Preliminary X-ray Crystallographic Data on Mouse Submaxillary Gland Renin and Renin-Inhibitor Complexes*

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We have crystallized renin from the submaxillary gland of male mice, both in its native state, and in binary complex with transition-state analog inhibitors. The best of the many crystal forms examined consisted of tetragonal bipyramids with space group symmetry P412121 (or its enantiomorph) and unit cell dimensions a = b = 91.4 Å, c = 211.6 Å; a = β = γ = 90°. This tetragonal form was compatible with both inhibited and uninhibited renin. Two of the inhibitors used were synthesized as iodinated analogs; their binary complexes with renin may serve as single-site rational heavy atom derivatives. X-ray data beyond 2.8-Å resolution have been collected by oscillation photography using the Cornell High Energy Synchrotron Source in Ithaca, NY.

Hypertension is a leading public health problem in industrialized societies, contributing, among other things, to cardiovascular disease, stroke, and renal failure (1). Novel strategies for the treatment of hypertension have recently centered on the renin-angiotensin system through the inhibition of angiotensin II production (2). Angiotensin II is a potent octapeptide hormone which both constricts the vasculature, and induces fluid retention through the stimulated release of aldosterone (3). The additive effect of more fluid in less space leads to higher blood pressure. The hormone angiotensin II is produced from an inactive circulating protein prohormone, angiotensigen, in two steps; a specific cleavage to the inactive Nα-terminal decapeptide angiotensin I by the action of renin, and the subsequent removal of the COOH-terminal dipeptide of angiotensin I by angiotensin-converting enzyme (4). Inhibitors of angiotensin-converting enzyme, a zinc di-carboxypeptidase, have already proven themselves clinically as safe and effective antihypertensives. It is generally thought that angiotensin-converting enzyme inhibitors function by preventing angiotensin II formation, demonstrating the usefulness of the renin-angiotensin system as a drug target (4, 5), but it is possible that a more complicated set of interactions may be in operation involving inhibition of other enzymes (e.g. Ref. 6). Renin inhibitors would better define the clinical importance of angiotensin II by blocking the renin-angiotensin system at the earlier step of angiotensin I production, bypassing angiotensin-converting enzyme-dependent effects altogether. The fact that renin is a more selective enzyme than angiotensin-converting enzyme in principle provide the opportunity for more selective inhibition.

At present, renin inhibitors potent enough to lower blood pressure are peptidyl in nature and not orally active. This situation, strongly reminiscent of the early work on angiotensin-converting enzyme inhibition (4), makes them difficult to work with in a clinical setting. One of the objectives of our renin structural program is to provide a basis for the design of non-peptidyl inhibitors with more favorable pharmacokinetic properties. To meet this objective, we have purified (7) and crystallized renin from the submaxillary gland of the male mouse (a rich source of the enzyme), both in its native state and in binary complex with a series of state-containing peptidyl inhibitors synthesized at Merck (8).

Mouse submaxillary gland renin is strongly homologous in sequence (9, 10) with human kidney renin (11, 12), the presumed medicinal target enzyme. Nearly 70% of the amino acid residues of the two enzymes are identical, so that structural insights from the one should be readily applicable to the other. Moreover, mouse submaxillary gland renin can be more readily obtained in quantity (7, 13). Mouse submaxillary gland renin is a member of the class of aspartyl proteases which includes pepsin (14) and a number of microbial enzymes (15–18), whose structures have been solved crystallographically. Comparisons between these molecules and mouse submaxillary gland renin should contribute to a better understanding of the mechanism of action of the aspartyl proteases, the high substrate selectivity of renin as compared to the rest of the class, and the anomalous pH optimum for renin activity (19).

EXPERIMENTAL PROCEDURES

Mouse submaxillary gland renin was isolated and purified as described by Poe et al. (7). In a typical crystallization experiment, a frozen 3-ml fraction of mouse submaxillary gland renin from the final purification step was thawed out which contained approximately 7 mg of the enzyme (as determined spectrophotometrically (20) assuming an absorption coefficient ε280 = 10.0, in 50 mM sodium acetate, at pH 5.38, 200 mM sodium chloride, saturated in cholesterol. The solution was concentrated to a volume of approximately 0.5 ml by ultrafiltration over an Amicon YM-10 membrane. The retained solution was dialyzed overnight with stirring at 4 °C versus 100 ml of 5 mM sodium acetate, 100 mM sodium chloride, pH 5.38, in a dialysis bag with a 12,000-dalton cut off. To prevent the growth of microorganisms all solutions were made in 0.01% sodium azide.

