Thermochimica Acta, 172 (1990) 1–10
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

THE USE OF CALORIMETRY AND THERMODYNAMIC ANALYSIS IN PROBING THE MOLECULAR DETAILS OF BIOCHEMICAL PROCESSES: ACTIVATION OF PHOSPHOLIPASE A_2*

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SUMMARY

In this paper we describe the overall strategy used in deducing a molecular model for the activation of phospholipase A_2 on lipid bilayer vesicles. The overall reaction includes two irreversible processes, enzyme activation and lipid hydrolysis, which complicate interpretation of the experimental data. Our approach includes the use of calorimetry to define the thermodynamics of some reversible subprocesses and to describe the thermotropic behavior of the lipid substrate; fluorescence spectroscopy to monitor equilibrium and irreversible changes in the enzyme and vesicle; and a pH stat to monitor the hydrolysis time course. Application of thermodynamic constraints and computer simulation of possible models allows elimination of several models for the activation process. The only model which is compatible with all the data is one in which the soluble, monomeric enzyme binds passively to the membrane surface, forms dimers reversibly and then spontaneously undergoes a quasi-irreversible change in structure to become maximally active. The degree of active enzyme-substrate association must be defined in steady-state rather than equilibrium thermodynamic terms. Furthermore, the rate of spontaneous activation is strongly coupled to dynamic structural changes in the vesicles which depend upon temperature and vesicle composition. The nature of the coupling between lipid structure changes and enzyme activation are being investigated using fluorescence spectroscopy and microcalorimetry.

I. INTRODUCTION

The sensitivity and sophistication of calorimetric techniques have now reached the level where they can be important tools in the delineation of the molecular details of biological processes. The important advances have been the development of heat leak microcalorimetry and the development of high sensitivity differential scanning calorimetry. The former type of instrumentation has been used to define the thermodynamics of complex equilibrium systems involving proteins and their various ligands. An example is the detailed thermodynamic description of coupled proton and inhibitor binding to ribonuclease A (1). High sensitivity differential scanning calorimetry (DSC) has been essential in establishing two principles of protein thermodynamic stability (2). First, it is the only means by which the validity of the two-state approximation for protein unfolding can be established (3). Second, it demonstrated directly that a very large heat capacity increase occurs upon protein unfolding (4), confirming the importance of the hydrophobic effect in protein stability as described by Kauzmann (5).

* Presented at the 7th International Symposium on Microcalorimetric Applications in Biology, Egham, U.K., 9–11 April 1990, and Dedicated to Ingemar Wadsö on the Occasion of his 60th Birthday.

0040-6031/90/$03.50

These studies used calorimetry as the primary technique to describe the thermodynamics of simple, reversible processes. This is not always possible when the system becomes more complex and may involve nonequilibrium processes. In these latter cases the use of microcalorimetry is, however, useful in defining the thermodynamics of the reversible processes which set rigorous, quantitative constraints upon descriptions of the overall reactions being investigated by a variety of other techniques. This calorimetric information used in conjunction with that obtained with other techniques, the rigorous application of the precepts of thermodynamics and extensive computer modelling allows one to probe deeply into the molecular nature of the overall process. In this paper we discuss this multifaceted approach by way of a brief review of our application of calorimetry and thermodynamic and kinetic analysis to the study of the activation of phospholipase A_2.

II. DESCRIPTION OF THE SYSTEM

Phospholipase A_2 (PLA_2) is a ubiquitous, small molecular weight enzyme (approximately 14,000 daltons) which catalyzes the hydrolysis of the sn-2 bond of phospholipids. This enzyme is readily obtainable in large quantities from snake venom and animal pancreas and is also found in platelets and a variety of other systems (6). In most cases Ca^{2+} is an absolute requirement for catalytic activity. A most interesting property of phospholipase A_2 is that its catalytic activity is most pronounced toward aggregated substrates of the phospholipid, as demonstrated by the fact that the enzyme's intrinsic activity toward micelles is several fold greater than it is toward monomeric substrate (7). It has also been shown that the enzyme can catalyze the hydrolysis of phospholipid bilayers, but this activity is strongly dependent on the dynamic structural state of the lipid bilayer.

