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High and ultra low doses of mercuric chloride affect α -amylase starch interaction through two different binding sites of the enzyme

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ABSTRACT

Ultra low doses of mercuric chloride, called potencies, promote activity of diastase or α -amylase in terms of breakdown of starch, a polysaccharide into a disaccharide maltose in a cell-free medium in test tubes. *Merc cor* or HgCl_2 in high doses inhibits the enzyme activity. The objectives of the present study are to see (i) whether the high and ultra low dose effects of HgCl_2 involve different binding sites of the enzyme and (ii) to find an explanation for the low dose effect of HgCl_2 in spite of absence of its original molecules. *Merc cor* mother tincture (147 mM HgCl_2 solution in distilled water) was used undiluted in this experiment. *Merc cor* 200cH and 1000cH, in 90% ethanol and ethanol, also 90%, were diluted with deionized, distilled water 1:1000 to minimize ethanol content in test solutions. The control consisted of distilled water in lieu of *Merc cor* or ethanol. An isothermal calorimetric (ITC) instrument was used to

measure the interaction between soluble starch and α -amylase with each potency of *Merc cor*, its mother tincture, ethanol and control. Soluble starch 90 μM was injected into 300 μl of 15 μM α -amylase at 6 μl / injection. Twenty injections, one every 2 min, were given. The enzyme substrate interaction in terms of heat released (exothermic) or absorbed (endothermic) were monitored by the ITC instrument. All ITC measurements were analysed by an in-built software Origin 7. The data are presented in figures. While *Merc cor* mother tincture shows endothermic reaction, all its potencies, ethanol and water control show exothermic reactions. There is wide variation in enthalpy (ΔH) entropy (ΔS) and binding constant (K) among the *Merc cor* and ethanol treated samples and also control. It is concluded that mother tincture and potencies produce different effects on the enzyme substrate interaction. Potencies show wide variation in ΔH , ΔS and K values. It appears from the results that the drugs used in homeopathy produce dual action on proteins. At high doses they act on a binding site(s) but at ultra low doses they act on a different binding site(s).

KEYWORDS

Starch, α -amylase, *Merc cor*, potencies, HgCl_2 , ITC, enthalpy, entropy, binding site

INTRODUCTION

Drugs at ultra low doses have been used in homeopathy for more than a couple of centuries. According to homeopathic principle symptoms produced by high doses of a drug on a healthy individual would be alleviated in a patient suffering from any disease but showing similar high dose symptoms, by ultra low doses of the same drug. However, the low dose effect becomes questionable when the dose crosses the Avogadro number resulting in virtual absence of original drug molecules. Mercuric chloride is a known inhibitor of starch-digesting enzyme α -amylase. This drug has been used in homeopathy for treatment of patients suffering from various ailments [1,2]. In a series of experiments Boyd (1954) demonstrated that ultra low dose of mercuric chloride, called *Merc cor* 200cH

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promoted activity of the enzyme diastase. His experiments were followed up by some other scientists. The objectives of the present study are (1) to see whether the high and ultra low dose effects of mercuric chloride involve different binding sites of α -amylase and (2) to find out an explanation for

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the low dose effect inspite of the absence of original drug molecules.

Using a new technology of isothermal calorimetry (ITC) we have been able to demonstrate α -amylase-starch interaction with high and ultra low doses of mercuric chloride in a cell-free medium. The results proved beyond doubt the effectiveness of an ultra low dose of HgCl_2 and also helped in finding an explanation for the initiation of action of low doses. ITC is a thermodynamic technique which helps in measuring directly the heat evolved during a chemical reaction. In the present case the reaction occurs between α -amylase and soluble starch resulting in breakdown of starch into product maltose. The heat evolved during the formation of one mole of the product is actually ΔH , the molar variation of enthalpy of the reaction. The reaction may cause release of heat (exothermic) or absorption of heat (endothermic). In the titration experiment a small amount of a substrate is injected at regular intervals into a measurement cell containing the enzyme. The heat released or absorbed during the reaction at each injection is monitored. We would see here how the intervention of a third substance, here *Merc cor* 200cH, 1000 cH and its diluent medium aqueous ethanol could alter the reaction in terms of heat generation.

