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ON THE ENTHALPY OF BINDING OF ADP TO HEAVY MEROMYOSIN

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The binding of ADP to heavy meromyosin has been studied by microcalorimetry. Minute amounts of myokinase interfere with binding measurements, but by selection of appropriate conditions, we can estimate that the value of the apparent $\Delta H_{binding}$ lies between -1.0 and -3.0 kcal per mole of ADP bound (0.3 M KCl, 2 mM $MgCl_2$, 20 mM Tris, pH 8.00, 20°C). Values of $\Delta H_{binding}$ reported to date are an order of magnitude larger, and we suggest that these values are artifactual results due to myokinase contamination.

INTRODUCTION

Increasing attention is being focused on the role of the muscle protein myosin in the transduction of chemical to mechanical energy. In terms of the 1971 model of Lymn and Taylor (1), transduction occurs in concert with the actin-induced dissociation of ATP[1] hydrolysis products from the myosin-product complex. Bagshaw and Trentham (2) have provided free energy calculations which suggest that this model is reasonable. In an extension of their thermodynamic analysis, we have obtained a microcalorimetric estimate of the enthalpy of dissociation of ADP from myosin. For experimental convenience, this value was determined in studies of the heat of binding of ADP to heavy meromyosin (HMM). In the course of this work, a considerable disparity was found between our results and the results of other calorimetric studies. The dual purpose of this paper, then, is to report the values we have found and to explain the observed discrepancy.

METHODS AND MATERIALS

Myosin was prepared and characterized by the methods of Goodno and Swenson (3). HMM was prepared and stored by the methods of Lowey and Cohen (4), with a digestion time of 90 sec. ATPase activities were assayed by the method of Lowey and Luck (5).

HMM solutions were prepared for calorimetric studies by exhaustive dialysis at 4° C against a buffer consisting of 0.3 M KCl, 20 mM Tris-HCl, and 2 mM $MgCl_2$ (pH 8.00 ± 0.01 at 20°C). AMP, ADP, and ATP were obtained from P -L Biochemicals. Nucleotide solutions for calorimetry were prepared in the outer dialysate and adjusted to pH 8.00 ±

[1] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; IMP, inosine monophosphate; HMM, heavy meromyosin; ΔH_b, apparent heat of binding (includes heat of buffer protonation); $\Delta H_{dissociation}$, apparent heat of dissociation (includes heat of buffer protonation); Tris, tris(hydroxymethyl) aminomethane.

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0.05 at 20°C before use. Nucleotide purity was verified by thin-layer chromatography on polyethyleneimine-cellulose plates. AMP-deaminase and myokinase (both from Sigma Chemical Co.) were prepared for calorimetric studies by exhaustive dialysis against the same buffer used for HMM. AMP-deaminase was assayed by the method of Harris and Suelter (6), and myokinase was assayed in a coupled system containing AMP-deaminase.

Calorimetry

The construction of our heat-burst microcalorimeter has been described elsewhere (7, 8). In an experiment, protein and nucleotide solutions were loaded into separate compartments of the calorimetric vessel from 0.5 ml syringes. After 1–2 hr, thermal equilibration was manifested by a flat voltage-time trace, and mixing of the two solutions was accomplished by rotation of the vessel. Mixing was complete within a single rotation. Upon re-establishment of the baseline voltage, a second rotation was performed to obtain a correction for viscous heating. Data were obtained by integration of the voltage-time traces, whose areas were standardized against the heat of neutralization of Tris by the procedure of Crowder et al. (8). All standardizations and experiments were carried out at 20°C ± 0.1, and the heat of neutralization of Tris was taken to be −11.5 kcal/mole. (Literature values bracket this value within an interval of about ± 0.5 kcal/mole.)

Calculations

The heat of binding ADP to HMM was calculated from the following equation:

$$\Delta H_b = \frac{Q}{n_s \cdot f},$$

in which Q is the corrected heat of mixing of HMM and ADP, n_s is the number of equivalents of binding sites, and f is the fraction of nucleotide-binding sites occupied. Q values were obtained from averaged triplicate experiments at each ADP concentration, and values of n_s and f were calculated on the basis of $K_b = 1 \times 10^5\ M^{-1}$ and n = 2 sites per molecule (typical values from [5]). The molecular weight of HMM was taken to be 320,000 daltons (4).

