Microcalorimetric Investigation of the Interaction of Calmodulin with Seminalplasmin and Myosin Light Chain Kinase*

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Flow microcalorimetric titrations of calmodulin with seminalplasmin at 25 °C revealed that the high affinity one-to-one complex in the presence of Ca2+ (Comte, M., Mal noe, A., and Cox, J. A. (1986) Biochem. J. 240, 567-573) is entirely enthalpy-driven (ΔH° = -95 kJ·mol⁻¹; ΔS° = 0 J·K⁻¹·mol⁻¹; ΔC° = 0 J·K⁻¹·mol⁻¹) and is not influenced by the proton or Mg²⁺ concentration. The Sr²⁺- and Cd²⁺-promoted high affinity complexes are also exothermic for ~49 and ~45 kJ·mol⁻¹, respectively. The observed low affinity interaction in the absence of divalent ions displays no enthalpy change. No enthalpy changes are observed when calmodulin and seminalplasmin are mixed in the presence of millimolar concentrations of Mg²⁺, Zn²⁺, or Mn²⁺. Enthalpy titrations of the 1:1 calmodulin-seminalplasmin complex with Ca²⁺ and of partly Ca²⁺-saturated calmodulin with seminalplasmin revealed that only the species calmodulin-Ca₉₂ is fully competent for high affinity interaction with seminalplasmin. Binding of the second Ca²⁺ is strongly enhanced (K₉ = 5 x 10⁶ M⁻¹) as compared to that in free calmodulin (K₉ = 2.6 x 10⁶ M⁻¹). This is essentially due to the concomitant strongly exothermic step of isomerization of the calmodulin-seminalplasmin complex from its low to its high affinity form. Binding of the remaining two Ca²⁺ to the high affinity seminalplasmin-calmodulin complex displays the affinity constants and endothermic enthalpy change as in free calmodulin. A microcalorimetric study on the complex formation between Ca²⁺-saturated calmodulin and turkey gizzard myosin light chain kinase revealed that the interaction is strongly exothermic with an important overall gain of order (ΔH° = -95 kJ·mol⁻¹; ΔS° = -122 J·K⁻¹·mol⁻¹) and occurs with significant proton uptake (0.44 H⁺ per mol at pH 7.5). The observed low affinity interaction (K = 2.2 x 10⁵ M⁻¹) in the absence of Ca²⁺ (Mamar-Bachi, A., and Cox, J. A. (1987) Cell Calcium 8, 473-482) displays neither a change in enthalpy nor in protonation.

Calmodulin (CaM) often couples the action of Ca²⁺-mobilizing agonists acting outside the cell to cellular responses such as contraction, secretion, and proliferation (for review, see Refs. 1-3). As part of the activation mechanism, CaM binds 3 Ca²⁺ ions before it undergoes critical conformational changes which allow it to form a high affinity (Kninie = 10⁸ M⁻¹), 1:1 complex with the target enzymes (for review, see Ref. 4). Blumenthal and Stull (5) showed that in the case of skeletal muscle myosin light chain kinase (MLC kinase) hydrophobic forces and electrostatic interactions are primarily responsible for stabilizing the CaM-target complex. In a general way, this was confirmed by studies with so-called model peptides, i.e. small natural and synthetic peptides that form complexes with CaM which resemble those formed with the bulky target enzymes (6-8). A flow microcalorimetric study of the interaction of melittin, the best known model peptide, with CaM-Ca₉ (3) showed that at 25 °C the complex is primarily stabilized by hydrophobic interactions. However, melittin has four drawbacks as a model for CaM-regulated enzymes: 1) the peptide itself and its complex with CaM are strongly hemolytic; 2) strong nonspecific Ca²⁺-independent CaM-(melittin)₉ complexes are formed; 3) the positive charges are clustered, not scattered over the CaM-binding segment as in other CaM-target peptides (10), and 4) CaM-Ca₉-melittin, complex formation is endothermic (9), whereas it is exothermic in the case of at least one enzyme, i.e. MLC kinase(5). The newly discovered model peptide seminalplasmin (SP), an antimicrobial peptide found in seminal fluid, binds to CaM with a slightly higher affinity than melittin (10), is much less hemolytic than melittin, and is in its primary structure, depicted in Fig. 1, much more characteristic (10) of the small positively charged amphiphilic α-helical segments, i.e. the CaM-binding domains, present in the target enzymes (11-13).

