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Substrate Specificity of Recombinant Human Renal Renin: Effect of Histidine in the P_2 Subsite on pH Dependence

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ABSTRACT: Steady-state kinetic analysis of human renin demonstrates the histidine proximal to the substrate scissile peptide bond contributes to the unique specificity and pH dependence of this aspartyl protease. Recombinant human renal renin purified from mammalian cell culture appears to be indistinguishable from renin isolated from human kidney with respect to specific activity (1000 Goldblatt units/mg). Recombinant renin contains carbohydrate covalently attached to asparagines at positions 5 and 75 (renin numbering) and disulfide linkages at Cys-51/Cys-58, Cys-217/Cys-221, and Cys-259/Cys-296. Renin pH dependence was evaluated between pH 4.0 and 8.0 by using a synthetic substrate identical with the amino terminus of porcine angiotensinogen (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-*His*-Leu*Leu-Val-Tyr-Ser, where the asterisk indicates the scissile peptide bond and the proximal histidine is in italics) and an analogous tetradecapeptide where the proximal histidine was substituted with glutamine. Comparison of the pH profiles shows the catalytic efficiency (V/K_m) and maximal velocity (V) of renin are greater above pH 6.5 with the substrate containing histidine proximal to the scissile peptide bond, but below pH 5.0 these parameters are greater with the glutamine substrate analogue. Solvent isotope effects show that proton transfer contributes to the rate-limiting step in catalysis with both substrates and that the proximal histidine does not serve as a base in the catalytic mechanism. Molecular modeling indicates the substrate histidine could hydrogen bond to Asp-226 of the enzyme (renin numbering), thus perturbing the ionization of the catalytic aspartyl groups (Asp-38 and Asp-226). This enzyme-substrate complex would enable the proximal histidine to "direct" catalysis and account for the activity of renin at physiological pH, which is uncommon among structurally homologous cellular aspartyl proteases. Thus, interactions in the renin-substrate complex rather than amino acid substitutions between renin and cellular aspartyl proteases appear to account for renin activity at physiological pH.

Renin (EC 3.4.23.15) is a plasma aspartyl protease that regulates the initial step in the production of the potent pressor octapeptide angiotensin II (Ondetti & Cushman, 1982). Renin selectively cleaves the decapeptide angiotensin I from the amino terminus of the α_2-globulin angiotensinogen. The angiotensinogen specificity and strategic role of renin in the regulation of blood pressure make renin an attractive target for inhibitors that could be powerful antihypertensive agents.

Despite the interest in renin inhibitors, little is known about the catalytic mechanism of renin relative to other proteases. Pepstatin inhibition (Aoyagi et al., 1972) and specific modification with diazoacetyl-D,L-norleucine methyl ester (Inagami et al., 1974; McKown & Gregerman, 1975) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (Misono & Inagami, 1980) have established that renin is functionally homologous to aspartyl proteases. Cloning of the human renin gene (Imai et al., 1983) and its expression in mammalian cell culture (Carilli et al., 1988) have permitted X-ray diffraction studies of the enzyme (Sielecki et al., 1989). The crystal structure demonstrates human renal renin is structurally homologous to cellular aspartyl proteases. However, the structure-function relationships that account for renin's restricted substrate specificity and preference for neutral pH relative to cellular aspartyl proteases remain undefined.

The pH dependence of recombinant human renal renin has been evaluated with a synthetic tetradecapeptide identical with the amino terminus of porcine angiotensinogen (PTDP,[1] Table I) and an analogous substrate (HP2Q, Table I), where glut-

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[1] Abbreviations: PTDP, porcine tetradecapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser); HP2Q, tetradecapeptide of sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-Gln-Leu-Leu-Val-Tyr-Ser; P_2-His, histidine in the P_2 position of PTDP or angiotensinogen; K_m Michaelis constant; V, maximal velocity; V/K_m, catalytic efficiency; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; BSA, bovine serum albumin; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; MES, 2-(*N*-morpholine)ethanesulfonic acid; v, initial rate of product formation; S, substrate concentration; γ, kinetic parameter (V/K_m or V); c, pH-independent value of the kinetic parameter; K_a, dissociation constant for a group that ionizes at low pH; K_b, dissociation constant for a group that ionizes at high pH; CHO, Chinese hamster ovary; $^D(V/K_m)$, ratio of V/K_m determined in H_2O to V/K_m determined in D_2O; $^D(V)$, ratio v determined in H_2O to V determined in D_2O.

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Table I: Substrates Used To Evaluate the pH Dependence of Recombinant Human Renal Renin

substrate[b]	position[a]													
	P_{10}	P_9	P_8	P_7	P_6	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'
PTDP	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val	Tyr	Ser
HP2Q	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	Gln	Leu	Leu	Val	Tyr	Ser

[a] Nomenclature of Schechter and Berger (1967). [b] PTDP, porcine tetradecapeptide, identical with the amino terminus of porcine angiotensinogen; HP2Q, PTDP with glutamine substituted for histidine in the P_2 position.

amine has been substituted for histidine proximal to the scissile peptide bond (the P_2 position[2]). Comparison of the pH profiles demonstrates histidine in the P_2 position of the substrate (P_2-His) significantly contributes to the catalytic efficiency (V/K_m) and maximal velocity (V) of renin at physiological pH. A catalytic mechanism is presented that accounts for the unique specificity and pH dependence of renin through *substrate-directed* catalysis involving P_2-His.

Experimental Procedures

Synthesis of Renin Substrates. PTDP, HP2Q, and the decamer standards were assembled on an Applied Biosystems (Foster City, CA) 430A automated synthesizer according to the procedure of Barany and Merrifield (1980). Using 0.5 mmol of methylbenzhydrylamine resin (Omni Biochem, National City, CA), amino acids (Applied Biosystems) were sequentially incorporated with 2 equiv of the preformed symmetrical anhydrides. Histidine and glutamine residues were double coupled. Cleavage of the peptide from the resin and amino acid side chain deprotection were accomplished by treatment with 90% HF/anisole for 60 min at 0 °C. Peptides were purified on a Waters (Bedford, MA) reversed-phase C-18 column (μBondapak, 19 × 150 mm), and fractions of greater than 98% purity were pooled and lyophilized. Peptide composition was verified by amino acid analysis and fast-atom bombardment mass spectroscopy.

