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THE INTERACTION OF MALONALDEHYDE WITH MYOSIN AND ITS SUBFRAGMENTS: A MICROCALORIMETRIC STUDY

F. SARMIENTO *, M. NOGUEIRA **, M.I. PAZ-ANDRADE * and M.N. JONES ***

Department of Biochemistry and Molecular Biology, Biomolecular Organisation and Membrane Technology Group, School of Biological Sciences, University of Manchester, Manchester M13 9PT (Gt. Britain)

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ABSTRACT

The enthalpies of interaction between malonaldehyde and myosin (M) and its subfragments, heavy meromyosin (HMM) and light meromyosin (LMM) in aqueous solution (pH 6.8) at 25 °C have been measured as a function of malonaldehyde concentration by microcalorimetry. The interaction enthalpies are all endothermic reaching saturation values of approximately 60 J g^{-1} (M) and 110 J g^{-1} (HMM). The enthalpies of interaction between malonaldehyde reactive amino acids have been measured under the same conditions and used to derive contributions of the reactive side chains of amino acid residues to the overall enthalpies of the myosins–malonaldehyde interactions. It is found that the enthalpies of reactions with the amino acid side chains in myosins cannot account for the large endothermic interaction enthalpies observed with malonaldehyde, but that the reactions initiate the denaturation of the myosins resulting in absorption of heat. Microcalorimetry is demonstrated to be a useful method of investigating the interaction of malonaldehyde with myosins on a relatively short time scale (20–30 min).

INTRODUCTION

Malonaldehyde (propanedial) is a product of the auto-oxidation of polyunsaturated fatty acids. It is a highly reactive dialdehyde and gives rise to adverse effects during food storage [1]. The interaction of malonaldehyde with proteins in general [2–7] and particularly with food proteins [8–11] is of considerable interest and it has been shown that malonaldehyde will react preferentially with certain amino-acid residues, notably with histidine, arginine, tyrosine, methionine and lysine residues in myosin [8]. These

* Permanent address: Departmento de Fisica Aplicada, Universidad de Santiago, 15706 Santiago de Compostela, Spain.
** Permanent address: Departmento de Bioquimica, Facultad de Biologia, Universidad de Santiago, 15706 Santiago de Compostela, Spain.
*** Author to whom correspondence should be addressed.

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reactions can lead to the unfolding of the native protein and there is also evidence that malonaldehyde introduces intermolecular cross-links with the formation of protein dimers and trimers [4,6]. The interaction of malonaldehyde with proteins is a complex process and the nature of the reactions is not fully understood. In aqueous solution malonaldehyde exists predominantly as the enol tautomer giving rise to nucleophilic attack on the amino-acid side chains of the protein. Arginine can be converted to δ-*N*-(2-pyrimidinyl)-ornithine in concentrated acid [12] and the ε-NH_2 group of lysine residues in polylysine are converted to aminopropenol residues by malonaldehyde [13].

Many of the studies of malonaldehyde reactions with proteins have been concerned with effects arising over relatively long time scales (hours to days). In this work we have used microcalorimetry to investigate the interaction of malonaldehyde with myosin (M), light meromyosin (LMM) and heavy meromyosin (HMM) over a short time scale (~ minutes) and to aid interpretation of the results measurements have also been made of the enthalpies of interaction of some amino acids with malonaldehyde.

EXPERIMENTAL

Materials

Malonaldehyde was freshly prepared by acid–catalysed hydrolysis of 1,1,3,3-tetraethoxypropane (Sigma Chemical Co. Ltd.) according to the method of Buttkus [8] and finally made up in 0.026 M phosphate buffer pH 6.8 containing 0.45 M potassium chloride. L-Histidine (product no. H-8000), L-methionine (product no. M-9625) were from Sigma and L-arginine and L-lysine were from British Drug Houses. Rabbit muscle myosin (product no. M 1636), light meromyosin (product no. M 9139) and heavy meromyosin (product no. M 9014), obtained in 50% glycerol solution from Sigma, were dialysed for 72 h against phosphate buffer (pH 6.8, 0.45 M KCl) before use using Spectrapor membrane tubing (molecular weight cut-off 6000-8000).