RESULTS

At low concentrations of ADP (Fig. 1A) the heat of binding develops instantaneously, within the response time of the apparatus (about 5 sec). At elevated ADP concentrations it was found that this instantaneous heat development was followed by an extended phase of slow heat liberation (Fig. 1B and 1C). The apparent heats of binding, ΔH_b^1, are tabulated in Table I.

It was deemed useful to compare results with those of Yamada et al. (9). These workers, however, did not normalize their data on the basis of the number of moles of ADP actually bound to HMM. In order to compare results, we have analyzed their data under the same assumptions we have used for our calculations. The normalized ΔH_b

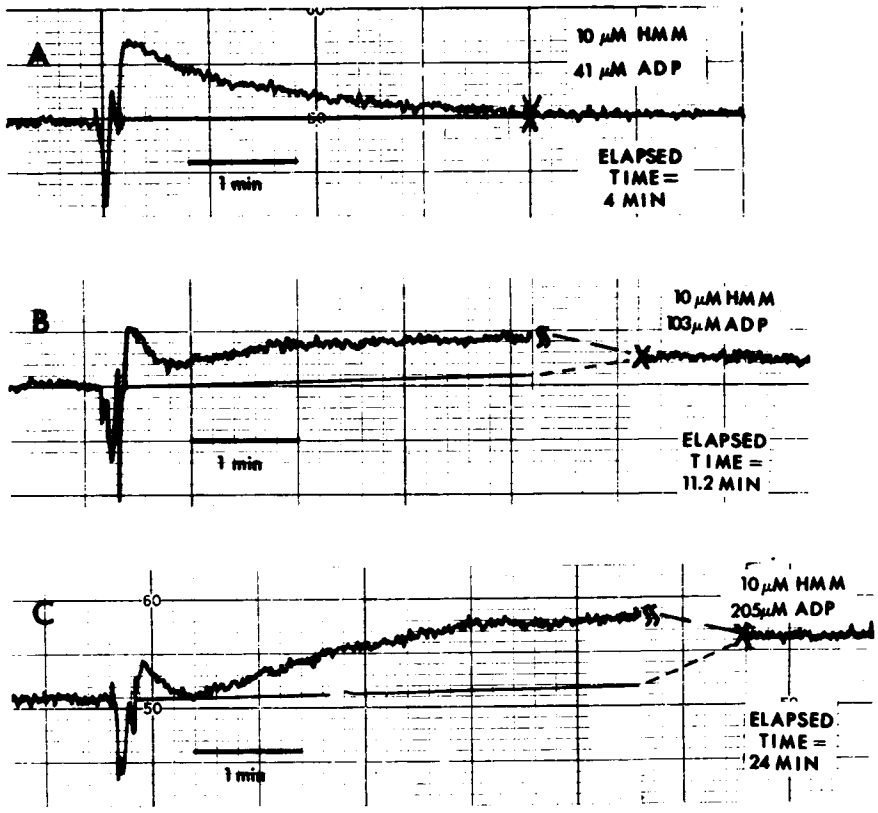

Fig. 1. Recorder traces produced by the mixing of 20 μM HMM with different concentrations of ADP. The concentration of ADP increases from A to C, with the final [HMM] and [ADP] indicated at the right of each trace. The elapsed time from mixing to re-establishment of baseline is indicated at the lower right of each trace. Traces B and C were truncated at their plateaus in order to fit them into the figure. The baseline is indicated beneath each peak, and re-establishment of the baseline is denoted by X. Baseline differences render comparison of the rapid phases of these curves difficult.