Expression and Purification of Recombinant Human Renal Renin. The gene for human preprorenin (Imai et al., 1983) was cloned between a mouse metallothionein promoter and a SV40 polyadenylation site in a bovine papilloma virus vector. The mouse mammary tumor cell line C127 was transfected with the vector, and clones that were positive for renin activity were grown to confluence in Dulbecco's Modified Eagles medium containing fetal calf serum. Cells were switched to media without calf serum and grown to confluence and the media pooled; 44 L of media was concentrated to 1.4 L, solid trypsin (Sigma, St. Louis) added to 0.5 mg/L original volume, and the mixture incubated at 4 °C for 5 min. Conversion of prorenin to renin was terminated by the addition of PMSF to 100 μM and SBTI to a 4-fold molar excess over trypsin. Trypsinized renin was applied to a Sephadex G-25 column (Pharmacia, Piscataway, NJ; 10 × 38 cm) equilibrated with chromatography buffer (20 mM Tris-HCl, pH 8.0, and 100 μM PMSF). The flow-through effluent was applied to a Q-Sepharose column (Pharmacia, 2.5 × 31 cm) equilibrated in chromatography buffer. Renin was eluted with a linear 0–500 mM NaCl gradient and brought to 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The enzyme was applied to a Con-A–Sepharose column (Sigma, 5.0 × 3.0 cm) equilibrated in chromatography buffer with 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and eluted with 0.5 M methyl α-D-mannoside (Sigma). Exogenous carbohydrate was removed by Sephacryl S-200 chromatography (Pharmacia, 5.0 × 89 cm, chromatography buffer and 150 mM NaCl), and the enzyme was dialyzed against 10 volumes of 50% glycerol, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl before storing at −20 °C. Protein concentrations were measured according to the method of Bradford (1976), and purity was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970).

Measurement of Recombinant Human Renal Renin Activity. Renin activity was measured by HPLC analysis of a peptide assay, similar to the method of Poorman et al. (1987). Renin was incubated with PTDP in dilution buffer (100 mM sodium phosphate, pH 6.50, 1 mg/mL BSA, and 100 μM PMSF) at 37 °C, and the reaction was quenched at various times by the addition of 0.02 volume of concentrated HCl. Fifty microliters of the assay was added to 100 μL of 90% acetonitrile, and 50 μL was injected onto a C-8 microbore column (Alltech, Deerfield, IL; 2.1-mm i.d.) equilibrated in 15% acetonitrile and 0.1% TFA. PTDP and the decamer product (angiotensin I) were separated by a linear 15–35% acetonitrile gradient. The rate of product formation (nanomoles of angiotensin I per minute) was determined by HPLC analysis of the assay at various time points. The amount of decamer formed was determined by comparison of the decamer peak area to an angiotensin I standard curve. A tetrapeptide product peak was not observed and assumed to coelute with the injection peak. The concentration of the standard was determined from the extinction coefficient of tyrosine ($\epsilon_{274.6}$ = 1420 M^{-1} cm^{-1}) at pH 6.50. The specific activity of recombinant human renal renin is expressed as Goldblatt units (the amount of renin required to produce 120 μg of angiotensin I from human angiotensinogen per hour per milliliter) per milligram and was determined by comparison of activity (nanomoles of angiotensin I per minute) with the World Health Organization standard renin (lot C070575).

Determination of Disulfide Linkages. The disulfide content of renin was determined by Ellman's analysis and peptic mapping under reducing and nonreducing conditions. Purified renin (1 mg/mL) was treated with Ellman's reagent (Sigma), and free sulfhydryls were assayed by an increase in absorbance at 410 nm. Renin was digested with pepsin (Boehringer Mannheim, Indianapolis, IN) at a 50:1 ratio, respectively, in 100 mM ammonium acetate, pH 2.5, at 37 °C overnight. The reaction was stopped by direct application onto an Aquapore RP-300 C-8 microbore column (Applied Biosystems, 2.1-mm i.d.) equilibrated in 0.1% TFA, and peptides were separated by a 0–70% acetonitrile gradient over 120 min. The proteolytic fragments were collected and dried before resuspending in 100 mM Tris-HCl, pH 8.5. For reducing conditions, dithiothreitol (Aldrich, Milwaukee, WI) was added to 20 mM and the mixture incubated for 1 h at 37 °C before HPLC analysis. The peptides corresponding to shifts in the elution profile relative to the nonreducing conditions were collected manually and subjected to N-terminal sequencing.

Determination of Glycosylation Sites. Renin glycosylation sites were identified by tryptic mapping before and after treatment with glycopeptidase F (Boehringer Mannheim). Purified renin (0.25 mg/mL) was dissolved in 0.5 M Tris-HCl, pH 8.5, containing 6 M guanidine. Renin was reduced by adding dithiothreitol to 10 mM and incubating for 2 h at 37

[2] Nomenclature of Schechter and Berger (1967).

°C. Iodoacetic acid was added to 25 mM and incubated at 4 °C overnight. Reduced and alkylated renin was dialyzed against 0.167 M Tris-HCl, pH 8.5, containing 2 M guanidine for 6 h and against 100 mM ammonium bicarbonate, pH 7.9, overnight at room temperature before digestion with trypsin (1:25, trypsin:renin) at 37 °C overnight. The reaction was stopped by direct application onto the C-8 microbore column, and peptides were separated, collected, and dried as for the disulfide determinations. Fifty micrograms of tryptic renin peptides was resuspended in 200 mM sodium phosphate, pH 6.5, and incubated with 1 unit of glycopeptidase F overnight at 37 °C. The deglycosylated peptides were separated as above, and the peptides corresponding to shifts in the elution profile relative to glycosylated peptides were collected and subjected to N-terminal sequencing.