Bilayer phospholipid vesicles can be readily prepared by a number of procedures (8,9). This report will focus on small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) made from dipalmitoylphosphatidylcholine (DPPC) as substrate. The heat capacity function for LUV (approximate diameter = 100 nm) is shown in figure 1. The temperature of the heat capacity maximum is 41 °C, the half-width is 1.0 °C and the enthalpy change approximately 7 kcal/mole lipid. Experimental data indicates that this gel-liquid crystalline transition is not first order, but is better described as a continuous order transition with little latent heat (11). This experimental description of the transition is consistent with the results of Monte Carlo calculations by Mouritsen and his group (12).

It has been clearly established that significant activity of PLA_2 toward LUV is only observed in the phase transition region (13). A characteristic time course for the catalytic reaction is shown in figure 2, where following a long latency phase, the enzymatic activity increases by up to 3 orders of magnitude very abruptly. The time, τ, required to reach this

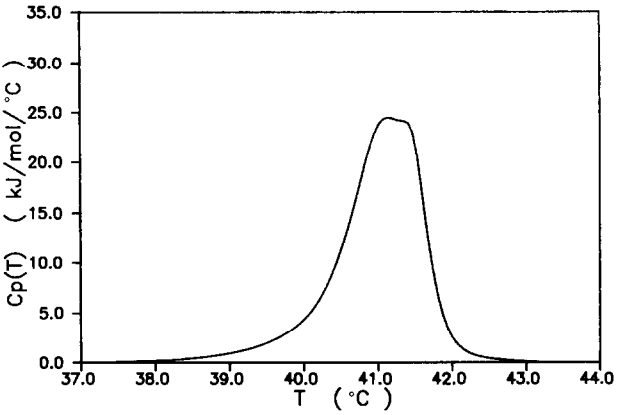

Fig. 1. Heat Capacity function of DPPC large unilamellar vesicles made by extrusion (13). This scan was performed with a DSC based on the heat-leak principle (14) at a scan rate of 10 °C/hr.

critical point is a function of enzyme and substrate concentration. The temperature dependence of τ (figure 3) mimics the inverse of the heat capacity function of the substrate. This strong correlation between τ^{-1} and $C_p(T)$ suggests that rate of apparent activation of the enzyme is

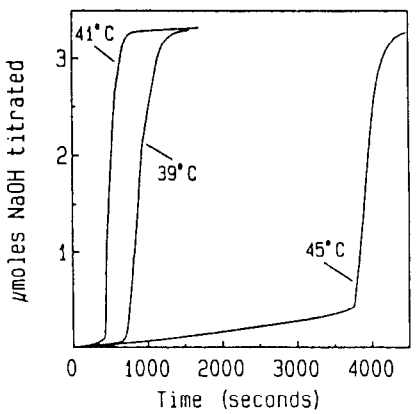

Fig. 2. Time courses of hydrolysis of DPPC LUV by AppD49 PLA_2 at three temperatures. (Reproduced from reference 20 with permission.)

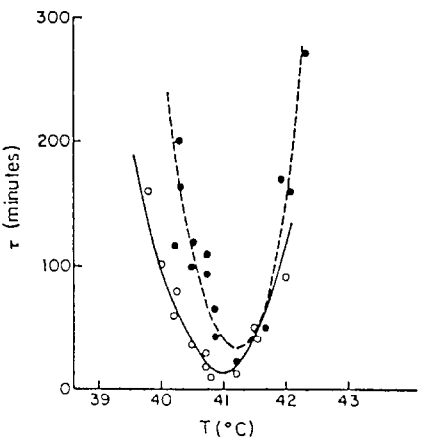

Fig. 3. The temperature of τ at two substrate concentrations. (Reproduced from reference 13 with permission.)

quantitatively related to the structural fluctuations of lipid vesicle. In order to test this hypothesis, it is necessary to establish an appropriate model for the reaction process. This model must be thermodynamically sound and describe all kinetic, structural and thermodynamic data in both qualitative and quantitative terms.

III. ESTABLISHMENT OF THERMODYNAMIC CONSTRAINTS

We first established some thermodynamic constraints to the overall set of reactions by defining experimentally the thermodynamics of the binding of calcium to the free enzyme. For the porcine pancreatic PLA_2, calorimetric studies showed the dissociation constant was approximately 0.3 mM with a ΔH of -18 kJ/mol (14) at 25 °C. This result demonstrates that, while specific binding of Ca^{2+} to the enzyme occurs, it does not apparently induce any thermodynamically significant structural change in the enzyme, and that the calcium affinity does not change significantly over the range of temperature used in our studies.

