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# KINETICS OF INHIBITION OF ANGIOTENSIN CONVERTING ENZYME BY CAPTOPRIL AND BY ENALAPRIL DIACID

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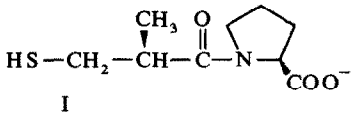
**Abstract**—Using a continuous spectrophotometric assay, inhibition of angiotensin converting enzyme by captopril and by the active diacid derivative of enalapril was reinvestigated. The onset of inhibition was comparatively slow, but the inhibition achieved was stronger than previous estimates: approximate $K_i$-values were 0.3 nM for captopril and 0.06 nM for enalapril diacid. The rate-constants for association and dissociation of these enzyme-inhibitor complexes were estimated, and half-times of approximately 12 min for the captopril complex and 60 min for the enalapril diacid complex were calculated. The rate of dissociation of the captopril–enzyme complex was measured directly by reacting the thiol group in free captopril with 5,5′-dithiobis(2-nitrobenzoic acid) and observing the reactivation of the enzyme; a half-time of approximately 30 min was obtained. Therefore the release of these inhibitors from the enzyme may be slow enough to affect the duration of their hypotensive action.

Captopril (I) and the diacid derivative (II) of enalapril (MK 421) are powerful inhibitors of angiotensin converting enzyme (reviewed in [1]) and are effective for treating certain types of hypertension. Reported $I_{50}$ concentrations are 23 nM for captopril [2] and 1.2 nM for enalapril diacid [3], with $K_i$ for captopril being 1.7 nM [2].



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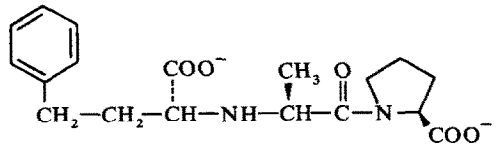

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Holmquist *et al.* [4] have developed a continuous spectrophotometric assay for angiotensin converting enzyme, using the synthetic substrate 2-furanacryloyl-L-phenylalanyl-glycyl-glycine (FA-PGG*): the sensitivity of the assay approaches that of the discontinuous fluorimetric assay of Piquilloud *et al.* [5] and is much more convenient and informative. Using this assay the time-dependent onset of inhibition by low concentrations of captopril and of enalapril diacid has now been observed; this phenomenon was previously noticed by Stone (personal communication) using the discontinuous fluorimetric assay. The rate of reactivation of the captopril-inhibited enzyme was also measured in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to react with free captopril.

* Abbreviations: FA-PGG, 2-furanacryloyl-L-phenylalanyl-glycyl-glycine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

## MATERIALS AND METHODS

Rabbit-lung acetone powder, dithiothreitol and DTNB were obtained from Sigma and FA-PGG from Bachem; captopril was a gift from Squibb. *N*-[1-(R,S)-Carboxy-3-phenylpropyl]-L-alanyl-L-proline was synthesized in the Chemical Research Laboratory of the Wellcome Research Laboratories. Only the S isomer (enalapril diacid) is an active inhibitor of angiotensin converting enzyme [3]; for convenience the RS isomer mixture will be referred to as RS-enalapril diacid.

Angiotensin converting enzyme was purified from rabbit-lung acetone powder by the method of Cheung and Cushman [6], but using Sephacryl S300 (Pharmacia) in place of Biogel P300. It was assayed by following the decrease in absorbance at 335 nm upon hydrolysis of FA-PGG [4]; $\Delta E_{335}^{1\,mM}$ was found to be 2.1 $cm^{-1}$. Semi-micro cuvettes contained, in 0.5 ml, 0.2 mM FA-PGG, 0.285 M NaCl and 50 mM Tris–HCl buffer, pH 7.5, thermostatted at 30° in a Gilford 250 spectrophotometer.

From the published specific activity with this substrate [4], the concentration of enzyme in the assays was 0.15–0.4 nM, and initial rates were not greater than 0.009 absorbance units per minute. No corrections have been applied for depletion of the inhibitor-concentrations due to binding to enzyme.

## RESULTS

Inhibition by captopril was, at first, variable and generally much weaker than expected from the published value of $I_{50}$ of 23 nM [2]. However, in the presence of 20 $\mu$M dithiothreitol, near-total inhibition was given after 3 min by 10 nM captopril. This concentration of dithiothreitol, alone, gave 22% inhibition. With captopril concentrations of 1–10 nM, time-dependent inhibition was observed (Fig. 1a). RS-enalapril diacid also inhibited in a strong and time-dependent manner (Fig. 1b), and did so in the absence of dithiothreitol.