Kinetic Studies. The 100 mM buffers used in the pH dependence studies were as follows: sodium acetate at pH 4.00, 4.50, 5.00, and 5.50; sodium citrate at pH 4.25, 4.75, and 5.25; MES at pH 5.75, 6.25, and 6.75; sodium phosphate at pH 6.00, 6.50, 7.00, 7.50, and 8.00; and Tris-HCl at pH 7.25 and 7.75. pH was measured with an Orion Model 611 pH meter (Orion Research Inc., Cambridge, MA) at room temperature. Stock solutions of the substrate were prepared by dissolving the peptide in dimethyl sulfoxide, diluting 1 to 10 in the appropriate buffer, and filtering through a 0.2-μm filter. Stock solutions of the substrate for measuring isotope effects were prepared in 100 mM sodium phosphate, pH 6.50, dissolved in D_2O (99%, MSD Isotopes, Montreal, Canada). The pD of the D_2O solutions was determined by adding 0.4 to the observed pH (Bell, 1959). The concentration of the substrate was established from a standard curve constructed from the integrated peak areas of varying concentrations of tetradecapeptides determined from $\epsilon_{274.6} = 2840\ M^{-1}\ cm^{-1}$ at pH 6.50. The stock solutions were used to prepare the assay solutions, which contained 0.1 mg/mL BSA, 100 μM PMSF, and varying substrate concentrations (5–300 μM); 48 μL of assay solution was preincubated at 37 °C, 2 μL of enzyme added and mixed (the final renin concentration varied between 5 and 25 nN), and the reaction quenched at various times by the addition of 1 μL of concentrated HCl. The nanomoles of product per minute was analyzed as above. The decamer was the only product observed with PTDP or HP2Q by HPLC analysis. A standard assay (200 μM PTDP in 100 mM sodium phosphate, pH 6.50, 0.1 mg/mL BSA, 100 μM PMSF) was performed in parallel with each experiment to standardize the initial velocity determinations. The molar activity of renin was determined from the initial velocity of the standard assays (nanomoles of angiotensin I per minute) and the assay concentration of the enzyme (Bradford, 1976).

Data from the initial velocity studies were fit to

$$v = VS/(K_m + S) \quad (1)$$

where v is the initial rate of product formation, S is the substrate concentration, V is the maximal velocity, and K_m is the Michaelis constant, by using the nonlinear regression analysis program of R. J. Leatherbarrow (ENZFITTER, Elsevier-BIOSOFT).

The ionization constants for the maximal velocity and catalytic efficiency were determined from

$$\log y = \log [c/(1 + H/K_a)] \quad (2)$$

$$\log y = \log [c/(1 + (H/K_a)) + (H/K_b)] \quad (3)$$

where y is V or V/K_m, c is the pH-independent value of V or V/K_m, and K_a and K_b are the dissociation constants of the groups that ionize at low and high pH, respectively (Cleland, 1979; modified for the IBM PC by Dr. Charles Grissom, Berkeley, CA).

Modeling of Renin–Angiotensinogen Complex. The crystal structure of the aspartyl protease from *Cryphonectria (Endothia) parasitica* (endothia pepsin) complexed with a renin inhibitor containing histidine in the P_2 position (1.86-Å resolution, graciously provided by Dr. Huey-Shengh Sheih, Monsanto Co., St. Louis) was used as a template to model angiotensinogen in the active site of renin. Overlapping of the renin crystal structure with cellular aspartyl protease crystal structures demonstrates the main-chain atoms are in homologous conformations in this enzyme family (Sielecki et al., 1989). Amino acid substitutions between the active sites of renin and endothia pepsin were made and refined by using the molecular modeling program PROTEUS (Dr. Henry Dayringer, Monsanto Co., St. Louis).

Table II: Purification of Recombinant Human Renal Renin from C-127 Cell Culture Media

purification step	vol (mL)	total protein (mg)	total (GU)[a]	sp act. (GU/mg)
concentrated C-127 media	1400	1100	14000	13
trypsinized media	1400	1100	110000	99
Sepharose G-25	1400	1100	100000	94
Q-Sepharose	140	180	100000	580
Con-A–Sepharose	110	79	76000	960
Sephacryl S-200	220	59	62000	1000
dialysis	69	50	51000	1000

[a] Goldblatt unit (GU) is defined as the amount of renin necessary to produce 120 μg of angiotensin I from human angiotensinogen per hour per milliliter.

RESULTS

Purification and Structural Characterization. Recombinant human renal renin purified from mammalian cell culture appears to be indistinguishable from renin purified from human kidney with respect to covalent modifications and specific activity. The purification procedure is summarized in Table II. The final enzyme preparation was judged to be at least 95% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the first 15 cycles of N-terminal amino acid sequencing gave single amino acids corresponding to the sequence of mature human renin (Imai et al., 1983). Recombinant human renal renin has 100% of the specific activity of a World Health Organization standard preparation (1000 Goldblatt units/mg) and is stable for months when stored at −20 °C.

Glycopeptidase F treatment of recombinant renin identified two N-linked glycosylation sites at Asn-5 and Asn-75 [numbering of Imai et al. (1983)]. Renin purified from human kidney is glycosylated (Yokosawa et al., 1980), but the residues that are covalently modified have not been identified. Recombinant human renin purified from CHO cell culture for X-ray diffraction studies is glycosylated only at Asn-75 (Sielecki et al., 1989), but differing degrees of glycosylation have been reported for recombinant renin expressed in CHO cells (Fritz et al., 1986). Although renin glycosylation appears to be variable, covalent attachment of carbohydrate does not appear to influence enzymatic activity (Carilli et al., 1988).

