

## ITC VII - Enzyme Kinetics Assays (see also Protein-small molecule interactions)

Andujar-Sanchez M., Las Heras-Vazquez F. J., Clemente-Jimenez J. M., Martinez-Rodriguez S., Camara-Artigas A., Rodriguez-Vico F., and Jara-Perez V. (2006) Enzymatic activity assay of D-hydantoinase by isothermal titration calorimetry. Determination of the thermodynamic activation parameters for the hydrolysis of several substrates. *J Biochem Biophys Methods* **67**, 57-66.

**Abstract:** Isothermal titration calorimetry (ITC) has been applied to the determination of the activity of D-hydantoinase (EC 3.5.2.2) with several substrates by monitoring the heat released during the reaction. The method is based on the proportionality between the reaction rate and the thermal power (heat/time) generated. Microcalorimetric assays carried out at different temperatures provided the dependence of the catalytic rate constant on temperature. We show that ITC assay is a nondestructive method that allows the determination of the catalytic rate constant ( $k_{cat}$ ), Michaelis constant ( $K_M$ ), activation energy and activation Gibbs energy, enthalpy and entropy of this reaction.

Bianconi M. L. (2003) Calorimetric determination of thermodynamic parameters of reaction reveals different enthalpic compensations of the yeast hexokinase isozymes. *J Biol Chem* **278**, 18709-18713.

**Abstract:** The change in enthalpy and rate constants for the reactions of yeast hexokinase isozymes, PI (Hxk1) and PII (Hxk2), was determined at pH 7.6 and 25 degrees C by isothermal titration calorimetry. The reactions were done in five buffer systems with enthalpy of protonation varying from -1.22 kcal/mol (phosphate) to -11.51 kcal/mol (Tris), allowing the determination of the number of protons released during glucose phosphorylation. The reaction is exothermic for both isozymes with a small, but significant ( $p < 0.0001$ ), difference in the enthalpy of reaction ( $\Delta H_R$ ), with an  $\Delta H_R$  of -5.1 +/- 0.2 (mean +/- S.D.) kcal/mol for Hxk1, and an  $\Delta H_R$  of -3.3 +/- 0.3 (mean +/- S.D.) kcal/mol for Hxk2. The  $K_M$  for ATP determined by ITC was very similar to those reported in the literature for both isozymes. The effect of NaCl and KCl, from 0 to 200 mM, showed that although the rate of reaction decreases with increasing ionic strength, no change in the  $\Delta H_R$  was observed suggesting an entropic nature for the ionic strength. The differences in  $\Delta H_R$  obtained here for both isozymes strongly suggest that, besides glucose phosphorylation, another side reaction such as ATP hydrolysis and/or enzyme phosphorylation is taking place.

Bianconi M. L. (2007) Calorimetry of enzyme-catalyzed reactions. *Biophys Chem* **126**, 59-64.

**Abstract:** This mini-review shows the valuable contributions of Professor Julian Sturtevant to the current applications of calorimetry to the study of enzyme-catalyzed reactions. The more recent applications of calorimetric techniques such as isothermal titration calorimetry and flow calorimetry to the study of enzyme kinetics, as well as the advantages on using calorimetric techniques in the determination of kinetic parameters of enzymes, is also discussed here.

Cai L., Cao A., and Lai L. (2001) An isothermal titration calorimetric method to determine the kinetic parameters of enzyme catalytic reaction by employing the product inhibition as probe. *Anal Biochem* **299**, 19-23.

**Abstract:** An isothermal titration calorimetric (ITC) method was developed to measure the kinetic parameters of ribonuclease A catalytic hydrolysis of cytidine 2',3'-cyclic monophosphate. Employing the inhibition of product as a probe, the  $K(m)$ ,  $K(i)$ ,  $k(c)$ , and  $\Delta H(m)$  can be determined by two simple calorimetric measurements. First, the substrate was titrated into the cell containing high concentration of enzyme. The molar reaction heat was calculated from the titration peak area divided by substrate moles per titration, and the initial catalytic reaction rate in the presence of various concentrations of product can be calculated from the peak height and the molar reaction heat. From Michaelis-Menten function in the presence of inhibitors, the relationship between  $K(m)$  and  $K(i)$  can be obtained. Then, the dissociation constant, which is equal to  $K(i)$ , was measured by a regular ITC experiment. Thus,  $K(m)$  and  $k(c)$  can be calculated. The method developed here can be applied in other enzyme catalytic systems with inhibitive products.

Cai L., Cao A., and Lai L. (2002) Monitoring the kinetics and thermodynamics of interfacial enzymatic catalysis by differential scanning calorimetry. *Biochem Biophys Res Commun* **297**, 446-451.

**Abstract:** Using phase transition profile as an indicator of thermodynamic property and phase transition

heat as the second indicator of the percentage of substrates unhydrolyzed, differential scanning calorimetry has been used to observe in detail the kinetics and thermodynamics of phospholipase A(2)-catalyzed 1,2-dipalmitoyl-sn-glycero-3-phosphocholine large unilamellar vesicle (LUV) hydrolysis. Phase transition profiles show that the original LUV almost completely changes into a novel aggregate at the end of the latency, followed by an abrupt activation of the reaction. The phase transition profiles are asymmetric between the heating and cooling curves, indicating a thermodynamic mesostatic property of the system. The reaction in activated phase follows a single first-order kinetics and all of the substrates in vesicles can be hydrolyzed. All these evidences indicate that the products and substrates can freely exchange between the outer and the inner layers of the vesicles and the membrane of the vesicle in the activated phase is permeable. This permeability favors the exchange of the substrates and products, thus, resulting in the activation of the fast reaction.

