Probing the Renin Active Site by Collisional Quenching of Endogenous Fluorescence*

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The structural and enzymatic aspects of renin are of great interest in hypertension research. In this paper, we examine the solution accessibility of the three tryptophan (Trp) residues of mouse submaxillary gland renin by solute collisional fluorescence quenching. Our studies indicate that there are two "classes" of Trp residues in renin: class I, a class of Trp residues which are at or near the surface of renin and fully accessible to the fluorescence quencher iodide; and class II, a class of Trp residues which are, for practical experimental conditions, totally inaccessible to the aqueous solution. The former class contains 2 Trp residues, while only a single Trp is identified in the latter class. The presence of a tetradecapeptide substrate or a nonhydrolyzable substrate analogue (peptide H-77) lowers the accessibility of iodide to the class I Trp residues. These data indicate that the class I Trp residues are at or near the peptide-binding site of renin. In addition, the finding that the class I Trp residues are quantitatively quenched more efficiently than the Trp model compound indole suggests that the environment of the class I tryptophans may be positively charged, and thus have a higher "local" concentration of iodide. These data, taken together with the available sequence and computer-generated three-dimensional structure of renin, permit us to speculate that the class I Trp residues are Trp-39 and Trp-300. This solution study of renin structure is discussed in light of the known information about renin catalysis and physiology.

The formation of the peptide angiotensin I from the prohormone angiotensinogen is catalyzed in a highly specific manner by the enzyme renin. Because it is the first and rate-limiting step in plasma angiotensin I formation and the subsequent release of aldosterone, it plays a central role in the physiological control of blood pressure. Renins from canine, murine, and human sources have been purified, and biochemical and immunological characterizations of these enzymes have been performed (1–6). Recently, the amino acid sequence of a mouse submandibular gland renin was determined (7). In addition, a computer-generated model for mouse renin structure has become available (8). These two studies provide the rationale for detailed examination of the solution structure and function of mouse renin, the first of which is reported in this paper.

We have used the intrinsic fluorescence of the tryptophan residues of renin in a study to determine the location of the 3 Trp residues in murine submandibular gland renin. Our results are consistent with data on the known structure of the renin active site and provide strong support for the current concepts of renin structure.

EXPERIMENTAL PROCEDURES

Materials—Renin was purified from mouse submandibular gland according to the method of Cohen et al. (1). Potassium iodide (Baker Analyzed) was the highest purity available. Indole was purchased from Sigma and was stored desiccated away from light. The renin substrate analogue H-77 (9) was obtained from Ferring Pharmaceutical. The renin substrate tetradecapeptide was obtained from Sigma. Stock solutions of KI, protected from light and colorless, were used promptly. Formation of I₂ was not found to be significant since S₂O₇²⁻ had no effect on the quenching experiments.†

Methods—Fluorescence measurements were made with a Perkin-Elmer model MP-4 fluorescence spectrometer. Indole was dissolved in the indicated buffers and the concentration determined by the absorbance at 280 nm assuming an extinction coefficient of 5600 m⁻¹ cm⁻¹. A solution of 0.20–1.00 ml was used for fluorescence measurements at room temperature (23°C). Typically, 10–15 µl of a concentrated solution of quenching compound was added to the 1-cm fluorescence cuvette, the contents mixed, and the fluorescence emission spectrum recorded. The fluorescence emission wavelength was 346 nm; the bandwidth varied from 5 to 10 nm for both excitation and emission. The observed fluorescence and the iodide concentration were corrected and calculated for sample dilution. For all quenching experiments, the initial and final absorbance at the excitation wavelength were always <0.06, and, thus, no correction for inner filter effects was necessary. Iodide concentrations were converted to activities using available data on the activity coefficients of potassium iodide in aqueous solution (10). The quenching constant (Kᵢₒ) for a simple, single fluorophor, homogenous system is calculated using equation 1,

\[ \frac{F₀}{F} = 1 + Kᵢₒq₀ \]

where \( Kᵢₒ = kᵢₒ \cdot F₀ \) is the fluorescence intensity (in arbitrary terms) in the absence of quencher, \( F \) is the fluorescence in the presence of quencher, \( kᵢ \) is the bimolecular collisional rate constant, \( Jᵢ \) is the unquenched fluorescence lifetime, and \( q₀ \) is the quencher activity.

RESULTS

Fluorescence Spectroscopy of Renin—The emission and excitation spectra of renin were determined as an initial starting

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point for these experiments. Fig. 1 shows these spectra. The excitation spectra shows a peak near 280 nm indicative of a substantial contribution of tryptophan fluorescence to the overall spectrum. The emission spectrum is centered at 338 nm (using the half-height method of calculation) (11). These data indicate that the use of 280 nm as an excitation wavelength and 340 nm as an emission wavelength should provide an accurate estimation of the fluorescence emission intensity of the tryptophan residues for the subsequent collisional quenching experiments.