Ellman's analysis established there were no free sulfhydryl groups in recombinant human renal renin. Peptic mapping under reducing and nonreducing conditions identified disulfide bonds at Cys-51/Cys-58 and Cys-259/Cys-296. According to the sequence of human renin (Imai et al., 1983), two other cysteines are at positions 217 and 221. From the results of

Table III: pH Dependence of Kinetic Constants for Renin with PTDP and HP2Q

	PTDP		HP2Q	
buffer[a] (100 mM)	V (s^{-1})	K_m (μM)	V (s^{-1})	K_m (μM)
sodium acetate, pH 4.00	0.048	75	0.21	92
sodium citrate, pH 4.25	0.056	41	0.36	150
sodium acetate, pH 4.50	0.093	45	0.50	140
sodium citrate, pH 4.75	0.33	120	0.75	230
sodium acetate, pH 5.00	0.26	54	0.92	240
sodium citrate, pH 5.25	0.95	120	ND[b]	ND[b]
sodium acetate, pH 5.50	0.84	54	0.74	140
MES, pH 5.75	0.97	22	ND[b]	ND[b]
sodium phosphate, pH 6.00	0.98	12	0.91	110
MES, pH 6.25	1.1	16	ND[b]	ND[b]
sodium phosphate, pH 6.50	1.1	15	0.92	200
MES, pH 6.75	0.92	19	ND[b]	ND[b]
sodium phosphate, pH 7.00	0.90	21	0.46	230
Tris-HCl, pH 7.25	0.73	36	0.44	120
sodium phosphate, pH 7.50	1.1	60	0.43	210
Tris-HCl, pH 7.75	0.69	80	0.30	290
sodium phosphate, pH 8.00	0.79	28	0.22	180

[a] See Experimental Procedures for preparation of buffers. [b] ND, not determined.

Table IV: pK- and pH-Independent Values Obtained from pH Dependence of Kinetic Parameters with PTDP and HP2Q

	V/K_m		V		
substrate	pK_a	pK_b	pK_a	pK_b	c[a] (s^{-1})
PTDP	6.3 ± 0.1	6.9 ± 0.1	5.3 ± 0.1		1.0 ± 0.1
HP2Q			4.4 ± 0.1	7.4 ± 0.1	0.9 ± 0.1

[a] pH-independent value of V.

the Ellman's test, these must also be in a disulfide linkage. This has been confirmed from the crystal structure of human renal renin (Sielecki et al., 1989). Mouse submaxillary gland renin contains a partially buried free sulfhydryl group, but it does not appear to influence enzyme activity (Misono & Inagami, 1980). Sequence alignment of human renin with porcine pepsin (Tang & Wong, 1987) shows that the three disulfide linkages are conserved in the same location of renin and pepsin, which reinforces the assumption that the cellular aspartyl proteases are structurally homologous.

pH Dependence with PTDP and HP2Q. The kinetic constants V and K_m for renin with PTDP and HP2Q determined between pH 4.0 and 8.0 are compared in Table III. The error with either substrate was generally less than 20%. At pH 5.75, V and K_m for PTDP are in good agreement with the values determined by radioimmunoassay (Cumin et al., 1987). Figure 1 shows the pH dependence of renin with PTDP and HP2Q. The fit of the data to eq 2 or 3 demonstrates that even though buffers of varying ionic strength were used (Table III), the pH dependence of both V/K_m and V is independent of ionic strength.

When the values for V/K_m with PTDP were fit to eq 3, pKs of 6.3 and 6.9 were obtained (Figure 1A; Table IV). The slope of the curves above and below the maxima is approximately one, indicating each pK corresponds to a single ionizable group. The maxima of the curve between the pKs shows that one group must be protonated and another unprotonated to obtain the greatest catalytic efficiency. When HP2Q was the substrate (Figure 1A), V/K_m was pH independent and generally less than the catalytic efficiency with PTDP. Thus, the formation of the enzyme–substrate complex from free renin and substrate is enhanced between pH 5.0 and 8.0 when histidine is in the P_2 position of the substrate.

The values of V with PTDP were best fit by eq 2, while the values of V with HP2Q were best fit by eq 3 (Figure 1B). With PTDP, a single ionizable group with a pK of 5.3 must

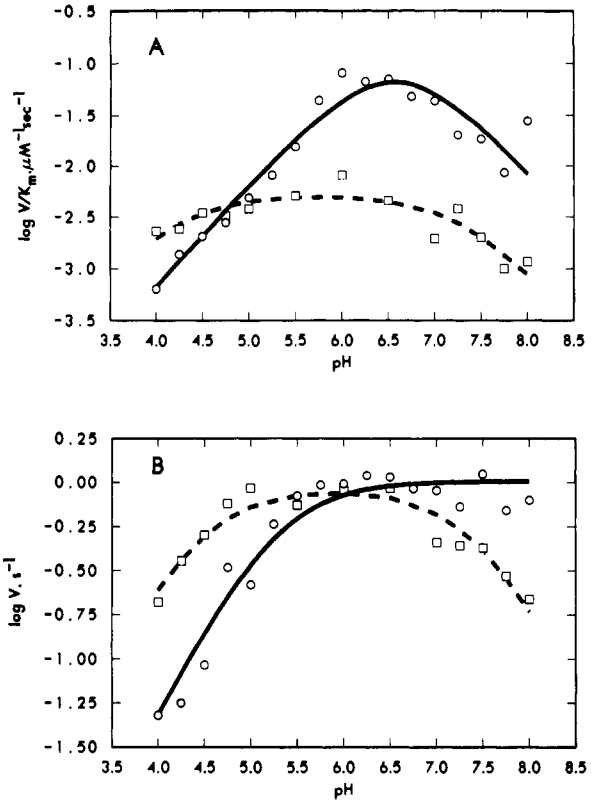

FIGURE 1: pH dependence of the catalytic efficiency (V/K_m; A) and maximal velocity (V; B) of recombinant human renal renin with PTDP (open circles, solid line; see Table I) and HP2Q (open squares, dashed line; see Table I).