Noncalorimetric studies using the pancreatic enzyme are difficult because few physical observable changes upon binding of the enzyme to zwitterionic substrate. The best available evidence showing that the protein does interact with the zwitterionic substrate outside the phase transition region are the calorimetric results of Lichtenberg, et al. (13) which demonstrated that binding of the enzyme to LUV occurs with a stoichiometry of approximately 25 lipids per enzyme and an enthalpy change of -32 kJ/mol. These results indicated no thermodynamically significant structural change in the enzyme upon interaction with substrate. Unfortunately, the association constant could not be determined (except to note that it was significantly less than 10^{+3} M^{-1}) because of the limiting sensitivity of the calorimeter. However, PLA_2 from A. piscivorus piscivorus (AppD49) exhibits an extremely large fluorescence change upon interaction with SUV in the gel state (15). The binding to SUV was measured fluorometrically and an association constant was estimated to be on the order 10^4 M^{-1}. The binding is strongly coupled to the pretransition of the lipid matrix. The use of a nonhydrolyzable lipid in which the fatty acid at the sn-2 position was attached to the glycerol backbone by an ether rather than an ester linkage allowed study of the binding of the enzyme to lipid in the presence to Ca^{2+}. It was found that calcium does not affect the binding of the protein to the substrate, indicating that the initial substrate binding step is Ca^{2+} independent.

IV. KINETIC STUDIES

The Ca^{2+} dependence of enzymatic activity has been interpreted in terms of the existence of two Ca^{2+} binding sites, one of which is very weak (16). However, recent studies in our laboratory have shown that a major effect of Ca^{2+} at high concentration (>0.1 mM) is to relieve product inhibition (B. Lathrop, unpublished). It appears the product, a fatty acid which is negatively charged at pH 8.0, interacts very strongly with the enzyme in the absence of a

competitive cation. We have shown, however, that this product inhibition could be relieved by Mg^{2+}, although Mg^{2+} cannot support catalysis by itself. Evaluation of the Ca^{2+} dependence of activity toward SUV in the presence of Mg^{2+} demonstrated that only about 20 μM Ca^{2+} was required to achieve half maximal activity for both the AppD49 and porcine pancreatic PLA_2. On the other hand, the apparent Ca^{2+} dissociation constants for pancreatic and AppD49 PLA_2 are 0.3 and 1.6 mM (18), respectively. Thus Ca^{2+} binding to the active form of the enzyme has an association constant one to two orders of magnitude greater than that of the free enzyme. Insofar as Ca^{2+} does not affect the initial binding of the substrate to the vesicle, this increase in Ca^{2+} affinity upon activation is thermodynamic proof of a structural change in the enzyme-substrate complex following the initial binding step.

The activation of phospholipase A_2 on the surface of the vesicle is complex (see fig. 2). The time required to achieve maximal activity of the enzyme has been shown to be inversely dependent on substrate concentration and directly dependent upon enzyme concentration. A number of thermodynamic and kinetic models have been proposed, most of them based upon qualitative interpretation of available experimental data. Romero et al. (17) proposed that the activation process for porcine pancreatic PLA_2 involved dimerization of the monomeric enzyme on the vesicle surface. This was based on quantitative analysis of the enzyme and substrate concentration dependence of the early portion time course of the reaction process. Subsequently, an activated form of porcine pancreatic PLA_2 (18,19) was isolated and purified. This enzyme is acylated during the hydrolytic process and readily dimerizes in solution. Presumably, it is the acylation of the enzyme that enhances dimer formation in solution. Similar results were obtained with the AppD49 enzyme (18).

A dramatic irreversible increase in the fluorescence emission of the AppD49 is associated with the sudden increase in the rate of hydrolysis (20). Real-time, simultaneous measurement of the enzyme fluorescence and amount of product formation showed that the fluorescence change preceded the onset of rapid hydrolysis (figure 4A), and that the rate of hydrolysis and the magnitude of the fluorescence change were in temporal correspondence (figure 4B). Furthermore, the time at which the fluorescence change took place is in exact correlation with a change in the fluorescence of a probe of membrane structure. These results suggested that the sequence of events in PLA_2 activation are first binding of the enzyme to the substrate surface, slow hydrolysis of the lipid, a global change in the structural organization of the lipid matrix with simultaneous activation of the enzyme and, finally, rapid hydrolysis of the remaining phospholipid. The question which now remained was whether a quantitative scheme for the activation of PLA_2 that unified this broad range of experimental observations could be developed.

