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Dithiothreitol Augments Renin Activity by an Action on Renin Substrate[1] (39632)

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Renin is not generally believed to be a sulfhydryl-dependent enzyme. Thus hog renin was not inhibited by *p*-chloromercuribenzenesulfonic acid or *N*-ethylmaleimide (NEM) which block free SH-groups (1). Furthermore, treatment of renin with dithiothreitol (DTT, Cleland's reagent) did not influence its pressor potency, as determined by direct assay in the rat (1). Hog renin was also reported to be resistant to treatment with *p*-chloromercuribenzoate (PCMB) and iodoacetamide (2). Human renin acting on hog substrate has similar properties since it was not inhibited by iodoacetamide, merthiolate, or phenylmercuriacetate (3). However, Waldhausl and Lewandowski (3) reported that human renin is greatly potentiated by DTT and dithioerythritol (DTE), and suggested that the results with DTT indicated that unprotected SH-groups existed within the active center of the enzyme. These conflicting results and the uncertainty in the literature on the importance of SH-groups in the renin–angiotensin reaction led us to investigate the effects of DTT on bovine renin. Preliminary studies had indicated that DTT potentiates the reaction of bovine renin with homologous substrate but not with tetradecapeptide substrate (TDP). The present work shows that DTT has different effects on the renin–angiotensin reaction depending on the substrate utilized. In addition, the evidence indicates that the potentiating effect of DTT on angiotensin generation is due to an effect on the substrate and not on the enzyme.

Methods. Preparation of bovine serum substrate. Substrate was prepared according to the method of Lever *et al.* (4). The final preparation was precipitated between 1.5 and 2.3 *M* ammonium sulfate and dialyzed against PO_4 buffer (150 m*M*, pH 7.0) containing EDTA (10 m*M*). The material was then frozen in 1.0-ml aliquots.

Preparation of bovine renin. Bovine kidneys were obtained from a local slaughterhouse. The cortex was separated and frozen. After thawing, 100-g lots were homogenized in a Waring Blendor with 1 liter of H_2O. Further treatment, to be described in detail elsewhere, included acid treatment, ammonium sulfate fractionation, batch adsorption, and elution from DEAE cellulose and from kaolin, and finally acetone treatment.

Renin assay. Bovine renin was incubated for 30 min at 37° with bovine or hog substrate (200 pmole/ml) in a volume of 250 μl. The final concentration of PO_4 was 75 m*M* and of EDTA was 5.0 m*M*. DTT or NEM was added at the beginning of the incubation in some experiments. After incubation, 2.0 ml of Tris buffer (100 m*M*, pH 7.4) was added and the tubes were placed on ice. All assays were done in duplicate or triplicate. Aliquots of 50 μl were used for radioimmunoassay of angiotensin I. Renin assays were linear with enzyme concentration within the range of samples used.

Pretreatment experiments. In some experiments, either substrate or enzyme was preincubated for 10 min at 37° with DTT and then diluted with H_2O to lower the DTT concentration. Aliquots of the pretreated enzyme or substrate were then used in the final reaction mixture at the usual conditions.

Radioimmunoassay. Angiotensin I was assayed by the radioimmunoassay technique of Haber *et al.* (5), scaled down to a 200-μl volume. The isotope, antibody, and standards were obtained from New England Nuclear. The range of standards was from 25 to 1600 pg. The supernatant after charcoal

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treatment was added to 5.0 ml of Aquasol and counted in a Packard Tri-Carb liquid scintillation counter.

Materials. Hog substrate was obtained from Miles Laboratories; DEAE-cellulose, NEM, and DTT from Sigma Chemical Co.; kaolin from Fisher Scientific.

Results. Effect of DTT on the kinetics of the renin–angiotensin reaction. Bovine renin was reacted with increasing concentrations of bovine substrate in the presence or absence of DTT (10 m*M*). Angiotensin generation was potentiated by the presence of DTT. The results, plotted according to the method of Lineweaver and Burk, show that DTT increases the V_{max} without changing the K_m(Fig. 1). When a similar experiment was done with hog substrate, the potentiating effect of DTT was much more dramatic. Again, the V_{max} increased while the K_m remained unchanged (Fig. 2).