Table V: Solvent Isotope Effects for Renin with PTDP and HP2Q[a]

	substrate	
parameter	PTDP	HP2Q
K_m, H_2O	15 ± 3.0 μM	200 ± 28 μM
K_m, D_2O	24 ± 4.7 μM	89 ± 13 μM
V, H_2O	1.1 ± 0.089 s^{-1}	0.92 ± 0.081 s^{-1}
V, D_2O	0.60 ± 0.062 s^{-1}	0.50 ± 0.035 s^{-1}
$^D(V/K_m)$[b]	1.16	0.82
$^D(V)$[c]	1.82	1.84

[a] 100 mM $NaPO_4$, pH 6.50, 1 mg/mL BSA, 100 μM PMSF, 37 °C. [b] The ratio of V/K_m in H_2O to V/K_m in D_2O. [c] The ratio of V in H_2O to V in D_2O.

be unprotonated to achieve the optimal V values (Table IV). This is unusual when compared to the pH dependence of V for porcine pepsin (Dunn et al., 1987; Pohl & Dunn, 1988), bovine chymosin (Dunn et al., 1987; Suzuki et al., 1989), and endothia pepsin (Dunn et al., 1987), which generally fits a bell-shaped curve at more acidic pH. The data for HP2Q (Figure 1B; Table IV) fit a bell-shaped curve with pKs of 4.4 and 7.4, which approximates the results obtained with the aspartyl proteases cited above. Thus, the maximal velocity of renin is greatest above pH 6.5 when histidine is in the P_2 position and greatest below pH 5.5 when glutamine is in the P_2 position.

Solvent Isotope Effects. To evaluate whether V is describing the rate of substrate to product conversion or the rate of product release, solvent isotope effects were measured for both PTDP and HP2Q at pH 6.5 (Table V). No isotope effect on V/K_m was expected or observed with PTDP or HP2Q since it is unlikely that the formation of the renin–substrate complex from free enzyme and free substrate involves proton exchange. A significant isotope effect on V was measured with both substrates, indicating proton transfer contributes to the rate-

limiting step in catalysis, and it is unlikely that the release of product from renin is rate-limiting. It has been suggested from crystal structures of aspartyl protease–inhibitor complexes that the scissile peptide bond may undergo torsional strain during catalysis, which could also contribute to limiting the rate of hydrolysis (Suguna et al., 1987a). The magnitude of $^{D}(V)$ is the same with both substrates, indicating P_2-His is not serving as a base during catalysis. In addition, the equivalence of the pH-independent values for V with PTDP and HP2Q (Table IV) indicates the rate-limiting step(s) is (are) the same for both substrates.

Discussion

The comparison of renin pH dependence with PTDP and HP2Q demonstrates the importance of the histidine proximal to the substrate scissile peptide bond to renin's activity at physiological pH. The pH preference of renin in unusual among cellular aspartyl proteases, which generally prefer acidic pH. The catalytic mechanisms proposed for aspartyl proteases assign a water molecule hydrogen-bonded to two active-site aspartic acids (Asp-38 and Asp-226 in renin) as the nucleophile. The ability of water to act as a nucleophile is dependent on the aspartyls being in opposite ionization states (James & Sielecki, 1985; Pearl, 1987; Suguna et al., 1987a). These ideas are consistent with the structural, kinetic, and chemical modification studies performed on pepsin and the fungal aspartyl proteases (Polgar, 1989). Crystallographic analysis (Sielecki et al., 1989) and chemical modification studies (Inagami et al., 1974; McKown & Gregerman, 1975; Misono & Inagami, 1980) of renin are consistent with the paradigm of water as the nucleophile and the active-site aspartyls as essential for catalysis. Even though renin is structurally and chemically homologous to cellular aspartyl proteases, its activity above acidic pH is an enigma and does not fit the proposed aspartyl protease mechanisms.

Amino acid substitutions between renin and cellular proteases are a logical choice to explain the unique pH dependence of renin. A replacement that could affect the electrostatic microenvironment of the active site occurs at position 317 (renin numbering), where human, mouse, and rat renin have an alanyl residue (Inagami, 1989) and the aspartyl proteases that prefer acidic pH have aspartic acid (Tang & Wong, 1987). However, changing Ala-317 to Asp appears to have little effect on the pH dependence of human renin (Yamauchi et al., 1988). Another substitution that could perturb the electrostatic microenvironment of the active site occurs at position 229 (renin numbering), where human renin has an alanyl residue and the cellular aspartyl proteases preferring acidic pH have serine or threonine. Structural analysis of fungal aspartyl proteases illustrates that the hydroxyl moiety of threonine participates in the extensive hydrogen bonding between the catalytic aspartyls and active-site residues (James & Sielecki, 1983; Suguna et al., 1987b). However, renin from rat and mouse have threonine and serine, respectively, at position 229 (Inagami, 1989). Since these nonhuman renins also prefer neutral pH (Inagami, 1989), it appears Ala-229 cannot account for renin's pH dependence. In addition, chemical modification of mouse submaxillary gland renin demonstrates that histidine, tryptophan, and amino groups are not essential for renin activity (Misono & Inagami, 1980). Therefore, active-site amino acid substitutions between renin and cellular aspartyl proteases that prefer acidic pH do not appear to account for the pH dependence of renin.

