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Disulphide Bond Reduction of RNase A by Drug Metosartan a Comparative Study

Eswari Beeram[1*], Kamala Katepogu[1], Bukke Suman[1], Divya Bysani[1] and Thyagaraju Kedam[1]

[1]Department of Biochemistry, Sri Venkateswara University, Tirupathi-517502, India.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

RNase A is the most experimental protein in the 20th century. Disulphide bonds are necessary for enzymatic action of many proteins as it is also required for this protein. RNaseA kinetic studies is performed with the drug metosartan using RNA as the substrate Metosartan, is a drug used as blocker in excretion and found to contain inhibitory property on RNaseA. Protein degradation and thiol titration assay has found to be that the drug has reducing property on RNaseA.

Keywords: RNaseA; SDS PAGE; enzyme kinetics; beta blocker; Angiotensin receptor type I.

1. INTRODUCTION

Metosartan is the drug normally used as antihypertensive agent that act on heart and reduces the work load of the heart. Its components telmisartan is a angiotensin type I receptor blocker known to induce apoptosis in urinary bladder and metoprolyl is the beta receptor blocker. The previous studies of Eswari Beeram et al. has shown that the drug inhibits RNaseA [1]. So, study of the enzyme kinetics at different substrate concentrations with and

**Corresponding author: E-mail: eshu.sonu@gmail.com;*

without drug was proven to be challengeable in order to find the type of inhibition.

RNase A was isolated by Rene´ Dubos, crystallized by Moses Kunitz, sequenced by Stanford Moore and William Stein, and synthesized in the laboratory of Bruce Merrifield, all at the Rockefeller Institute/University [2]. It consists of four disulphides in its native position at 26–84, 40–95, 58–110, and 65–72 [3] which is necessary for three dimensional structure of RNase A.and the function of it. The 3D crystal and aqueous form of the RNaseA was previously determined.

It consists of three alpha helices, helix I (residues 3–13), helix II (residues 24–34) and helix III (50–60), and an antiparallel Beta sheet comprising Beta strand I (43–49), beta strand II (61–63), beta strand III (72–74), beta strand IV (79–87), beta strand V (96–111), and beta strand VI (116–124). There are four proline residues: Pro42, Pro93, Pro114, and Pro117. In the native state, the peptide bonds preceding Pro93 and Pro114 adopt the *cis* conformation [3]. So, the activity of the RNase A in reduced form is necessary to find out whether the disulphide bond determined 3D structure is necessary for the activity or not. But the disulphide bonds are not present in the active site.

RNaseA was known to exist in monomer, dimer, trimer and tetramer but the enzyme is highly active in the trimer state and doesn't contain tryptophan. So, oxidation with performic acid is sufficient to cleave the –s-s- bonds, but in many proteins which contain tryptophan it is more complicated as the reduction of indole group leads to undesired products [4]. The reduction of disulphide bonds is necessary to form intermediates between the folded and unfolded forms of the enzyme [5].

Glutathione is one of the peptide that interacts with protein in most of the biological systems and the concentration of it was found to be 8 mM [6]. So, it is necessary to study whether the pancreatic ribonuclease is active after reduction or not. Methylthioinosinedicarboxaldehyde is one of the compound known to inhibit pancreatic RNaseA by the formation of schiffs base with €-aminogroups of lysine residues that are close to the catalytic center [7] but it's inhibition by drugs is yet to be studied. Denaturing SDS PAGE was used as one of the technique used for the study and also the method of folin lowry for the estimation of RNase to find out the effect of drug on RNaseA.

2. MATERIALS AND METHODS

2.1 Protein Quantification by Lowry Method

The method was followed as that of Stephen P et al. [8] which is explained in brief here. The RNase A along with 200 µl of TCA was incubated in water bath at 65° c for 15 min, was centrifuged at 12,000 rpm and the supernatant was discarded. Pellet retained was resuspended in 0.5 ml of lowry reagent and incubated with the drug for prescribed time period and centrifuged at 12,000 rpm for 20 min. The supernatant was retained and estimated by lowry method or stored at -20°c.