Attempts were made to estimate the final steady-state velocities and the half-times for the approach to these velocities. This was possible in only a narrow range of inhibitor concentrations. Results with 4 nM captopril and 2 nM S-enalapril diacid are given in Table 1. The results are not highly accurate, especially for the percent activity left; this will therefore apply also to $I_{50}$, $K_D$, $k_{off}$ and $t_{1/2}$ for dissociation of the enzyme-inhibitor complexes. Inhibition by captopril and related compounds is competitive [2]; enalapril diacid, being an analogue of known competitive inhibitors, was also assumed to be competitive in the calculations.

Dithiothreitol, which was present in the assays with captopril itself gave 22% inhibition. If this was competitive with substrate and captopril (which is likely as inhibition is probably via zinc-binding) then revised parameter values for captopril-inhibition are: $k_{on} = 3.45 \times 10^9\ M^{-1}\cdot sec^{-1}$; and $K_D = 0.29$ nM: the other parameters are not affected.

Although these inhibitors are likely to bind to zinc ions in the enzyme, addition of 0.1 mM $ZnCl_2$ to the assay did not significantly affect the inhibition.

The rate of release of captopril from the enzyme can be estimated by reacting free captopril with thiol-reactive compounds: enzyme should regain its catalytic activity as captopril leaves the enzyme. When sufficient DTNB was added to the assay cuvette to react with all the captopril and dithiothreitol present, no measurable activation took place. However, when enzyme was inhibited by incubation with captopril and dithiothreitol, addition of DTNB did reactivate the enzyme as measured by removing and assaying aliquots. The reactivation was apparently still incomplete when the experiment was terminated after nearly 90 min (Fig. 2). The half-times for reactivation were similar with and without added $ZnCl_2$ (29 and 37 min respectively). Oxidised glutathione (0.56 mM) also reactivated the captopril-

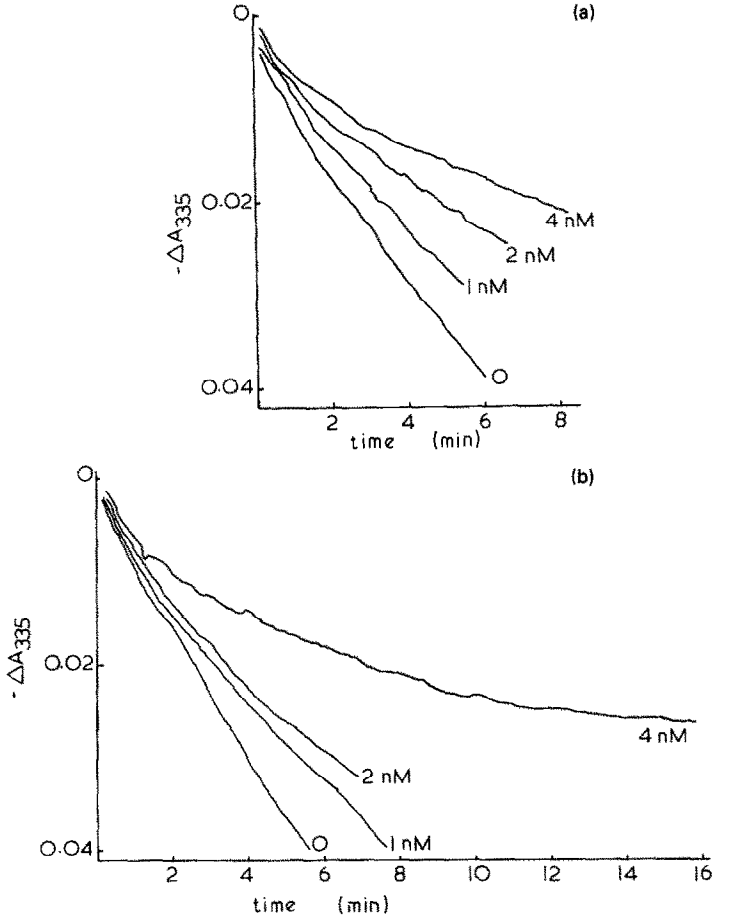


Fig. 1. Effects of captopril and RS-enalapril diacid on assays of angiotensin-converting enzyme. (a) Captopril; (b) RS-enalapril diacid. Cuvettes, thermostatted at 30°, contained the indicated concentration of inhibitor, 0.2 mM FA-PGG, 0.285 M NaCl, 50 mM Tris–HCl buffer, pH 7.5 and, in (a) only, 20 $\mu$M dithiothreitol. Enzyme was added last to start the reaction.