Pretreatment of renin and substrate with DTT. Since the effect of DTT was much more effective on hog substrate than on bovine substrate and since other experiments (not shown) did not indicate any effect of DTT on the reaction with tetradecapeptide substrate, it seemed likely that the effect of DTT was exerted on the substrate and not on the enzyme. Therefore, experiments were carried out in which the enzyme or the substrate was pretreated with DTT (to reduce SH-groups) and then diluted so that

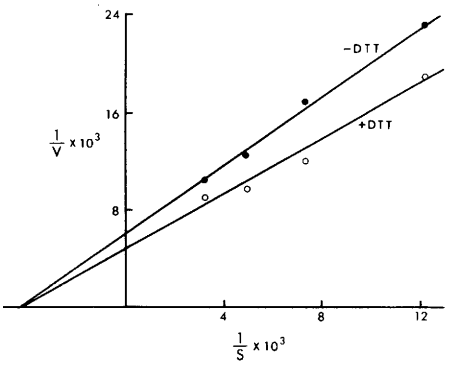

FIG. 1. Effect of dithiothreitol (DTT) on the reaction between bovine renin and bovine substrate. Lineweaver–Burk plot of the results in the absence or presence of DTT (10 m*M*). Ordinate: $1/V \times 10^3$, where V is expressed in micrograms of angiotensin per hour per milligram. Abscissa: $1/S \times 10^3$, where S is expressed in picomoles per milliliter.

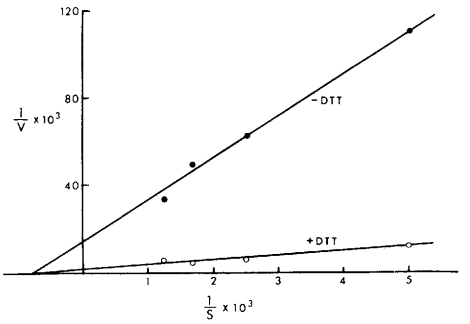

FIG. 2. Effect of DTT (10 m*M*) on the reaction between bovine renin and hog substrate. Lineweaver–Burk plot with units expressed as Fig. 1.

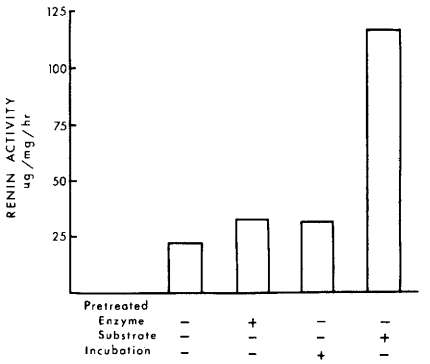

FIG. 3. Effect of DTT pretreatment on the reaction between bovine renin and hog substrate. Renin or substrate was preincubated with DTT (10 m*M*) and then diluted so that the final DTT concentration was 0.3 m*M*. Pretreatment of the enzyme (column 2) caused a slight increase in renin activity compared to the control (column 1), while pretreatment of the substrate caused a large increase (column 4). Addition of DTT (0.3 m*M*) during the final incubation (column 3) (no preincubation) caused a slight increase in renin activity. Renin activity is expressed as micrograms of angiotensin per milligram of protein per hour.

the concentration of DTT in the final assay mixture was low. Pretreatment of the enzyme with DTT (10 m*M*) followed by dilution so that the final DTT concentration during incubation was 0.3 m*M* produced a slight increase in renin activity, but this did not exceed the value obtained with 0.3 m*M* DTT present only during the incubation (Fig. 3). However, pretreatment of the substrate (DTT, 10 m*M*) followed by dilution to the same final DTT concentration (0.3 m*M*) produced a striking potentiation (Fig. 3).

Effect of NEM. Since DTT is still present in the incubation mixture after pretreatment of the enzyme and dilution, the small potentiating effect could still have been exerted through an effect on the substrate. To test for this possibility, use was made of NEM, which reacts with free SH-groups (as in DTT) and does not inhibit renin (1). Addition of NEM (5.0 m*M*) to the final incubation reaction did not affect the renin activity, while DTT (0.5 m*M*) produced a twofold potentiation. However, when both NEM and DTT were present, there was no potentiation, indicating that NEM had reduced the concentration of DTT to an ineffective value (Fig. 4). When hog substrate was pretreated with DTT (10 mM) and then diluted to yield a final DDT concentration of 0.5 m*M*, there was a dramatic potentiation (sevenfold). Addition of NEM (5.0 m*M*) to this reaction mixture, at a concentration shown to completely block the potentiating effect of 0.5 m*M* DTT, caused a slight reduction in angiotensin generation. The decrease was similar to the decrease caused by the addition of NEM to the reaction mixture containing 0.5 m*M* DTT (no preincubation) (Fig. 4). Therefore, in this condition (excess NEM present) there was probably little free DTT during the reaction and yet the dramatic potentiation due to DTT pretreatment was evident. Since the enzyme in this experiment had not been exposed to an effective concentration of DTT, the augmented renin activity can only be due to an effect of DTT on the substrate.

