APPLICATION OF PULSE RADIOLYSIS TO THE STUDY OF PROTEINS

CHYMOTRYPSIN AND TRYPSIN

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ABSTRACT The one-electron reduction of chymotrypsin, trypsin, and their zymogens have been studied by pulse radiolysis. The optical spectra of the transient products from the two active enzymes display a pH-dependent band at 360 nm, associated with the histidine-electron adduct. The yield of the histidyl radical as a function of pH is consistent with a $pK_a < 4.5$, which suggests that the radical is located at the enzyme active site. The histidines of the proenzymes chymotrypsinogen and trypsinogen are unreactive towards the hydrated electron. We conclude that formation of the histidine-electron adduct at the serine protease active site is sensitive to the physical alterations which accompany protease activation.

INTRODUCTION

The relationship between an enzyme's catalytic functions and its three-dimensional structure continues to be a preeminent problem in enzymology. The structural data obtained in recent years by X-ray crystallography have contributed greatly to the exposition of this relationship. However, these data have so far yielded primarily static pictures of enzymes, and ambiguities remain in understanding the dynamic basis of protein catalysis. In this report we shall show that the technique of pulse radiolysis can be utilized to obtain information about the dynamic structural properties of proteins—in particular, the serine proteases.

The serine proteases are among the most extensively investigated of all proteins. (For a most recent review see Kraut, 1.) The reaction pathway in the catalytic hydrolysis of peptides and esters is understood in general terms. The crystal structures of a number of enzymes in this class have been elucidated, and recent low-temperature work has allowed the isolation of reactive intermediates (2). The active sites of these enzymes are all thought to contain an aspartic acid-histidine-serine triad called the "charge relay system" (3). The dynamic consequences of this structural feature, found in chymotrypsin (3, 4), trypsin (5, 6), and elastase (7), are not adequately understood. Using ^{13}C nuclear magnetic resonance, (NMR) Hunkapiller and co-workers (8) have assigned the low pK_a of ~ 3.8 to the histidine side chain of this grouping in α-lytic protease, leaving the higher pK_a of 6.8, known from enzyme kinetic studies, to the aspartic acid side chain. These assignments can be rationalized in terms of the charge

relay system. In support of this conclusion, Markley and Porubcan (9) have determined a pK_a of ~4.5 for the active site histidine of porcine trypsin. In contrast, Robillard and Shulman (10), on the basis of their proton nuclear magnetic resonance studies, have been led to the conclusion that the active site histidine of bovine chymotrypsin has a pK_a of 7.5.

Many of the serine proteases are found as inactive precursors which can be activated by limited proteolysis (11). The structural changes accompanying this activation have been investigated for chymotrypsin (12) and trypsin (13, 14). The results of these studies have been recently reviewed by Stroud et al. (15). While structural differences have been observed between the inactive proenzymes and their activated forms, changes at the active site appear to be minor, and not identical in the two systems. Thus, the correlation between structural changes and enzyme activation is not properly understood.

Pulse radiolysis (16, 17) permits production of hydrated electrons within less than a microsecond; combined with a fast response detection system it offers an effective tool for studying rapid electron transfer reactions, and the properties of transient radicals together with their parent molecules. The atomic groupings in proteins most reactive with the hydrated electron have been established with amino acids and small peptides; these are cystine disulfide bridge, the imidazolium side chain of histidine, the peptide carbonyl group, and the aromatic side chain of tyrosine and tryptophan.

The reactions of linear disulfides with the hydrated electron have rate constants in the range of 2×10^9–4×10^{10} M^{-1} s^{-1} (18, 19). The radical anion $RSSR^-$ and its conjugate acid RSSRH have absorption bands centered near 410 nm ($\epsilon_{max} \simeq 1 \times 10^4$) and 385 nm ($\epsilon_{max} \simeq 7 \times 10^3$), respectively. The disulfide radical anion decays by the reaction $RSSR^- \rightarrow RS\cdot + RS^-$, with a first-order rate constant between 2×10^5 and 2×10^6 s^{-1} (19). The reaction of the hydrated electron with the protonated imidazolium of histidine is characterized by a rate constant of $\simeq 4 \times 10^9$ M^{-1} s^{-1}. The neutral imidazole is much less reactive, $k < 1.4 \times 10^6$ M^{-1} s^{-1} (20, 21). The histidine-electron adduct absorbs with a maximum ($\epsilon_{max} \simeq 2 \times 10^3$ M^{-1} cm^{-1}) near 360 nm. Because the protonated imidazole reacts more rapidly, the absorbance yield at 360 nm is pH-dependent, and the pK_a of the histidine side chain can be determined even when other reactive sites are available on the molecule (21). The reactions of the peptide carbonyl group and of aromatic amino acid side chains with the hydrated electron are characterized by rate constants on the order of 5×10^8 M^{-1} s^{-1} (22, 23).