D'Amico S., Sohler J. S., and Feller G. (2006) Kinetics and energetics of ligand binding determined by microcalorimetry: insights into active site mobility in a psychrophilic alpha-amylase. *J Mol Biol* **358**, 1296-1304.

**Abstract:** A new microcalorimetric method for recording the kinetic parameters  $k(\text{cat})$ ,  $K(\text{m})$  and  $K(\text{i})$  of alpha-amylases using polysaccharides and oligosaccharides as substrates is described. This method is based on the heat released by glycosidic bond hydrolysis. The method has been developed to study the active site properties of the cold-active alpha-amylase produced by an Antarctic psychrophilic bacterium in comparison with its closest structural homolog from pig pancreas. It is shown that the psychrophilic alpha-amylase is more active on large macromolecular substrates and that the higher rate constants  $k(\text{cat})$  are gained at the expense of a lower affinity for the substrate. The active site is able to accommodate larger inhibitory complexes, resulting in a mixed-type inhibition of starch hydrolysis by maltose. A method for recording the binding enthalpies by isothermal titration calorimetry in a low-affinity system has been developed, allowing analysis of the energetics of weak ligand binding using the allosteric activator chloride. It is shown that the low affinity of the psychrophilic alpha-amylase for chloride is entropically driven. The high enthalpic and entropic contributions of activator binding suggest large structural fluctuations between the free and the bound states of the cold-active enzyme. The kinetic and thermodynamic data for the psychrophilic alpha-amylase indicate that the strictly conserved side-chains involved in substrate binding and catalysis possess an improved mobility, responsible for activity in the cold, and resulting from the disappearance of stabilizing interactions far from the active site.

Freyer M. W. and Lewis E. A. (2008) Isothermal titration calorimetry: experimental design, data analysis, and probing macromolecule/ligand binding and kinetic interactions. *Methods Cell Biol* **84**, 79-113.

**Abstract:** Isothermal titration calorimetry (ITC) is now routinely used to directly characterize the thermodynamics of biopolymer binding interactions and the kinetics of enzyme-catalyzed reactions. This is the result of improvements in ITC instrumentation and data analysis software. Modern ITC instruments make it possible to measure heat effects as small as 0.1 microcal (0.4 microJ), allowing the determination of binding constants,  $K$ 's, as large as  $10(8) - 10(9)\text{M}(-1)$ . Modern ITC instruments make it possible to measure heat rates as small as 0.1 microcal/sec, allowing for the precise determination of reaction rates in the range of  $10(-12)$  mol/sec. Values for  $K(\text{m})$  and  $k(\text{cat})$ , in the ranges of  $10(-2) - 10(3)$  microM and 0.05 - 500 sec $(-1)$ , respectively, can be determined by ITC. This chapter reviews the planning of an optimal ITC experiment for either a binding or kinetic study, guides the reader through simulated sample experiments, and reviews analysis of the data and the interpretation of the results

Henzler K., Haupt B. and Ballauff M. (2008) Enzymatic activity of immobilized enzyme determined by isothermal titration calorimetry. *Anal Biochem* **378**, 184-189.

**Abstract:** The activity of adsorbed beta-glucosidase onto spherical polyelectrolyte brushes (SPBs) is investigated by UV-Vis spectroscopy and isothermal titration calorimetry (ITC). By comparing the results of these two methods, we demonstrate that ITC is a precise method for the study of the activity of immobilized enzymes. The carrier particles used for immobilization here consist of a polystyrene core onto which poly(acrylic acid) chains are grafted. High amounts of enzyme can be immobilized in the brush layer at low ionic strength by the polyelectrolyte-mediated protein adsorption (PMPA). Analysis of the activity of beta-glucosidase was done in terms of Michaelis-Menten kinetics. Moreover, the enzymatic activity of immobilized enzyme is studied by ITC using cellobiose as substrate. All data show that ITC is a general method for the study of the activity of immobilized enzymes

Jeoh T., Baker J. O., Ali M. K., Himmel M. E., and Adney W. S. (2005) beta-d-Glucosidase reaction kinetics from isothermal titration microcalorimetry. *Anal Biochem* **347**, 244-253.

**Abstract:** The cellobiase activities of nine thermal stable mutants of *Thermobifida fusca* BglC were assayed by isothermal titration microcalorimetry (ITC). The mutations were previously generated using random mutagenesis and identified by high-temperature screening as imparting improved thermal stability to the beta-d-glucosidase enzyme. Analysis of the substrate-saturation curves obtained by ITC for the wild-type enzyme and the nine thermally stabilized mutants revealed that the wild type and all the mutants were subject to binding of a second substrate molecule. Furthermore, the "inhibited" enzyme-substrate complexes were shown to retain catalytic activity. In the case of three of the BglC mutants (N178I, N317Y/L444F, and N317Y/L444F/A433V), binding of a second substrate molecule resulted in improved cellobiose turnover rates at lower substrate concentrations. No correlation between denaturation temperatures of the mutants and activity on cellobiose at 25 degrees C was evident. However, one particular mutant, BglC S319C, was significantly improved in both thermal tolerance and cellobiase activity with respect to those of the wild-type BglC. The triple mutant, N317Y/L444F/A433V, had a 5 degrees C increase in denaturation temperature while maintaining activity levels similar to that of the wild type at higher substrate concentrations. ITC provided a highly sensitive and nondestructive means to continuously monitor the reaction of BglC with cellobiose, resulting in abundant data sets that could be rigorously analyzed by fitting to known enzyme kinetics models. One distinct advantage of using data from the ITC was the empirical validation of the pseudo steady state assumption, a necessary condition for obtaining solutions to the proposed mechanisms.