Malonaldehyde assay

The malonaldehyde concentrations were determined using 2-thiobarbituric acid (TBA) according to the method of Buttkus [8]. Standard curves of absorbance at 535 nm of the TBA-malonaldehyde reaction product against malonaldehyde concentration were prepared every few days.

Microcalorimetry

Enthalpy measurements were made with an LKB 10700 batch microcalorimeter which utilizes the twin-vessel principle, each vessel being divided

into two compartments [14]. The instrument was operated at 25°C and calibrated electrically. Aliquots of the required myosin or amino acid and malonaldehyde solutions were placed in the reaction vessel and aliquots of the corresponding amounts of buffer and malonaldehyde were placed in the reference vessel. The additions were made by weight using 2-ml syringes. On rotation of the calorimeter the heats of dilution of the malonaldehyde solutions in the reaction and reference vessels are identical. The heats of dilution of the myosins and amino acids under these conditions were negligible. The experiments were carried out over a range of malonaldehyde concentrations up to 100 mM keeping the myosin or amino acid concentrations constant. Immediately after the thermograms had been obtained (approx. 30–40 min) the contents of the reaction and reference vessels were removed and assayed for malonaldehyde. The differences in malonaldehyde concentration were used to calculate the amount of malonaldehyde that had reacted with the myosin.

Spectroscopy

The absorption spectra of the products of the reaction between malonaldehyde and the amino acids were measured in the range 240–500 nm using a Cary 219 spectrophotometer in the double beam mode with a buffer (pH 6.8 phosphate) blank. An LKB Ultraspec 4050 spectrophotometer was used for the measurement of the absorbance at 535 nm to assay the malonaldehyde.

RESULTS

Figures 1–3 show the enthalpies of interaction of M, HMM and LMM with malonaldehyde at 25°C as a function of malonaldehyde concentration.

Also shown in Figs. 1–3 are the moles of malonaldehyde per mole of myosin lost during the reaction determined by assaying the malonaldehyde in the reaction mixture at the end of the experiments. It should be noted that these data were collected within a period of 30–40 min after mixing, and thus relate to the initial interaction. The thermograms gave conventional peaks coming back to the base line within this time period and gave no indication of a continuing reaction. This does not necessarily imply that the reactions between the myosins and malonaldehyde had gone to completion but it does mean that after the initial reaction any subsequent processes were proceeding at a sufficiently slow rate that no heat could be detected over a short time scale. It should also be noted that the concentrations of malonaldehyde on a molar basis are in excess of those of the myosins. At a 50 mM malonaldehyde concentration the molar ratios (malonaldehyde : myosin) in the reaction mixture are $7.4 \times 10^4 : 1$ (M), $5.9 \times 10^4 : 1$ (HMM) and

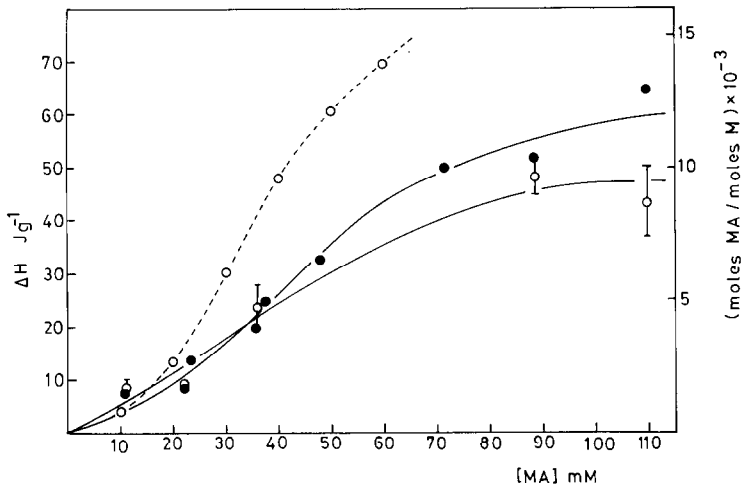


Fig. 1. Enthalpy of interaction between myosin and malonaldehyde and moles of malonaldehyde reacted per mole of myosin (right hand axis) in aqueous solution at 25 °C, pH 6.8. ●, Enthalpy (J per g myosin); ○, moles malonaldehyde per mole myosin. The myosin concentration was 0.68 μM. The broken line was calculated as described in the text.