MATERIALS AND METHODS

Porcine pancreatic α -amylase and soluble starch were obtained from SRL, Mumbai. The enzyme activity has been mentioned as 1-25 units/mg. *Merc cor* 200 cH, a product of Dr. Reckeweg, Germany, was purchased. The ethanol content of drug was 90%. We prepared

90% ethanol. Both ethanol and the drug were diluted with sterile deionized distilled water 1:1000 just before the experiment to minimize the ethanol content so as to eliminate the alcohol effect of either the drug and the medium [3]. Using a UV-VIS spectrophotometer (Shimadzu, model-UV-2401 PC) optical density (OD) of drug and ethanol solutions was measured at 220.5nm. Calorimetric measurements were made by ITC 200, GE Healthcare, Biosciences Ltd, Sweden. Reaction cells (0.2 ml) were filled with degassed solutions and equilibrated at the indicated temperature. Stirring speed was 400 rpm and thermal power was recorded every 2 s. Both the sample and reference cells containing buffer only were maintained at a constant temperature of 36°C. Once the thermal equilibrium was reached injections of the substrate solution (90 μM soluble starch) was started every 2 min. Twenty injections were given, and the amount of starch solution released per injection was 6 μl into 300 μl of 15 μM α -amylase. The drug, ethanol or water control were mixed with α -amylase in the proportion of 1:10. Starch solution was in sodium acetate buffer having pH 4.7.

RESULTS

Optical Densities of drug and control having ethanol content at 90%, 70%, 50%, 25%, 10% and 5% were 1.66, 1.29, 0.46, 0.18 and 0.09, respectively. After dilution with distilled water 1:1000 the O.D. of the drug and ethanol, which were in 90% ethanol, was 0.03. The calorimetric trace in terms of heat flow as a function of time is shown in Figure 1a for α -amylase + water control and soluble starch. Figure 1b shows heat released per mole of starch during interaction with α -amylase in relation to molar ratio (starch / α -amylase). Figure 2a shows heat versus time for α -amylase+*Merc cor* 200 cH, and 2b heat change versus molar ratio of starch/ α -amylase. Similarly, Figure 3a represents heat rate versus time and 3b does heat change versus molar ratio of starch/ α -amylase for starch+ α -amylase + *Merc cor* 1000 cH. Figure 4a stands for heat rate vs time and 4b for heat change vs molar ratio of starch/ α -amylase + starch +

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147mM HgCl_2 solution. Figure 5a represents heat rate versus time, and 5b heat change versus molar ratio of starch/ α -amylase for starch + α -amylase + ethanol. The amount of *Merc cor* 200 cH, *Merc cor* 1000 cH and HgCl_2 solution mixed with α -amylase was same as in the control. ITC data were analysed for binding constant (K), change in enthalpy (ΔH) and in entropy (ΔS) by a software origin 7.

ITC data indicate single site binding of starch to amylase for all the samples. While the control (Fig. 1a,b), *Merc cor* 200 cH (Fig. 2a,b) and *Merc cor* 1000 cH (Fig. 3a,b) and ethanol (Fig 5 a,b) show enzyme-substrate reaction as exothermic, 147 mM HgCl_2 solution or *Merc cor* mother tincture (θ) (Fig. 4a,b) shows endothermic reaction. HgCl_2 shows strong enzyme starch interaction and positive ΔH and ΔS values. In all other cases these values are negative. ΔS indicates change in entropy. ΔH represents change in enthalpy. The

interactions together bring about very strong stabilizing force for proteins. These weak interactions are at the heart of the enzyme catalysis. Water directly participates in hydrolysis [4]. We have already observed that free water molecules and hydrogen bond strength form the physical basis of homeopathic potencies [5-11]. Formation of weak bonds between substrate and enzyme results desolvation of the substrate. Enzyme substrate interactions replace most or all of the hydrogen bonds between the substrate and water [4]. It is possible that water molecules released during desolvation may bind to free water molecules from *Merc cor* potencies at one substrate molecule, and this leads to a chain reaction involving other molecules through the process of proton hopping and opposite movement of OH groups. Higher H bond strength of OH groups is redistributed resulting in an equilibrium. All these events contribute to enhancement of enzyme substrate interaction.