Table 1. Estimated steady-state $I_{50}$ values, rates of onset of inhibition and calculated rate-constants for dissociation of enzyme-inhibitor complexes

| | Captopril (4 nM) | S-Enalapril diacid* (2 nM) |
|---|---|---|
| $t_{1/2}$ for onset of inhibition (min) | 1.79 ± 0.11 (4) | 2.83 ±0.20 (4) |
| $k_{on}$ ($M^{-1}\cdot sec^{-1}$) | $2.69 \times 10^{6}$† | $3.40 \times 10^{6}$ |
| % Activity left | 13.25 ± 2.4 (8) | 4.53 ± 1.4 (4) |
| $I_{50}$ (nM) Means | 0.61 | 0.095 |
| $I_{50}$ (nM) 95% Confidence limits | 0.32–0.94 | 0.001–0.197 |
| $K_D$(nM) | 0.367† | 0.057 |
| $k_{off}$ ($sec^{-1}$) | $9.87 \times 10^{-4}$ | $1.94 \times 10^{-4}$ |
| $t_{1/2}$ for dissociation of E–I (min) | 11.7 | 59.5 |

* Added as mixture of R + S isomers (4 nM).

† The effect of dithiothreitol (20 μM), which was present in the experiments with captopril, has not been taken into account in the above calculations (see Text for revised values).

Means ± standard errors are given where applicable with the number of observations in brackets. The means were used to derive the other values. From $I_{50}$, $K_D$ (i.e. $K_i$) was calculated assuming competitive inhibition; and from $K_D$ and $k_{on}$, $k_{off}$ was calculated

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI$$

$$K_D = \frac{k_{off}}{k_{on}}$$

($K_D$ is the equilibrium dissociation-constant, and $k_{on}$ and $k_{off}$ are rate-constants).

In converting $I_{50}$ to $K_D$, and 'half-time for onset of inhibition' to $k_{on}$, it was assumed that the ratio of free to substrate-bound enzyme was 3:2, calculated from the Michaelis equation with the substrate-concentration 0.2 mM and the reported $K_m$ of 0.3 mM [4]. Conditions were as in Fig. 1. In some of the experiments 0.1 mM $ZnCl_2$ was present (see text).

inhibited enzyme. In the absence of enzyme, free captopril reacted rapidly with DTNB, the reaction being complete within 6 min.

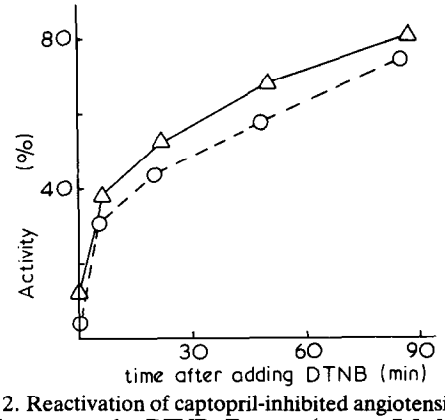


Fig. 2. Reactivation of captopril-inhibited angiotensin-converting enzyme by DTNB. Enzyme (approx 7.5 nM) was incubated in 110 μl at 25.3° with 4.5 mM sodium phosphate, 11.4 mM Tris–HCl, 68 mM NaCl, 23 nM captopril, 45 μM dithiothreitol, pH 7.6, either without (0) or with (△) 0.45 mM $ZnCl_2$. After 11.5–16 min, one aliquot of 20 μl was removed and assayed immediately, and after 19.5 min the reactivations were started by adding 15 μl of 1 mM DTNB in 45 mM Tris–HCl/0.27 M NaCl/10% ethanol, to give concentrations (before disulphide-exchange) of 3.9 mM sodium phosphate, 16.2 mM Tris–HCl, 97 mM NaCl, 19.5 nM Captopril, 39 μM dithiothreitol and 143 μM DTNB, and 0.39 mM $ZnCl_2$(△). Aliquots of 20 μl were removed for assay at the times shown.

## DISCUSSION

The continuous spectrophotometric assay employed here demonstrated that enalapril diacid, and captopril when kept reduced, inhibited angiotensin converting enzyme even more powerfully than had been reported previously, when somewhat different assay-conditions were used [2, 3]; and that at low concentrations of inhibitor the inhibition developed comparatively slowly.

The kinetic estimates for the half-times of the enzyme-inhibitor complexes (11.7 min for captopril and 59.5 min for enalapril diacid) depend upon the values for $K_D$; these are only approximate. The direct determination of the half-time for dissociation of the enzyme-captopril complex using DTNB (Fig. 2), which gave approx 30 min, suggests that the $K_D$ for captopril (Table 1) may have been overestimated. Improvements on these estimates should be possible using a method appropriate for tight-binding inhibitors such as that of Henderson [8]. These half-times are comparable to those for the decay of activity *in vivo*, measured as inhibition of the pressor response of angiotensin I in rats and dogs [7]; however, this may be fortuitous, as there is unlikely to be a linear relationship between inhibition of the enzyme and of pressor response.

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