TABLE I. Calorimetric Results for the Binding of ADP to HMM at 20°C

Protein conc. (μM)	ADP conc. (μM)	Q (cal × 10^{-5})	ΔH_m* (kcal/mole)	f†	ΔH_b (kcal/mole)
10.08	20.5	− 1.6 ± 2.7	− 1.6 ± 2.7	0.507	− 1.6 ± 2.7
10.08	41	− 5.7 ± 0.7	− 5.7 ± 0.7	0.725	− 3.9 ± 0.5
10.08	103	−21.0 ± 6	−21.0 ± 6	0.895	−11.7 ± 3.3
10.08	205	−79 ± 16	−78 ± 16	0.949	−41.2 ± 8.5

*Values have been corrected for heats of dilution of HMM and ADP.
†Calculated using $K_b = 1 \times 10^5\ M^{-1}$.
Values are given for the concentration of protein and nucleotide after mixing (final volume = 1.00 ml). ΔH_m gives the heat of mixing per mole of heavy meromysin; f, the fraction of nucleotide-binding sites occupied; and ΔH_b, the heat per mole of ADP bound.

values are presented in Table II. Whereas our initially small ΔH_b values increase with increasing $\frac{[ADP]}{[HMM]}$, the values of Yamada et al. are large and essentially constant.

In addition to the heat of ADP binding, we have determined ΔH values for several reactions which could potentially compete with the binding process (Tables III and IV):

$$(1)\quad ATP \longrightarrow ADP + P_i$$
$$(2)\quad 2ADP \longrightarrow AMP + ATP$$
$$(3)\quad AMP \longrightarrow IMP + NH_4^+.$$

Our ΔH values for reaction (1) are in close agreement with the value obtained by Podolsky and Morales (10) under similar conditions. These values (about -16 kcal/mole) stand in sharp contrast to the values obtained by Yamada et al. (-39 to -44 kcal/mole). No suitable literature values could be found for comparison with our ΔH values for reactions (2) and (3).

DISCUSSION

At low mole ratios of ADP to HMM, our ΔH_b values can be completely explained by the release of 0.1 to 0.3 H^+ upon binding of ADP by HMM, followed by protonation of the buffer. This model is in accord with the results of Bagshaw and Trentham (11). At low mole ratios, our ΔH values are an order of magnitude smaller than the values of Yamada et al., yet at higher ratios, our values approach and exceed theirs (cf. Tables I and II). A clue to the origin of the effect which equalizes our values is seen in the changing of the shape of the voltage-time trace as ADP concentration is increased from 41 μM (Fig. 1A) to 205 μM (Fig. 1C). At the elevated ADP concentrations heat is developed in two phases: an instantaneous one and an extended one. We have observed that as [ADP] is increased above saturation of the HMM, the size of the spontaneously developed heat remains small, while the extended heat increases. Experience with calorimetric enzyme assays indicates to us that such extended heat development is typical of a slow reaction. The studies of Bagshaw and Trentham (11) indicate that the binding of ADP (exponential rate constant = 12 sec^{-1}) should be fast relative to the response time of our apparatus (5 sec). Thus, for the binding of ADP, we should observe only an instantaneous heat production, followed by exponential decay (as seen in Fig. 1A).

In order to elucidate the source of the slowly developed heat, we have measured the ΔH values of the reactions catalyzed by two common contaminants of myosin preparations: myokinase and AMP-deaminase (Table IV). As shown in Table V, these heats of reaction are insufficient to account for more than a small fraction of the heats we observe upon mixing of HMM and ADP. Though the ΔH of ADP dismutation is negligible, the ΔH of hydrolysis of the resulting ATP is large (Table III). Thus, the probable source of the slow heat production is the HMM-catalyzed hydrolysis of a small amount of ATP which is produced by myokinase-catalyzed ADP dismutation. Table VI shows that the observed heats fall well within the theoretical maximum heats predicted by this model.

Two lines of evidence suggest that the results of Yamada et al. (9) are due to the same sort of process: (a) Their preparative procedure was inadequate to remove the

TABLE II. Calorimetric Data from the Work of Yamada et al.

Protein conc. (μM)	ADP conc. (μM)	ΔH_m (kcal/mole)	f*	ΔH_b* (kcal/mole)
41	41	23	0.41	28
41	82	38	0.71	27
41	165	50	0.90	28

*Calculated on the basis of $K_b = 1 \times 10^5\ M^{-1}$.
Results are adjusted for the amount of nucleotide actually bound. The definitions of ΔH_m, f, and ΔH_b are the same as in Table I. Solvent and temperature were also identical to those for Table I.