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free energy change is given by ΔG , which is calculated from ΔH and ΔS . In each figure, binding constant (K), change in enthalpy (ΔH), change in entropy (ΔS) and ΔG are given. The best -fit parameters are recorded in the figures (b) in the form of non-linear regression.

DISCUSSION

The binding of starch molecules to protein molecules is reversible and non covalent in nature. The binding site is actually the catalytic site or active site of the enzyme. The surface of the active site is lined with amino acid residues whose substituent groups bind the substrate and catalyze its chemical transformation. Water is the most abundant in the living system forming hydration shell of all bio-molecules including proteins and starch as in the present study. Four types of weak interactions occur within and between bio-molecules in aqueous solution: ionic, hydrophobic and van-der-wals interactions, and hydrogen bonds. These weak

This type of transient protein ligand binding is critical to life and allows an organism to respond rapidly to change in metabolic and environmental conditions [4]. Our present study involves single binding site. However, the site is not same for all the drugs as the binding in case of HgCl_2 solution(θ) is endothermic as opposed to *Merc cor* potencies, ethanol and their control which are exothermic in nature. So, in this case the mother tincture produces the opposite effect as compared to its potencies. *Merc cor* mother tincture confers inhibitory effect on α -amylase. Uncompetitive inhibitors bind only enzyme substrate complex (ES) at a site distant from the active site. In case of mixed inhibition an inhibitor binds to either EE or ES at a site distant from where the substrate binds [4]. HgCl_2 molecules bind to a site different from active site of α -amylase. Thus it is either an uncompetitive or mixed inhibitor. In homeopathy, the mother tincture produces symptoms in healthy individuals, and these symptoms are ameliorated by its potencies when the patient shows similar symptoms due to a disease. The K value or binding constant shows affinity of a protein (here α -amylase) for a ligand here starch. In the present study this

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affinity shows marked variation from each other mainly due to the action of mother tincture and the different potencies of the drug on the enzyme. In the present study the variation in K value is of several orders of magnitude. Here, *Merc cor* 1000 cH shows the highest affinity. This suggests that a potentized drug changes or enhances the affinity between protein and ligand.

The heat evolved during the formation of one mole of product represent ΔH , the molar variation of the enthalpy of reaction. During a chemical reaction at a constant temperature, here 36°C , the free energy change, ΔG , is determined by ΔH which reflects the kinds and numbers of chemical bonds and non covalent interactions, broken and formed.

$\Delta G = \Delta H - T \Delta S$ where T is the absolute temperature.

In our study, the mother tincture of *Merc cor* and its potencies like 200 cH and 1000 cH show marked variation from each other with respect to ΔH and ΔS . This indicates that the potencies show differences in reaction as compared to the control, alcohol and mother tincture.

Randomness of the components of the chemical system is represented by entropy, S. Here the components were α -amylase and starch. Any change in randomness is expressed as ΔS . In our study mother tincture of *Merc cor* shows a positive value for ΔS which means an increase in randomness. The higher is the value of ΔS , the greater is the distance between components [4]. In our study *Merc cor* 1000 cH, *Merc cor* 200 cH

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show very low ΔS values and this means these potencies have brought the components like starch and the enzyme closer to each other. This suggests that by bringing the molecules close to each other, potencies have helped in stronger reaction.

CONCLUSION

1. *Merc cor* mother tincture and its potencies interact at two different binding sites of α -amylase during titration with starch.
2. Drugs used in homeopathy appear to produce dual action on proteins. At high doses they act on a binding site(s) and at ultra low doses they act on a different site(s).
3. Free water molecules and hydrogen bond strength in the drug contribute to the alteration of hydrolysis.