A catalytic mechanism for renin hydrolysis of angiotensinogen or PTDP that uses *substrate-directed* catalysis through P_2-His of the substrate to explain renin pH dependence and specificity is presented in Figure 2. The binding of substrate displaces most of the solvent molecules from the active site (Figure 2A,B), with the exception of the nucleophile between Asp-38 and Asp-226. In addition, an ion pair between P_2-His of the substrate and Asp-226 of renin is formed. Model building of the renin–angiotensinogen complex (Figure 3) indicates the hydrogen bond between P_2-His and Asp-226 is conceivable, and diffraction studies demonstrate that P_2-His can be oriented toward the active site in endothia pepsin–renin inhibitor complexes (Sali et al., 1989). This P_2-His/Asp-226 interaction may also account for the enhanced selectivity of renin inhibitors containing histidine at the P_2 position (Hui et al., 1987; Cumin et al., 1987a; Maibaum & Rich, 1988; Hanson et al., 1989). An additional hydrogen bond could exist between P_2-His and Ser-89, but the interaction does not seem to be critical since mouse and rat renin have glycine at position 89 (Inagami, 1989). Assuming a P_2-His/Asp-226 ion pair, the V/K_m pH dependence curve for PTDP in Figure 1A could describe the ionization of Asp-226 ($pK = 6.3$) and P_2-His ($pK = 6.9$), which must be unprotonated and protonated, respectively, for the optimal rate of enzyme–substrate complex formation. The assignment of the pKs is supported by (1) the pH *independence* of V/K_m when HP2Q is the substrate (Figure 1A), (2) P_2-His being the only substrate residue interacting with the renin substrate cleft (residues P_4–P_3', Table I; Sielecki et al., 1989) that would be expected to ionize between pH 4.0 and 8.0, and (3) there being no other renin residues neighboring P_2-His that would be expected to ionize in this pH range (Figure 3). Human angiotensinogen has histidine in the P_3' site, but the preference of human renin for porcine tetradecapeptide over the human substrate (Cumin et al., 1987) indicates the P_3' imidazole is of less importance to renin specificity than the one conserved at the P_2 position.

The consequence of the Asp-226/P_2-His pairing would be the "surrogate" protonation of Asp-226 at physiological pH through the hydrogen bond with P_2-His. This could satisfy the requirements that one of the aspartyl groups must be ionized (Asp-38) and the other "protonated" (Asp-226) for the nucleophile to attack the scissile peptide bond. In addition, the ion pairing could perturb the ionization of the active-site aspartyls. A neighboring positive charge (P_2-His) would be expected to lower the pK of Asp-226 from that observed in the free enzyme ($pK = 6.3$, PTDP curve Figure 1A) or the renin–HP2Q complex ($pK = 7.4$, HP2Q curve Figure 1B). Lowering the pK of Asp-226 could raise the pK of neighboring Asp-38 in the enzyme–substrate complex from 4.4 (HP2Q curve, Figure 1B) to 5.3 (PTDP curve, Figure 1B). Since it is unlikely that Asp-38 and Asp-226 undergo a significant conformational change upon binding of the substrate (i.e., no inversion of pK relative to the apoenzyme), the pK of Asp-226 in the renin–PTDP complex should be between 5.3 and 6.3. According to the mechanism in Figure 2, Asp-226 remains ionized throughout PTDP hydrolysis since it shares the P_2-His proton through a hydrogen bond. Abstraction of the nucleophilic solvent proton by Asp-38 (Figure 2B) would promote attack of the nucleophile on the scissile peptide bond (Figure 2C), stabilize the resulting oxyanion tetrahedral transition state (Figure 2D), and allow Asp-38 to serve as an acid by donating a proton to the amino terminus of the product P_1'-leucine (Figure 2D). Therefore, renin hydrolysis of angiotensinogen or PTDP occurs as long as Asp-38 ($pK = 5.3$) is unprotonated and protonated P_2-His ($pK > 8.0$) maintains the ion pair with Asp-226. Renin hydrolysis of HP2Q (Figure 1B, HP2Q curve) is optimal when Asp-38 ($pK = 4.4$) is unprotonated and Asp-226 ($pK = 7.4$) is protonated.