TABLE III. Heats of Hydrolysis of ATP

Solvent	Quantity of ATP hydrolyzed (μmole)	Number of experiments	Q (cal $\times 10^{-3}$)	ΔH_r* (kcal/mole)
0.3 M KCl, 20 mM Tris, 2 mM $MgCl_2$, pH 8.0†	0.125	2	-2.22 ± 0.29	-17.7 ± 4.1
0.3 M KCl, 20 mM Tris, 2 mM EDTA, pH 8.0	0.126	2	-1.92 ± 0.17	-15.3 ± 1.3

*Heats of dilution were negligibly small, thus these values need no correction.
†Identical to the solvent used by Yamada et al.
The indicated quantities of ATP were hydrolyzed with 12.5 μM HMM in a volume of 1 ml of the indicated solvent at 20°C. Q is the measured heat. ΔH_r is the molar heat of ATP hydrolysis.

TABLE IV. Enthalpies of Reactions Catalyzed by Residual Enzyme Contaminants Commonly Found in Heavy Meromyosin Preparations

Reaction	μmoles of substrate	Number of experiments	Q (cal $\times 10^{-5}$)	ΔH (kcal/mole of substrate)
2 ADP → AMP + ATP	0.43	3	-3.0 ± 0.2	-0.07 ± 0.005
AMP → IMP	0.69	3	-36.8 ± 0.10	-0.53 ± 0.15

TABLE V. Comparison of Calculated and Observed Heats of Mixing ADP and HMM in the Presence of Contaminating Traces AMP-Deaminase and Myokinase

Protein conc. (μM)	Q calculated for ADP → IMP (cal $\times 10^{-5}$)	Q_{obs} (cal $\times 10^{-5}$)
20.5	−0.7	−1.6
41	−1.4	−5.7
103	−3.5	−21
205	−7.0	−79

TABLE VI. Comparison of Theoretical and Observed Heats of Mixing ADP and HMM

ADP conc. (μM)	$Q_{theoretical}$ (cal X 10^{-5})	Q_{obs} (cal X 10^{-5})
20.5	− 36	−1.6
41	− 73	−5.7
103	−182	−21
205	−363	−79

Theoretical maximum values are based on the total conversion of ADP to AMP through the recycling of ADP by HMM/myokinase. The enthalpy of ATP hydrolysis was taken to be −17.7 kcal/mole.

myokinase. HMM was prepared by the method of Lowey and Cohen (4), followed by fractionation in 45–55% saturated ammonium sulfate. Lowey and Luck (5) point out that the Lowey preparation fails to remove considerable amounts of myokinase, and Kress et al. (12) show that myokinase begins to salt out near 54% saturation in ammonium sulfate. (b) They obtain extremely large values for the enthalpy of ATP hydrolysis, and they report reasonable values only after subtracting the enthalpy of ADP binding as a control. This result can be explained by the hypothesis that the ADP is not simply binding, but is also undergoing dismutation, followed by HMM-catalyzed cleavage of the resulting ATP.

In contrast to the work of Yamada et al., our studies have used HMM prepared from the 35–45% fraction of myosin, and we have worked at fourfold lower total protein concentration. Even with these precautions, the use of ADP concentrations on the order of 100 μM gives rise to slow heat development characteristic of a reaction following the rapid binding. The slow heat evolution can be explained by the presence of myokinase contamination with a specific activity of less than 10^{-3} μmole min^{-1} per mg of HMM. Indeed, assays of the myokinase activity in our usual myosin preparations yield values in this range. We have recently obtained an HMM preparation with no detectable myokinase activity, and, in agreement with the stated hypothesis, this preparation gives only a rapid phase of heat production in the ADP binding experiment.

In conclusion, very small amounts of myokinase contamination in myosin and HMM preparations pose a threat to calorimetric studies on the binding of ADP. At ADP concentrations which are expected to be well below the K_m value of myokinase, ΔH_b approaches values between −1.0 and −3.0 kcal/mole of ADP bound (current studies are aimed at determination of more precise values in the absence of myokinase). Thus, we find that the dissociation of ADP from myosin is a slightly endothermic process (under the solvent conditions studied) with $\Delta H_{dissociation}$ between +1.0 and +3.0 kcal per mole.

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