Liang Y., Du F., Zhou B. R., Zhou H., Zou G. L., Wang C. X., and Qu S. S. (2002) Thermodynamics and kinetics of the cleavage of DNA catalyzed by bleomycin A5. *Eur J Biochem* **269**, 2851-2859.

**Abstract:**  $\mu$ calorimetry and UV-vis spectroscopy were used to conduct thermodynamic and kinetic investigations of the scission of calf thymus DNA catalyzed by bleomycin A5 (BLM-A5) in the presence of ferrous ion and oxygen. The molar reaction enthalpy for the cleavage, the Michaelis-Menten constant for calf thymus DNA and the turnover number of BLM-A5 were calculated by a novel thermokinetic method for an enzyme-catalyzed reaction to be  $-577 \pm 19$  kJ.mol<sup>-1</sup>,  $20.4 \pm 3.8$   $\mu$ m and  $2.28 \pm 0.49 \times 10^{-2}$  s<sup>-1</sup>, respectively, at 37.0 degrees C. This DNA cleavage was a largely exothermic reaction. The catalytic efficiency of BLM-A5 is of the same order of magnitude as that of lysozyme but several orders of magnitude lower than those of TaqI restriction endonuclease, NaeI endonuclease and BamHI endonuclease. By comparing the molar enthalpy change for the cleavage of calf thymus DNA induced by BLM-A5 with those for the scission of calf thymus DNA mediated by adriamycin and by (1,10-phenanthroline)-copper, it was found that BLM-A5 possessed the highest DNA cleavage efficiency among these DNA-damaging agents. These results suggest that BLM-A5 is not as efficient as a DNA-cleaving enzyme although the cleavage of DNA by BLM-A5 follows Michaelis-Menten kinetics. Binding of BLM-A5 to calf thymus DNA is driven by a favorable entropy increase with a less favorable enthalpy decrease, in line with a partial intercalation mode involved in BLM-catalyzed breakage of DNA.

Lonhienne T., Baise E., Feller G., Bouriotis V., and Gerday C. (2001) Enzyme activity determination on macromolecular substrates by isothermal titration calorimetry: application to mesophilic and psychrophilic chitinases. *Biochim Biophys Acta* **1545**, 349-356.

**Abstract:** Isothermal titration calorimetry has been applied to the determination of the kinetic parameters of chitinases (EC 3.2.1.14) by monitoring the heat released during the hydrolysis of chitin glycosidic bonds. Experiments were carried out using two different macromolecular substrates: a soluble polymer of N-acetylglucosamine and the insoluble chitin from crab shells. Different experimental temperatures were used in order to compare the thermodependence of the activity of two chitinases from the psychrophile *Arthrobacter* sp. TAD20 and of chitinase A from the mesophile *Serratia marcescens*. The method allowed to determine unequivocally the catalytic rate constant  $k_{cat}$ , the activation energy  $E(a)$  and the thermodynamic activation parameters ( $\Delta G(\#)$ ,  $\Delta H(\#)$ ,  $\Delta S(\#)$ ) of the chitinolytic reaction on the soluble substrate. The catalytic activity has also been determined on insoluble chitin, which displays an effect of substrate saturation by chitinases. On both substrates, the thermodependence of the activity of the psychrophilic chitinases was lower than that observed with the mesophilic counterpart.

Lonhienne T. G. and Winzor D. J. (2004) A potential role for isothermal calorimetry in studies of the effects of thermodynamic non-ideality in enzyme-catalyzed reactions. *J Mol Recognit* **17**, 351-361.

**Abstract:** Attention is drawn to the feasibility of using isothermal calorimetry for the characterization of enzyme reactions under conditions bearing greater relevance to the crowded biological environment, where kinetic parameters are likely to differ significantly from those obtained by classical enzyme kinetic studies in dilute solution. An outline of the application of isothermal calorimetry to the determination of enzyme kinetic parameters is followed by considerations of the nature and consequences of crowding effects in enzyme catalysis. Some of those effects of thermodynamic non-ideality are then illustrated by means of experimental results from calorimetric studies of the effect of molecular crowding on the kinetics of catalysis by rabbit muscle pyruvate kinase. This review concludes with a discussion of the potential of isothermal calorimetry for the experimental determination of kinetic parameters for enzymes either in biological environments or at least in media that should provide reasonable approximations of the crowded conditions encountered in vivo.

Lonhienne T. G. and Winzor D. J. (2002) Calorimetric demonstration of the potential of molecular crowding to emulate the effect of an allosteric activator on pyruvate kinase kinetics. *Biochemistry* **41**, 6897-6901.

**Abstract:** A method based on isothermal calorimetry is described for the direct kinetic assay of pyruvate kinase. In agreement with earlier findings based on the standard coupled assay system for this enzyme in the presence of a fixed ADP concentration, the essentially rectangular hyperbolic dependence of initial velocity upon phosphoenolpyruvate concentration is rendered sigmoidal by the allosteric inhibitor phenylalanine. This effect of phenylalanine can be countered by including a high concentration of a space-filling osmolyte such as proline in the reaction mixtures. This investigation thus affords a dramatic example that illustrates the need to consider potential consequences of thermodynamic nonideality on the kinetics of enzyme reactions in crowded molecular environments such as the cell cytoplasm.

Lonhienne T. G., Reilly P. E., and Winzor D. J. (2003) Further evidence for the reliance of catalysis by rabbit muscle pyruvate kinase upon isomerization of the ternary complex between enzyme and products. *Biophys Chem* **104**, 189-198.