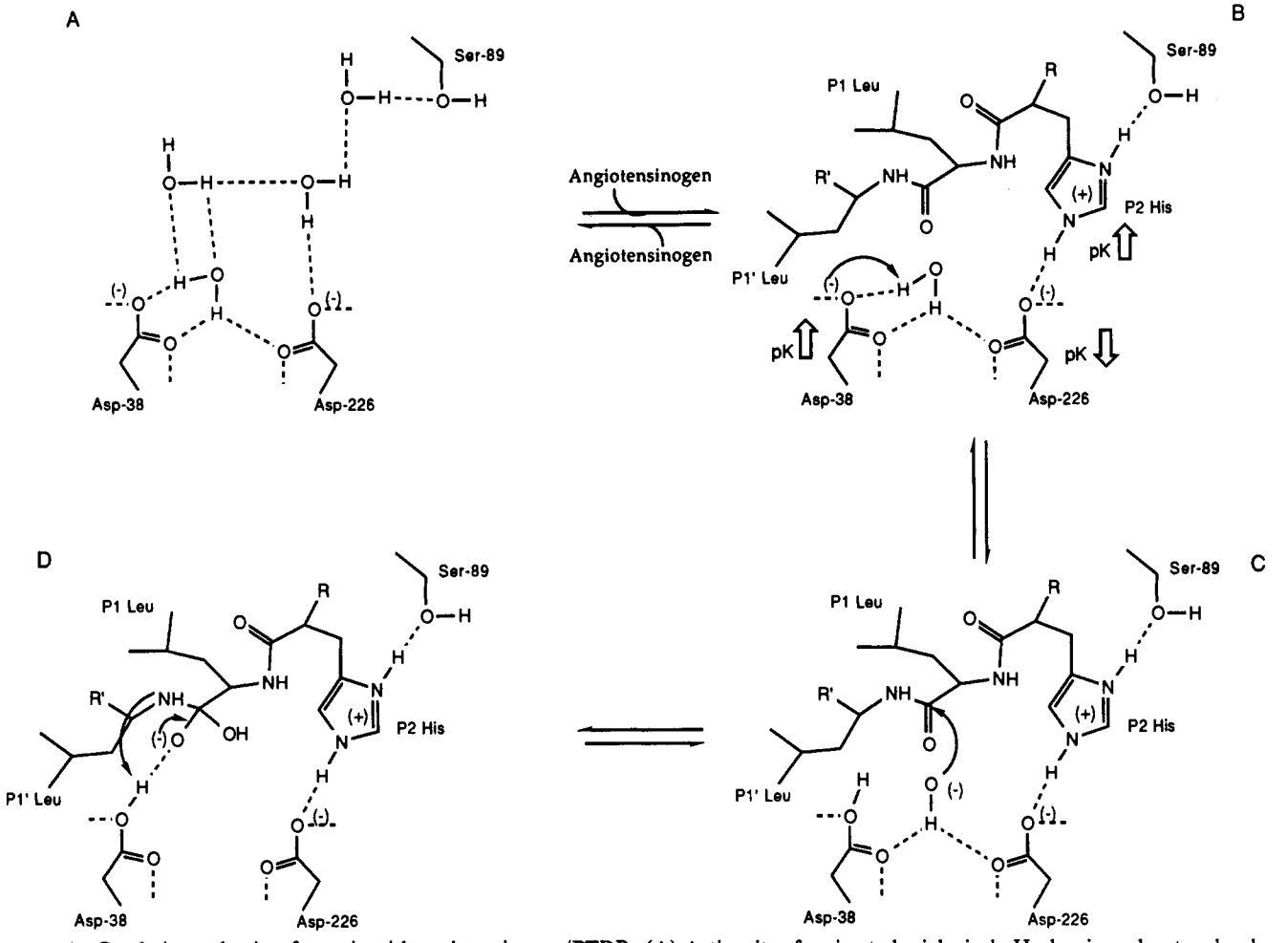

FIGURE 2: Catalytic mechanism for renin with angiotensinogen/PTDP. (A) Active site of renin at physiological pH, showing solvent molecules hydrogen bonded to the ionized catalytic aspartic acids (Asp-38 and Asp-226, renin numbering) and Ser-89 of the "flap" region. (B) P_2–P_1' residues of angiotensinogen/PTDP bound in the active site of renin. The protonated P_2-His could hydrogen bond to Ser-89 and the unprotonated Asp-226. (C) Attack of the nucleophile on the P_1 carbonyl carbon. (D) Tetrahedral oxyanion transition state. Protonated Asp-38 helps to stabilize the oxyanion and serves as an acid upon collapse of the transition state.

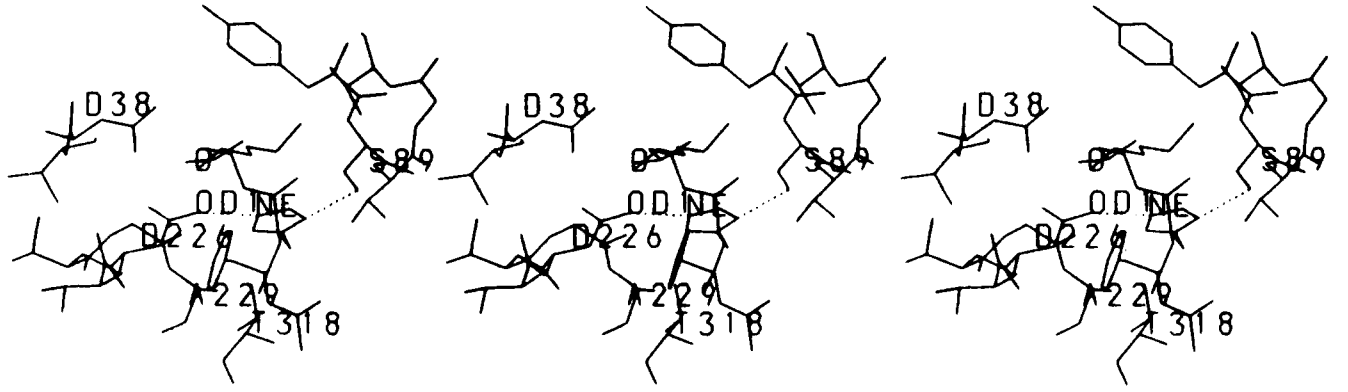

FIGURE 3: Stereo diagram of a renin–angiotensinogen/PTDP active-site model. The images on the left and center are for normal stereo vision; the images on the right and center are for inverted stereo vision. The P_1 carbonyl oxygen of the substrate (O) is positioned between Asp-38 (D38) and Asp-226 (D226), the ϵ-nitrogen of P_2-His is in a hydrogen bond with the δ-oxygen of Asp-226 (OD1), and the δ-nitrogen of P_2-His (unlabeled) is in a hydrogen bond with the oxygen of the Ser-89 (S89) hydroxyl. Also labeled are Ala-229 (A229) and Thr-318 (T318). The nucleophilic water is not shown. See Experimental Procedures for a description of the model construction.

Histidine in the P_2 position allows renin to be active in a pH range uncommon for aspartyl proteases (Figure 4). The optimal pH range of pepsin falls between pH 2 and 3 (Denburg et al., 1968; Cornish-Bowden & Knowles, 1969), while chymosin (Suzuki et al., 1989), the fungal aspartyl proteases (Dunn et al., 1987), and renin with HP2Q as the substrate are most active between pH 4 and 7. The differences between the pH-dependent activity of pepsin and chymosin, fungal aspartyl proteases, and renin with HP2Q appear to be due at least in part to enzyme stability since pepsin unfolds above pH 6 (Edelhoch, 1958). However, the degree of solvation in the active site of the enzyme–substrate complex could also affect the ionization of the active-site carboxylates (Creighton, 1984) and hence the pH optimum for catalysis. In contrast, the pH dependence of renin toward angiotensinogen appears to be due to P_2-His in the substrate.