Complex formation of CaM with its targets characteristically increases the affinity of CaM for Ca²⁺ by virtue of free energy coupling (14-18). Direct binding studies of the interactions between Ca²⁺, CaM, and turkey gizzard myosin light chain kinase revealed that the affinity increase appears to occur during binding of the third Ca²⁺ (19). A microcalorimetric investigation of the interactions in the CaM-Ca₉-melittin complex (9) indicated that only CaM-Ca₉ is capable of high affinity interaction with melittin. The strong affinity increase of Ca²⁺ for melittin is entirely entropy-driven with a ΔH° in the last two binding steps 2.5 kJ·site⁻¹ more endothermic than in the absence of melittin. Hence, in this case, the strengthening of the linkage between CaM and Ca²⁺ is entirely entropy-driven, and occupation of all the Ca²⁺-binding sites is required for complex formation. In the case of trifluoperazine too, free energy coupling increases the affinity of CaM for Ca²⁺ up to the fourth binding step, but the affinity increase is both enthalpy- and entropy-driven (20).

In this report, we describe the thermodynamic parameters of the interaction between CaM and SP, the effect of some divalent cations on this interaction, the influence of SP on
smooth muscle MLC kinase, taken fromMeans phenyl-Sepharose according to the method described by Gopalakrishna and Anderson (21), followed by hydroxylapatite chromatography. Metal removal from CaM was carried out as previously described (22). The CaM concentration was determined by spectrophotometry using a specific extinction coefficient of 34 and 35, respectively. The Ca2+-binding properties of CaM, and the enthalpy and entropy contributions in the process of free energy coupling. We also present the first microcalorimetric data on the interaction using a specific extinction coefficient of 

Table 1

Thermodynamic parameters for the interaction of CaM with MLC kinase, SP, and melittin (ME) &nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&n

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At 4 different temperatures from 25 to 40 °C, the \( \Delta H' \) for the interaction of SP with CaM-Ca (in PIPES buffer containing 500 \( \mu \)M Ca\(^{2+} \)) is \(-50 \pm 1 \) kJ \( \cdot \) mol\(^{-1} \), indicating that the heat capacity change, \( \Delta C_p' \), defined according to Kirchhoff's formula (\( \Delta C_p' = d\Delta H'/dT \)) can be considered as negligible in this temperature range.

**Ca\(^{2+} \)** Dependence of and Free Energy Coupling in the SP-CaM-Ca\(_2\) Complex—Similar to melittin and other CaM targets, SP is expected to introduce a marked increase in the affinity of CaM for Ca\(^{2+} \) which raises the question of what thermodynamic force is responsible for this affinity increase. In order to determine at which level of Ca\(^{2+} \) saturation the high affinity CaM-SP is formed, the enthalpy titration was measured at 25 °C after mixing 50 \( \mu \)M CaM with 50 \( \mu \)M SP, both pre-equilibrated in buffers containing varying concentrations of Ca\(^{2+} \) in order to obtain varying degrees of saturation of CaM (Fig. 3, o). Interestingly, at a ratio of two Ca\(^{2+} \) per CaM, the specific complex is already fully formed with a \( \Delta H' = -48 \) kJ \( \cdot \) mol\(^{-1} \) (i.e. close to the one in Fig. 2). The net intersection of the profile at the 2 Ca\(^{2+} \)/1 CaM equivalence point indicates that the second stoichiometric Ca\(^{2+} \)-binding constant is much higher than in free CaM (K\(_2 \approx 5 \times 10^{10} \) M\(^{-2} \)). The enthalpy increases linearly with the Ca\(^{2+} \) saturation from 0 to 2, indicating that: 1) the CaM-Ca\(_2\) species is fully capable to interact with high affinity with SP, 2) the thermodynamic parameters of binding of the third and fourth Ca\(^{2+} \) are not different from those in free CaM, and 3) at ratios of Ca\(^{2+} \)/CaM lower than 2, complex formation with SP favors an instantaneous redistribution of all CaM between the metal-free protein and the active species CaM-Ca\(_2\). Due to free energy coupling, CaM takes up some Ca\(^{2+} \) under the experimental conditions of Fig. 3. This endothermic reaction smooths the enthalpy profile at the equivalence point of 2 Ca\(^{2+} \) per CaM.