2.2 SDS Page

The procedure was followed as that of laemmali [9] (1970).

2.3 Enzyme Kinetics

1 ml of enzyme was added to 1ml of different concentrations of RNA (4.8 mM,1.2 mM,0.6 mM and 0.3 mM) and monitored with UV- Visible spectrophotometer for every minute with the and without the drug. Michaelis menton graph was obtained from the data.

2.4 Titration of RNaseA and Measurement of Enzyme Activity

The protocol was followed as that of Sho takahas [10]. Titration of thiol groups was highly sensitive at PH 8.0 in the presence of denaturant. 2 g of urea, 0.2 ml of 2N tris buffer (PH 8.0) and 0.1 ml of 2x10-3 M DTNB in 0.1 N tris buffer (PH 6.5) was added in the sequence. 2 ml of the above solution was taken and the content of thiol groups was calculated from the absorbance at 412 nm of the mixture.

3. RESULTS

3.1 Reduction of RNaseA by Drug Metosartan

As mentioned in Fig. 1A, exactly at 3rd hr the protein concentration was less compared to the 1st and 2nd hr and again [11] there is increase in the concentration at 4th hr with respect to 3rd hr and less than 1st and 2nd hrs. so we may conclude from Fig. 1 the protein is either cleaved or lost its structure. From 1B it is clear

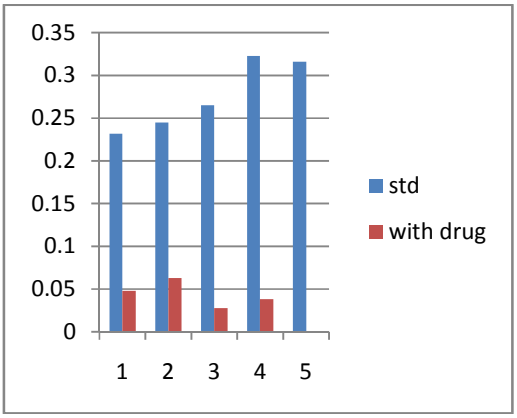
that the 3rd hr sample was similar to that of control and from the Fig. 1c it is clear that the reduction of disulphide bonds present in the protein may be one of the reason for the reduced concentration as it dissociate in to monomer.

The thiol reduction of RNaseA has shown that the activity was same or equal for all the 3 hrs so, it indicates that disulphide bonds doesn't contribute to the active site of the enzyme.

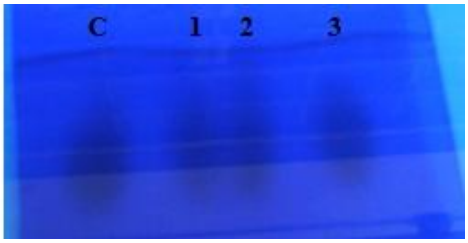
3.2 Enzyme Kinetics with Different Concentrations of Substrate with and without Drug

Except for the highest concentration of substrate, the activity of enzyme was less or equal for the remaining substrate concentration in the presence and absence of the drug.

For the 1.2 mM concentration the enzyme activity was high without drug and next to it is 0.3 mM concentration.



(1A)