Several groups have investigated the reaction of the hydrated electron with proteins (24–31). With disulfide-containing proteins a transient species is produced having an absorption band centered near 410 nm, indicating the radical $RSSR^-$. Bisby and coworkers (30), working with pancreatic ribonuclease, reported an additional band centered near 360 nm. It was observed at low pH, but was absent under alkaline conditions. They suggested that this absorbing species was the protonated radical RSSRH. We have concluded, however, that this 360-nm band is due to the histidine-electron adduct (31). The absorbance yield at 360 nm was found to be pH-dependent and in ribonuclease characterized by a pK_a of $\simeq 5.9$, close to the acid dissociation

constants of the two active-site histidines (32–34). The decay rate of the 360-nm band is approximately three orders of magnitude smaller than that expected of the radical disulfide, and that observed with the 410-nm band in the same protein. Finally, the transient spectra of α-casein, which contains histidine but no cystine, show the 360-nm band, while the 410-nm band characteristic of $RSSR^-$ is absent.

The pH-dependent formation of the histidine-electron adduct in ribonuclease A and α-casein suggests that pulse radiolysis is an additional tool for determining histidine pK_a's in proteins. However, not all protein imidazole groups appear to be reactive toward the hydrated electron (31). Thus, there is no evidence for the reaction of the histidine in ribonuclease, which titrates with a pK_a near 7. No 360-nm band was observed with lysozyme, known to have one histidine on the surface of that molecule (35). Nor have we observed the 360-nm band with lactalbumin, a protein containing three histidine residues (M. Faraggi, M. H. Klapper, and L. M. Dorfman, unpublished results). It appears, therefore, that the three-dimensional structure of the polypeptide chain is a controlling factor in the reactivity of the histidine residue.

In this report we shall extend our observations to the reactions of the hydrated electron with the serine proteases α-chymotrypsin, trypsin, and with their proenzymes chymotrypsinogen, trypsinogen.

METHODS

Bovine pancreatic three times crystallized salt-free α-chymotrypsin and trypsin, and five times crystallized chymotrypsinogen and trypsinogen were purchased from Worthington Biochemical Corp. (Freehold, N.J.) and used with no further purification. The pulse radiolysis instrumentation has been described elsewhere (36). Pulses of 500-ns duration (hydrated electron concentration $\sim 5\ \mu M$) were used for the determination of transient spectra, while kinetic measurements of electron decay utilized 100-ns pulses. Irradiated solutions contained 1 mM phosphate buffer, 0.4 m *t*-butanol as the scavenger for the OH radical, and 0.05 – 0.8 mM protein. Oxygen was removed by gently bubbling argon through the solution for approximately 30 min. The protein concentrations were sufficiently high to insure that $\geq 90\%$ of the hydrated electrons formed in solution reacted with the protein. To establish these combination yields, the decay rate of the electron was monitored at 550 nm ($\epsilon_{550} = 10^4\ M^{-1}\ cm^{-1}$), and comparisons were made between solutions with and without protein. The fraction of electrons reacting with the protein was calculated as the ratio $(k'_p - k'_b)/k'_p$, where k'_p and k'_b are the pseudo first-order decay constants obtained from sample and blank experiments, respectively. The second-order rate constants for the reaction of hydrated electron and protein were determined from the slope of the linear relationship between the pseudo first-order decay constant k'_p and protein concentration.