**Abstract:** Isothermal calorimetry has been used to examine the effect of thermodynamic non-ideality on the kinetics of catalysis by rabbit muscle pyruvate kinase as the result of molecular crowding by inert cosolutes. The investigation, designed to detect substrate-mediated isomerization of pyruvate kinase, has revealed a 15% enhancement of maximal velocity by supplementation of reaction mixtures with 0.1 M proline, glycine or sorbitol. This effect of thermodynamic non-ideality implicates the existence of a substrate-induced conformational change that is governed by a minor volume decrease and a very small isomerization constant; and hence, substantiates earlier inferences that the rate-determining step in pyruvate kinase kinetics is isomerization of the ternary enzyme product complex rather than the release of products.

Magalhaes M. L. and Blanchard J. S. (2005) The Kinetic Mechanism of AAC(3)-IV Aminoglycoside Acetyltransferase from *Escherichia coli*. *Biochemistry* **44**, 16275-16283.

**Abstract:** The aminoglycoside 3-N-acetyltransferase AAC(3)-IV from *Escherichia coli* exhibits a very broad aminoglycoside specificity, causing resistance to a large number of aminoglycosides, including the atypical veterinary antibiotic, apramycin. We report here on the characterization of the substrate specificity and kinetic mechanism of the acetyl transfer reaction catalyzed by AAC(3)-IV. The steady-state kinetic parameters revealed a narrow specificity for the acyl-donor and broad range of activity for aminoglycosides. AAC(3)-IV has the broadest substrate specificity of all AAC(3)'s studied to date. Dead-end inhibition and ITC experiments revealed that AAC(3)-IV follows a sequential, random bi-bi kinetic mechanism. The analysis of the pH dependence of the kinetic parameters revealed acid- and base-assisted catalysis and the existence of three additional ionizable groups involved in substrate binding. The magnitude of the solvent kinetic isotope effects suggests that a chemical step is at least partially rate limiting in the overall reaction.

Morin P. E. and Freire E. (1991) Direct calorimetric analysis of the enzymatic activity of yeast cytochrome c oxidase. *Biochemistry* **30**, 8494-8500.

**Abstract:** The kinetic and thermodynamic parameters associated with the enzymatic reaction of yeast cytochrome c oxidase with its biological substrate, ferrocytochrome c, have been measured by using a titration microcalorimeter to monitor directly the rate of heat production or absorption as a function of time. This technique has allowed determination of both the energetics and the kinetics of the reaction under a

variety of conditions within a single experiment. Experiments performed in buffer systems of varying ionization enthalpies allow determination of the net number of protons absorbed or released during the course of the reaction. For cytochrome c oxidase the intrinsic enthalpy of reaction was determined to be -16.5 kcal/mol with one (0.96) proton consumed for each ferrocyanochrome c molecule oxidized. Activity measurements at salt concentrations ranging from 0 to 200 mM KCl in the presence of 10 mM potassium phosphate, pH 7.40, and 0.5 mM EDTA display a biphasic dependence of the electron transferase activity upon ionic strength with a peak activity observed near 50 mM KCl. The ionic strength dependence was similar for both detergent-solubilized and membrane-reconstituted cytochrome c oxidase. Despite the large ionic strength dependence of the kinetic parameters, the enthalpy measured for the reaction was found to be independent of ionic strength. Additional experiments involving direct transfer of the enzyme from low to high salt conditions produced negligible enthalpy changes that remained constant within experimental error throughout the salt concentrations studied (0-200 mM KCl). These results indicate that the salt effect on the enzyme activity is of entropic origin and further suggest the absence of a major conformational change in the enzyme due to changes in ionic strength.(ABSTRACT TRUNCATED AT 250 WORDS).

Oh B. C., Chang B. S., Park K. H., Ha N. C., Kim H. K., Oh B. H., and Oh T. K. (2001) Calcium-dependent catalytic activity of a novel phytase from *Bacillus amyloliquefaciens* DS11. *Biochemistry* **40**, 9669-9676.

**Abstract:** The thermostable phytase from *Bacillus amyloliquefaciens* DS11 hydrolyzes phytate (myo-inositol hexakisphosphate, IP6) to less phosphorylated myo-inositol phosphates in the presence of Ca<sup>2+</sup>. In this report, we discuss the unique Ca<sup>2+</sup>-dependent catalytic properties of the phytase and its specific substrate requirement. Initial rate kinetic studies of the phytase indicate that the enzyme activity follows a rapid equilibrium ordered mechanism in which binding of Ca<sup>2+</sup> to the active site is necessary for the essential activation of the enzyme. Ca<sup>2+</sup> turned out to be also required for the substrate because the phytase is only able to hydrolyze the calcium-phytate complex. In fact, both an excess amount of free Ca<sup>2+</sup> and an excess of free phytate, which is not complexed with each other, can act as competitive inhibitors. The Ca<sup>2+</sup>-dependent catalytic activity of the enzyme was further confirmed, and the critical amino acid residues for the binding of Ca<sup>2+</sup> and substrate were identified by site-specific mutagenesis studies. Isothermal titration calorimetry (ITC) was used to understand if the decreased enzymatic activity was related to poor Ca<sup>2+</sup> binding. The pH dependence of the V<sub>max</sub> and V<sub>max</sub>/K<sub>m</sub> consistently supported these observations by demonstrating that the enzyme activity is dependent on the ionization of amino acid residues that are important for the binding of Ca<sup>2+</sup> and the substrate. The Ca<sup>2+</sup>-dependent activation of enzyme and substrate was found to be different from other histidine acid phytases that hydrolyze metal-free phytate.