The ability of renin to promote the formation of an ion pair between P_2-His and Asp-226 may be due to a threonine that

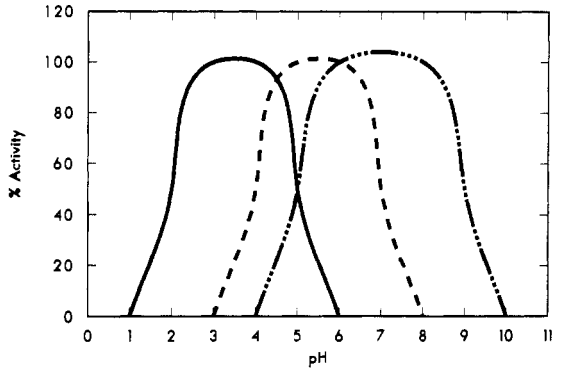

FIGURE 4: pH optima for aspartyl proteases. Percent enzyme activity is plotted as a function of pH for pepsin (—); chymosin, fungal aspartyl proteases and renin with HP2Q (- - -); and renin with angiotensinogen/PTDP (-···-). See text for references.

is conserved at position 318 (Figure 3). Valine is found in the corresponding position in porcine pepsin and chymosin, while isoleucine is found in endothia pepsin and penicillopepsin (Tang & Wong, 1987). It has been suggested that Ser-214, which neighbors Asp-102 and is conserved in trypsin and related serine proteases, helps to stabilize the Asp-102/His-57 ion pair (Brayer et al., 1978). Perhaps Thr-318 plays an analogous role in the enzyme–substrate complex of renin, but this awaits further experimentation. The aspartyl protease from *Rhizopus chinensis* also has threonine at the position corresponding to 318 in renin, but the imidazole of P_2-His in an inhibitor complexed with the enzyme does not interact with the catalytic aspartyls (Suguna et al., 1987a). However, the inhibitor used in the diffraction studies contained phenylalanine in the P_1' position, which could sterically interfere with the interaction between P_2-His and Asp-218 (*Rhizopus* protease numbering). In addition, the carboxylate of Asp-79 in the fungal enzyme forms a hydrogen bond with the P_2 peptide amide. The residue corresponding to Asp-79 in renins is Ser/Thr-90 (Inagami, 1989), which may be unable to extend into a hydrogen bond with the P_2 peptide amide, thus permitting the flexibility necessary to form the P_2-His/Asp-226 ion pair. A high-resolution crystal structure of renin complexed with an inhibitor containing P_2-His would resolve these assumptions.

This is the first known example of the participation of a substrate neighboring group in natural enzymatic catalysis, but it differs mechanistically from the "anchimeric assistance" described for nonenzymatic catalysis (March, 1968) and "substrate-assisted" catalysis designed into subtilisin (Carter & Wells, 1987). Since P_2-His is not required for renin catalysis but perturbs the ionization of the essential enzyme residues, its involvement in catalysis is best described as *substrate-directed* rather than *substrate-assisted*. The activity of renin toward HP2Q at acidic pH demonstrates this enzyme can function as a "classical" aspartyl protease and is consistent with its structural homology to cellular aspartyl proteases. Thus, the activity of renin at physiological pH appears to be more attributable to interactions in the enzyme–substrate complex rather than amino acid substitutions between renin and cellular aspartyl proteases.

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Comparative Study on the Chromophore Binding Sites of Rod and Red-Sensitive Cone Visual Pigments by Use of Synthetic Retinal Isomers and Analogues†

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ABSTRACT: A comparative study on the chromophore (retinal) binding sites of the opsin (R-photopsin) from chicken red-sensitive cone visual pigment (iodopsin) and that (scotopsin) from bovine rod pigment (rhodopsin) was made by the aid of geometric isomers of retinal (all-trans, 13-cis, 11-cis, 9-cis, and 7-cis) and retinal analogues including fluorinated (14-F, 12-F, 10-F, and 8-F) and methylated (12-methyl) 11-*cis*-retinals. The stereoselectivity of R-photopsin for the retinal isomers and analogues was almost identical with that of scotopsin, indicating that the shapes of the chromophore binding sites of both opsins are similar, although the former appears to be somewhat more restricted than the latter. The rates of pigment formation from R-photopsin were considerably greater than those from scotopsin. In addition, all the iodopsin isomers and analogues were more susceptible to hydroxylamine than were the rhodopsin ones. These observations suggest that the retinal binding site of iodopsin is located near the protein surface. On the basis of the spectral properties of fluorinated analogues, a polar group in the chromophore binding site of iodopsin as well as rhodopsin was estimated to be located near the hydrogen atom at the C_{10} position of the retinylidene chromophore. A large difference in wavelength between the absorption maxima of iodopsin and rhodopsin was significantly reduced in the 9-cis and 7-cis pigments. On the assumption that the retinylidene chromophore is anchored rigidly at the α-carbon of the lysine residue and loosely at the cyclohexenyl ring, each of the two isomers would have the Schiff-base nitrogen at a position altered from that of the 11-cis pigments. Thus, the remarkable red-shift in the absorption spectrum of iodopsin as compared with that of rhodopsin could be attributed to a difference between the two pigments in the location of a negative charge directly hydrogen bonded to the Schiff-base nitrogen.

In contrast with the extensive works on rod visual pigment (rhodopsin), far less studies have been conducted on cone pigments, which are responsible for photopic vision. This is because of the difficulty in obtaining large quantities of cone cells without contamination of rod cells and because of the lower stability of the cone pigments (Wald et al., 1955; Okano et al., 1989). Among several cone visual pigments, a red-sensitive pigment in chicken retina is relatively well characterized and named iodopsin[1] (Wald et al., 1955). Iodopsin is different in physical and chemical properties from rhodopsin as follows: First, the absorption maximum of iodopsin is located at a longer wavelength [562 nm, Wald et al. (1955);

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[1] In a previous paper (Okano et al., 1989), the term chicken red was used to indicate the chicken red-sensitive cone visual pigment for contrast with other chicken cone pigments. Instead of chicken red, the specific name iodopsin was used in this paper because iodopsin is the only cone pigment used in this experiment and because this is convenient for designating the isomers and analogues, for example, 9-*cis*-iodopsin.

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