$6.5 \times 10^4 : 1$ (LMM), which correspond to malonaldehyde to amino acid residue molar ratios of 18 : 1 (M), 21 : 1 (HMM) and 55 : 1 (LMM).

Figures 4 and 5 show the enthalpies of interaction of histidine, lysine,

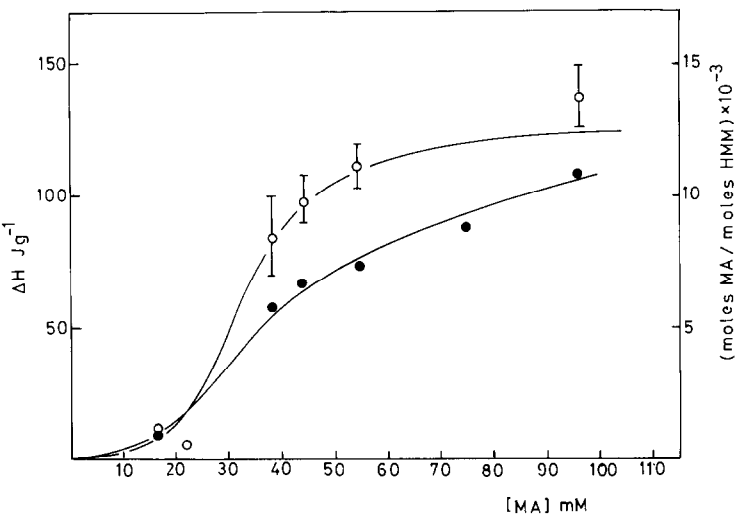


Fig. 2. Enthalpy of interaction between heavy meromyosin and malonaldehyde and moles of malonaldehyde reacted per mole of myosin (right hand axis) in aqueous solution at 25 °C, pH 6.8. ●, Enthalpy (J per g HMM); ○, moles malonaldehyde per mole HMM. The HMM concentration was 0.83 μM.

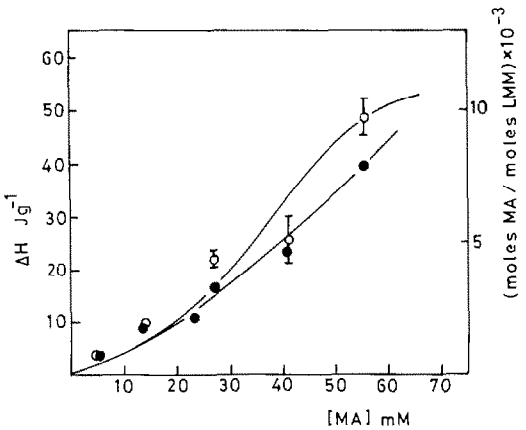


Fig. 3. Enthalpy of interaction between light meromyosin and malonaldehyde and moles of malonaldehyde reacted per mole myosin (right and axis) in aqueous solution at 25 °C, pH 6.8. ●, Enthalpy (J per g LMM); ○, moles malonaldehyde per mole LMM. The LMM concentration was 0.77 μM.

arginine and methionine as a function of malonaldehyde concentration; apart from histidine the reactions are all endothermic. The reactions give rise to significant changes in the absorbance spectrum of malonaldehyde as shown in Fig. 6; the malonaldehyde absorbance at 350 nm shifts to 400 nm on reaction with the amino acids and for each the absorbance of the peak at 400 nm increases sigmoidally with increase in malonaldehyde concentration (inset Fig. 6).