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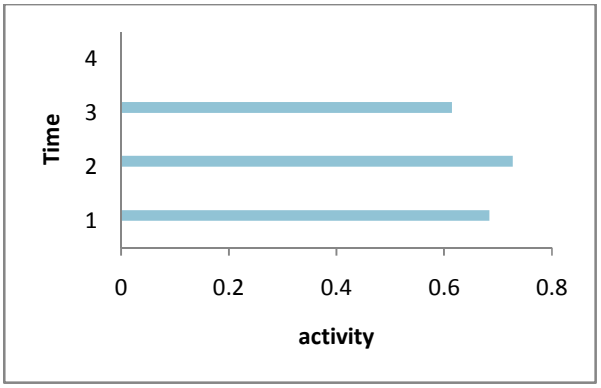
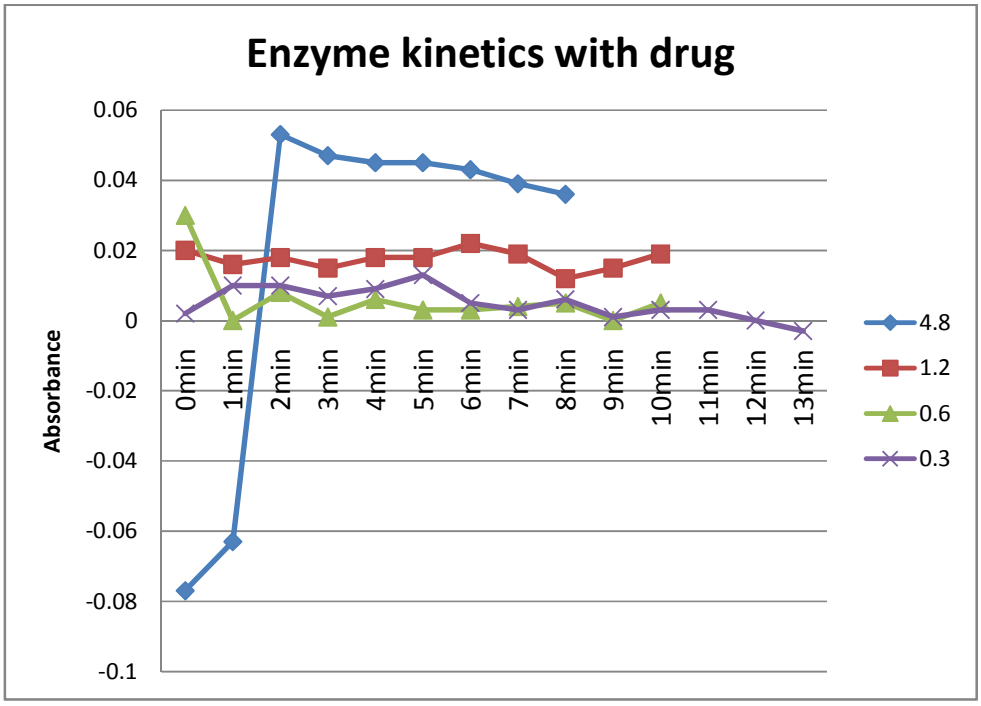
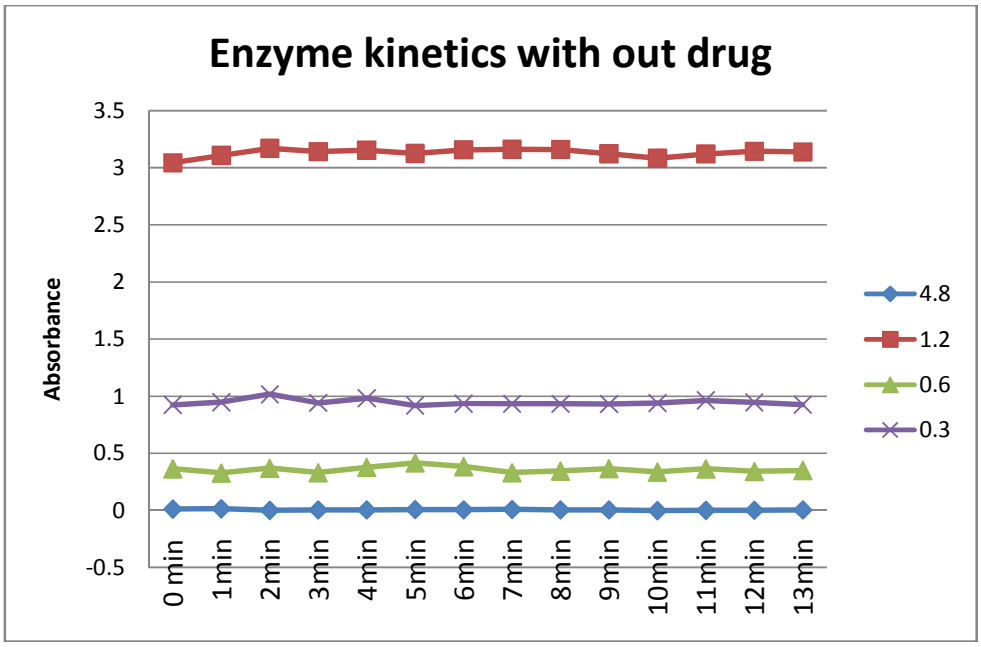