RESULTS

The reaction of the hydrated electron with the four proteins trypsin, α-chymotrypsin, trypsinogen, and chymotrypsinogen is diffusion-controlled, as indicated by the magnitudes of the second-order rate constants, $1.1–4.5 \times 10^{10}\ M^{-1}\ s^{-1}$. Others (24, 25, 37) have reported similar results for trypsin and chymotrypsin.

With all four proteins, at high and low pH the transient spectra obtained within 1 μs of the pulse showed a large band centered near 410 nm, indicating the formation of

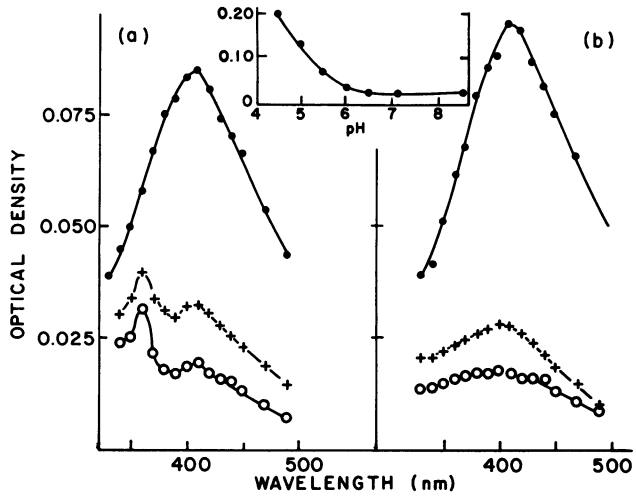

FIGURE 1 Transient spectra of 4×10^{-4}M α-chymotrypsin in argon-saturated solutions containing 0.4 M t-butanol and 10^{-3}M phosphate buffer. Initial concentration of the hydrated electron 6.1×10^{-6}M. Temperature 25 ± 1°C. (●–●), t = 0 μs after pulse; (+–+), t = 200 μs after pulse; (o–o), t = 1,800 μs after pulse. (*a*) pH 4.5. (*b*) pH 7.1. (insert) effect of pH on the 360 nm absorbance at 200 μs after subtraction of residual $RSSR^-$ absorbance.

$RSSR^-$ (Figs. 1–3). No distinct 360-nm band was observed, although a shoulder at this wavelength was seen in the low pH trypsin spectrum (Fig. 2). In the microsecond time range ($t \leq 10$ μs) the 410 nm band decreased in intensity with no observed shifts at any solution pH, suggesting the absence of the neutral radical RSSRH.

At longer times ($t \geq 200$ μs) the trypsin and chymotrypsin transient spectra displayed a distinct band centered near 360 nm, known from previous work to be due to the histidine-electron adduct (see Introduction). By assuming that both 410 and 360-nm bands are Gaussian with respect to wave number, the magnitude of the latter band could be estimated. The insets of Figs. 1 and 2 show the effect of pH on the 360-nm absorbance; the yield goes up as the pH is lowered. Unfortunately, the reaction could not be studied below pH 4.2 due to the competition of hydronium ion for the electron: $e^-_{aq} + H_3O^+ \rightarrow H + H_2O$. One way to minimize the importance of this competing reaction is to increase the protein concentration. We were, however, limited by the decreased intensity of the analyzing light at higher protein levels.

As discussed in the Introduction, the pH dependence of the 360-nm absorbance is due to the greater reactivity of the imidazolium ion relative to the uncharged imidazole. Thus, the pK_a of histidine can be determined by pulse radiolysis. Because we were not able to measure absorbance yields below pH 4.2, an unambiguous pK_a could not be assigned for trypsin or chymotrypsin. An estimate was based on the following assumptions: the extinction coefficient of the histidine-electron adduct in proteins is identical with that of small peptides (2,000 M^{-1} cm^{-1}; 20, 21); the maximum yield of the histidine adduct in trypsin and chymotrypsin is 50%. (The fraction of protein-attached electrons in the histidine-electron adduct was found in earlier experiments to be 85%