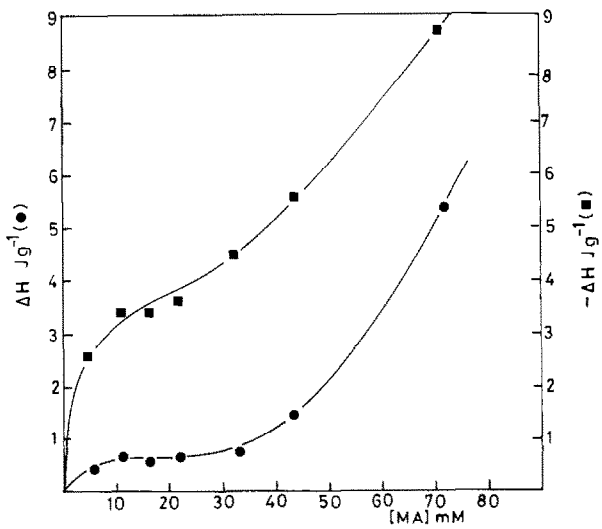


Fig. 4. Enthalpy of interaction between lysine (●) and histidine (■), (right hand axis) with malonaldehyde at 25 °C, pH 6.8. The lysine concentration was 34.2 mM and the histidine concentration was 10.5 mM.

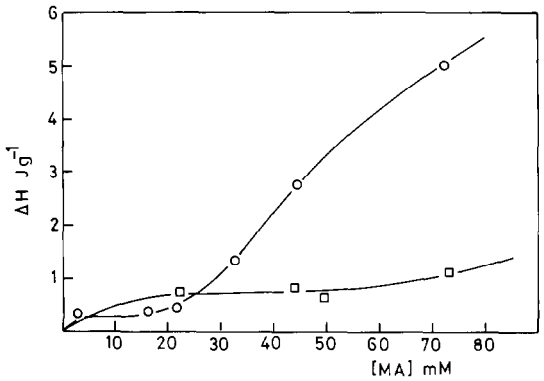


Fig. 5. Enthalpy of interaction between arginine (○) and methionine (□) with malonaldehyde at 25 °C, pH 6.8. The arginine concentration was 7.2 mM and the methionine concentration was 33.5 mM.

Figure 7 shows the heats of the reactions of malonaldehyde with histidine and methionine as a function of the moles of malonaldehyde reacted. The slopes of the lines give values of -1.61 ± 0.24 and 0.55 ± 0.08 kJ mol^{-1} for the enthalpies of reaction with histidine and methionine, respectively. If it is assumed that the reactions result in a single product, from the initial concentrations of amino acid and malonaldehyde and the final malonalde-

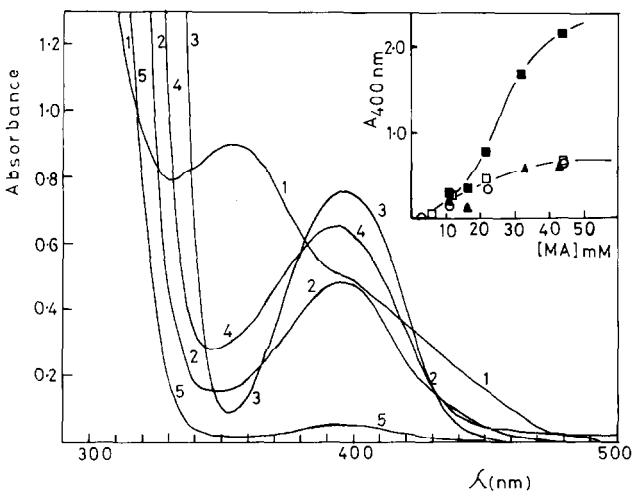


Fig. 6. Absorption spectra of malonaldehyde and malonaldehyde–amino acid mixtures in aqueous solution pH 6.8. Curve 1, malonaldehyde (44 mM); curve 2, malonaldehyde (10.9 mM) plus lysine (35.2 mM); curve 3, malonaldehyde (10.8 mM) plus histidine (11.1 mM); curve 4, malonaldehyde (22.1 mM) plus arginine (7.39 mM); curve 5, malonaldehyde (2.76 mM) plus methionine (34.3 mM). The inset shows the absorbance at 400 nm as a function of malonaldehyde concentration at constant amino acid concentration. ▲, Lysine (34.3 mM); ■, histidine (10.7 mM); ○, arginine (7.2 mM); □, methionine (33.5 mM).