We also measured the enthalpy change of Ca\(^{2+} \) binding to an equivalent mixture of metal-free CaM and SP at 25 °C. Fig. 3 (+) shows that Ca\(^{2+} \) binding to the complex is accompanied by a production of heat which is linear up to 2 Ca\(^{2+} \) per complex (\( \Delta H'_{\text{max}} = -40 \) kJ \( \cdot \) mol\(^{-1} \)) followed by a decrease in enthalpy. Again, the sharp inflection point is indicative of a high K\(_2 \) value. These data can be interpreted as follows: from 0 to 2 Ca\(^{2+} \), two reactions take place, namely endothermic Ca\(^{2+} \) binding to CaM, followed by exothermic high affinity complex formation with SP. The linearity stems from the fact that any CaM-Ca\(_2\) formed is immediately redistributed to CaM and CaM-Ca\(_2\), the latter binding SP with high affinity. From 2 to 4 Ca\(^{2+} \) and above, 2 more Ca\(^{2+} \) bind endothermically to CaM, while for SP binding the same heat development is always observed. Assuming that the enthalpy change of Ca\(^{2+} \) binding is the same as for free CaM, i.e. endothermic for 4.25 kJ-site\(^{-1} \) in PIPES buffer (22), we “corrected” the enthalpy profile for this endothermic reaction bearing in mind that: 1) up to 2 Ca\(^{2+} \) per complex, all added Ca\(^{2+} \) binds due to free energy coupling (correction: +8.5 kJ at the ratio 2) and 2) for 2 to infinite total Ca\(^{2+} \) per complex we calculated (as in Ref. 22) the degree of saturation (\( v \)) assuming a K\(^v \) value of 10\(^-6\) M\(^{-1}\) for the last two binding steps (correction: +8.5 kJ + v \times 8.5 kJ). Fig. 3 (---) shows that the curve thus calculated, with a plateau at \(-48 \) kJ \( \cdot \) mol\(^{-1} \), is nearly identical with that obtained with Ca\(^{2+} \)-pre-equilibrated solutions of CaM and SP (Fig. 2, o). In order to test the validity of our deduction from the experiments of Fig. 3, we measured the enthalpy titration profile upon increasing the ratio of SP/CaM when CaM was saturated with 2.0 Ca\(^{2+} \). Fig. 2 shows that the latter curve is nearly superposable with the one in the case of CaM saturated with Ca\(^{2+} \), thus confirming that CaM-Ca\(_2\) is fully active in binding SP with high affinity.

**Enthalpy Titration of CaM-MLC Kinase Interaction in the Presence or Absence of EGTA**—As detailed in calorimetric studies with model peptides allowed us to define the optimal conditions for the study of the interaction between CaM and the physiological target MLC kinase, which was available to us in limited amounts. Fig. 4 shows the production of heat when MLC kinase is titrated with CaM at 25 °C in 50 mM PIPES, pH 7.5, 150 mM NaCl in the presence or absence of Ca\(^{2+} \). In the presence of Ca\(^{2+} \), the profile is strictly linear and shows a strict 1:1 stoichiometry in the complex. At 25 °C, binding is strongly exothermic with \( \Delta H' \) equal to \(-80 \) kJ mol\(^{-1} \). A preliminary experiment yielded the same \( \Delta H' \) at 40 °C. In 50 mM Tris buffer, pH 7.5, at 25 °C under otherwise identical conditions, a \( \Delta H' \) value of \(-64 \) kJ mol\(^{-1} \) was determined (not shown). This buffer dependency of \( \Delta H' \) is due to absorption of protons by the CaM-MLC kinase complex and, given the strong buffering capacity of the reaction medium, to subsequent proton release from the buffer. It was calculated (as in Ref. 22) that the proton uptake by the complex amounts to 0.44 H\(^+\) mol\(^{-1} \) and the corrected \( \Delta H' \) equals \(-85 \) kJ mol\(^{-1} \).

In the absence of Ca\(^{2+} \) (i.e. with both proteins pre-equilibrated in buffer containing 2 mM EGTA), the enthalpy change is barely measurable in both PIPES and Tris buffer, pH 7.5, although there is a very slight exothermic effect. It should be noted that, in the absence of Ca\(^{2+} \), MLC kinase binds CaM in a 1:1 complex with an affinity constant of 2 \( \times 10^{9} \) M\(^{-1}\) (19),