Pey A. L. and Martinez A. (2005) The activity of wild-type and mutant phenylalanine hydroxylase and its regulation by phenylalanine and tetrahydrobiopterin at physiological and pathological concentrations: An isothermal titration calorimetry study. *Mol Genet Metab* **86**, 43-53.

**Abstract:** The activity of phenylalanine hydroxylase (PAH) is regulated by the levels of both the substrate (l-Phe) and the natural cofactor (6R)-tetrahydrobiopterin (BH(4)). It has recently been observed that many PAH mutants associated with BH(4)-responsive phenylketonuria display abnormal kinetic and regulatory properties as shown by standard kinetic analyses. In this work, we have developed a high-sensitive and high-throughput activity assay based on isothermal titration calorimetry (ITC) in order to study the kinetic properties of wild-type PAH (wt-PAH) and the BH(4)-responsive c.204A>T (p.R68S) mutant at physiological and superphysiological concentrations of l-Phe and BH(4). Compared to wt-PAH, the p.R68S mutant showed reduced apparent and equilibrium binding affinity for the natural cofactor and increased affinity and non-cooperative response for l-Phe, together with a strong substrate inhibition that is alleviated at high BH(4) concentrations. For both wt-PAH and mutant, the apparent affinity for BH(4) decreases at increasing l-Phe concentrations, and the affinity for the substrate also depends on the cofactor concentration. Our results indicate that the activity landscape for wt and mutant enzymes is more complex than expected from standard kinetic analyses and highlight the applicability of this ITC-based assay to characterize the activity and regulation of PAH at a wide range of substrate and cofactor concentrations. Moreover, the results aid to understand the activity dynamics of wild-type and mutant PAH under physiological and pathological conditions, as well as BH(4)-responsiveness in certain PKU mutations.

Poduch E., Bello A.M., Tang S., Fujihashi M., Pai E.F., Kotra L.P. (2006) Design of inhibitors of orotidine monophosphate decarboxylase using bioisosteric replacement and determination of inhibition kinetics. *J Med Chem.* **49**, 4937-45.

**Abstract:** Inhibitors of orotidine monophosphate decarboxylase (ODCase) have applications in RNA viral, parasitic, and other infectious diseases. ODCase catalyzes the decarboxylation of orotidine monophosphate (OMP), producing uridine monophosphate (UMP). Novel inhibitors 6-amino-UMP and 6-cyano-UMP were designed on the basis of the substructure volumes in the substrate OMP and in an inhibitor of ODCase, barbituric acid monophosphate, BMP. A new enzyme assay method using isothermal titration calorimetry (ITC) was developed to investigate the inhibition kinetics of ODCase. The reaction rates were measured by monitoring the heat generated during the decarboxylation reaction of orotidine monophosphate. Kinetic parameters ( $k(\text{cat}) = 21 \text{ s}^{-1}$ ) and  $K_M = 5 \text{ }\mu\text{M}$ ) and the molar enthalpy ( $\Delta H(\text{app}) = 5 \text{ kcal/mol}$ ) were determined for the decarboxylation of the substrate by ODCase. Competitive inhibition of the enzyme was observed and the inhibition constants ( $K_i$ ) were determined to be  $12.4 \text{ }\mu\text{M}$  and  $29 \text{ }\mu\text{M}$  for 6-aza-UMP and 6-cyano-UMP, respectively. 6-Amino-UMP was found to be among the potent inhibitors of ODCase, having an inhibition constant of  $840 \text{ nM}$ . We reveal here the first inhibitors of ODCase designed by the principles of bioisosterism and a novel method of using isothermal calorimetry for enzyme inhibition studies.

Quesada-Soriano I., Leal I., Casas-Solvas J. M., Vargas-Berenguel A., Baron C., Ruiz-Perez L. M., Gonzalez-Pacanowska D. and Garcia-Fuentes L. (2008) Kinetic and thermodynamic characterization of dUTP hydrolysis by Plasmodium falciparum dUTPase. *Biochim Biophys Acta* **1784**, 1347-1355.

**Abstract:** Deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase) catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate and plays an important role in nucleotide metabolism and DNA replication controlling relative cellular levels of dTTP/dUTP, both of which can be incorporated into DNA. Isothermal titration calorimetry has been applied to the determination of the kinetic and thermodynamic parameters of the trimeric Plasmodium falciparum dUTPase, a potential drug target against malaria. The role of divalent ions in binding, and inhibition by different uridine derivatives has been assessed. When dUTP hydrolysis in the presence of EDTA was evaluated, a 105-fold decrease and a 12-fold increase of the  $k(\text{cat})$  and  $K_m$  values, respectively, were observed when compared with the dUTP.Mg<sup>2+</sup> complex. Calculation of the activation energy,  $E(a)$ , and the thermodynamic activation parameters showed that the energetic barrier was approximately 4-fold higher when Mg<sup>2+</sup> was depleted. Other divalent ions such as Co<sup>2+</sup> or Mn<sup>2+</sup> can substitute the physiological cofactor, however the  $k(\text{cat})$  was significantly reduced compared to dUTP.Mg<sup>2+</sup>. Binding and inhibition by dU, dUMP, dUDP, and alpha,beta-imido-dUTP were analysed by ITC and compared with data obtained by spectrophotometric methods and binding equilibrium studies. Product inhibition ( $K_{ip} \text{ dUMP: } 99.34 \text{ }\mu\text{M}$ ) was insignificant yet  $K_i$  values for dUDP and alpha,beta-imido-dUTP were in the low micromolar range. The effect of ionic strength on protein stability was also monitored. DSC analysis evidenced a slight increase in the unfolding temperature,  $T_m$ , with increasing salt concentrations. Moreover, the thermal unfolding pathway in the presence of salt fits adequately to an irreversible two-state model (N3 $\rightarrow$ 3D)

Saboury A. A. and Moosavi-Movahedi A. A. (1997) A simple novel method for determination of an inhibition constant by isothermal titration calorimetry. The effect of fluoride ion on urease. *J Enzyme Inhib* **12**, 273-279.

