Original Article

Extreme dilutions of mercuric chloride produce prolonged binding interaction with α -amylase and higher heat change than blank solventsPriya Mondal ¹, Indrani Chakraborty ², Nirmal Chandra Sukul * ^{1,3}, Atheni Konar ¹, Tandra Sarkar ¹, Anirban Sukul ¹¹Sukul Institute of Homeopathic Research, Santiniketan, West Bengal, India.²Department of Zoology, Jogamaya Devi College, Kolkata, West Bengal, India.³Department of Zoology, Visva-Bharati University, Santiniketan, West Bengal, India.* ncsukul@gmail.com<https://orcid.org/0000-0001-5888-3369>**Abstract**

Background: High and ultra-low doses of HgCl_2 modulate α -amylase starch interaction through two different binding sites of the enzyme. *Mercurius corrosivus* at ultra-high dilution (UHD) is reported to have a long duration of action for 30-60 days. **Objectives:** (i) To see whether the mother tincture and UHDs of mercuric chloride could directly act on α -amylase without intervention of its substrate starch. (ii) To see whether the MT and UHDs produce longer binding interaction with α -amylase than the control. **Methods:** Mercuric chloride MT (0.15 M) or *Merc cor* θ and its three UHDs, 30, 200, 1000 cH called potencies were in 90% ethanol. Blank 90% ethanol and deionised and distilled (DD) water were used as controls. All ethanol based ligands were diluted with DD water to reduce EtOH content to 0.09%. Using an ITC instrument each ligand was injected once at 10 μl into 15 μM α -amylase at 25°C and the reaction was observed for 33.3 min. **Results:** While *Merc cor* θ produced endothermic reaction, all other ligands did exothermic reaction. All ligands sustained heat change for the entire period of observation (33.3 min). **Conclusions:** *Merc cor* θ and potencies produced exactly the opposite reaction at the protein binding sites. *Merc cor* 200 cH and 1000 cH produced highest heat change (6 micro cal/sec) during interaction with α -amylase.

Keywords: Mercuric Chloride, High Dilution, α -amylase, Binding interaction, Isothermal titration calorimetry

Introduction

Material doses of drugs used in allopathy have a short duration of action, maybe a few hours. Here the drug effect manifests itself after a lag period, increases gradually, reaches a peak, then declines and finally disappears. The entire process is related to absorption of drug molecules, their rise in plasma and final clearance from the body [1]. But ultra-high dilutions (UHD) of drugs used in homeopathy have a long duration of action spanning over several days [2-3]. *Mercurius corrosivus* is one such drug prepared from HgCl_2 by successive dilution followed by succussion. A single dose of a UHD of *Merc cor*, called potency, has been reported to have a duration of action of 30-60 days [2]. Do the potencies of *Merc cor* show any semblance of prolonged effect in *in vitro* tests? The present study is designed to address this question.

We have recently demonstrated that extremely low concentrations of drugs interact at the binding sites of a protein [4-6]. These experiments were conducted by isothermal titration calorimetry (ITC) following multiple injection method. In the present study we have adopted a single injection method of ITC using the mother tincture (MT) of *Merc cor* and its three potencies as ligands and α -amylase as

a protein. This protein is a starch digesting enzyme found mostly in saliva and intestinal secretions [7]. Three potencies of *Merc cor* like 30 cH, 200 cH and 1000 cH were used in the present study. Besides these potencies, ethanol, the vehicle of the potencies, water (control) and *Merc cor* θ (0.15 M HgCl₂ solution in water) were used. Before the ITC experiments, all the three potencies of *Merc cor* and ethanol were diluted with DD water 1:1000 so that ethanol content in all the samples was reduced to the level of 0.09%. Thus they were all virtually water. In the previous study we tested *Merc cor* θ , 200 cH and 1000 cH on the α -amylase starch interaction using the multiple injection method of ITC [5]. The potencies were not directly tested on the enzyme in that study. In this study we would see whether *Merc cor* θ and its three potencies directly act on the binding sites of the enzyme excluding any starch interaction.

In the case of the multiple injection method, the ligand is injected into the protein solution every 2 min and the heat change is monitored after each injection. Usually 20-40 injections are given. The heat change should diminish after each injection, and for this the concentrations of the ligand and protein should be properly adjusted. In the single injection method one can compare easily the change in the heat rate with different drugs interacting on a protein. In the present study we mainly focused on the change in heat rate with different potencies of *Merc cor* interacting with α -amylase. ITC experiment helps in obtaining accurate data on ligand protein interaction under optimal conditions in a single experiment [8].