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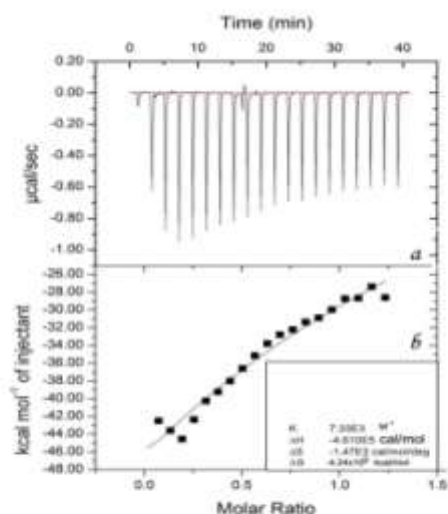


Fig. 1 a) In the heat rate vs. time titration curve, each peak represents an injection of lig (starch) into the sample cell containing α -amylase. b) Typical representation of ITC showing heat released per mole of starch during interaction of α -amylase in relation to molar ratio, starch/ α -amylase (control with water only).

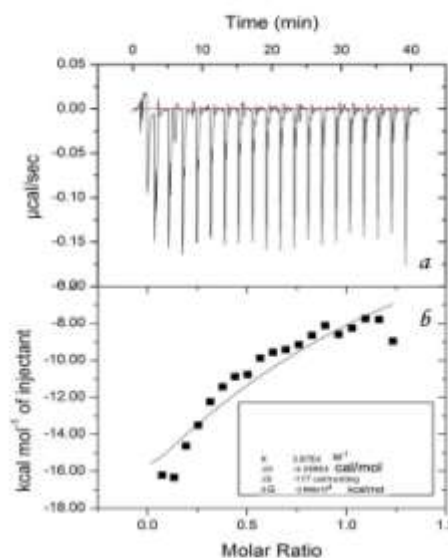


Fig. 2 a) In the heat rate vs. time titration curve, each peak represents an injection of ligand (starch) into the sample cell containing α -amylase+Merc 200 cH. b) Heat change vs. molar ratio of starch/ α -amylase+Merc 200 cH.

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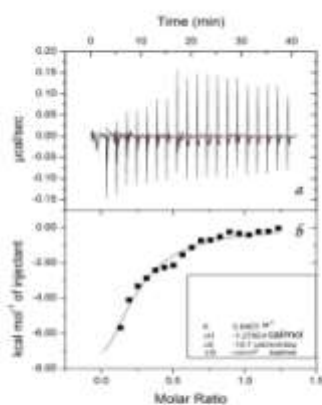


Fig. 3 a) is the heat rate vs. time titration curve, each peak represents an injection of ligand (methyl) into the sample cell containing α -styrene-Merc con 1000 (3). b) Heat change vs. molar ratio of methyl α -styrene-Merc con 1000 (4).

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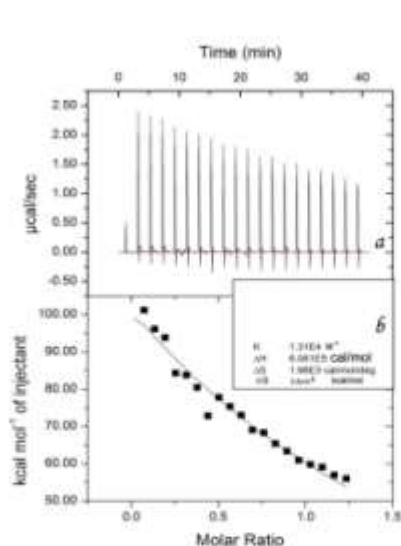


Fig. 4a: In the heat rate vs. time titration curve, each peak represents an injection of ligand (starch) into the sample cell containing α -amylase+Merc or Mother tincture (O). **b)** Heat change vs. molar ratio of starch/ α -amylase+Merc or Mother tincture (O).

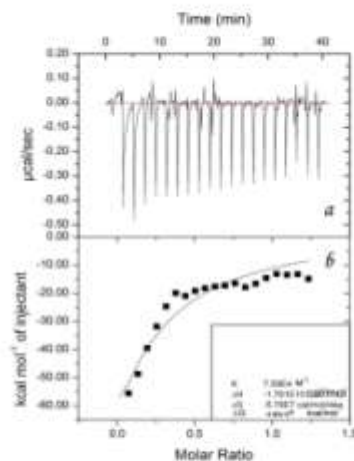


Fig. 5a: In the heat rate vs. time titration curve, each peak represents an injection of ligand (starch) into the sample cell containing α -amylase+Alcohol. **b)** Heat change vs. molar ratio of starch/ α -amylase+Alcohol.

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