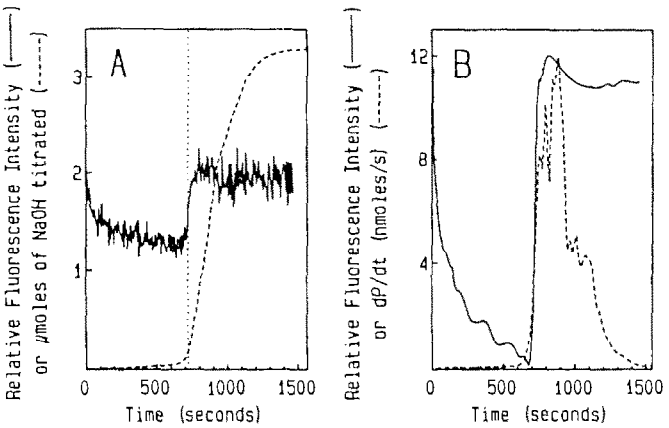

Fig. 4. Correlation of A. piscivorus piscivorus PLA_2 fluorescence with the time course of DPPC LUV hydrolysis. A, superposition of the time courses of vesicle hydrolysis (dashed line) and enzyme fluorescence (solid line). B, the derivative of the hydrolysis time course (dP/dt) was calculated from the data in A. The enzyme fluorescence was rescaled and smoothed to demonstrate more clearly the temporal correlation of the fluorescence change with dP/dt. (Reproduced from reference 20 with permission.)

V. COMPUTER SIMULATIONS

Computer simulations of various models which had placed upon them severe thermodynamic constraints were undertaken (J. Bell and R. Biltonen, submitted). These models included a product-induced phase change of the lipid bilayer. That is, abrupt activation occurs when a critical concentration of products is achieved within the substrate vesicle. Three types of reversible activation models, one in which the presence of the product only enhanced substrate binding of the enzyme and two in which the presence of the product induced a conformational change in and activation of the enzyme, were considered. In these three models, the enzyme does not remain active once it dissociates from the substrate surface. Two irreversible activation models in which the product-induced phase change enhanced to the rate of activation were considered. The primary experimental distinction between the models was that in both activation models, τ could be a minimum at finite substrate concentration under certain conditions, but no conditions under which τ would exhibit a minimum could be found for the equilibrium models. Experimental determination of τ as a function of substrate concentration showed clearly that such a minimum exists (figure 5). Therefore, activation by an equilibrium process appears to be incapable of explaining the experimental time course data.

A necessary condition for the existence of this minimum in τ for the irreversible activation models is that the apparent binding of the active enzyme to the substrate surface must be defined in steady-state rather than equilibrium terms. Further detailed analysis of qualitative behavior of the substrate dependence of τ and other parameters verified that only the dimeric activation model was consistent with all of the experimental data. It thus appears that after the enzyme binds to the vesicle a dimer is formed which is then activated. Furthermore, the enzyme remains active once it dissociates from the substrate surface.

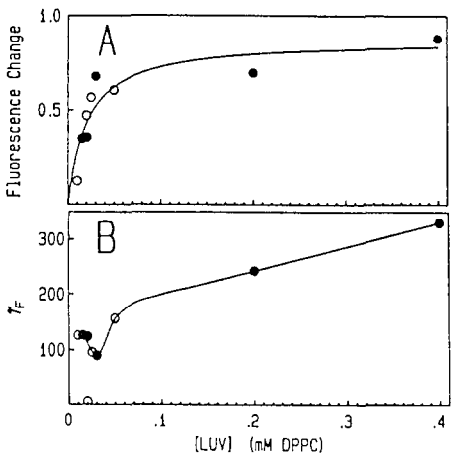

Fig. 5. Substrate dependence of the magnitude and time of onset of A. piscivorus piscivorus phospholipase A_2 fluorescence change during hydrolysis of LUV. A, the magnitude of the change in fluorescence. B, the time of onset of the increase in enzyme fluorescence intensity (τ_F) for the experiments indicated in A is the time at which the fluorescence time course in the presence of vesicles deviated from the time course in the absence of vesicles. For both panels, the solid circles and open circles represent points from experiments in the same series. (Reproduced from reference 20 with permission.)