**Abstract:** A simple novel method was introduced for determination of an inhibitor binding constant ( $K_i$ ) and enthalpy of binding by isothermal titration calorimetry technique. This method was applied to the binding of fluoride ion, as an inhibitor, with the active sites of jack bean urease at  $\text{pH} = 7.0$  (Tris  $30 \text{ mM}$ ) and  $T = 300 \text{ degrees K}$ . The dissociation equilibrium constant measured by this method was markedly consistent with the inhibition constant obtained from assay of enzyme activity in the presence of fluoride ion.

Saboury A. A., Divsalar A., Jafari G. A., Moosavi-Movahedi A. A., Housaindokht M. R., and Hakimelahi G. H. (2002) A product inhibition study on adenosine deaminase by spectroscopy and calorimetry. *J Biochem Mol Biol* **35**, 302-305.

**Abstract:** Kinetic and thermodynamic studies have been made on the effect of the inosine product on the activity of adenosine deaminase in a  $50 \text{ mM}$  sodium phosphate buffer,  $\text{pH } 7.5$ , at  $27 \text{ degrees C}$  using UV

spectrophotometry and isothermal titration calorimetry (ITC). A competitive inhibition was observed for inosine as a product of the enzymatic reaction. A graphical-fitting method was used for determination of the binding constant and enthalpy of inhibitor binding by using isothermal titration calorimetry data. The dissociation-binding constant is equal to 140  $\mu\text{M}$  by the calorimetry method, which agrees well with the value of 143  $\mu\text{M}$  for the inhibition constant that was obtained from the spectroscopy method.

Saboury A. A., Divsalar A., Ataie G., Amanlou M., Moosavi-Movahedi A. A., and Hakimelahi G. H. (2003) Inhibition study of adenosine deaminase by caffeine using spectroscopy and isothermal titration calorimetry. *Acta Biochim Pol* **50**, 849-855.

**Abstract:** Kinetic and thermodynamic studies were made on the effect of caffeine on the activity of adenosine deaminase in 50 mM sodium phosphate buffer, pH 7.5, using UV spectrophotometry and isothermal titration calorimetry (ITC). An uncompetitive inhibition was observed for caffeine. A graphical fitting method was used for determination of binding constant and enthalpy of inhibitor binding by using isothermal titration microcalorimetry data. The dissociation-binding constant is equal to 350  $\mu\text{M}$  by the microcalorimetry method, which agrees well with the value of 342  $\mu\text{M}$  for the inhibition constant that was obtained from the spectroscopy method. Positive dependence of caffeine binding on temperature indicates a hydrophobic interaction.

Sarraf N. S., Saboury A. A., and Moosavi-Movahedi A. A. (2002) Product inhibition study on carbonic anhydrase using spectroscopy and calorimetry. *J Enzyme Inhib Med Chem* **17**, 203-206.

**Abstract:** Kinetic and thermodynamic studies have been made on the effect of the p-nitrophenol product on the activity of bovine carbonic anhydrase in 50 mM Tris buffer pH 7.5, at 300K using UV spectrophotometry and isothermal titration calorimetry (ITC). A competitive inhibition was observed for p-nitrophenol as a product of the enzymatic reaction. A graphical fitting method was used for determination of the binding constant and enthalpy of inhibitor binding using ITC data. The dissociation binding constant was 0.10mM by the calorimetric method, which is in good agreement with the value of 0.11 mM for the inhibition constant obtained from the spectrophotometric method.

Spencer S. D. and Raffa R. B. (2004) Isothermal titration calorimetric study of RNase-A kinetics (cCMP -- > 3'-CMP) involving end-product inhibition. *Pharm Res* **21**, 1642-1647.

**Abstract:** PURPOSE: Isothermal titration calorimetry (ITC) and progress curve analysis was used to measure the enzyme kinetic parameters ( $K_M$  and  $k_{cat}$ ) of the hydrolysis of cCMP by RNase-A, a reaction that includes end-product competitive inhibition by 3'-CMP. METHODS: The heat generated from injection of 9-15  $\mu\text{l}$  cCMP (20 mM) into bovine pancreatic RNase-A (600 nM) in 50 mM  $\text{Na}^+$  acetate buffer (pH 5.5; 37 degrees C) was monitored for 1500-2000 s. Thermal power ( $dQ/dt$ ), equal to  $(1)/\Delta H_{app} \times d(\text{cCMP})/dt$  was recorded every 1 s. The end-product inhibition constant ( $K_p$ ) and enthalpy of the inhibitor binding interaction was obtained from the saturation data of 60 sequential injections of 3'-CMP (1.2 mM) into 0.05 mM RNase-A. The data of the plot of  $-d[\text{cCMP}]/dt$  against  $[\text{cCMP}]$  were fitted to kinetic equations incorporating  $K_p$  to yield  $K_M$  and  $k_{cat}$ . RESULTS:  $\Delta H_{app}$  for each run was obtained by integration of the progress curve. The plot of  $-d[\text{cCMP}]/dt$  against  $[\text{cCMP}]$  yielded the kinetic parameters  $K_M = 105.3 \mu\text{M}$ , 121.6  $\mu\text{M}$ , and 131.3  $\mu\text{M}$ ;  $k_{cat} = 1.63 \text{ s}^{-1}$ , 1.56  $\text{s}^{-1}$ , and 1.71  $\text{s}^{-1}$ . The end-product bound with 1:1 stoichiometry and  $K_p = 53.2 \mu\text{M}$ . CONCLUSIONS: The combination of progress curve analysis and ITC allowed rapid and facile measurement of the kinetic parameters for catalytic conversion of cCMP to 3'-CMP by RNase-A, a reaction complicated by end-product inhibition.