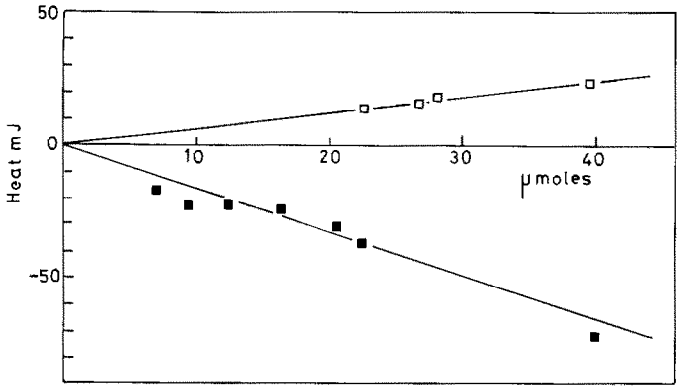


Fig. 7. Heat of reaction (mJ) as a function of the amount of malonaldehyde reacted (μmol) in aqueous solution at 25 °C, pH 6.8 ■, histidine (10.52 mM); □, methionine (33.5 mM).

hyde concentration the equilibrium constants were found to be 0.031 ± 0.002 and 0.020 ± 0.005 for histidine and methionine respectively. For lysine and arginine we were not able to satisfactorily assay for the amounts of malonaldehyde reacted with the thiobarbituric acid assay. Tyrosine residues in proteins are also potential malonaldehyde-reactive sites [8], however, owing to the limited solubility of tyrosine in high ionic strength buffer it was not possible to make calorimetric measurements on this reaction.

DISCUSSION

The enthalpies of interaction of myosin and its subfragments with malonaldehyde are all endothermic and are of magnitude comparable to the enthalpies of interaction of linoleic acid [15], a potential precursor of malonaldehyde in the autoxidation of lipids, but linoleic acid interacts exothermically with HMM in contrast to malonaldehyde [15]. Figure 1 (broken line) shows the enthalpy of interaction of myosin with malonaldehyde (ΔH_M) calculated from the interaction enthalpies of its subfragments ΔH_{HMM} and ΔH_{LMM} according to the equation

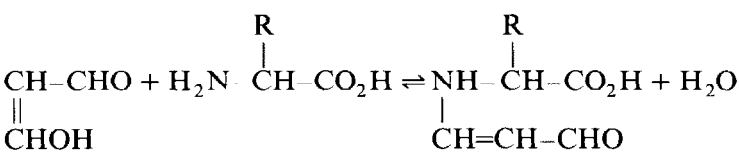
$$\Delta H_M (\mathrm{J\ g^{-1}}) = 0.3\Delta H_{LMM} + 0.7\Delta H_{HMM}$$

where the weight fractions were calculated from the following molecular weights 480 000 (M), 340 000 (HMM) and 140 000 (LMM) [16]. The experimental curve for myosin falls below that predicted from the enthalpies of interaction with its subfragments suggesting that the subfragments do not behave independently within the myosin molecule.

A surprising observation is that the number of moles of malonaldehyde lost during the reactions with the myosins exceeds the number of likely reaction sites. For example the number of amino acid residues in myosin is

4141 [16] yet at a malonaldehyde concentration of 100 mM approximately 9000 malonaldehyde molecules appear to have reacted. Malonaldehyde could react preferentially with the following numbers of residues lys (442), his (77), arg (206), met (110) and tyr (96); thus even assuming that malonaldehyde reacted with the peptide bonds plus these reactive side chains (5071 reactive sites) it would not be possible to account for the loss of malonaldehyde. Furthermore peptide bond cleavage in proteins by malonaldehyde is not extensive even in very acid solution [12]. It is possible that some malonaldehyde is consumed by cross-linking reactions [13] with the myosin but to account for the amount lost it would seem to be necessary to postulate that reaction with protein initiates some side reaction which consumes malonaldehyde, possibly a polymerization. However, as heats of polymerization are generally exothermic, e.g. acrolein which is structurally related to the enolic forms of malonaldehyde has a heat of polymerization of -81.5 kJ mol^{-1} monomer [17], it seems unlikely that any such polymerization could be very extensive since the enthalpies of malonaldehyde interactions with the myosins are endothermic.

