 1983). In addition we noticed an anomalous phase where heat produced becomes very small or even heat is absorbed. This heat is also characterized by an increase in heat capacity, which is obtained by the dependence of ΔH on temperature. This characteristic phase of positive enthalpy and heat capacity changes should be involved in the mechanism of regulation of contraction because these anomalous phases are associated exclusively with the proteins having regulatory functions (Yamada, 2004). We may call these regulatory calcium sites as "active" calcium sites of troponin C. Thus microcalorimetric titrations of EF-hand calcium-binding proteins resulted in the notion that calcium binding to the "active" calcium sites induce characteristically anomalous enthalpy and heat-capacity changes (Yamada, 2004).

In rabbit skeletal muscle the characteristic phase of positive enthalpy and heat capacity changes is associated with calcium binding to one of the two high-affinity calcium sites of the C-terminal domain (Yamada and Kometani, 1982). Calorimetric studies have also shown that, in frog skeletal troponin C, one of the N-terminal low-affinity calcium-binding sites is the "active" calcium site (Imaizumi and Tanokura, 1990). This feature is in good agreement with the results of structural studies of the N-terminal domain of skeletal troponin C originated from the reconstituted chicken troponin C (Slupsky, Kay, Reinach, Smillie and Sykes, 1995). The structure of the calcium-saturated N-terminal domain is "open" and the hydrophobic pocket is exposed to the solvent compared with the apo N-terminal domain. The frog troponin C has the same number of amino-acid residues as chicken troponin C, which has three amino-acid residues more than rabbit skeletal troponin (van Eerd, Canopy, Ferraz and Pechere, 1978).

The hydrophobic effect is an important factor in the organization of the molecules in biology (Tanford, 1973). A group of hydrophobic residues exposed to the solvent on calcium binding to the "active" site of troponin C may interact with the hydrophobic NH_2-terminal regions of troponin I (see Yamada, 1999).

Anomalously positive enthalpy change (heat absorption) has also been detected in ATP cleavage reaction on myosin (Kodama and Woledge, 1979; Kodama, 1981; also see

Kodama, 1985). In this case, however, entropy change is positive and heat-capacity change is negative, indicating that hydrophobic residues are retracted from the surface to the interior of the molecule.

Microcalorimetric studies of various kinds of calcium-binding proteins that have specific functions in muscle cells have indicated that there could be a fundamental difference in the mechanism of regulation of contraction by the calcium-troponin-tropmyosin system among skeletal muscles of different species and between cardiac and skeletal muscle (Yamada, 2004). Specific features attained by structural studies should fit in with the outline provided by calorimetry.

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DISCUSSION

Rall: You call the calcium binding site in calcium binding proteins that exhibits anomalous heat production you have as the "active" site. What do you mean by active site?

K. Yamada: By "active site" I mean, in a very tentative sense, the single Ca-binding site of troponin C which has an thermodynamically anomalous nature, and is most likely to be involved in the mechanism of regulation. It should be the same as the "regulatory" site.

Kushmerick: Does the delayed splitting of PCr caused the ADP coming from slow creatine kinase reaction.

K. Yamada: The delay in creatine kinase reaction is unlikely to occur during contraction at low temperature. This is because 1) muscles develop force which is very well maintained even with long tetanus, and 2) the amount of delayed PCr splitting does not increase with the duration of contraction longer than about 1s.