Materials and Methods

Mercuric chloride obtained from SRL Mumbai, was dissolved in DD water to prepare *Merc cor* θ or 0.15 M HgCl₂ solution. Three potencies of *Merc cor* like 30 cH, 200 cH and 1000 cH were prepared in our laboratory following the standard method of successive dilution 1:100 of *Merc cor* θ followed by succussion in 30, 200 and 1000 steps, respectively [6,9]. Alpha-amylase obtained from SRL was dissolved in DD water to prepare 15 μ M solution. The enzyme activity was 10-25 units/mg. *Merc cor* potencies were preserved in 90% ethanol. Just before the ITC experiment the three potencies of *Merc cor* and 90% ethanol were diluted with DD water 1:1000 to minimize ethanol content to a negligible amount of 0.09%. At this dilution, blank ethanol does not produce any tangible biological effect, but the drugs at UHDs do retain their effect [10]. Optical density (OD) of these diluted potencies and ethanol was measured by a UV-VIS spectrophotometer (Shimadzu, model UV 2401-PC) and found to be 0.03 at 220.5 nm. Control was DD water.

Interaction between α -amylase and its ligands *Merc cor* θ (0.15 M HgCl₂ solution in water), its potencies, ethanol and water was measured by an instrument ITC 200, GE Healthcare, Biosciences Ltd, Sweden. In this ITC experiment 10 μ l of each drug or control (water) was injected once into a measurement cell containing 300 μ l of 15 μ M α -amylase solution in water, and binding reaction was recorded for 2000 seconds in terms of release of heat (exothermic) or absorption of heat (endothermic). Both the sample and reference cells containing water only were maintained at a constant temperature of 25 °C. Injection of a drug solution or control was started after thermal equilibrium had been established. After each experiment the instrument was thoroughly rinsed with DD water as a routine procedure.

Results

Results are presented in Figures 1-6 and Table 1. Figure 1 shows heat rate (μ cal/sec) versus time in min up to 33.3 min or 2000 sec following a single injection of ligand, here 10 μ l of DD water into 15

μM α -amylase in DD water, pH 6.9. Solid line represents original data and line dotted with solid squares stands for data transformed by processing with the help of a software Origin 7. After the single injection, reaction due to protein ligand binding led to a heat change of $0.4 \mu\text{cal/sec}$ which continued for 20 min. Then it came back to a slightly lower level (Fig 1). Reaction was exothermic.

Fig. 1 - α -amylase + Water. Solid line: Change in heat rate in relation to time in min following a single injection of $10 \mu\text{l}$ of deionized and distilled (DD) water into $15 \mu\text{M}$ α -amylase in DD water, pH 6.9 in an ITC instrument (ITC 200, GE Healthcare, Sweden). Solid square line: Fit to the data with Origin 7. Heat change $0.4 \mu\text{cal/sec}$ for 33.3 mins. Exothermic reaction. Heat change calculated with reference to base line.

The best fit thermodynamic parameters like binding constant (K), change in enthalpy (ΔH), change in entropy (ΔS), number of binding sites and Gibbs free energy change (ΔG calculated from the equation $\Delta G = \Delta H - T\Delta S$, where T is absolute temperature in Kelvin) are given in Table 1. Figure 2 shows heat rate versus time in min after a single injection of $10 \mu\text{l}$ of aqueous ethanol (0.09%) into $300 \mu\text{l}$ of $15 \mu\text{M}$ α -amylase. The heat change ($0.5 \mu\text{cal/sec}$) was sustained for 33.3 min with minor fall after 20 min like water. Reaction was exothermic. Thermodynamic parameters like K , ΔH , ΔS and ΔG are given in Table 1. Figure 3 shows heat rate versus time in min after a single injection of $10 \mu\text{l}$ of *Merc cor* 30 cH in 0.09% EtOH into $15 \mu\text{M}$ α -amylase. Heat change was $0.5 \mu\text{cal/sec}$ which was maintained for the entire period of observation. Reaction was exothermic with a single binding site. Figure 4 shows heat rate versus time in min from a single injection of $10 \mu\text{l}$ of *Merc cor* 200 cH in 0.09% EtOH to $15 \mu\text{M}$ α -amylase. Heat change was large, $6 \mu\text{cal/sec}$ which was maintained throughout the period of 33.3 min observed. Reaction was exothermic with one binding site. Figure 5 shows heat rate versus time in min after a single injection of *Merc cor* 1000 cH in 0.09% EtOH to $300 \mu\text{l}$ of $15 \mu\text{M}$ α -amylase in water. Heat change was large, $6 \mu\text{cal/sec}$ which was maintained for 33.3 min observed. Reaction was exothermic with one binding site. Figure 6 shows heat rate versus time in min after a single injection of *Merc cor* θ (0.15 M HgCl_2 solution in water) into $300 \mu\text{l}$ of $15 \mu\text{M}$ α -amylase in water. Heat change was large, $4 \mu\text{cal/sec}$ which was maintained for 33.3 min. Reaction was endothermic with one binding site.