Todd M. J. and Gomez J. (2001) Enzyme kinetics determined using calorimetry: a general assay for enzyme activity? *Anal Biochem* **296**, 179-187.

**Abstract:** Two techniques for determining enzyme kinetic constants using isothermal titration calorimetry are presented. The methods are based on the proportionality between the rate of a reaction and the thermal power (heat/time) generated. (i) An enzyme can be titrated with increasing amounts of substrate, while pseudo-first-order conditions are maintained. (ii) Following a single injection, the change in thermal power as substrate is depleted can be continuously monitored. Both methods allow highly precise kinetic characterization in a single experiment and can be used to measure enzyme inhibition. Applicability is demonstrated using a representative enzyme from each EC classification, including (i) oxidation-reduction activity of DHFR (EC 1.5.1.3); (ii) transferase activity of creatine phosphokinase (EC

2.7.3.2) and hexokinase (EC 2.7.1.1); (iii) hydrolytic activity of *Helicobacter pylori* urease (EC 3.5.1.5), trypsin (EC 3.4.21.4), and the HIV-1 protease (EC 3.4.21.16); (iv) lyase activity of heparinase (EC 4.1.1.7); and (v) ligase activity of pyruvate carboxylate (EC 6.4.1.1). This nondestructive method is completely general, enabling precise analysis of reactions in spectroscopically opaque solutions, using physiological substrates. Such a universal assay may have wide applicability in functional genomics.

Todorova N. A. and Schwarz F. P. (2008) Effect of the phosphate substrate on drug-inhibitor binding to human purine nucleoside phosphorylase. *Arch Biochem Biophys* **480**, 122-131.

**Abstract:** The thermodynamics of the drug-inhibitors acyclovir, ganciclovir, and 9-benzylguanine binding to human purine nucleoside phosphorylase (hsPNP) were determined from isothermal titration calorimetry as a function of the substrate phosphate ion (Pi) concentration from 0 to 0.125 M and temperature from 15 degrees C to 35 degrees C. At 25 degrees C and with an increase in the Pi concentration from 0 to 50mM, acyclovir binding becomes more entropically-driven and ganciclovir binding becomes more enthalpically-driven. At 25 degrees C, the tighter 9-benzylguanine binding reaction goes from an enthalpically-driven reaction in the absence of Pi to an entropically-driven reaction at 10 mM Pi, and the enthalpically-driven nature of the binding reaction is restored at 75 mM Pi. Since the dependencies of the driving-nature of the binding reactions on Pi concentration can be simulated by Pi binding to its catalytic site, it is believed that bound Pi affects the interactions of the side-chains with the ribose catalytic site. However, the binding constants are unaffected by change in the bound Pi concentration because of enthalpy-entropy compensation. The enzymatic activity of hsPNP was determined by an ITC-based assay employing 7-methylguanosine and Pi as the substrates. The heat of reaction determined from the assay increased by 7.5 kJ mol<sup>-1</sup> with increase in Pi concentration from 50 to 100mM and is attributed to weak binding of the Pi to a secondary regulatory site. Although the binding constants of acyclovir and ganciclovir at 20 microM hsPNP were in agreement with the inverse inhibition constants determined from the ITC enzyme inhibition assays at 60 nM, the binding constant of 9-benzylguanine, which interacts with Phe159 from an adjacent subunit, decreased from 5.62 x 10<sup>5</sup> M<sup>-1</sup> to 1.14 x 10<sup>5</sup> M<sup>-1</sup>. This reduction in the 9-benzylguanine binding affinity along with a 7-fold increase in the specific activity of hsPNP at 14.5 nM results from partial dissociation of the hsPNP trimer into monomers below the 60 nM level

Watt G. D. (1990) A microcalorimetric procedure for evaluating the kinetic parameters of enzyme-catalyzed reactions: kinetic measurements of the nitrogenase system. *Anal Biochem* **187**, 141-146.

**Abstract:** The mechanism of nitrogenase catalysis, as evaluated from steady-state kinetic measurements, is presently unresolved primarily due to conflicting results regarding the reaction order of the nitrogenase reductant, S<sub>2</sub>O<sub>2</sub>-4, at high concentrations. A microcalorimetric method was developed and is described which measures the rate of heat production (and hence the rate of reactant disappearance or product formation) as a function of time. Because each substrate reaction order has a unique profile for the rate of heat production with time, the described procedure provides a means for establishing the substrate reaction order for the enzyme-catalyzed reaction under consideration by visual inspection of the resulting thermogram. The rate constant and other kinetic parameters are obtained from analysis of the shape of the thermogram and thermodynamic parameters are evaluated from either the shape of or the area bound by the thermogram. Application of this procedure to the nitrogenase system has confirmed one-half- and first-order reaction orders under limiting conditions for the S<sub>2</sub>O<sub>2</sub>-4 and MgATP substrates during the enzyme-catalyzed reaction for this important biological process. From a single thermogram, the enthalpy of reaction and the kinetic rate law are readily evaluated. The procedure is completely general in nature and is applicable to any chemical or biochemical system that evolves heat.