![Graph](image-url)
and, that under the experimental conditions of Fig. 4 (x), most of the protein (over 80%) would be expected to exist in the low affinity complex at a ratio of 1:1. Unfortunately, in contrast to the case of ion binding studies (30), the unitary entropy and heat capacity changes, as well as their hydrophobic and vibrational contributions, cannot be determined in peptide binding studies. Within the temperature range studied here, the heat capacity change is zero for both MLC kinase and SP binding to Ca$$^{2+}$$-saturated CaM. This means that in the case of MLC kinase the affinity decreases at increasing temperature (by a factor of 2 per 7°C), whereas the affinity of CaM for SP does not vary within the indicated temperature range. Using a method based on enzyme activation, Blumenthal and Stull (5) measured a $$\Delta H^\circ$$ = -42 kJ•mol$$^{-1}$$; $$\Delta S^\circ$$ = 42 J•K$$^{-1}$$•mol$$^{-1}$$ for skeletal muscle MLC kinase, i.e., substantially less enthalpy-driven than in our study. This obviously is due to the structural differences between the enzymes and/or the presence of the substrates, which can alter the interaction between MLC kinase and CaM (16, 17).

A surprising observation in this and the previous microcalorimetric study on the interaction of CaM with “model” peptides is that the latter may not be very good models for the interaction of CaM with target enzymes. From different enzyme studies (4, 19), the picture emerged that activation curves as a function of free Ca$$^{2+}$$ display a strong positive cooperativity which is a reflection of the binding isotherm of Ca$$^{2+}$$ to the complex enzyme-CaM. This apparent homotropic cooperativity is due to the stabilization of the complex from its low to its high affinity form in the third Ca$$^{2+}$$-binding step. In other words, the enzymes affect the third Ca$$^{2+}$$-binding constant much more than the first, second, and fourth. In the case of melittin, stabilization occurs in the fourth Ca$$^{2+}$$-binding step (9). The fact that the fourth constant is 2 to 3 orders of magnitude higher than in free CaM implies that the real high affinity complex is only formed with the species CaM-Ca$$^{4+}$$. In the case of SP (this study), complex stabilization already occurs when 2 Ca$$^{2+}$$ are bound to CaM, meaning that in the presence of SP the second constant is 2 orders of magnitude higher than in free CaM. Interestingly, the enthalpy change of the latter reaction is the same as in free CaM, but a strongly exothermic isomerization obligatorily follows. It is not clear from this study if the first Ca$$^{2+}$$-binding constant is also dramatically increased (this would necessitate direct determination of the binding constants as was done for MLC kinase(19)). The “successful” enthalpy profile correction in Fig. 2 indicates that the third and fourth Ca$$^{2+}$$-binding constants, as well as their enthalpy values, are not different from those in free CaM. In conclusion, energy coupling in the complex target-CaM-Ca$$^{4+}$$ occurs at different n values in different target systems, which may lead to the selective and sequential activation of CaM-regulated processes.

In spite of the structural similarities between the model peptides and amino acid segments of natural targets (10), enthalpy and entropy contributions in the complex formation between Ca$$^{2+}$$-saturated CaM and the target are very dissimilar: in the case of melittin, the reaction is strongly endothermic and thus necessitates a large increase in entropy. In the case of SP, the global entropy is zero and the reaction is essentially driven by the negative enthalpy change. Finally in the case of the natural target MLC kinase the interaction is so exothermic that it compensates for the quite strong global entropy decrease. Part of the differences may already be accounted for by the fact that melitin and SP binding to Ca$$^{2+}$$-saturated CaM at pH 7.5 is not accompanied by an overall change in the protonation state, whereas in the MLC kinase-CaM complex an ionizable group displays an increased affinity for H$^+$. The uptake of 0.44 H$^+$ by His or Lys (the most likely candidates at pH 7.5) is exothermic and can account for the differences between SP and MLC kinase. Interestingly, this uptake of protons seems to play a crucial role in the enzymatic activation of the MLC kinase-CaM complex (5, 31). Structural differences also may account for the differences in enthalpy changes: circular dichroism measurements have shown that 19 amino acid residues become helical in the complex with melittin (32), but only 11 residues in the case of SP (10) and 12 in the synthetic peptide of smooth muscle MLC kinase (11). Finally, it should be pointed out that in the intact enzymes communication must exist between the CaM-binding domain and the catalytic center, involving additional enthalpy and entropy changes, which does not occur in model peptides, even in those excised from the natural target enzymes. Microcalorimetric investigations comparing the synthetic peptides of CaM-regulated enzymes with the enzymes themselves should provide clues as to the conformational changes involved in this communication process.

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