VI. COUPLING OF LIPID STRUCTURAL CHANGES

The models of PLA_2 activation that have been considered assume a structural change in the vesicle structure as the cause of the sudden burst in activity. That such a change occurs was suggested by previous experiments using membrane fluorescence probes (20,21) and temporally correlating the putative change in the structure of the vesicle with the sudden burst in activity. Experiments over a broad range of conditions further showed that τ was observed at a constant mole-fraction of hydrolysis. Subsequent calorimetric experiments of vesicles containing various amounts of product suggested that above a concentration of products of about 10 mol-percent compositional phase separation occurs within the 2-dimensional lipid matrix.

The existence of a product-induced reorganization of the lipid substrate at a well-defined concentration of fatty-acid and lysolecithin was confirmed by experiments using a fatty-acid labelled with the fluorescence probe, pyrene (Qiang Yuan, unpublished). In this experiment pure DPPC vesicles containing approximately 0.1 mole-percent pyrene labelled fatty acid, was used as the substrate. The concentration distribution of pyrene is characterized by its fluorescence emission spectrum; as the concentration of the pyrene increases, molecules in the excited state tend to form a complex with unexcited molecules. This complex, called an excimer, has a characteristic emission spectrum distinguishable from the monomer fluorescence spectrum and thus the excimer/monomer fluorescence ratio (E/M) is a measure of the concentration of pyrene-labelled molecules (21). After addition of PLA_2 to the vesicles, the E/M ratio slowly changed until it abruptly increased, reached a maximum and then decreased. This result demonstrated that a fatty acid rich phase was formed at a low mole fraction of product and confirms the basic assumption that a product-induced compositional phase change does indeed occur and presumably promotes the enzyme activation process.

The product-induced phase change of the lipid is now being thermodynamically characterized using a titration calorimeter. In this experiment the substrate is thermally-equilibrated in the calorimeter and at time zero a small amount of the enzyme is added. The calorimetric signal, figure 6, is a direct measure of the rate of heat production by the system.

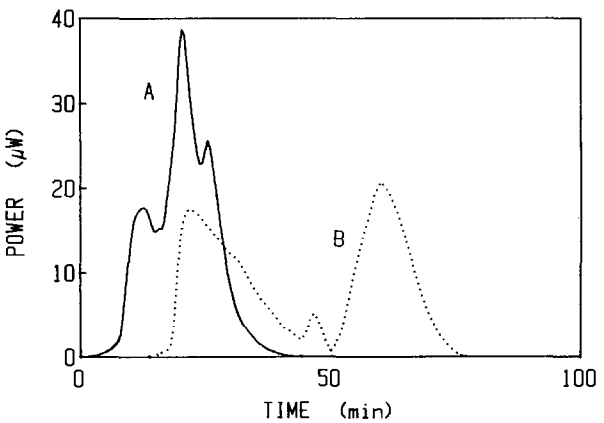

Fig. 6. Rate of heat production upon addition of PLA_2 to 1.0 mM DPPC LUV at 39°C [Ca^{2+}] = 10 mM (A) [E] = 6.6 μg/ml (B) [E] = 0.66 μg/ml. A Thermal Activity Monitor (LKB) with perfusion cell was used in these experiments.

The time course of this signal indicates three sequential processes. Preliminary interpretation of the data is that the first process was the lipid structural change since its magnitude is enzyme concentration independent. The second process reflects of the rate of enzyme catalyzed hydrolysis since its magnitude was dependent on enzyme concentration and the type of buffer used. The last process was likely Ca^{2+}-palmitate precipitation. These calorimetric results are consistent with and support the existence of a product-induced vesicle structural change as was assumed in the analysis of the kinetic data. More importantly, it appears that a more complete thermodynamic and kinetic description of all the processes involved in the hydrolysis of lipid can be obtaining using calorimetric procedures.

VII. CONCLUDING REMARKS

A thermodynamic description of specific subprocesses of PLA_2 activation were essential in the development of an understanding of the overall mechanism of the reaction. They were necessary to define specific thermodynamic constraints required to describe and interpret the experimental data qualitatively and quantitatively. It is clear that the use of calorimetry, thermodynamic analysis and computer modelling can yield detailed information of biochemical processes. It is anticipated that studies similar to that described will allow one to deduce molecular details of other processes of biochemical interest.

Acknowledgement: I would like to take this opportunity to thank Ingemar Wadsö of the University of Lund for his continued encouragement of calorimetric experimentation of biological systems. I also thank John Bell, Brian Lathrop, Qiang Ye, Qiang Yuan and Kim Thompson for providing access to unpublished data discussed herein. The work was supported by the National Institutes of Health, National Science Foundation and Office of Naval Research.

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