Ligands injected, 10µl each	KM ⁻¹ (Binding constant) ×10 ⁵	ΔH Cal/mol ×10 ⁴	ΔS Cal/mol/de g	ΔG Cal/mol ×10 ³	Nº of binding sites, reaction
Water (Control) 55 mM	1.75	-5.58	-163	-5.04×10 ²	1, Exothermic, heat change 0.4µcal/sec for 20 min, then back to ground level
Ethanol (0.09%)	1.75	-1.91	-4.02	-1.90×10 ²	1, Exothermic, heat change 0.5 µcal/sec for 20 min, then back to ground level
<i>Merc cor</i> 30 cH (0.09%EtOH)	1.75	6902	47.1	5.33	1, Exothermic, heat change 1µcal/sec continued for 2000s
<i>Merc cor</i> 200 cH (0.09%EtOH)	1.75	-4.24×10	-1.40×10 ³	-3.73×10 ²	1,Exothermic, heat change 6 µcal/sec continued for 33.3 min
<i>Merc cor</i> 1M 0.09%EtOH	1.75	-9.83	-306	-9.73×10 ²	1, Exothermic, heat change 6 µcal/sec continued for 33.3 min
<i>Merc cor</i> θ	1.94	-1.52×10	-485	-1.32×10 ²	1, Endothermic, heat change 4 µcal/sec continued for 2000 sec

Table 1- Thermodynamic parameters of interactions between 15 µM α-amylase in water and ligands like water, 0.09% ethanol, *Merc cor* 30 cH, 200 cH, 1000 cH, and mother tincture θ (0.15 M HgCl₂ in water at 25 °C. Each ligand was injected once at 10 µl into 300 µl of α-amylase. Except water control and *Merc cor* θ, all ligands were in 0.09% EtOH.

Fig. 2 - alpha-amylase + Ethanol 0.09%. Solid line: Change in heat rate in relation to time in min after a single injection of aqueous ethanol (0.09%) into 15µM α-amylase in DD water, pH 6.9 in an ITC instrument. Solid square line: Fit to the data analysed by Origin 7. Heat change 0.5 µcal/sec for 33.3 mins. Exothermic reaction. Heat change calculated with reference to base line

Fig. 3 - alpha-amylase + Merc cor 30. Solid line: Change in heat rate versus time in min from a single injection of *Merc cor 30* cH in 0.09% EtOH in 15µM α-amylase in DD water, pH 6.9. Solid square line: Fit to the data processed by Origin 7. Heat change 0.5 µcal/sec for the entire period of 33.3 min observed. Exothermic reaction. Heat change calculated with reference to base line.

Fig. 4 - alpha-amylase + Merc cor 200. Solid line: Change in heat rate versus time in min from a single injection of *Merc cor 200* cH in 0.09% EtOH in 15µM α-amylase in water, pH 6.9. Solid square line: Fit to the data processed by Origin 7. Heat change 6 µcal/sec maintained throughout the period of 33.3 min observed. Exothermic reaction. Heat change calculated with reference to base line.

Fig. 5 - alpha-amylase + Merc cor 1000 . Solid line: Change in heat rate versus time in min from a single injection of *Merc cor 1000* cH in 0.09% EtOH in 15 μ M α -amylase in water, pH 6.9. Solid square line: Fit to the data processed by Origin 7. Heat change 6 μ cal/sec observed throughout the cut off period of 33.3 min. Exothermic reaction. Heat change calculated with reference to base line. Heat change calculated with reference to base line.