Renin inhibitors used are listed in Table 1; they were synthesized as described in Ref. 8.1 Binary mouse submaxillary gland renin-inhibitor complexes were prepared by adding 1.2–1.8 eq of inhibitor complexes were prepared by adding 1.2–1.8 eq of inhibitor dissolved in 0.05 ml of N,N-Dimethylformamide to the starting thawed fractions before proceeding as above. At these levels, N,N-Dimethylformamide did not interfere with the enzymatic activity of mouse submaxillary gland renin. Excess inhibitor was lost in the concentration and dialysis steps, so that all starting solutions contained binary 1 to 1 complexes.

Crystallizations were performed using the hanging drop vapor
Crystallography of Renin and Renin-Inhibitor Complexes

The abbreviations used are: Boc, tert-butyloxycarbonyl; Ibu, isobutyryl (2-methylpropanoyl); Sta, statyl (3S,4S)-4-amino-3-hydroxy-5-methyl-heptanoyl; IPh, p-iodophenylalanyl.

The inhibitors used in this study were:

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Structure</th>
<th>IC50</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Boc-His-Pro-Phe-His-Sta-Leu-Phe-NH2</td>
<td>4.9</td>
<td>8</td>
</tr>
</tbody>
</table>
| 2         | Boc-His-Pro-Phe-IPh-Sta-Leu-Phe-NH2 | 0.62 | -*
| 3         | Boc-His-Pro-Phe-His-Sta-Leu-Tyr-NH2 | 100.0| 8    |
| 4         | Boc-His-Pro-Phe-Phe-Sta-Leu-Phe-NH2 | 5.0  | -b   |
| 5         | Ibu-His-Pro-Phe-Sta-Leu-Phe-NH2    | 15.0 | 8    |
| 6         | Boc-His-Pro-Phe-Sta-Leu-IPh-NH2    | 11.0 | -b   |

* - M. Poe, and J. Boger, unpublished results.

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**TABLE I**

Summary of crystal forms and crystallization conditions for mouse submaxillary gland renin and mouse submaxillary gland renin-inhibitor complexes.

PEG 6000 is polyethylene glycol with an average Mw = 6000.

<table>
<thead>
<tr>
<th>Form</th>
<th>Complex</th>
<th>Morphology</th>
<th>Crystallization conditions*</th>
<th>Space group</th>
<th>Cell parameters</th>
<th>Zb</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None, 1, 2, 3</td>
<td>Tetragonal bipyramids</td>
<td>15 mg/ml; 30% PEG 6000, 30 mM sodium acetate, pH 5.9; 1 week; 25°C; macro seeds needed.</td>
<td>P44212 or is enantiomorph</td>
<td>a = b = 91.4 Å, c = 211.6 Å, α = β = γ = 90°</td>
<td>2-3</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>Chunky rods</td>
<td>15 mg/ml; 6% PEG 6000, 6 mM sodium acetate, pH 5.9; 2 months; 25°C</td>
<td>P21212</td>
<td>a = 94.7 Å, b = 114.2 Å, c = 148.6 Å, α = β = γ = 90°</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>Plates</td>
<td>15 mg/ml; 8.3% PEG 6000, 100 mM sodium acetate, pH 5.8; 1 month; 25°C</td>
<td>P21212</td>
<td>a = 98.1 Å, b = 130.2 Å, c = 265.4 Å, α = β = γ = 90°</td>
<td>8+</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>Thick plates</td>
<td>15 mg/ml; 12% PEG 6000, 30 mM sodium acetate, pH 5.9; 1 mM myristic acid; 2 weeks; 25°C</td>
<td>Uncharacterized</td>
<td>grows from precipitate.</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>None</td>
<td>Irregular thick rods</td>
<td>15 mg/ml; 5% PEG 6000, 20 mM sodium acetate, pH 7.3; 2-3 months; 4°C, a = 67.1 Å, b = 67.9 Å, c = 100.8 Å, β = 98.5°, α = γ = 90°</td>
<td>P21</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>None</td>
<td>Plates</td>
<td>10 mg/ml; 20% PEG 6000, 25 mM sodium acetate, pH 5.2; 1-2 months; 4°C</td>
<td>P2122 (marginal)</td>
<td>a = 94 Å, b = 106 Å, c = 188 Å, α = β = γ = 90°</td>
<td>4-6</td>
</tr>
<tr>
<td>G</td>
<td>None, 1</td>
<td>Hexagonal plates</td>
<td>5 mg/ml; 7% PEG 6000, 100 mM sodium acetate, pH 6.2; 1 month; 25°C</td>
<td>Uncharacterized</td>
<td>grows from precipitate.</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>None</td>
<td>Thin rods</td>
<td>10 mg/ml; 5% PEG 6000, 20 mM sodium acetate, 80 mM sodium cacodylate, pH 5.9; 3 months; 4°C</td>
<td>Uncharacterized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>None</td>
<td>Clusters of needles</td>
<td>15 mg/ml; 7.5% PEG 6000, 60 mM sodium acetate, 40 mM sodium cacodylate, pH 5.9; 1 week; 25°C</td>
<td>Uncharacterized</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Crystallization conditions include: the protein concentration, the precipitant and its concentration, the buffer and its concentration, any additives and their concentration, pH, crystallization time, crystallization temperature, and whether seeds were used.