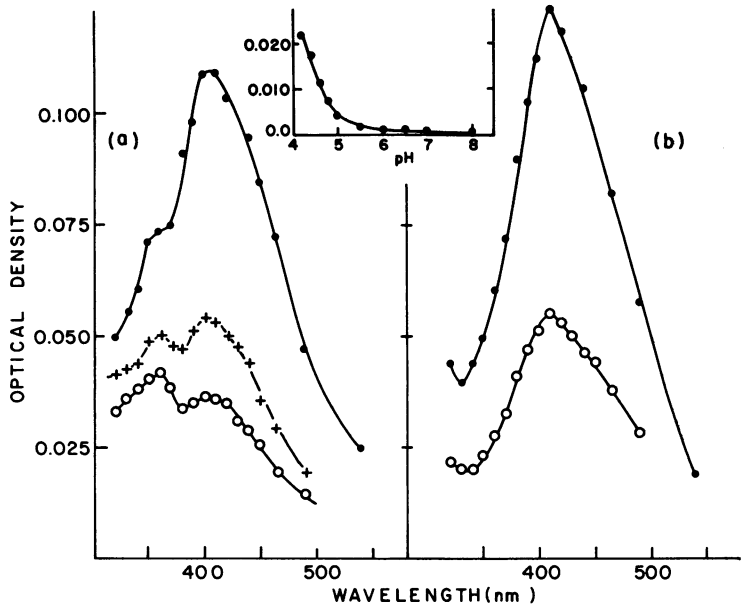

FIGURE 2 Transient spectra of 5 x 10^{-5}M trypsin: conditions were identical to those of Fig. 1 except the pH's of the two experiments were 4.4 and 8.4.

for pancreatic ribonuclease A at pH 4.3 (31). The fractions obtained with α-chymotrypsin at pH 4.5, and trypsin at pH 4.4 were 20% and 32%, respectively.) The pK_a's thus estimated for both proteins are ~4.3. Although the potential for error in this estimate is large, the data clearly indicate pK_a's below 4.5. Chymotrypsin and trypsin have 1 and 2 histidines, respectively, with titration midpoints near 7. We found no 360 nm absorption change associated with this pK_a.

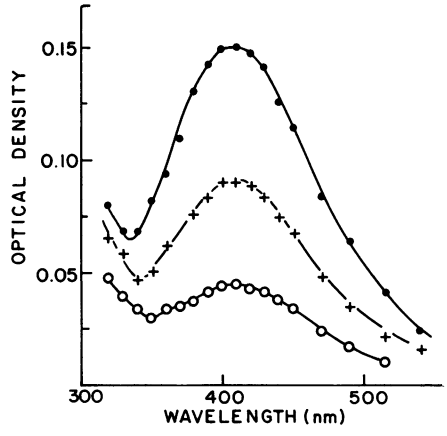

FIGURE 3 Transient spectra of 5 x 10^{-5}M chymotrypsinogen: conditions were identical to those of Fig. 2.

In contrast to the results obtained with the two active proteases, no 360-nm band was observed with either chymotrypsinogen (Fig. 3) or trypsinogen down to the lowest pH tested, 4.2. Since it is known that the histidines of both proenzymes titrate with midpoints near pH 7 (38), we conclude that our inability to see the histidine-electron adduct in the zymogens is not due to an acid pK_a shift of the histidines we observed in the active enzymes.

DISCUSSION

We have shown previously that the diffusion-controlled reaction of the hydrated electron with proteins results in the formation of disulfide and/or histidine adducts (31). Aromatic amino acid side chains may also react with the hydrated electron to produce species absorbing in the ultraviolet below 350 nm (21, 28). Experimental limitations precluded our observation of these reactions, if they occurred to the small extent expected from the 10–50-fold lower reactivities. Because the yield of the histidine adduct depends on solution pH, the pK_a of the reactive histidine group(s) in small peptides and proteins may be determined by pulse radiolysis (28, 31). The pK_a's for the histidines that react in bovine α-chymotrypsin and trypsin are below pH 4.5, comparable to the active site histidine pK_a's assigned by Hunkapiller et al. (8) in α-lytic protease, and by Markley and Porubcan (9) in porcine trypsin. These latter assignments were made on the basis of nuclear magnetic resonance experiments. Thus, the pulse radiolysis results represent an independent confirmation of the low apparent pK_a for the active-site histidine in serine proteases.