The enthalpies of reaction of malonaldehyde with the amino acids can be used to estimate the contribution of malonaldehyde reactions with the corresponding amino acid residues in the myosins. The enolate of malonaldehyde most probably reacts both with the side chains and with the amine group on the α-carbon to form an enamine [18]. The reaction with the α-carbon amine group might be written



by analogy with the reaction between malonaldehyde and glycine to form *N*-prop-2-enal amino acetic acid [18]. For lysine this reaction will also occur with the ε-amino group so that the observed enthalpy of reaction with lysine gives, to a reasonable approximation, twice the enthalpy of the above reaction. Thus by taking half the enthalpy of reaction with lysine from the enthalpies of reaction for the other amino acids the side-chain contributions can be obtained, which can be used to estimate the contributions of the side-chain reactions in the myosins to the overall enthalpy of interaction. The results of such a calculation for myosin are shown in Fig. 8. The percentage by weight of lysyl, histidyl, arginyl and methionyl residues in myosin are 11.8%, 2.2%, 6.7%, and 3.0% respectively [16]. As Fig. 8 shows, histidyl and lysyl residues make the major contributions to the interaction enthalpies but the enthalpies are of opposite sign. Calculations for HMM and LMM give very similar results to those for myosin. It is clear by comparing the data in Fig. 1 with those in Fig. 8 that the enthalpies of reaction of malonaldehyde with the amino acid residues make an almost

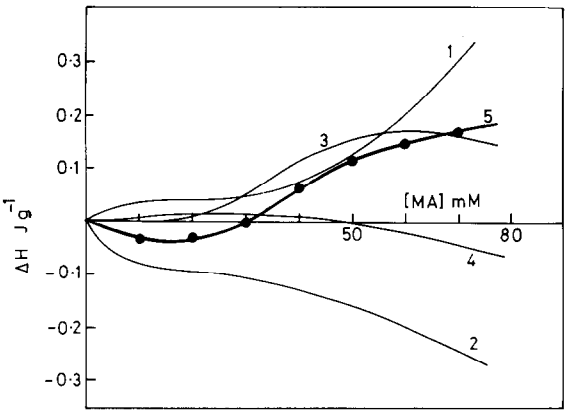


Fig. 8. Contributions to the enthalpy of interaction between malonaldehyde and myosin from reactions with amino acid residues in aqueous solution at 25 ° C, pH 6.8. Curve 1, lysyl; curve 2, histidyl; curve 3, arginyl; curve 4, methionyl; curve 5, total enthalpy contributions.

negligible contribution to the overall enthalpy of interaction between malonaldehyde and myosin. This arises because of the relatively small weight fractions of the residues and the cancellation of the exothermic reaction with histidyl residues by the endothermic reactions with the other amino acid residues.

The large endothermic interaction enthalpies of malonaldehyde with the myosins cannot be accounted for by reaction enthalpies with individual amino acid residues known to be reactive to malonaldehyde. Data are not available for interaction with tyrosyl residues present to the extent of 3.3% by weight which are reactive to malonaldehyde [8] in myosin. However, it would seem unlikely that a contribution from this amino acid would significantly change the above conclusions. It follows that the endothermic enthalpies of malonaldehyde–myosin interactions must arise from other processes such as unfolding (denaturation) and/or aggregation initiated by reaction with malonaldehyde. Thermal denaturation of myosin and its subfragments, is endothermic and associated with enthalpy changes of 15–25 J g^{-1} at high ionic strength [19]. Ordered aggregation or gel "strengthening" which follows unfolding is a relatively slow process (~ hours) [20] so that thermal effects arising from this source would not be detectable in the time scale of our experiments. We can thus conclude that reactions between malonaldehyde and reactive side chains of amino acid residues while not in themselves giving rise to substantial thermal effects initiate unfolding of myosin and its subfragments, which results in substantial absorption of heat. The enthalpies of malonaldehyde-induced unfolding are comparable to those observed on interaction with linoleic acid [15] and larger than those associated with thermal unfolding [19].

The results demonstrate that microcalorimetry is a useful tool for the detection of the interaction between malonaldehyde and myosins on a relatively short time scale (20–30 min). Previous studies [1,2,8] have been largely concerned with effects over several hours or days. It is clear however, that the malonaldehyde initiates effects which are detectable by microcalorimetry within minutes.

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