(1C)

Fig. 1. Reduction of disulphide bonds of RNase A by metosartan: 1A indicates the protein estimated by lowry method after extraction with TCA. It indicates that at exactly 3rd hr the protein concentration was low compared to 1st and 2nd hr even though the concentration of protein used was same. It indicates dissociation of RNaseA in to monomer. 1B indicates SDS PAGE of the control (RNase A) and drug treated RNase A. 1C indicates thiol content of RNase A. So, as a whole it indicates that the drug reduces RNaseA and recovery from it was seen again by 4th hr



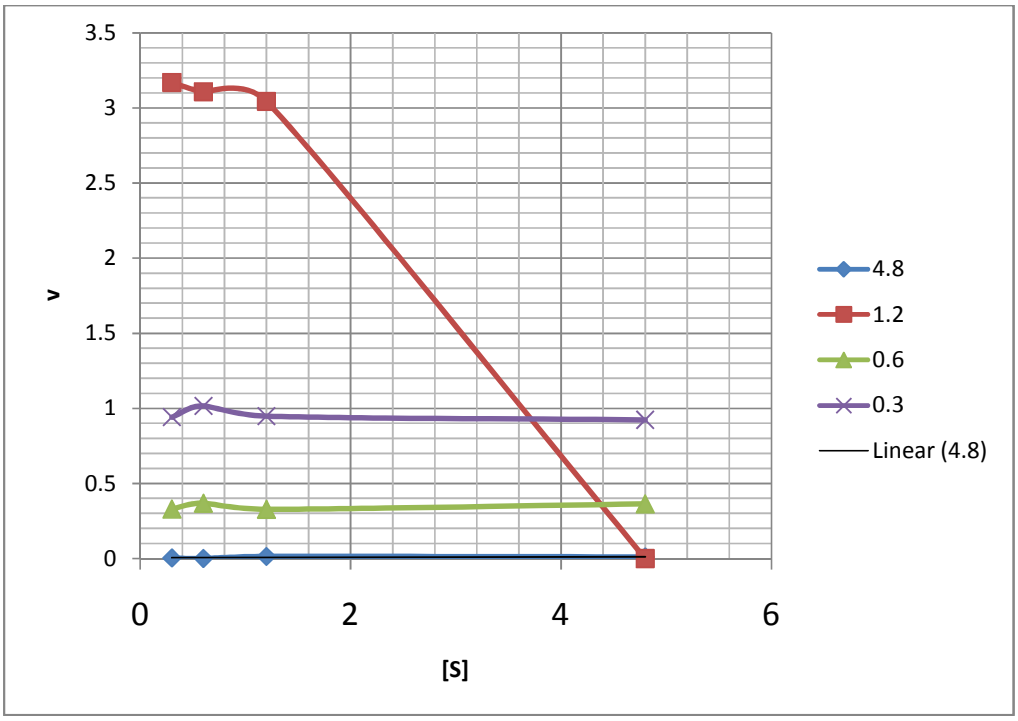
(2A)