Worsham L. M., Langston K. G., and Ernst-Fonberg M. L. (2005) Thermodynamics of a protein acylation: activation of *Escherichia coli* hemolysin toxin. *Biochemistry* **44**, 1329-1337.

**Abstract:** HlyC, hemolysin-activating lysine-acyltransferase, catalyses the acylation (from acyl-acyl carrier protein [ACP]) of *Escherichia coli* prohemolysin (proHlyA) on the epsilon-amino groups of specific lysine residues, 564 and 690 of the 1024 amino acid primary structure, to form hemolysin (HlyA). Isothermal titration calorimetry was used to measure the thermodynamic properties of the protein acylation of proHlyA-derived structures, altered by substantial deletions and separation of the acylation sites into two different peptides and site directed mutation analyses of acylation sites. Acylation of proHlyA-derived proteins catalyzed by HlyC was overall an exothermic reaction driven by a negative enthalpy. The reaction, whose kinetics are compatible to a ping-pong mechanism, is composed of two partial reactions. The first,

the formation of an acyl-HlyC intermediate, was entropically driven, most likely by noncovalent complex formation between acyl-ACP and HlyC; enthalpy-driven acyl transfer followed, resulting in acyl-HlyC and ACPSH product formation. The second partial reaction was an energetically unfavorable acyl transfer from acyl-enzyme intermediate to the final acyl acceptor, a proHlyA derivative. Overall the acylation of proHlyA-derived proteins catalyzed by HlyC was driven by the energetics of the acyl enzyme intermediate reaction. Of the two acylation sites, intactness of the site equivalent to proHlyA K564 was more important for acylation reaction thermodynamic stability.

Wright E. and Serpersu E. H. (2005) Enzyme-substrate interactions with an antibiotic resistance enzyme: aminoglycoside nucleotidyltransferase(2'')-Ia characterized by kinetic and thermodynamic methods. *Biochemistry* **44**, 11581-11591.

**Abstract:** Aminoglycoside nucleotidyltransferase(2'')-Ia is one of the most often detected enzymes in aminoglycoside-resistant bacteria. Despite its prevalence, little biochemical and biophysical work has been reported for this enzyme. In the current study, substrate specificity and temperature dependence of  $k(\text{cat})$  are determined by kinetic assays. Dissociation constants and thermodynamic properties of enzyme-substrate complexes are determined by isothermal titration calorimetry, electron paramagnetic resonance, and fluorescence spectroscopy. Kinetic studies show that aminoglycosides with 2'-NH(2) are better substrates (higher  $k(\text{cat})/K(\text{m})$ ) than ones with 2'-OH when magnesium(II) is used as the catalytically required divalent cation. The activity is reduced 10-fold for substrates with 2'-NH(2) when manganese(II) replaces magnesium as the required metal. However, kanamycin A, which has a 2'-OH, shows a much smaller decrease in activity when manganese substitutes for magnesium as the divalent cation. Temperature dependence studies show the activation energy of catalysis to be 19.2 kcal/mol and the temperature optimum between 30 and 32 degrees C. The binding of the aminoglycoside substrate tobramycin to the enzyme occurs with a favorable enthalpy which compensates for a large entropic penalty to yield a negative  $\Delta G$  value for the complex formation. Enthalpy of binding is less exothermic in the presence of metal-nucleotide. However, due to the more favorable entropy, a more favorable  $\Delta G$  is observed for the formation of the enzyme-metal-nucleotide:aminoglycoside complex. Tobramycin binds to ANT(2'') with a dissociation constant of 0.6  $\mu\text{M}$ , which is further reduced by 3-fold when metal-nucleotide is present. Binding of ATP to the enzyme is determined to be very weak in the absence of a divalent cation, and becomes 2 orders of magnitude tighter when magnesium or manganese is present. Binding studies also show that, in addition to binding to the enzyme in the form of metal-nucleotide complex, a second catalytically required metal binds to an additional site on the enzyme.

Xia L., Yuwen L., Jie L., Huilin L., Xi Y., Cunxin W., and Zhiyong W. (2004) Kinetic studies on Na<sup>+</sup>/K<sup>+</sup>-ATPase and inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase by ATP. *J Enzyme Inhib Med Chem* **19**, 333-338.

**Abstract:** Na<sup>+</sup>/K<sup>+</sup>-ATPase (EC 3.6.1.3) is an important membrane-bound enzyme. In this paper, kinetic studies on Na<sup>+</sup>/K<sup>+</sup>-ATPase were carried out under mimetic physiological conditions. By using microcalorimeter, a thermokinetic method was employed for the first time. Compared with other methods, it provided accurate measurements of not only thermodynamic data ( $\Delta H_m$ ) but also the kinetic data ( $K_m$  and  $V_{\text{max}}$ ). At 310.15K and pH 7.4, the molar reaction enthalpy ( $\Delta H_m$ ) was measured as  $-40.514 \pm 0.9 \text{ kJmol}^{-1}$ . The Michaelis constant ( $K_m$ ) was determined to be  $0.479 \pm 0.020 \text{ mM}$  and consistent with literature data. The reliability of the thermokinetic method was further confirmed by colorimetric studies. Furthermore, a simple and reliable kinetic procedure was presented for ascertaining the true substrate for Na<sup>+</sup>/K<sup>+</sup>-ATPase and determining the effect of free ATP. Results showed that the MgATP complex was the real substrate with a  $K_m$  value of about 0.5mM and free ATP was a competitive inhibitor with a  $K_i$  value of 0.253 mM.