**Fluorescence Collisional Quenching**—The collisional quenching of renin fluorescence was examined to determine the solvent accessibility of those tryptophans which contribute significantly to renin’s intrinsic fluorescence. Fig. 2 shows the effect of increasing iodide activity on renin fluorescence. Indole fluorescence quenching was measured under identical buffer and temperature conditions as a model substance. The fluorescence of indole was quenched in a kinetically simple (linear) fashion as expected for a process involving a water-soluble, freely solvent-accessible fluorophor. The quenching of renin is clearly more complex and includes two types of component to the total fluorescence. Assuming, for simplicity, only two “types” of tryptophan residue environments, we obtain a very satisfactory fit to the data (as shown in Fig. 2) using the $K_Q$ and $f_i$ values. The renin tryptophans in the largest class (class I) have a $K_Q$ value which is twice the value for indole, indicating these tryptophans are more easily quenched than even those of free indole in solution. On the other hand, certain tryptophan residue(s) have collisional quenching constants which are experimentally indistinguishable from zero, i.e. they are nearly totally inaccessible to the iodide quencher.

Class I represents a 0.77 fractional contribution to the total fluorescence which mathematically represents 2.3 of the 3 tryptophans in renin. The nonintegral value presumably arises from the imprecisions of the fitting process of our data to equation 2 or to the invalidity of the necessary simplifying assumption that all 3 tryptophans contribute equally to the overall fluorescence (i.e. that they have equal quantum yields).

**Effect of Substrate or Substrate Analogues on Renin Tryptophan Quenching**—To investigate the possible presence of tryptophan residues at or near the active site of renin, we performed experiments to determine if the renin substrates or substrate analogues might perturb the quenching of renin. If iodide quenching was reduced by the presence of these peptides, we could infer that tryptophans were involved in substrate binding, stabilization, or destabilization (enzymatic hydrolysis step) in the enzymatic mechanism of renin action.

The tetradecapeptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-Leu-Leu-Leu-Val-Tyr-Ser is a substrate for renin and is hydrolyzed to Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu and Leu-Val-Tyr-Ser (12). The presence of the tetradecapeptide at $10^{-7}$ M has a dramatic effect on the ability of iodide to quench renin (Fig. 3). Quantitatively, the $K_Q$ for the class I Trp residues changes from 34 to 18 M$^{-1}$. The class II Trp residues have such a small $K_Q$ that it is difficult to determine an effect of the tetradecapeptide substrate on iodide quenching. These results clearly indicate that the class I Trp residues are at or near the peptide-binding site (active site) of renin.

To determine if a nonhydrolyzable substrate analogue could protect tryptophan fluorescence in a manner similar to the tetradecapeptide substrate, we incubated renin with compound H-77 (9). This compound is the (6-13)-octapeptide of equine angiotensinogen containing a reduced (-CH$_2$-NH-) bond at the renin cleavage site (9) and is a potent in vivo inhibitor on canine renin ($K_I = 2.4 \times 10^{-8}$ M). Fig. 3 shows that this compound is capable of reducing the efficiency of iodide quenching in a manner analogous to the tetradecapeptide substrate. The $K_Q$ for fluorescence collisional quenching changes from 34 M$^{-1}$ in the absence of H-77 to 14 M$^{-1}$ in the presence of H-77. These data support and extend the data
DISCUSSION

Renin is a member of the aspartyl protease family. There is a 42% homology between mouse submandibular gland renin and porcine pepsin (6). The active site amino acid residues primarily involved in the hydrolysis of substrate (residues 31–35 and 215–217) are completely conserved as in other aspartyl proteases. Despite these similarities, however, renin is uniquely different from other aspartyl proteases in several aspects. Renin is extremely specific for its substrate, while other aspartyl proteases display a broad range of specificity. The pH optimum of renin and angiotensinogen is much less acidic than other aspartyl proteases. These functional differences may be explained by differences in substrate-binding sites and tertiary structures of these enzymes. Unfortunately, crystallization and x-ray diffraction studies on renin have not been accomplished. Using computer graphics, a model of the three-dimensional structure of mouse renin has been prepared based on the known structures of other aspartyl proteases (8). Although a similar model has been recently proposed for human renin (12, 13), this study examined mouse renin and the discussion will focus on the mouse enzyme (8). Like other aspartyl proteases, the substrate-binding site of renin is most likely formed as a cleft between two lobes of the enzyme molecule. At the central part of the binding site, two aspartyl residues converge on the substrate and are primarily involved in the hydrolysis of the substrate. Computer graphic data also indicate several important differences between renin and other aspartyl proteases. At the edge of the active site cleft, renin contains highly basic residues as well as a surface loop and a flap which are distinct from other aspartyl proteases. These proposed structural data provide the rationale for our detailed examination of the solution structure and function of renin.