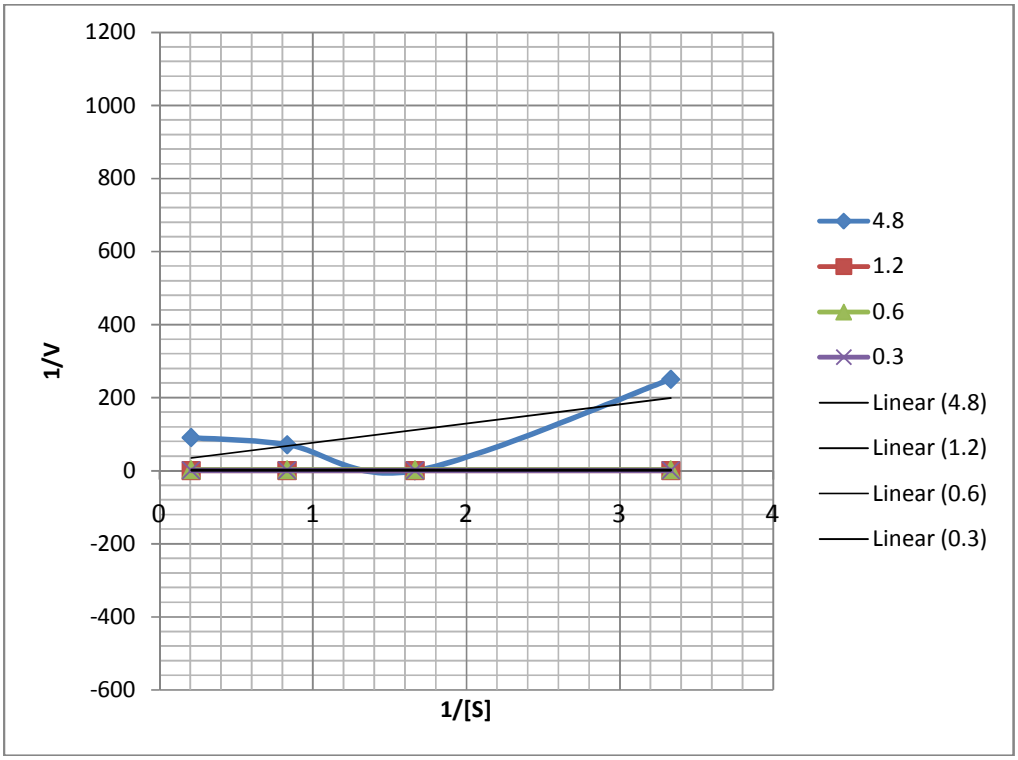
(2B)

Fig. 2. Time dependent assay of Enzyme RNaseA: Fig. 2A indicates the graph obtained in the presence of drug and 2B indicates the graph without any drug. From the graph it is known that the enzyme activity was more or less same even though different substrate concentrations are used. In the graph 4.8,1.2,0.6, 0.3 indicates substrate concentration in mM

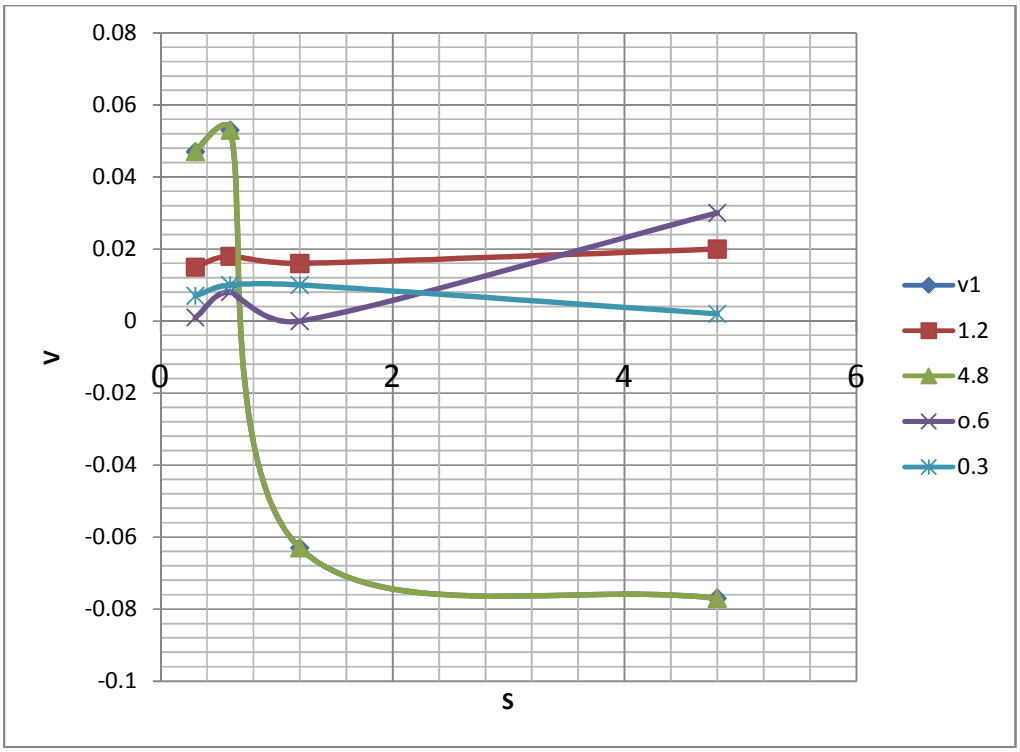
3.3 Michaelis – Menten Graph for without Drug:



3.4 Lineweaver Burk Plots for with out Drug:

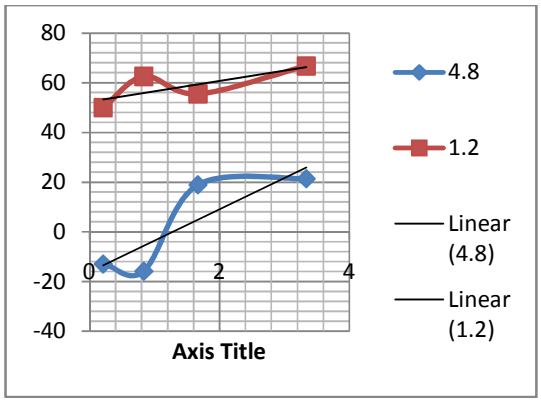


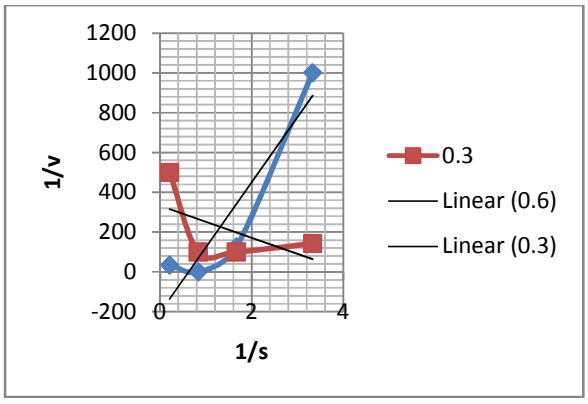
3.5 Michaelis – Menten graph for with Drug:



From the graph 3.5 and 3.6 we can conclude that the drug allosterically inhibit RNase A in addition to reduction of it to monomer.

3.6 Lineweaver Burk Plots for with Drug:





4. DISCUSSION

From the previous data we can conclude that the disulphide bonds is required for biological activity of many proteins but here in this context for RNaseA the disulphide bonds are not required for the activity of enzyme but required for the maintainance of trimer state. Histidine is present at the active site of the enzyme. So, the imidazole groups participate in the reaction of catalytic activity. From our work we may conclude that the drug metosartan reduces the RNaseA by attacking the disulphide bonds and inhibiting the enzyme allosterically in the testis (1,11) found by titrating the enzyme with the agents that determine the thiol content and

michaelis- menton graph. SDS PAGE has shown that the enzyme is dissociating in to monomer instead of dimer or trimer and tetramer as explained by smearing of the sample.

5. CONCLUSION

We conclude that metosartin inhibits the activity of RNAse A allosterically by reducing the disulphide bonds present on it. The reduction of disulphides may dissociate the enzyme to its monomer which is not highly active hence reducing the catalytic activity of RNase A.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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