As mentioned in the Introduction, not all protein histidines are reactive toward the hydrated electron, and this is true for trypsin and chymotrypsin as well. Those histidines not at the active site, and which have pK_a's closer to 7, are not seen in the pulse radiolysis experiment. Chymotrypsinogen and trypsinogen contain no reactive histidines. Solvent inaccessibility of the imidazole side chain does not appear to be a reasonable explanation of the observed variability in histidine reactivity. The single histidine of lysozyme, solvent-accessible in the crystal structure (35), does not produce a histidine-electron adduct (31). In contrast to this observation, the yield of histidine plus disulfide electron adducts is greater in chymotrypsin than would be expected on the basis of intrinsic reactivities, amounts, and solvent accessibilities of those protein groups which are most reactive. If intrinsic reactivities are similar in proteins and small molecule models, and the hydrated electron reacts directly only with those groups accessible to the solvent, then the expected partitioning of the hydrated electron amongst the various groups may be computed (Table I). The calculated results imply that most of the electrons will react with peptide carbonyls, and that ~20% will combine directly with disulfides and imidazoles. However, we have observed approximately 60% bound to these two groupings.

It is known from studies with oligopeptides that electrons attached initially to the carbonyl oxygen can migrate to other loci (39, 40), and that the intramolecular hydrogen bond between peptide units may serve as a path for electron migration (41). We,

TABLE I

YIELDS OF ELECTRON ATTACHMENT TO DIFFERENT SITES IN α-CHYMOTRYPSIN

Protein component	M_i No. of the component in contact with the solvent*	k_i Second-order rate constant for the reaction of e^-_{aq} with the component	Fraction of e^-_{aq} located on the component.			
			Calculated‡		Observed	
			Acid	Alkaline	pH = 4.5	pH = 8.4
Carbonyls	104	5×10^8	71%	78%		
Disulfides	1	1.3×10^{10}	18%§	20%§	40%	60%
Histidines	1	4×10^9 ‖	6%	0	20%	0
Aromatic amino acids	5	3×10^8	2%	2%		

*Obtained from Birktoft and Blow (44).

‡Calculated by $f = k_i M_i / \Sigma k_i \cdot M_i$; k_i is the intrinsic rate constant of the i^{th} component with M_i units accessible to the hydrated electron.

§Assuming one disulfide bridge.

‖ Value for the protonated imidazole.

therefore, propose that the hydrated electron attaches initially to the protein surface, then rapidly migrates into the protein, and that it settles finally into a potential sink provided by either imidazole or disulfide, with the yields of each determined competitively. Grossweiner (42) has also proposed internal electron migration in proteins to interpret photochemical data. Thus, two explanations may be forwarded for the variable reactivity of histidines in proteins. A particular histidine may be unreactive either because there is no "electron path" leading to it from the protein surface, or because the potential sink provided by the imidazole side chain is not low enough for effective competition with the disulfide bond, or with other potential sites.

The reactivities of the imidazoles in chymotrypsin and trypsin and their contrasting unreactivity in the corresponding proenzymes are particularly noteworthy. These results suggest that formation of the histidine electron adduct is sensitive to the physical changes at the serine protease active site, which accompany conversion of the proenzyme to its catalytically active form. Parenthetically, it is suggestive that the only other reactive histidine(s) we have encountered so far in disulfide-containing proteins appear to be at the active site of ribonuclease. We cannot now explain the observed reactivity alterations upon zymogen activation. However, Birktoft and co-workers (43) have proposed that upon activation of chymotrypsinogen the hydroxyl group of the active site serine 195, hydrogen-bonded to histidine 57, is rotated very close to the disulfide bond of cysteines 42 and 48. The resultant disulfide-serine-histidine triad, not present in the zymogen, may serve as an electron path in chymotrypsin.

Irrespective of the detailed mechanisms that will be required to explain the results we have obtained to date, the apparent reactivities of histidine residues in globular proteins clearly reflect some topological properties of the folded polypeptide chain. Thus, pulse radiolysis may serve as an additional tool for dynamic protein structure studies.

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DISCUSSION

PECHT: In view of the recently determined three-dimensional structure of the copper-containing blue proteins—plastocyanin and azurin, where two of the metal ligands are histidines—would you also expect the imidazole residue to be an effective electron-transferring group in an outer sphere type of a reaction? Furthermore, if this is the case, would you expect to find intermediates with the electron on the imidazole?