Fluorescence of Renin—The intrinsic fluorescence of any protein, in the absence of fluorescent prosthetic groups, is composed of contributions from tryptophan, tyrosine, and phenylalanine. In general, due to a number of factors, tryptophan fluorescence is most commonly studied, because phenylalanine has a very low quantum yield and because tyrosine fluorescence is frequently very weak due to quenching. However, in any given case, one must demonstrate that the observed fluorescence does not contain contributions from tyrosine or phenylalanine in order to provide useful data for subsequent structure-function interpretation. The conclusion that the fluorescence observed for renin is largely due to tryptophan residues is based on two kinds of data: the excitation-emission wavelength dependence and pH effects on fluorescence.

Fig. 1 shows the fluorescence excitation-emission characteristics of renin. Several points are noteworthy: excitation is centered at 280 nm, the absorption maximum of tryptophan; the emission maximum also occurs at the expected maximum for tryptophan, 340 nm; and the shoulder of excitation above 300 nm clearly represents tyrosine absorbance, but extrapolation of this peak to 280 nm indicates that 15% of the 280 nm absorbance can be attributed to tyrosine.

In addition, since tyrosine fluorescence is quenched by titration of the phenolhydroxyl of tyrosine, a process which typically occurs above pH 10, the pH dependence of the fluorescence for a given protein can give some indication of the relative contribution of tyrosine fluorescence to the overall process. Using an excitation of 280 nm and emission of 340 nm, we found 8% change in renin fluorescence between pH 7 and 10. Thus, it appears that the use of 280 nm excitation and 340 nm emission for studying renin fluorescence and fluorescence quenching can be used to accurately examine the environments of the tryptophans of renin.

Iodide Quenching of Renin—Quenching of intrinsic fluorescence in proteins is an established technique for learning about the environments of Trp residues in the native state in solution. In the case of mouse renin, there are 3 Trp residues: Trp-39, Trp-190, and Trp-300. Using computer graphics techniques, the work of Blundell et al. (see Fig. 2 of Ref. 8) suggests that Trp-39 (an invariant residue among aspartyl proteases) is present at the inner surface of the active site cleft at substrate P1. Trp-300 is also present adjacent to the active site cleft at specificity subsite P1, with access to the aqueous environment of this region. Trp-190, on the other hand, may be within the hydrophobic core of the enzyme.

With these predicted environments as a reference, the interpretation of renin quenching by iodide becomes an important exercise. The curvilinear nature of renin quenching indicates two very distinct kinds of Trp environments. The largest class of Trp residues is quenched easily by iodide, indicating aqueous accessibility of approximately 2 Trp residues. On the other hand, one class of Trp residues is largely inaccessible to even the highest concentrations of iodide. Using the above computer graphics data, we can conclude that our solution conformational experiments are consistent with the notion that Trp-39 and Trp-300 are at or near the protein surface, while Trp-190 is largely inaccessible.

The observation that the $K_q$ for iodide quenching of renin was greater than for free indole in solution warrants comment. The simplest interpretation is that the environment of Trp-39 and Trp-300 is positively charged, causing the iodide anion to be relatively more concentrated near these Trp residues and, thus, to be more effective at quenching. Returning again to the model of Blundell et al. (8), it is intriguing to note that renin, in fact, contains several positively charged residues at the edges of the active site cleft where Trp-39 and Trp-300 are found. Specifically, the extended surface flap contains Lys-81, Arg-79, and His-74 as well as an additional surface region including Lys-239, Lys-241, Arg-242, and His-244. None of these residue positions are occupied by basic amino acids in the homologous aspartyl proteases of which the primary structure is known.

The significance of the basic amino acid cluster is unknown, but Blundell et al. (8) have suggested that they may be important for renin zymogen processing or in renin binding to angiotensinogen, a large protein ($M_r \sim 57,000$) which is a specific substrate for renin. It is likely that this specific protein-protein interaction involves more than a renin active site-angiotensinogen substrate site interaction.

Conclusion—The solution conformation of mouse submaxillary gland renin was probed by the solute fluorescence quenching technique. A solvent-accessible, surface location was found for 2 Trp residues, presumably Trp-39 and Trp-300. A solvent-inaccessible location is suggested for 1 Trp residue, presumably Trp-190. In addition, the quantitative analysis of Trp fluorescence quenching is consistent with the known presence of cationic amino acids near the substrate-binding cleft of renin.

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Renin Structural Studies