*Z is molecules/asymmetric unit. Mornon et al. (27) have reported crystals in space group P21 with cell dimensions a = 98.4 Å, b = 104.4 Å, c = 77.4 Å, β = 101.2°, α = γ = 90°.

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**TABLE II**

Summary of crystal forms and crystallization conditions for mouse submaxillary gland renin and mouse submaxillary gland renin-inhibitor complexes.
Crystallography of Renin and Renin-Inhibitor Complexes

FIG. 1. Crystals of form A of mouse submaxillary gland renin. The bar corresponds to 1 mm. a, data quality single crystal resulting from the seeding process. b, seed crystals grown spontaneously.

Large crystals suitable for x-ray diffraction were transferred into protein-free stabilizing solutions which were generally at 10–25% higher precipitant concentration than the reservoir solutions used in the crystallizations. These were subsequently mounted in glass capillaries for data collection. Precession photographs for surveys were obtained using conventional nickel-filtered copper Kα radiation. Large scale data collection was by oscillation photography using focused, monochromated 1.5-Å synchrotron x-rays at the Cornell High Energy Synchrotron Source (CHESS) in Ithaca, NY.

RESULTS AND DISCUSSION

Mouse submaxillary gland renin crystals were first reported by Cohen et al. (25), but were not characterized crystallographically. Recently, Mornon et al. (26) have described a mouse submaxillary gland renin crystal form with four independent molecules per asymmetric unit which diffracts to 5-Å resolution. We have obtained multiple crystal forms of mouse submaxillary gland renin in its native state, and in binary complex with several inhibitors synthesized at Merck. As shown in Table II, very small differences in crystallization conditions or in the nature of the inhibitors used can shift growth to one or another crystal form. This suggests that the region around the binding pocket is involved in crystal packing. The best of these forms, A in Table II, is tetragonal, in space group P41212 (or its enantiomorph), with unit cell dimensions: \( a = b = 91.4 \, \text{Å}, \ c = 211.6 \, \text{Å} \), and volume, \( V = 1,718,000 \, \text{Å}^3 \). Fig. 2 is an (0kI) precession photograph of our form A crystals taken at CHESS. Assuming \( M_r = 36,500 \) (10) and two molecules per asymmetric unit, one can calculate the crystal volume/unit of protein molecular weight, \( V_m = 3.026 \, \text{Å}^3 \) dalton\(^{-1} \) corresponding to 60% solvent content. This is within the range of values which have been observed with protein crystals (27), although three molecules per asymmetric unit can also be accommodated, giving \( V_m = 2.018 \, \text{Å}^3 \) dalton\(^{-1} \) (39% solvent). If one assumes \( M_r = 38,000 \) (9, 26), then \( V_m = 2.907 \, \text{Å}^3 \) dalton\(^{-1} \) (57% solvent), and \( V_m = 1.938 \, \text{Å}^3 \) dalton\(^{-1} \) (36.5% solvent) for two and three molecules/asymmetric unit, respectively.

Mouse submaxillary gland renin crystals are particularly difficult to obtain and show moderate radiation sensitivity. With high-intensity synchrotron radiation, we have observed...
an increased lifetime per photon, as have others (28). We have now collected a complete set of oscillation photographs at CHESS, with reflections at 2.8-Å resolution and beyond (see Fig. 3) from crystals of a rational iodine heavy atom derivative in which mouse submaxillary gland renin was complexed with an inhibitor analog synthesized with p-iodo-Phe (compound 2 in Table I). A full description of the synthesis of this compound, and several characteristics of its interaction with mouse submaxillary gland renin will be described elsewhere. A Patterson search with the related microbial aspartyl protease from *Rhizopus chinensis* (15) as a structural probe has been carried out using the iodine derivative structure factors. As might be expected for a high symmetry space group with multiple molecules/asymmetric unit (29), these Patterson search results have not been decisive in solving the structure. Additional data is being collected on isomorphous heavy atom derivatives and on the "native" mouse submaxillary gland renin-inhibitor complex in order to solve the structure.

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REFERENCES