Fig. 6 - alpha-amylase + Merc cor MT. Solid line: Change in heat rate versus time in min from a single injection of *Merc cor* θ (0.15 M HgCl₂ solution in DD water) into 15 μ M α -amylase in DD water, pH 6.9. Solid square line: Fit to the data processed by Origin 7. Heat change 4 μ cal/sec which remained unchanged for the entire period of observation. Endothermic reaction. Heat change calculated with reference to base line.

Merc cor mother tincture (θ), potencies, ethanol and water control show marked difference from each other with respect to change in enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG) (Table 1). However, binding constant (K) does not show any major variation from each other (Table 1). While *Merc cor* 30 cH shows positive values for ΔH , ΔS and ΔG , all other ligands show negative values for the same. *Merc cor* 200 cH and 1000 cH show the highest heat change (6 $\mu\text{cal/sec}$) among all the ligands. *Merc cor* 1000 cH shows the highest negative ΔG value among the ligands (Table 1).

Discussion

In this study we see that heat change due to protein ligand interaction following a single injection was maintained for 33.3 min with a minor fall after 20 mins with both water control and ethanol solution used as ligands. For *Merc cor* θ , HgCl_2 molecules directly participated in binding with α -amylase. It is known that four types of weak interactions occur within and between bio-molecules in aqueous solution. These are ionic, hydrophobic, van-der-waals interactions and hydrogen bonds [7]. In our study the protein ligand interaction might have involved breaking of hydrogen bonds thereby releasing heat energy and producing exothermic reaction. It may be assumed that *Merc cor* potencies can induce a chain reaction of breakdown of hydrogen bonds for a longer time. In our earlier study using multiple injection of ligand, we observed that *Merc cor* potencies maintained continued exothermic interaction for 40 mins, but *Merc cor* θ , ethanol solution and water control showed gradual saturation with respect to protein ligand interaction [5]. In case of *Merc cor* θ , the reaction was endothermic in the present study by a single injection method (Table 1) as well as in earlier study by multiple injection method [5-6]. This means there was absorption of energy which might have been needed for the formation of H-bonds during binding of HgCl_2 molecules with α -amylase at a site different from the binding sites of *Merc cor* potencies.

In our earlier study by multiple injection method we observed exactly the same interaction between α -amylase and *Merc cor* mother tincture and its potencies. But in that experiment soluble starch was used as a third component. In both the experiments there was a single binding site, exothermic reaction with water, ethanol and *Merc cor* potencies and endothermic reactions with *Merc cor* mother tincture (θ). This shows that the presence of the substrate (starch) could not modify the *Merc cor* and the α -amylase interaction. Liquid form of homeopathic potencies available in the market may contain some impurities, but these impurities have never been reported to interfere with the therapeutic effect of potencies on patients. Results of the present experiment provide an explanation for that.

We have already observed that free water molecules and H-bond strength form the physical basis of the homeopathic potencies [11-17]. So, these two factors in *Merc cor* potencies might have played an important role in the protein (α -amylase) and ligand (*Merc cor*) binding. Water strongly influences structure and function of biomolecules including proteins, and the interactions mostly involve H-bonds. Strength of H-bonds between hydrating water and biomolecules is also an important issue [18]. It is suggested that hydration shell dynamics is essential to biomolecular functions, catalytic activity of enzymes and molecular recognition [19].

In homeopathy high and ultra-low doses of a drug produce exactly the opposite symptoms as reported from *in vivo* observations on healthy and sick individuals [9]. The same phenomenon is observed at the molecular level concerning protein (α -amylase) ligand (HgCl_2) binding as observed in the present study. It is interesting to note that ethanol and *Merc cor* potencies were same in chemical composition (0.09% ethanol), yet they produced different effects on α -amylase.

Conclusions

1. *Merc cor θ* and potencies initiate their interaction on binding sites of α -amylase.
2. *Merc cor θ* and potencies might have acted on two different binding sites of α -amylase and produce exactly the opposite reaction, endothermic and exothermic, respectively.
3. *Merc cor θ* and potencies produced higher heat changes during interactions with α -amylase when compared to water and ethanol control.

Acknowledgement

The work described here has been financially supported by the 'Sukul Institute of Homeopathic Research' including research fellowships for AK and PM. We are thankful to Mr. Souvik Roy, Technical Officer, DBT-IPLS, Calcutta University for his help in operating the ITC instrument.

Declaration

The authors declare that there is no conflict of interest regarding publication of this article.

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Received: Feb 11, 2020. Accepted: Jul 29, 2020.

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