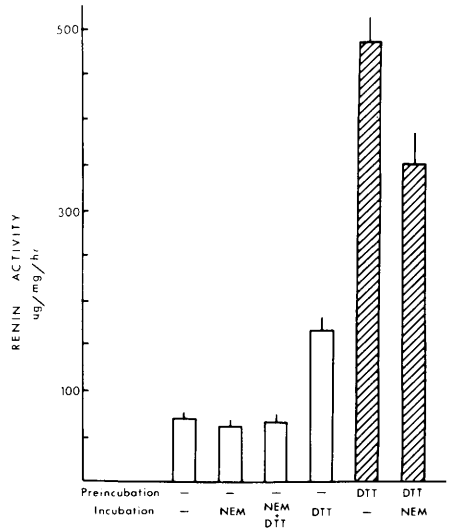

FIG. 4. Effect of NEM and DTT on reaction between bovine renin and hog substrate. When NEM was used, it was present during the final incubation at 5.0 m*M*. DTT was either added to the final incubation at 0.5 m*M* or preincubated at 10 m*M* for 10 min with the substrate followed by dilution so that the final concentration was 0.5 m*M*. Renin activity is expressed as in Fig. 3. The enzyme preparation used in this experiment was from a different batch from that used in Fig. 3 and had a specific activity about 2.5 times greater.

Discussion. The present study shows that dithiothreitol increases the amount of angiotensin generated by bovine renin acting on either bovine or hog substrate. The kinetic analysis suggests that this phenomenon is not due to an increased affinity of the enzyme for the substrate. Since the V_{max} was increased, it is possible that DTT reduced disulfide bonds or protected sulfhydryl groups either in the enzyme or in the substrate, which are important for the catalytic reaction. However, the preincubation experiments showed clearly that the effect was exerted on the substrate. Even when the effective DTT concentration was rendered negligible by the presence of an excess of NEM during the incubation, the influence of DTT pretreatment of the substrate on angiotensin generation was striking (Fig. 4). The concentration of DTT employed (10 m*M*) was chosen because Waldhausl and Lewandowski reported that higher concentrations caused gelation of their incubation mixture and they showed results with 10 m*M* DTT (Table 3 in Ref. 3) in comparison with other activators and inhibitors of the renin-substrate reaction.

These results may help clarify the inconsistent reports in the literature on the importance of SH-groups for renin activity. Since there are consistent reports that treatment of renin with organic mercurials or NEM does not inhibit its activity, this should have been a clue that renin is not a sulfhydryl-dependent enzyme. In fact, studies by Haas *et al.* with PCMB, iodoacetate, and cysteine led them to conclude, "A reactive thiol group is probably not involved directly in the catalytic activity of renin" (6). Their studies on the sulfhydryl groups of renin did suggest, however, that while the enzyme

was fully active in its reduced or oxidized state, it was more susceptible to denaturation in the reduced state (6). Therefore, the long-term influence of reduced sulfhydryl groups on renin and on its substrate may have opposite effects on the final generation of angiotensin.

It seems likely that renin substrates from various species contain SH-groups which are important for the renin–angiotensin reaction. Depending on the different methods of preparation and the history of the substrate prior to its use, there may be different amounts of free SH-groups at the time the reaction is studied. Thus, the apparent discrepancy concerning the role of SH-groups in renin is probably due to variations in the substrate and not the enzyme.

Summary. Dithiothreitol (DTT) increases the generation of angiotensin when bovine renin acts on bovine substrate or hog substrate. Kinetic analysis indicates that the V_{max} increases without a change in K_m. Pretreatment of bovine renin with DTT followed by dilution of the DTT to a low concentration does not augment renin activity, while pretreatment of the substrate and a similar dilution reveals marked potentiation. *N*-Ethylmaleimide (NEM) does not inhibit renin activity but does prevent the augmenting effect of DTT present during the incubation. It is concluded that DTT augments renin activity by an action on renin substrate and that renin does not fall in the category of sulfhydryl-dependent enzymes.

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